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1318. Erratum
NOVEL INHIBITORS OF LEUKOTRIENES SYNTHESIS IN A TREATMENT OF INFLAMMATORY PAIN

KRYSTYNA CEGIELSKA-PERUN*, EWA MARCZUK and MAGDALENA BUJALSKA-ZADROŻNY

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Abstract: A search of new analgesic and anti-inflammatory agents is still ongoing. It is well known that an activation of the leukotriene (LT) synthesis is involved in pain signaling. LTs belong to a class of potent proinflammatory mediators that are biosynthesized from arachidonic acid (AA) inter alia by 5-lipoxygenase (5-LOX) enzyme in association with 5-LOX-activating protein (FLAP). Therefore, the main goal of this article review was to present recent advances on new compounds influencing the LOX pathway, which are in clinical studies. The mechanisms of action and possible implementations of these molecules in a treatment of inflammatory pain are discussed.

Key words: leukotriene, lipoygenase inhibitor, 5-lipoxygenase-activating inhibitor, novel agent

A treatment of chronic pain is still a problem of medicine. As the effectiveness of available analgesics often seems not sufficient, nowadays, there is a search for compounds focused on new mechanisms of action. It was shown that an inhibition of lipoygenase pathway decrease alleviates not only inflammatory (1-4) or chronic (5, 6) but also acute pain (1, 7). Moreover, it was revealed that increased expression of leukotriene (LT) synthases [5-lipoygenase (5-LOX), 5-lipoygenase-activating protein (FLAP), LTA4 hydrolyase – LTA4h and LTC4 synthase – LTC4s] and leukotriene B4 type 1 receptors (BLT1) as well as cysteinyl LT type 1 receptors (CysLT1) occurs in spinal cord after peripheral nerve injury (8). Therefore, lipoygenase inhibitors seem to be promising target of pain therapy.

Leukotrienes (LTs) are considered to play a significant role among others in the pathomechanism of chronic inflammatory disorders, cardiovascular and neurodegenerative diseases and certain types of cancer (9-11). The oxidation of arachidonic acid (AA) by 5-LOX in the presence of 5-lipoxygenase-activating protein (FLAP), which increases the affinity of 5-LOX to AA, is one of the substantial pathways of LTs production (12). 5-LOX converts AA to leukotriene A4 (LTA4), which is further enzymatically transformed into leukotrienes C4 (LTC4), D4 (LTD4) and E4 (LTE4). This group of LTs is called cysteinyl leukotrienes, in contrast to leukotriene B4 (LTB4) which is formed from LTA4 by LTA4 hydrolase (13) (Fig. 1). There are three types of cysteinyl leukotrienes receptors: CysLT1 (located in leukocytes, airway smooth muscles, spleen), CysLT2 (heart, brain, central nervous system, placenta, spleen, leukocytes) (14) and GPR17 (brain, heart and kidney) (15), and three types of receptors for LTB4: BLT1 (located on leukocytes), BLT2 (leukocytes, spleen, liver, ovary) and peroxisome proliferator-activated receptors (PPARs) (expressed in nucleus) (16-18). The diversity of LT receptors occurrence may indicate a role of leukotrienes in numerous physiological and pathological conditions. CysLT1 receptors bind with high affinity to LTD4 and less affinity to LTC4 or LTE4. These receptors are involved in bronchoconstriction, mucus secretion and edema in the airways. Therefore, selective CysLT1 antagonists, such as zafirlukast, montelukast and pranlukast, block the proasthmatic action of the CysLT1. On the other hand, CysLT2 receptors contribute to inflammation, vascular permeability as well as tissue fibrosis. Specific antagonists of CysLT2 receptors have been not known so far, but these receptors bind with equal affinity to LTC4 and LTD4 and with less affinity to

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Leukotrienes B4 bind with BLT1, BLT2 and PPARs receptors, however with the higher affinity to BLT1 receptor (19). BLT1 receptors mediate of its chemoattractant and proinflammatory action (12, 14). Little is known about BLT2 physiological function, but recent studies had shown a protective role of the BLT2 receptor in intestinal and airway inflammation (20). Moreover, PPARs function as lipid homeostasis factors and controls the inflammatory responses (18).

Efforts of creating new drugs inhibiting LOX pathway are focused on three targets: inhibition of enzyme, blocking of leukotrienes receptors or inhibition of FLAP. This review summarizes the current state of knowledge on the new LTs inhibitors and possibility of their implementation in pain treatment.

**Non-selective inhibitor of COX1/2 and 5-LOX**

**UP446**

UP446 is a standardized blend of extracts from two botanical sources (*Scutellaria baicalensis* and *Acacia catechu*) that contains free B-ring flavonoids and flavans standardized to baicalin and catechin. In

![Cascade of leukotrienes production from arachidonic acid](image)

Figure 1. Cascade of leukotrienes production from arachidonic acid with combination of mechanism of action the new agents investigated in clinical trials
Selective 5-LOX inhibitors

**CJ-13610**

CJ-13610 with high selectivity blocks 5-LOX enzyme through a non-redox, non-iron chelating mechanism as a direct competitor of arachidonic acid (24). In preclinical studies, the compound showed antinociceptive and anti-inflammatory effects in the acute model of carrageenan-induced paw edema and in the chronic model using complete Freund’s adjuvant. Furthermore, the compound was tested in osteoarthritis pain model using a medial meniscal transaction. In this study, CJ-13610 showed efficacy in relieving pain after acute administration as well as after 4 days of treatment (25). CJ-13610 was investigated in order to estimate the pharmacokinetic properties in the I phase of clinical trials. Matthew (26) reported that the drug molecule was metabolized by CYP3A cytochrome, the predominant metabolic pathway was oxidation of the sulfur heteroatom and the pharmacokinetic profile of the CJ-13610 was predicted after single-dose administration in humans. Sutton (27) performed the pharmacokinetic studies of a controlled release form of CJ-13610. In order to improve the pharmaceutical properties of CJ-13610 the derivative compound, selective and non-redox type 5-LOX inhibitor – PF-4191834 was designed.

**PF-4191834**

PF-4191834 is a competitive inhibitor of the 5-LOX enzyme that is being developed as an oral anti-inflammatory therapy for the treatment of asthma. It was shown that PF-4191834 has strong inhibiting properties, with about 300-fold selectivity for 5-LOX over 12-LOX and 15-LOX and no influence on the COX enzymes in *in vitro* studies. Further *in vivo* study confirmed inhibition of leukotrienes (CysLT and LTB4) in the carrageenan-inflamed air-pouch model in rats in a dose-dependent manner after single and 7 days administration at a dose of 5 mg/kg. PF-4191834 also demonstrated strong efficacy in chronic inflammatory pain. This effect was similar to the effect observed by celecoxib, a selective COX-2 inhibitor (28). In spite of the fact that pre-clinical results were promising, the II phase of clinical trials was terminated in osteoarthritis patients due to serious adverse events (SAEs): gastric ulcer hemorrhage and acute hepatitis (29). In this study SAEs occurred in the group receiving combination of PF-4191834 with naproxen. Therefore, it remains unclear if those adverse effects were related to PF-4191834 alone or to combined administration with naproxen. However, PF-4191834 is being investigated in clinical trial in asthma patients and it successfully completed the II phase study without SAEs (29).

**Licofoleone (ML3000)**

Originally discovered by Merckle GmbH and developed by EuroAlliance for osteoarthritis treatment as a dual COX/5-LOX inhibitor, recently it was characterized as a FLAP inhibitor (29) and weak inhibitor of microsomal prostaglandin E synthase-1 (mPGES-1) (30). It has been widely examined in animal models of inflammation and pain, as...
well as it passed III phase of clinical trials for osteoarthritis treatment (31). In preclinical studies, it completely blocked secretion of PGE₂ and LTB₄ in carrageenan-induced rat paw edema model (32). Furthermore, licofelone was as effective as indomethacin in reducing hyperalgesia in carrageenan-, bradykinin-, and arachidonic acid-induced rat hind paw edema models (33). Compound demonstrated longer action and stronger antinociceptive effect in comparison to indomethacin and zileuton in rat model of incisional pain (34). It also showed neuroprotective (35), antipyretic (34), antithrombotic (36) and antibronchoconstrictive effect (37).

Clinical studies were performed in healthy adults in order to assess the tolerability of ML3000 and in OA patients in order to assess the safety and efficacy. In phase I of clinical trial licofelone was assessed at two doses of 200 and 400 mg twice a day (bis in die – bid) for 4 weeks in comparison to naproxen at a dose of 500 mg bid or placebo in 121 healthy volunteers. In the study it was found that gastric mucosa was normal in 93% and 89% of volunteers given licofelone at doses of 200 and 400 mg bid, respectively, in 90% given placebo and 37% given naproxen at a dose of 500 mg bid (38). Therefore, gastric toxicity of licofelone appeared to be at the similar level to placebo and less than of naproxen. Pharmacokinetic parameters were explored in other study (39). Licofelone was administrated to 18 healthy young males and females (the average age was 30.9) and elderly (the average age was 72.1) individuals twice a day at a dose of 200 mg for 5 days and at a single dose of 200 mg at day 6 of experiment. It was found that the maximum plasma concentration (C max) was 0.74 in 4 h after administration, the rate of systemic elimination – 11.1 ± 7.0 in young individuals and 8.7 ± 4.7 in elderly individuals, as well as the mean t₁/₂ value was 15% higher in the elderly study population.

Subsequent phase II of clinical studies evaluated the efficacy of this compound at various dosages in OA patients. In the first study, patients n = 107 received licofelone at doses of 100, 200 and 400 mg bid or placebo for 4 weeks (38). This study found that tested compound at doses of 200 or 400 mg bid was effective in relieving pain and stiffness as determined by WOMAC index, as well as it showed effectiveness in secondary endpoints, such as level of disability. In the second trial, 404 patients were treated with licofelone at the same doses as in the previous study and the effect of the drug was compared to placebo or diclofenac (50 mg, three times a day) (38). Licofelone resulted in superior analgetic effect and greater improvements in stiffness as well as disability in comparison with placebo, but no significant difference was noted between diclofenac and licofelone. The most frequent side effects were diarrhea and abdominal pain at a dose of 400 mg.

The safety and efficacy of licofelone at a dose of 200 mg bid or naproxen at a dose of 500 mg bid for 12 weeks was tested in phase III of study. In 148 OA subjects efficacy was similar in both groups (40). However, licofelone has proven to have better gastrointestinal tolerability (14% of licofelone-treated patients reported gastrointestinal side effects vs 26% of those treated by naproxen). In a 52-week, long term phase III study with a large cohort of patients (n = 710) the efficacy and safety of licofelone (100 or 200 mg bid) was compared to naproxen (500 mg bid) in knee OA patients (38). The results of the study revealed that licofelone had an improved efficacy throughout the 52 weeks, better gastrointestinal profile and less risk of hypertension aggravation comparing to naproxen.

This data suggest that licofelone may be a promising alternative to traditional non-steroidal anti-inflammatory drugs not only in osteoarthritis treatment, but also in co-treatment with other drugs from different pharmacological groups in reducing inflammatory and neuropathic pain. Double COX/LOX pathway inhibitors possess synergistic analgesic effect due to blocking of arachidonic acid metabolism and subsequent inflammatory process. Therefore, they inhibition of prostaglandins (products of COX) as well as leukotrienes (products of 5-LOX) synthesis may result in stronger anti-inflammatory effect. Moreover, double inhibiting strategy seems to be reasonable due to better gastrointestinal and cardiovascular tolerability, as well as significantly diminish the possibility of drug-drug interaction.

**CONCLUSIONS**

LOX pathways seem to be involved in pathogenesis of inflammatory pain. Moreover, data available in the literature indicate that not only 5-LOX inhibitors, but also FLAP inhibitors, may be promising in a treatment of this kind of pain. Noteworthy, they are also dual COX/LOX inhibitors, as they possess not only a more potent analgesic activity but also less side effects in comparison to selective and not selective COX-1/COX-2 inhibitors.
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The DSC quantitative studies are usually dealing with purity (1, 2) or crystalline and amorphous content of raw materials (3, 4). The first application is using the Van’t Hoff equation that describes lowering of the melting point of eutectic impurities. Such a solution can be useful, because there are impurities that don’t have UV/Vis absorbance, what leads to absence of signal in LC/UV methodologies. The use of DSC technique, solely to determine active pharmaceutical ingredient (API) content in the matrix, is rare. During the literature search only 3 publications were found. Two of them were dealing with content of paracetamol (PAR) in pharmaceutical formulations (5) and suppositories (6). The third described DSC application to acetylsalicylic acid (7). In all cases the authors used relationship between melting enthalpy ($\Delta H$) and concentration of active ingredient $\Delta H = kA$, where $k$ is calorimetric constant, and $A$ is the area under the DSC curve. The calibration curves were constructed from increasing weights of raw PAR (5), acetylsalicylic acid or PAR various concentrations in physical mixtures with different excipients (7) or suppositories (6). The obtained analytical results were comparable or even better (6) than those, used for reference purposes (mainly UV spectrophotometry). The main advantages of the method were that samples didn’t need to exceed 2.5-4.0 mg and the procedure did not require an extraction process. Moreover, it is rapid, sensitive and reproducible. Finally, it was concluded that the DSC could clearly be an alternative method for measuring the content of API in suppositories or tablets.

The physical and mechanical properties of tablets, and thus the bioavailability of the drug are a result of pharmaceutical manufacturing process. Correct pharmacological action depends not only on the equipment used, but also on the physical properties of the APIs and excipients. Increasing prevalence of poorly soluble APIs is the reason of using numerous strategies for enhancing their dissolution rate. Those methods may certainly modify the solid-state interactions between drug and excipients. Reducing the particle size, that causes a loss of the crystalline nature and increases surface area of the drug, could change their stability and/or lead to polymorphic transformation resulting in different behavior of the same drug (8-17).

This research was designed to show the interactions that are being able to modify the results of quantitative DSC measurements. Three commercially available tablets with paracetamol, different in

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**Analysis**

**The Use of the DSC Method in Quantification of Active Pharmaceutical Ingredients in Commercially Available One Component Tablets**

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**Abstract:** Beside many known applications of the differential scanning calorimetry method in the study of solid phase substances, there is a possibility to use it in quantitative analysis. For this purpose, a relationship between the signal value of analyte (enthalpy change $\Delta H$) and its concentration in the matrix is used. This paper shows how the choice of a matrix (used excipients) and the method of its preparation (as a non-micronized or micronized mixture) affect the final result of such determinations. The problem has been shown by measuring different, commercially available tablets of paracetamol. The calculated results obtained from micronized, respectively, cellulose and starch mixtures were ranging from 456.83 mg to 491.42 mg and 465.58 mg to 486.68 mg while for non-micronized, physical mixtures were distinctly different, and were ranging, respectively, from 433.27 mg to 455.12 mg and 449.04 to 473.36 mg. The DSC method was validated and as a reference, a validated TLC method with densitometric detection was used.

**Keywords:** differential scanning calorimetry, DSC, calorimetry, paracetamol, acetaminophen

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weight, type of excipients and composition, were
determined by DSC method. For this purpose five
calibration curves plotted from four different mix-
tures containing micronized and non-micronized
starch (Starch 1500) and cellulose (Vivapur 12) with
an increasing amounts of PAR were used. The
micronization process was applied to modify the
crystalline nature of binary mixtures under study.
Described phenomenon concerning PAR and micro-
crystalline cellulose, both in physical and grinded
mixtures, was previously reported (18). The fifth
calibration curve was prepared with pure raw PAR.
Appropriate calibration curves were further applied
to calculate the final experimental contents of PAR
(ODSC) in the tablets and were compared with those
declared (D) by the manufacturer using equation
\[A(\%) = \left(\frac{ODSC - D}{D}\right) \times 100.\]

 Whereas a purity, melting heat (\(\Delta H\)), and tem-
perature for the onset (\(T_{onset}\)) of melting undergo
small variations when the sample mass is changed
(19, 20) the research was carried out using compar-
able weights of samples (about 4.9 mg).

 The DSC method was validated according to
ICH QR1 guidelines (21) and compared with the
results previously obtained from validated quantita-
tive reference method of thin layer chromatography
coupled with densitometric detection.

EXPERIMENTAL

Materials

 Paracetamol powder – pure polymorphic form
I with estimated melting temperature at \(T_{onset} =
169.4^\circ C\) (Lot: 6135999 B163) was supplied by
POL.NIL Warsaw, Poland. The microcrystalline
cellulose Vivapur 12 was obtained from J.
Rettenmaier & Sohne, Weissenborn Germany (Lot:
5601290308) and the corn starch Starch 1500 was
from Colcorcon Ltd. UK (Lot: 500075).

 Paracetamol is a low potency, high dose drug,
so its concentration in tablets is usually far above
50%. The following tablets containing 500 mg of
PAR per tablet were used: Codipar manufactured by
GlaxoSmithKline, Poland (Lot: RG0159), com-
posed of microcrystalline cellulose, starch,
polyvinylpyrrolidone K-30, talc, magnesium
stearate, anhydrous colloidal silica, sodium starch
glycolate; Paracetamol manufactured by Aflofarm,
Poland (Lot: 01AF0211), composed of starch,
polyvinylpyrrolidone, crospovidone, stearic acid;
paracetamol manufactured by Polfa-Łódź, Poland
(Lot: 010911), composed of sorbitol (170 mg), pota-
to starch, povidone, magnesium stearate. All drugs
are commercially available.

Sample and model mixtures preparation

 Twenty tablets of each medical product under
the study were individually weighed and ground in
an agate mortar and pestle into fine powder. The
mean weights of Paracetamol Aflofarm, Codipar
GlaxoSmithKline and Paracetamol Polfa-Łódź
tablets were 550.24 mg (90.87% of PAR), 597.10
mg (83.74% of PAR) and 789.04 mg (63.368% of
PAR), respectively.

Differential scanning calorimetry

 The set of PAR mixtures with starch or micro-
crystalline cellulose (1000 mg each) at concentra-
tions of PAR from 30% to 90% (corresponding to
1.47-4.41 mg of PAR in the sample) were prepared
separately, gently homogenized and divided. One
part (500 mg) was ready for further experiments; the
other was micronized in an agate mortar and pestle
with some drops of methanol for 10 min.

 The samples of about 4.9 mg, both of tablets
and mixtures, were accurately weighed in aluminum
pans and sealed.

Thin layer chromatography

 The tablet powder portions equivalent to 26 mg of
PAR was accurately weighed and transferred into 50.0
mL volumetric flask. About 25.0 mL of ethanol-water
(1 : 1 v/v) was added and the mixture was sonicated for
15 min. Then, the mixture was diluted to volume with
the same solvent and mixed well to obtain sample stock
solution of 0.52 mg/mL. For the determination of PAR
in tablets, the stock solution was diluted to final con-
centration 0.026 mg/mL. The standard solution of PAR
at concentration of 0.026 mg/mL was also prepared.

Analysis of paracetamol

 Differential scanning calorimetry

 The DSC measurements were performed in
nitrogen atmosphere with a flow rate of 50 mL/min
using EXSTAR DSC 7020 apparatus (SII
NanoTechnology Inc.) equipped with DSC7020
electric cooling unit. The calibration was done with
indium and tin standards.

 The sealed pans were equilibrated at 30°C for 15
min and thereafter the melting behavior was analyzed
at heating rate of 10°C/min. The pure raw PAR was
investigated in increasing sample weights 1, 2, 3, 4, 5
and 6 mg with the same procedure. All measurements
were performed at least three times and averaged. The
tablets were examined at least six times.

Thin layer chromatography and densitometry

 Chromatography was performed on 10 cm x 12
cm aluminum (cut from 20 x 20 cm) TLC plates
coated with silica gel 60F254 (Merck, Germany) layers.

Aliquots of 10 µL of standard and sample solutions were applied as bands 8 mm wide, 8 mm apart and 10 mm from the bottom and the edge of the plate by use of the Linomat V (CAMAG, Switzerland) sample applicator.

Linear ascending development with 10 mL of chloroform–toluene–acetone–ethanol–diethylamine (4 : 1 : 3 : 1 : 1, v/v/v/v/v) as mobile phase was performed in a previously saturated chromatographic chamber (18 × 9 × 18 cm in size, Sigma-Aldrich, USA). The optimum saturation time was found to be 10 min. The development distance was approximately 115 mm. After development, the plates were dried at room temperature for 30 min. Densitometric scanning was performed with a CAMAG TLC Scanner 3 with winCATS 1.3.4 software in absorbance/reflectance mode at 235 nm. The slit dimensions were 6 × 0.4 mm and the scanning speed was 20 mm/s. The obtained value of retardation factor for PAR was found to be 0.61. For identification, absorption spectra within the range 200–400 nm were recorded.

The content of PAR in pharmaceutical preparations was computed by comparing the peak areas for standard and tested solutions.

RESULTS AND DISCUSSION

Evaluation of differences between micronized and non-micronized mixtures under study

Figure 1 shows the selected DSC curves of model micronized PAR mixtures both with microcrystalline cellulose Vivapur 12 (I) and starch Starch 1500 (II) compared to the corresponding curves of non-micronized mixtures at the same drug content in the sample. We cut all the DSC curves up to 120°C to make them more transparent. The depression of melting points towards lower temperatures is typical for binary mixtures when the concentration of one component is decreasing. However, one can see the differences in the peaks surface areas. For the same content of PAR in the sample the non-micronized mixtures show higher values of enthalpy than their micronized analogues.

The obtained ΔH enthalpies of all examined mixtures are presented in Table 1. The broadest differences are related to PAR/microcrystalline cellulose mixtures, and range from -22.05 mJ/mg to 104.37 mJ/mg. Smaller effects recur in PAR/starch mixtures, where the differences are in the range of 5.88 mJ/mg to 49.25 mJ/mg.

In order to plot appropriate calibration curves, the obtained ΔH enthalpies of micronized as well as non-micronized mixtures as a function of the increasing weights of PAR in a constant sample weight (about 4.9 mg) were used. The calibration curve of increasing weights of pure raw PAR (not featured) was plotted for comparison purposes.

Validation of DSC method

Validation of the developed method proved that it meets the acceptance criteria in the scope of specificity, sensitivity, linearity, precision, and accuracy. The validation data were summarized in Table 2.

The developed method was specific to studied components. No new chemical individuals or incompatibilities were found (see Fig. 1), but only

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Figure 1. Selected DSC curves of the different contents of PAR in (I) PAR/cellulose and (II) PAR/starch mixtures; continuous line: model micronized mixtures, dotted line: non-micronized mixtures; A, B, C – content of PAR in the sample.
small depressions of melting points towards lower temperatures which were explained above.

The linearity of calibration curves were studied on the working range of 1.47–4.41 mg for all mixtures under study and 1-6 mg for pure raw PAR. The linear relationships coefficients between peak areas and concentrations were calculated as \((r) = 0.9974\) and \((r^2)\) ranging from 0.9948 to 0.9980. For calibration curve plotted from increasing weights of pure raw PAR \((r)\) and \((r^2)\) were both over 0.99. All obtained intercepts were not statistically significant.

Sensitivity of the method was high. The LODs and LOQs (calculated from the calibration curve slope \((a)\) and the slope standard estimation error \((S_e)\), using formulas \(\text{LOD} = 3.3 \times S_e/a\) and \(\text{LOQ} = 10 \times S_e/a\) evaluated for micronized mixtures both of cellulose and starch were 0.28 mg, 0.27 mg and 0.84 mg, 0.81 mg, respectively, while for physical mixtures were 0.18 mg, 0.17 mg and 0.56 mg, 0.52 mg. The limits values calculated for raw PAR were 0.32 and 0.97 mg, respectively.

### Table 1. Effects of mixture composition on the area of melting peak ∆H (averaged from three determinations).

<table>
<thead>
<tr>
<th>Composition of the mixture</th>
<th>Content of PAR in the sample [mg]</th>
<th>∆H of PAR* [mJ/mg]</th>
<th>Differences between non micronized and micronized mixtures [mJ/mg]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Micronized mixtures</td>
<td>Non micronized mixtures</td>
<td></td>
</tr>
<tr>
<td>PAR/cellulose</td>
<td>1.47</td>
<td>156.31</td>
<td>260.68</td>
</tr>
<tr>
<td></td>
<td>1.96</td>
<td>276.36</td>
<td>343.65</td>
</tr>
<tr>
<td></td>
<td>2.45</td>
<td>356.48</td>
<td>436.92</td>
</tr>
<tr>
<td></td>
<td>2.94</td>
<td>472.36</td>
<td>537.04</td>
</tr>
<tr>
<td></td>
<td>3.43</td>
<td>579.67</td>
<td>649.41</td>
</tr>
<tr>
<td></td>
<td>3.92</td>
<td>676.20</td>
<td>736.96</td>
</tr>
<tr>
<td></td>
<td>4.41</td>
<td>833.49</td>
<td>811.44</td>
</tr>
<tr>
<td>PAR/starch</td>
<td>1.47</td>
<td>207.27</td>
<td>256.52</td>
</tr>
<tr>
<td></td>
<td>1.96</td>
<td>307.72</td>
<td>352.80</td>
</tr>
<tr>
<td></td>
<td>2.45</td>
<td>414.87</td>
<td>428.75</td>
</tr>
<tr>
<td></td>
<td>2.94</td>
<td>473.34</td>
<td>514.50</td>
</tr>
<tr>
<td></td>
<td>3.43</td>
<td>581.96</td>
<td>625.98</td>
</tr>
<tr>
<td></td>
<td>3.92</td>
<td>661.17</td>
<td>691.88</td>
</tr>
<tr>
<td></td>
<td>4.41</td>
<td>787.92</td>
<td>793.80</td>
</tr>
</tbody>
</table>

*∆H was recalculated to the content of PAR in the sample

### Table 2. Validation of the DSC method.

<table>
<thead>
<tr>
<th>Validation parameters</th>
<th>Micronized mixtures</th>
<th>Non micronized mixtures</th>
<th>raw PAR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PAR/cellul.</td>
<td>PAR/starch</td>
<td>PAR/cellul.</td>
</tr>
<tr>
<td>Specificity</td>
<td>specific</td>
<td>specific</td>
<td>specific</td>
</tr>
<tr>
<td>Slope ((a \pm S_a))</td>
<td>222.6 ± 7.2</td>
<td>190.7 ± 6.0</td>
<td>193.3 ± 4.2</td>
</tr>
<tr>
<td>Intercept ((b \pm S_b))</td>
<td>-175.8 ± 22.3</td>
<td>-70.0 ± 18.5</td>
<td>-28.7 ± 12.9</td>
</tr>
<tr>
<td>(r)</td>
<td>0.9974</td>
<td>0.9976</td>
<td>0.9988</td>
</tr>
<tr>
<td>(r^2)</td>
<td>0.9948</td>
<td>0.9951</td>
<td>0.9977</td>
</tr>
<tr>
<td>LOD [mg]</td>
<td>0.28</td>
<td>0.27</td>
<td>0.18</td>
</tr>
<tr>
<td>LOQ [mg]</td>
<td>0.84</td>
<td>0.81</td>
<td>0.56</td>
</tr>
<tr>
<td>Linearity [mg]</td>
<td>0.84 – 4.41</td>
<td>0.81 – 4.41</td>
<td>0.56 – 4.41</td>
</tr>
<tr>
<td>Precision (n=6) %RSD</td>
<td>1.76</td>
<td>1.84</td>
<td>1.85</td>
</tr>
<tr>
<td>Recovery (n=6) %RSD</td>
<td>1.86</td>
<td>1.90</td>
<td>1.94</td>
</tr>
</tbody>
</table>

\(S_a\) - standard deviation of slope; \(S_b\) - standard deviation of intercept
The method have a good precision, which was determined for one concentration level of 2.94 mg of PAR in the sample. The results were expressed as the relative standard deviation (%RSD). Estimated %RSD values for micronized mixtures did not exceeded 1.84 while for non-micronized mixtures 1.99. The precision of pure raw PAR calibration curve was 2.87%.

Basing upon PAR contents (O DSC) obtained experimentally and reference TLC (O TLC) values the mean recovery (%R) were calculated using formula %R = O_DSC/100% / O_TLC. The %R values obtained both for micronized and non-micronized mixtures of PAR/cellulose and PAR/starch were excellent and ranged from 1.86 to 1.95. The recovery calculated for calibration curve of raw PAR (2.16) was slightly higher than the one from micronized and non-micronized mixtures.

### Validation of TLC method

To confirm analytical data obtained from DSC studies the PAR was also determined in the same drugs using thin layer chromatography (TLC). The developed, especially for this purpose, method was specific against studied components. Good correlation between UV spectra acquired from the standard and the pharmaceutical preparations indicated that PAR spot was free of any interference that might be present in the analysis. Resolution of the peaks appearing on the chromatograms was 5.24. The chromatographic method with densitometry detection has high sensitivity (LOD = 104 ng/band, LOQ = 156 ng/band), good precision and intermediate precision (%RSD are 1.50 and 1.82, respectively). Recovery of tested substances was 98.95 %.

### Linearity range was 0.1040 µg per band to 0.8840 µg per band. The correlation coefficient (r) and determination coefficient (r^2) obtained for significance level 0.05 and n = 8 were close to 0.99 that proved a highly significant linear correlation. Residuals of the regression line were distributed at random around the zero line, without any trend.

The results of PAR determination in tablets by means of TLC are comparable to contents declared by manufacturer, regardless of their composition and manufacturing methods (Table 3).

### Determination of PAR in tablets by DSC

The validated methods described above were applied to quantitative determination of PAR in the

<table>
<thead>
<tr>
<th>Analgesic</th>
<th>PAR content</th>
<th>A(%)</th>
<th>O_TLC ± SD [mg/tablet]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(O_{DSC}) ± SD [mg/tablet]</td>
<td>[(O_{DSC}-D)/D]x10</td>
<td></td>
</tr>
<tr>
<td>Micronized mixtures</td>
<td>PAR/cellul.</td>
<td>PAR/starch</td>
<td>PAR/cellul.</td>
</tr>
<tr>
<td>Paracetamol</td>
<td>469.46 ± 5.94</td>
<td>486.68 ± 6.95</td>
<td>-6.11</td>
</tr>
<tr>
<td>Aflofarm</td>
<td>456.83 ± 2.25</td>
<td>465.58 ± 2.62</td>
<td>-8.63</td>
</tr>
<tr>
<td>Codipar GlaxoSmithKline</td>
<td>491.42 ± 2.15</td>
<td>484.42 ± 2.51</td>
<td>-1.72</td>
</tr>
<tr>
<td>Non micronized mixtures</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paracetamol</td>
<td>455.12 ± 6.84</td>
<td>473.36 ± 7.30</td>
<td>-8.98</td>
</tr>
<tr>
<td>Aflofarm</td>
<td>433.27 ± 2.59</td>
<td>449.04 ± 2.76</td>
<td>-13.35</td>
</tr>
<tr>
<td>Codipar GlaxoSmithKline</td>
<td>443.55 ± 2.47</td>
<td>455.91 ± 2.64</td>
<td>-11.29</td>
</tr>
<tr>
<td>Paracetamol</td>
<td>464.14 ± 7.01</td>
<td></td>
<td>-7.17</td>
</tr>
<tr>
<td>Polfa Łódź</td>
<td>445.44 ± 9.33</td>
<td></td>
<td>-10.91</td>
</tr>
<tr>
<td>Pure raw PAR</td>
<td>446.35 ± 15.48</td>
<td></td>
<td>-10.73</td>
</tr>
</tbody>
</table>

*Table 3. PAR contents of the analgesics under study by means of DSC method based on calibration curves obtained from the different mixtures of PAR/cellulose and PAR/starch or increasing weights of raw PAR (n = 6); D = 500 mg per tablet.*
commercially available drugs. The PAR contents obtained experimentally $O_{\text{DSC}}$ compared to the value declared by the manufacturer $D = 500 \text{ mg}$ and the results calculated from the reference TLC method are reported in Table 3.

When analyzing Table 3, it can be seen that the differences between contents of PAR in tablets under study were significant and were results of used calibration curves. The $A(\%)$ values in the group of model micronized mixtures were evidently smaller (from -1.72 to -8.63) than those calculated for non-micronized mixtures (from -5.33 to -13.35). The same behavior was found for PAR/starch both of micronized and non-micronized mixtures. The calculated PAR contents were generally higher than those calculated from their cellulose counterparts and were ranging from -3.12 to -6.88 and -5.33 to -10.20, respectively. The only exception were Polfa Łódź tablets where PAR content calculated for cellulose micronized mixtures was $-1.72$ vs. $-3.12$ obtained for micronized starch mixtures.

It may seem logical that the best results should be obtained when excipients used for the preparation of calibration curve are the same as in measured tablets. In fact, the PAR content measured for Paracetamol Aflofarm, where starch is the main excipient, is much closer to that declared by the manufacturer, when the PAR/starch micronized mixture is used ($A(\%) = -2.66$). The same content but obtained for PAR/cellulose micronized mixture was almost doubled ($A(\%) = -6.11$). Similar relationships were obtained for non-micronized mixtures. However, this does not happen in all cases. The main excipient forming the Codipar GlaxoSmithKline tablets is cellulose. The PAR contents calculated from PAR/starch calibration curves were clearly higher comparing to those obtained from PAR/cellulose both micronized and non-micronized mixtures: -6.88, -8.63 and -10.20, -13.35, respectively.

The Paracetamol Polfa Łódź is an example where neither cellulose nor starch but sorbitol were chosen. Comparing the PAR content obtained from micronized and non-micronized mixtures one can find significant differences between them. In our opinion the reason is the presence of multiple hydroxyl groups coming from sorbitol and increased ability to form hydrogen bonds between components under micronization process.

The last part of the Table 3 incorporates the quantitative results calculated using a calibration curve plotted from the increasing weights of pure raw PAR (from 1 mg to 6 mg). The idea was to show how the sample mass alter such measurements. In the course of the studies it was found that the mean value of $\Delta H$ enthalpy of 1 mg sample was 189.5 mJ/mg, while for 6 mg sample (recalculated to 1 mg) 176.5 mJ/mg. In this case the $A(\%)$ values were in the range from -7.17 to -10.91.

CONCLUSIONS

Differential scanning calorimetry is the first line technique indispensable for industrial quality control laboratories and, next to many routine applications, could be used in quantitative assays. However, there are several limitations concerning interactions between APIs and coexisting excipients. In the course of the studies it was shown that composition of the mixtures, the way of their preparation and the weights of samples alter the final results of such measurements. When using the DSC method, in 5 different ways (methodologies — calibration curves), 5 different contents of PAR for each pharmaceutical product were obtained. It was also shown that there is no apparent direction of the observed differences. The $A(\%)$ values, calculated using equation $A(\%) = [(O_{\text{DSC}}-D)/D]\cdot100$, obtained from micronized, respectively, cellulose and starch mixtures were ranging from -1.72 to -8.63 and -2.66 to -6.88 while for non-micronized mixtures were distinctly higher and were ranging, respectively, from -8.98 to -13.35 and -5.33 to -10.20. For this reason, using the DSC method in quantification of APIs in commercially available, one component tablets should be assessed individually and in each case requires appropriate empirical preliminary studies.

Acknowledgments

Authors would like to dedicate this article to Professor Jan Krzek, our always remembered advisor, mentor and teacher.

REFERENCES


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Solid pharmaceutical preparations can be considered as heterogeneous systems consisting of one or more active pharmaceutical ingredients (APIs) and several excipients. In such blends physical interactions are quite common. Some of them are deliberately invoked to produce a certain effect, for example, to aid processing, modify drug dissolution (oral modified release) or distribution in the body (parenteral modified release drugs). Other interactions in solid phase are unintended, usually causing the problems. The essence of physical interactions is that interacting molecules are not modified in any way. In the other words: new molecules, in chemical meaning, are not created.

Most poly active component drugs, currently available on the market, belong to the group of non-steroidal anti-inflammatory drugs (NSAIDs); a drug class also comprising combinations of antipyretics and/or analgesics (e.g., ibuprofen (IBU)) together with APIs from other pharmacological groups as antiallergics, sympathomimetics and antitussives. The physical interactions between active ingredients are rare. Considering ibuprofen for example, only few reports concerning incompatibilities with ketoprofen (1) and menthol (2, 3) were found. Much more publications are dealing with excipients. Magnesium stearate is commonly used in pharmaceutical manufacturing as a “flow agent” which helps ensure that the pressing process is smooth and the ingredients stay blended in the proper proportions. It was demonstrated (4) that the particles of magnesium stearate appear to adhere to the surfaces of the other components on mixing. All stearates were found to form simple eutectics with ibuprofen (5-9). Some interaction were also observed with polyvinylpyrrolidone (PVP) and calcium phosphate (8, 9). For other drugs new associative structures (8, 10) were reported when a surfactant, sodium dodecylsulfate, was added as well as interactions of lactose with drugs containing amino groups (11).

In our previous paper (12) we have discussed the use of DSC method in quantification of APIs in commercially available, one-component tablets. For this purpose, a relationship between the signal value of analyte (enthalpy change $\Delta H$) and its concentration in the matrix is used. However, there are several limitations of its application, concerning solid state interactions between APIs, other APIs and/or coexisting excipients. With respect to their physical properties, it is known that amorphization state and/or permanent particle deformation can produce relatively large areas of interparticle contact and thus high particle-particle bonding forces. Finally, it may affect the DSC quantitative measurements. The problem was shown using commercially available, different poly component tablets containing ibuprofen in the presence of pseudoephedrine hydrochloride or paracetamol and coexisting excipients.

Keywords: differential scanning calorimetry, DSC, paracetamol, ibuprofen
of their preparation (as non-micronized mixture, micronized mixture or pure raw analyte) and the weights of samples alter the final results of such measurements leading to different contents of PAR. It was also shown that there is no apparent direction of the observed differences, suggesting that the use of the DSC method for quantification of APIs in commercially available tablets should be assessed individually and requires appropriate empirical preliminary studies in each case.

The objective of the present study was to estimate the influence of interactions between two active agents, presented in the tablet, on their quantification. For that reason six commercially available tablets with IBU different in weight, type of excipients and composition, were determined by DSC method. Two calibration curves plotted from mixtures containing micronized and non-micronized starch (Starch 1500) with an increasing amounts of IBU were used. The micronization process was applied to modify the crystalline nature of mixtures under study. In this way the complex technological processes of their manufacturing were emulated. Appropriate calibration curves were further applied to calculate the final experimental contents of IBU (ODSC) in the tablets and were compared with those declared (D) by the manufacturer using equation A(%) = [(ODSC-D)/D]◊100.

EXPERIMENTAL

Materials

Ibuprofen powder – pure polymorphic form I with estimated melting temperature at T_{onset} = 76.1°C was obtained from Hubei Granules-Biocause Pharmaceutical Co., Ltd., China (LOT 400-0709065M). The corn starch Starch 1500 was from Colcorcon Ltd. UK (Lot 500075).

Ibuprofen is a low potency, high dose drug. Typical dose of IBU in one component drugs in Poland is 400 mg, however in two component drugs the dose does not exceed 200 mg.

The following two components tablets containing 200 mg of IBU and 30 mg of pseudoephedrine hydrochloride were used: Acatar Zatoki manufactured by US Pharmacia Ltd., Poland (LOT U1107221), the mean weight of tablet 530.36 mg, composed of cellulose (Elcema P-100 and Elcema F-150), corn starch, pregelatinized starch, Guar gum, talc, croscarmellose sodium, crospovidone, colloidal silica, hydrogenated vegetable oil; Ibuprom Zatoki manufactured by US Pharmacia Ltd., Poland (LOT U1205401), the mean weight of tablet 544.08 mg, composed of cellulose (Elcema P-100 and Elcema F-150), corn starch, pregelatinized starch, Guar gum, talc, croscarmellose sodium, crospovidone, colloidal silica, hydrogenated vegetable oil; Modafen manufactured by Zentiva, Czech Republic (LOT 3230912), the mean weight of tablet 575.18 mg, composed of microcrystalline cellulose, lactose monohydrate, corn starch, pregelatinized starch, sodium lauryl sulfate, povidone 25, stearic acid, sodium carboxymethyl starch, anhydrous colloidal silica; Nurofen Zatoki manufactured by Reckitt Benckiser, Poland (LOT AH006), the mean weight of tablet 374.22 mg, composed of calcium phosphate, microcrystalline cellulose, povidone, croscarmellose sodium, magnesium stearate.

Sample preparation

Twenty tablets of each medical product under the study were individually weighed and ground in an agate mortar and pestle into fine powder.

The set of IBU mixtures with starch Starch 1500 (1000 mg each) at concentrations from 10% to 60% (corresponding to 0.50-3.0 mg of drug in the sample) were separately prepared, gently homogenized and divided. One part (500 mg) of received physical (non-micronized) mixture was ready for further experiments; the other was micronized in an agate mortar and pestle with some drops of methanol for 10 min.

Method

The principle of the method is a relationship between the signal value of IBU (enthalpy change ΔH) and its concentration in the matrix. For this purpose both of micronized and non-micronized sets of mixtures were measured by means of DSC. In this way two calibration curves with an increasing amounts of IBU were plotted. Appropriate calibration curves were further applied to calculate the final experimental contents of IBU (ODSC) in the tablets and were compared with those declared (D) by the manufacturer using equation A(%) = [(ODSC-D)/D]◊100.
**Thermal analysis**

The DSC measurements were performed in nitrogen atmosphere with a flow rate of 50 mL/min using EXSTAR DSC 7020 apparatus (SII Nano-Technology Inc.) calibrated with indium and tin, and equipped with DSC7020 electric cooling unit.

The samples of micronized and non-micronized mixtures of about 5.0 mg were accurately weighed in aluminum pans and sealed. The pans were equilibrated at 30°C for 15 min and thereafter the melting behavior was analyzed at heating rate of 10°C/min. All measurements were performed at least three times and averaged. The tablets were examined at least six times.

**Validation of DSC method**

The method was validated for specificity, linearity, precision, limit of detection, and limit of quantification as well (13). The calculations were made using statistical program STATISTICA v.10.

**Specificity**

Specificity of the method was assessed by comparing the DSC heating traces of raw IBU, raw excipients and obtained both micronized and non-micronized mixtures. The \( T_{onset} \), \( T_{max} \) temperatures, and presence of new chemical individuals were taken into account.

**Linearity**

The calibration plots were constructed by analysis of seven (n = 7) different mixtures (both micronized and non-micronized), corresponding to content of IBU ranging from 0.50 mg to 3.00 mg. Determination of linearity was made via three replicates and assessed as

---

### Table 1. Effects of mixture composition on the area of melting peak \( ΔH \) (averaged from three determinations). The differences between \( ΔH \) of IBU of non micronized and micronized mixtures (B) as a function of IBU concentration in the sample shows linear dependence: \( y = 41.27 - 19.98 \cdot x \), \( r = 0.9976 \), \( r^2 = 0.9952 \).

<table>
<thead>
<tr>
<th>Composition of the mixture</th>
<th>Content of IBU in the sample [mg]</th>
<th>( ΔH ) of IBU* [mJ/mg]</th>
<th>B** [mJ/mg]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Micronized mixtures</td>
<td>Non micronized mixtures</td>
</tr>
<tr>
<td>IBU/starch</td>
<td>0.50</td>
<td>53.38</td>
<td>83.00</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>130.50</td>
<td>153.00</td>
</tr>
<tr>
<td></td>
<td>1.50</td>
<td>208.50</td>
<td>219.75</td>
</tr>
<tr>
<td></td>
<td>1.75</td>
<td>241.5</td>
<td>249.08</td>
</tr>
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<td></td>
<td>2.00</td>
<td>293.33</td>
<td>295.50</td>
</tr>
<tr>
<td></td>
<td>2.50</td>
<td>370.00</td>
<td>360.00</td>
</tr>
<tr>
<td></td>
<td>3.00</td>
<td>435.00</td>
<td>416.00</td>
</tr>
</tbody>
</table>

\*\( ΔH \) was recalculated to the content of IBU in the sample; **differences between \( ΔH \) of non micronized and micronized mixtures

---

### Table 2. Data validation of DSC method.

<table>
<thead>
<tr>
<th>Validation parameters</th>
<th>Micronized mixtures</th>
<th>Non-micronized mixtures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specificity</td>
<td>Specific</td>
<td>Specific</td>
</tr>
<tr>
<td>Slope (a ± S_a)</td>
<td>154.94 ± 2.74</td>
<td>134.96 ± 2.68</td>
</tr>
<tr>
<td>Intercept (b ± S_b)</td>
<td>-23.68 ± 5.27</td>
<td>17.59 ± 5.15</td>
</tr>
<tr>
<td>r</td>
<td>0.9992</td>
<td>0.9990</td>
</tr>
<tr>
<td>( r^2 )</td>
<td>0.9984</td>
<td>0.9980</td>
</tr>
<tr>
<td>LOD [mg]</td>
<td>0.12</td>
<td>0.14</td>
</tr>
<tr>
<td>LOQ [mg]</td>
<td>0.37</td>
<td>0.41</td>
</tr>
<tr>
<td>Linearity [mg]</td>
<td>0.37 – 3.00</td>
<td>0.41 – 4.41</td>
</tr>
<tr>
<td>Precision (n = 6)</td>
<td>%RSD</td>
<td>1.95</td>
</tr>
</tbody>
</table>

Regression equation \( y = ax + b \); \( S_a \) - standard deviation of slope; \( S_b \) - standard deviation of intercept.
a relationship between the area of DSC melting peak $\Delta H$ and content of IBU in mg per sample.

Linearity was reported as the linear calibration equations ($y = ax + b$) and the correlation coefficients $r$ and $r^2$.

Limits of detection (LOD) and quantification (LOQ)

LOD and LOQ were calculated from the calibration curve slope ($a$) and the slope standard estimation error ($S_e$), using formulas: $LOD = 3.3 \times S_e/a$ and $LOQ = 10 \times S_e/a$.

Precision

The repeatability of the method was determined by analysis of six ($n = 6$) replicates of samples from individual weighing. The study was done for one concentration level of 2.00 mg of IBU in the sample, and the results were expressed as the relative standard deviation (%RSD).

RESULTS AND DISCUSSION

The determined enthalpy changes $\Delta H$ of mixtures with increasing IBU contents (0.5 mg to 3.00 mg per sample giving the concentration from 10 to 60%) and starch, both micronized and non-micronized, are presented in Table 1.

One can see the differences between $\Delta H$ values. Within the range from 0.50 mg to 2.00 mg of IBU per sample (10 to 40%) the $\Delta H$ values of micronized mixtures were distinctly lower than corresponding non-micronized mixtures. For contents of IBU over 2.00 mg per sample (40%) the relationship was reversed and the micronized mixtures enthalpies were distinctly higher. Interestingly, the $\Delta H$ differences (labeled as $B$ in Table 1) and concentration of IBU are linearly correlated with strong regression coefficient $r = 0.9976$ and $r^2 = 0.9952$ ($y = 41.27 - 19.98 \times X$). At this point we can’t comment this phenomenon.

In order to plot appropriate calibration curves, the obtained $\Delta H$ enthalpies of micronized as well as non-micronized mixtures as a function of the increasing weights of IBU in a constant sample weight (5.0 mg) were used. The validation data were summarized in Table 2. It thus demonstrated that developed method meets the acceptance criteria in the scope of specificity, sensitivity, linearity and precision.

Table 3 shows the melting onset and maximum temperatures ($T_{\text{onset}}, T_{\text{max}}$) of ibuprofen in tablets under study. With two exceptions (Metafen and Nurofen Ultima), the temperatures are comparable to pure raw IBU and ranging from 75.9°C to 76.2°C and 76.0°C to 76.2°C for $T_{\text{onset}}$ and $T_{\text{max}}$, respectively. Metafen and Nurofen Ultima have significantly lower temperatures: 72.9°C, 76.8°C and 70.9°C, 75.4°C. It is interesting that they are both composed of IBU in the presence of paracetamol (PAR) and the concentrations of IBU per tablet are definitely lower than 29% and 23% while for the others are ranging from 34.77% to 53.43%.

The calculated calibration curves described above were applied to quantitative determinations of IBU. The final experimental contents $O_{\text{DSC}} \pm SD$ and $A(\%)$ are presented in Table 4.

For clarity of observation, all respective drugs under study were gathered in Table 5, to show which excipients could interfere with IBU. Components of particular importance were marked in the gray fields.

The content of IBU in presence of pseudoephedrine hydrochloride, in tablets (Acatar Zatoki and Ibuprom Zatoki) composed from starch, cellulose, croscarmellose sodium, colloidal silica, Guar gum, talc, crospovidone and hydrogenated vegetable oil were very close to those declared by the manufacturer. It suggests that there are no interactions between drug components and if they are, they

<table>
<thead>
<tr>
<th>Analgesic</th>
<th>$T_{\text{onset}}$ [°C]</th>
<th>$T_{\text{max}}$ [°C]</th>
<th>$\Delta H$ [mJ/mg]</th>
<th>% IBU/tablet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metafen</td>
<td>72.9</td>
<td>76.8</td>
<td>94.98</td>
<td>28.60</td>
</tr>
<tr>
<td>Nurofen Ultima</td>
<td>70.9</td>
<td>75.4</td>
<td>98.02</td>
<td>22.98</td>
</tr>
<tr>
<td>Acatar Zatoki</td>
<td>76.2</td>
<td>79.0</td>
<td>146.25</td>
<td>37.73</td>
</tr>
<tr>
<td>Ibuprom Zatoki</td>
<td>76.1</td>
<td>79.1</td>
<td>139.25</td>
<td>36.78</td>
</tr>
<tr>
<td>Modafen</td>
<td>75.9</td>
<td>79.2</td>
<td>130.50</td>
<td>34.77</td>
</tr>
<tr>
<td>Nurofen Zatoki</td>
<td>76.0</td>
<td>79.0</td>
<td>118.75</td>
<td>53.43</td>
</tr>
<tr>
<td>Pure Ibuprofen</td>
<td>76.1</td>
<td>79.0</td>
<td>130.00</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 5. The components of drugs under study. The presence of the ingredient in the drug was marked with "◊"; respective excipients that could interfere with IBU are in the gray fields.

<table>
<thead>
<tr>
<th>Drug name</th>
<th>Type of excipient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Metafen</td>
<td>x</td>
</tr>
<tr>
<td>Nurofen Ultima</td>
<td></td>
</tr>
<tr>
<td>Acatar Z.</td>
<td></td>
</tr>
<tr>
<td>Ibuprom Z.</td>
<td></td>
</tr>
<tr>
<td>Modafen</td>
<td></td>
</tr>
<tr>
<td>Nurofen Zatoki</td>
<td></td>
</tr>
</tbody>
</table>

1-povidone (PVP); 2-starch; 3-cellulose; 4-magnesium stearate; 5-sodium lauryl sulfate; 6-calcium phosphate; 7-calcium stearate; 8-Guar gum; 9-talc; 10-croscarmellose sodium; 11-guar gum; 12-sodium lauryl sulfate; 13-calcium phosphate; 14-lactose.

don’t affect the final result. In other tablets consisting of pseudoephedrine hydrochloride the effect of stearates and PVP (Nurofen Zatoki, Modafen), calcium phosphate (Nurofen Zatoki), surfactants (sodium lauryl sulfate) as well as lactose (Modafen) were leading to moderate interactions. The strongest interactions can be observed for tablets composed from IBU in the presence of PAR where magnesium stearate, stearates and PVP were found.

**CONCLUSIONS**

The substances found in tablets often interact between themselves. The manufacturers generally provide an information on the type of excipients used during their production, however, usually only for main components and in most cases, there are no data concerning their concentrations. For that reason, it is difficult to estimate the influence of solid phase interactions on quantitative measurements. However, some general remarks are possible. The calculated statistical parameters of DSC method show that with several limitations it is possible to quantify API in poly pharmaceutical tablets. Under conditions that were used in the research, it was found that some excipients such as stearates, povidone, sodium lauryl sulfate as well as lactose could modify the measurement results in various ways. On
the other hand, the second active ingredient (both PAR and pseudoephedrine hydrochloride) doesn’t seem to interfere. It should be emphasized that the described mutual solid state interactions can be observed during DSC measurements, while they do not change the quality of drug, they can sometimes alter the drug pharmacodynamics.

Acknowledgments

Authors would like to dedicate this article to Professor Jan Krzek, our always remembered advisor, mentor and teacher.

REFERENCES


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Terpenes are components of natural essential oils. These are organic compounds built of carbon, hydrogen and oxygen in an isoprene arrangement (C₅H₈). There are numerous classes, groups and subgroups of terpenes that can be distinguished and their division depends on the number of carbon atoms. They are broadly distributed in nature and have been used in medicine and cosmetics for a long time. Numerous preparations containing single terpenes or complexes of essential oils are applied on the skin in their pure form or in the form of ointments, gels, oily solutions, etc. The aim of this study was to visualize changes in the stratum corneum by terpinen-4-ol, racemic camphor, eucalyptol, racemic menthol and levomenthol incorporated in various concentrations into lipophilic, ethanolic or hydrophilic vehicle. Additionally, an irritation in vitro test was performed to determine the influence of individual formulations and pure vehicles on viability of skin cells.

**Experimental**

Terpenes formulations  
All chemicals were of Ph. Eur. or cosmetic grade. Terpenes formulations were made by dissolving terpenes in ethanol (40º), grapeseed oil or carbomer gel (Table 1). In the last case, some amounts
of ethanol 96° were used to dissolve the terpene before incorporation into the vehicle. No ethanol solutions of 5% menthol and 5% levomenthol and no hydrogel formulations of levomenthol were prepared either due to the poor solubility of terpene and their re-crystallization. Finished preparations were stored at 8°C and protected from light.

**Ex vivo observations by fluorescent microscopy**

The studies were approved by the local Ethics Committee. Human skin was collected from cadavers in the area of the chest and abdomen. It was then cleaned from any remains of fat and subcutaneous tissue and divided into smaller fragments. The tissue was wrapped in aluminium foil and frozen at -40°C. Before the start of tests, the skin was thawed at room temperature. Its surface was cleaned by 2-times sticking and removing fragments of adhesive tape.

To separate the epidermis with the stratum corneum from the dermis, a thawed and cleaned fragment of skin was immersed in a beaker with purified water at 65°C for 45 s. After separating the layers and drying the epidermis surface delicately using tissue paper, the tissue was placed on a glass microscope slide. A small amount (approx. 0.5-1.0 µL) of ethanol or oily solution, or a thin layer of hydrogel, was applied on the surface of isolated stratum corneum. Skin with applied substrate not containing terpenes was the control. The sample on a microscope slide was placed on a Petri dish in which tissue saturated with 0.9% sodium chloride solution had been placed to ensure a constant moisture level. The closed Petri dish was placed in an incubator and incubated at 37°C for 30 min. After this period of time, the dish with the sample was taken out and the excess of the preparation was delicately removed from the surface and dried using tissue paper. A fluorescent stain solution (sodium fluorescein – NaFl, rhodamine B hexyl ester – RBHE or sulforhodamine B – SRB) was applied where the preparation had been. The microscope plate was replaced in the Petri dish and incubated again at 37°C for 15 min, protecting it from light. Next, the sample surface was dried delicately using tissue paper and observed under a fluorescent microscope.

The samples were observed under an epi-fluorescent microscope (Nikon eclipse e-50i) with a mercury lamp, using appropriate filters and wavelengths for stains used in the test and under objectives of 10x, 40x and 100x. The main color of the obtained images depended on the kind of the applied stain and an appropriate filter in the fluorescent microscope. The green color was observed for NaFl, orange for derivatives of rhodamine, i.e. SRB and RBHE.

Given the equipment of the microscope in an automatic drive of the z axis, a series of photographs were taken at various depths of the sample, changing the position of the table by 0.5–1.0 µm on average. Following that, a subsequent series of photographs were put together to create 3D image. The image was edited and recorded using Nis Elements AR 3.2. software. Also, 3D images and graphs were constructed so as to present the intensity of fluorescence recorded for individual tests.

**In vitro observations by atomic force microscopy (AFM) and scanning electron microscopy (SEM)**

After a 30-minute application of formulations containing terpenes or control on forearm or abdominal region of adults, the remains of the preparation were removed using tissue paper. Next, the tape striping procedure was conducted, that is, fragments of adhesive tapes were applied on the skin surface and pressed at a constant pressure of 1 kg (10 N) for 10 s (3, 4). In this way, corneocytes were collected from further, deeper layers of the skin and directly observed under an AFM or SEM microscope. The microscopic observation involved accurate scanning of subsequent corneocyte samples. The images obtained reflected the surface area, interaction forces and the topography of the tested sample.

<table>
<thead>
<tr>
<th>Terpene</th>
<th>Concentrations in ethanol 40°C (w/w)</th>
<th>Concentrations in oil (w/w)</th>
<th>Concentrations in hydrogel (w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Terpinen-4-ol</td>
<td>0.5%, 1%, 5%</td>
<td>0.5%, 1%, 5%</td>
<td>0.5%, 1%, 5%</td>
</tr>
<tr>
<td>Camphor</td>
<td>0.5%, 1%, 5%</td>
<td>0.5%, 1%, 5%</td>
<td>0.5%, 1%, 5%</td>
</tr>
<tr>
<td>Menthol</td>
<td>0.5%, 1%</td>
<td>0.5%, 1%, 5%</td>
<td>0.5%, 1%, 5%</td>
</tr>
<tr>
<td>Levomenthol</td>
<td>0.5%, 1%</td>
<td>0.5%, 1%, 5%</td>
<td>-</td>
</tr>
<tr>
<td>Eucalyptol</td>
<td>0.5%, 1%, 5%</td>
<td>0.5%, 1%, 5%</td>
<td>0.5%, 1%, 5%</td>
</tr>
</tbody>
</table>
Conditions of AFM and SEM observations were presented earlier (5).

Irritation test

The visualization analyses were supplemented by the determination of the degree of skin irritation by tested preparations, expressed by the percentage of survivability of the epidermis model cells EpiDerm®-200. The test was performed according to the procedure “ECVAM: In vitro skin irritation test: Human skin model”.

The tested formulations were applied on the insert surface for 60 min. At the same time, a positive application of 5% SDS solution (i.e., sodium lauryl sulfate in water) and a negative (application of purified water) control sample has been done. After this time, inserts were washed a few times to remove the tested preparation, and placed in a fresh substrate. After 24 h of incubation, the medium was subjected to analysis for interleukin IL-1α content, and the tissue was incubated for another 18 h to perform an MTT test for cytotoxicity of the tested substance. The survivability of cells was defined for each insert as the percentage of the average survivability as compared to the negative control. The tested sample is defined as irritating if cell survivability is lower than 50%.

RESULTS AND DISCUSSION

Fluorescent microscopy (ex vivo studies)

Images of stratum corneum and single corneoocytes selected from among several hundred pictures and the intensity of fluorescence before and after the application of tested preparations are presented in Figure 1.

In the tests, grapeseed oil, ethanol 40° or hydrogel were used as vehicles. First, vehicles were applied on appropriately prepared skin which was stained with prepared fluorophores after incubation. The aim of this was to visualize the influence of vehicles themselves on stratum corneum.

It was found that ethanol 40° destroys the stratum corneum structure. This observation confirmed previous reports on the destructive effect of ethanol on stratum corneum cells, involving lipid extraction from intercellular spaces. At the same time, it was observed that the application of pure grapeseed oil and hydrogel did not have a significant influence on the stratum corneum structure.

In numerous tests, it was proven that the factor determining the intensity of the action of xenobiotics on the skin is, amongst other things, the type of the vehicle used (6, 7). As shown in the tests, the vehicle itself can have a destructive influence on the stratum corneum, while a combination of the vehicle with the xenobiotic interfering with the stratum corneum structure may cause even greater damage to the stratum corneum. The obtained in this study photographs revealed various degrees of destruction to the skin barrier, i.e., the stratum corneum, under the influence of terpenes depending on the substrate in which it was incorporated.

First, oil and ethanol solutions, as well as hydrogels with terpinen-4-oil at concentrations of 0.5%, 1% and 5%, were applied. The photographs taken indicate the destructive influence of this terpene on stratum corneum cells. It can be claimed that the degree of disturbing the ordered stratum corneum structure is the greatest when this compound was applied in ethanol while the application of oily solution and hydrogel at the same concentration did not induce such drastic changes.

Another terpene applied on the skin was (+)-camphor. Preparations were fixed with the content of 0.5, 1 and 5% of terpene in the same three vehicles. Various types of staining of stratum corneum cells were observed after prior application of 1% camphor preparations in various vehicles. Stratum corneum stained with RBHE, which is a lipophilic stain, therefore, if the stratum corneum is undamaged it has affinity to intercellular lipids, as a result it was stained in a uniform manner. It is probably caused by the destructive action of camphor in combination with ethanol on the corneoocyte shell.

In the case of staining the skin with NaFl, previously subjected to the action of racemic menthol solutions at the same concentration (0.5%) but in various vehicles, various fluorescence intensities were observed. It can be assumed that this is also caused by the influence of the vehicle on the stratum corneum structure. The least intense fluorescence was seen for the oil vehicle. This indicates the weakest influence of this vehicle on the menthol action intensity on the stratum corneum structure. Hydrogel has an indirect effect on the menthol action intensity.

Levomenthol was applied onto the skin in oil solution at concentrations of 0.5, 1, 5% and in the ethanol solution at concentrations of 0.5 and 1%. Due to the low solubility it was not possible to prepare ethanol solution at a concentration of 5%. While analysing photographs taken after the application of 1% ethanol solution of levomenthol, stronger staining of corneoocytes with SRB than in the case of skin subjected to the action of oil solution of this terpene was found. More intense fluorescence could have been caused by additional destruc-
Control samples

RBHE-stained spaces between corneocytes after the application of ethanol 40° (scale 10 µm).

Fluorescence intensity. RBHE-stained sample after ethanol 40° application.

RBHE-stained spaces between corneocytes after the application of oil (scale 10 µm).

Tested samples

RBHE-stained corneocytes and intercellular spaces after the application of 5% (+)-camphor in ethanol (scale 10 µm).

Fluorescence intensity. RBHE-stained sample after application of 5% (+)-camphor in ethanol.

RBHE-stained corneocytes and intercellular spaces after the application of 5% levomenthol oily solution (scale 10 µm).

Fluorescence intensity. RBHE-stained sample after the application of 5% levomenthol oily solution.
The influence of terpenes on human *stratum corneum* by fluorescence...

Figure 1. Fluorescent images of the *stratum corneum*.
tive influence of ethanol on the *stratum corneum* as described in the above cases.

Another tested terpene was eucalyptol. While comparing effects of this terpene in various vehicles, again, the basic difference between the photographs taken was the intensity of fluorescence. Allegedly, this was caused by greater absorption of the stain into corneocytes. Based on the photographs taken, it can be concluded that the ethanol solution contributes the most to the destruction of the *stratum corneum* struc-

![Figure 2. Results of irritation test](image2)

![Figure 3. Typical AFM imaging of the stratum corneum and data set](image3)
The influence of terpenes on human *stratum corneum* by fluorescence... 1069

ture causing greater penetration of the stain into corneocytes. Oil solution, on the other hand, has the weakest effect on the *stratum corneum* structure.

While comparing changes in the *stratum corneum* structure induced by terpene preparations at various concentrations, applied in the same vehicle, it can be unambiguously concluded that the terpene concentration has a positive influence on the induced negative effect on *stratum corneum*.

**Irritation test (in vitro tests)**

Tests were performed using the epidermis model EpiDerm. Preparations with the strongest destructive effect on the *stratum corneum ex vivo* and pure vehicles were used in the test. The results of the test, expressed in the form of percentage of survivability of the cells, are presented in Figure 2. 5% ethanol solutions of (+)-camphor and eucalyptol and hydrogel with 5% of eucalyptol can be classified as irritating (cell survivability < 50%).

**Atomic force microscopy (in vivo tests)**

AFM imaging was performed by collecting the *stratum corneum* after the application of terpene-containing and control preparations. The obtained sample images of corneocytes and the sets of data are presented in Figure 3. For all preparations tested, no significant and repeatable changes were observed in the topography and in the roughness of cells, and friction forces between the “blade” of the microscope and the cell structure.

**Scanning electron microscopy (in vivo tests)**

SEM imaging was performed by collecting the *stratum corneum* after the application of terpene-containing and control preparations. Sample images are presented in Figure 4. Only slight changes in *stratum corneum/corneocyte* morphology were observed, which were manifested, for example, by edge curling.

**CONCLUSIONS**

- Terpenes in ethanol solutions have stronger destructive properties on the *stratum corneum ex vivo* than terpenes dissolved in oil.
- Among tested compounds, (+)-camphor and eucalyptol exhibit the strongest action destroying the *stratum corneum* structure *ex vivo*, in particular, when ethanol is the vehicle.
- 5% ethanol solutions of eucalyptol and camphor and hydrogel with 5% eucalyptol had the
strongest irritating effect on stratum corneum cells in the irritation test in vitro.

- In the in vivo tests (AFM, SEM), no negative effect of terpenes on the stratum corneum and its cells was confirmed.
- For terpenes, the stratum corneum layer ex vivo and its in vitro equivalents may not be analyzed as models equivalent to in vivo tests.

REFERENCES


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Electron paramagnetic resonance (EPR) spectroscopy was used to examine free radical formation during thermal sterilization of pharmaceutical samples (1-8). The amounts of free radicals in drugs should be minimized, because of their high activity (9, 10). Free radicals participate in skin-ageing processes (11, 12). The aim of this work was to find thermal condition of storage of the two strong antibiotic ointments applied on skin in dermatology. The conditions resulted without free radicals contents in the fusidic acid and neomycin samples, were searched. Magnetic dipolar (13, 14) and spin-lattice interactions (13-15) in the samples storage at different temperatures were compared.

EXPERIMENTAL

Characterization of the antibiotic samples

Two tested dermatological ointments consisted of the active components - fusidic acid \((C_{31}H_{48}O_6)\) (16) or neomycin \((C_{23}H_{46}N_6O_{13})\) (16), and the non-active basis component as Vaselinum album (16, 17). The strong antibiotics: fusidic acid and neomycin are used in skin infections (18-20).

The natural antibiotic - fusidic acid acts against Gram-negative bacteria, \(\beta\)-hemolytic streptococci, enterococci \(Staphylococcus aureus\), \(Corynebacterium\) and \(Mycobacterium leprae\) (18-20). The aminoglycoside antibiotic – neomycin acts against Gram-negative bacteria, partially against Gram-positive bacteria, \(Enterobacter cloacae\), \(Escherichia coli\) and \(Proteus vulgaris\) (18-20).

Thermal treatment of the ointments

The ointments were stored at different temperatures. The samples were located in refrigerator at low temperature of 4°C. Next samples were stored at room temperature (23°C). The samples stored at room temperature, were next heated at temperatures: 35, 38, 40, 45 and 50°C, during 2 h. Heating of the...
ointments was performed in the professional thermal sterilizer with circulation of hot air, produced by Memmert GmbH + Co. KG (Germany).

For EPR examination, the ointment samples were put into thin walled glass tubes of high paramagnetic purity with the external diameter of 1 mm. The masses of the samples in tubes were determined by Sartorius CPA balance (Germany).

**EPR measurements and spectral analysis**

EPR spectra of the ointments were measured at room temperature. An X-band (9.3 GHz) electron paramagnetic resonance spectrometer of Radiopan (Poznań, Poland) with magnetic modulation of 100 kHz and the total microwave power of 70 mW was used. EPR spectra as the numerical data were collected by the Rapid Scan Unit of Jagmar (Kraków, Poland). The measuring time of the individual EPR line was 1 s. Microwave frequency and magnetic induction were obtained by the MCM101 recorder and the NMR magnetometer of EPRAD (Poznań, Poland), respectively. The influence of microwave power in the range from 2.2 mW (attenuation of 15 dB) to 70 mW (attenuation of 0 dB) was measured. The microwave power was obtained from the formula: attenuation [dB] = 10 lg M/M₀, where M was the measuring microwave power, and M₀ was the total microwave power (70 mW).

The parameters of the first-derivative EPR spectra: amplitudes (A), integral intensities (I), linewidths (ΔBpp), and g-factors, were analyzed. g-Factor was calculated from the electron paramagnetic resonance condition as (17, 18): $g = h\nu/\mu_B B_r$, where: $h$ – Planck constant, $\nu$ – microwave frequency, $\mu_B$ – Bohr magneton, $B_r$ – induction of resonance magnetic field.

Free radical concentrations (N) in the samples were determined as the values proportional to integral intensities (I) of the EPR spectra measured with the low microwave power of 2.2 mW. The spectra of the tested samples were compared with the EPR spectrum of the stable paramagnetic reference – ultramarine. The second reference – the ruby crystal permanently placed in the resonance cavity was also used. Free radical concentrations (N) were obtained according to the formula (19, 20): $N = N_u [(W_u A_u)/I_u]/[I/(W_A m)]$, where: $N_u$ - the number of paramagnetic centers in the reference - ultramarine;

![Figure 1. EPR spectra of the ointment containing fusidic acid stored at (a) 38°C and (b) 50°C. The measurements were performed with low microwave power of 2.2 mW. B is the magnetic induction](image-url)
W, W<sub>u</sub> - the receiver gains for the tested antibiotics and the ultramarine; A, A<sub>u</sub> - the amplitudes of the ruby signal for the examined samples and the reference - ultramarine; I, I<sub>u</sub> - the integral intensities for the samples and ultramarine, m - the mass of the samples in the tubes. The integral intensities (I) of the EPR lines were calculated by double integration of the first derivative curves.

For measurement of the EPR spectra, analysis and data presentation the professional spectroscopic programs of Jagmar (Kraków, Poland), LabVIEW 8.5 of National Instruments Firm (Texas, USA), Origin (USA) and Microsoft Excel (USA) programs, were used.

RESULTS AND DISCUSSION

Free radicals were not found in the tested antibiotic ointments stored both at low temperature 4°C and at room temperature (23°C). The ointments with both fusidic acid and neomycin did not generated EPR signal via microwave absorption. So, the storage of the studied dermatological antibacterial drugs in refrigerator at temperature 4°C or at room temperature (23°C), may be recommended.

Free radicals were formed in these ointments at all the tested elevated temperatures: 35, 38, 40, 45 and 50°C. Their EPR spectra were observed even at low microwave power (2.2 mW). The exemplary EPR spectra of the ointment containing fusidic acid stored at temperatures: 38 and 50°C, are shown in Figure 1a-b. The exemplary EPR spectra of the ointment contains neomycin stored at the same temperatures, are shown in Figure 2a-b. g-Factors near 2 characteristic for free radicals were obtained for these lines.

Parameters of the EPR spectra depended on storage temperature. Amplitudes (A), integral intensities (I), and linewidths (ΔB<sub>pp</sub>) of the samples with fusidic acid and neomycin stored at elevated temperatures, were compared in Tables 1 and 2, respectively. The EPR spectra of the examined antibiotics
were broad lines. The linewidths ($\Delta B_{pp}$) of the EPR spectra of the ointment containing fusidic acid were in the range 0.46-0.67 mT (Table 1). The linewidths ($\Delta B_{pp}$) of the EPR spectra of the ointment containing neomycin were in the range 0.64-1.05 mT (Table 2). The narrower and the broader EPR lines were observed for fusidic acid and neomycin samples stored at 35 and 38°C, respectively. Dipolar interactions between free radicals were responsible for line broadening (13). Distances between unpaired electrons and molecular structure of the samples effected dipolar interaction (13).

Free radical concentrations ($N$) in the ointments containing fusidic acid and neomycin for the samples storage at elevated temperatures are presented in Figures 3a-b, respectively. Storage temperature strongly influenced free radical concentrations ($N$) in the antibiotic samples. Free radical concentrations ($N$) was the highest for ointments with fusidic acid (Fig. 3a) and neomycin (Fig. 3b) stored at 50°C. The relatively lower thermal free radical production characterized fusidic acid (Fig. 3a) and neomycin (Fig. 3b) samples stored at 38 and 35°C, respectively. EPR investigations pointed out that both ointments with fusidic acid and neomycin as the active components should not be stored or applied on skin at elevated temperatures: 35, 38, 40, 45 and 50°C, because of free radical contents (Fig. 3a-b).

Free radicals detected by EPR measurements in the examined antibacterial ointments were the final product of thermal treatment of both the active substance and the basis. Our earlier studies of Vaselinum album (2) showed the existence of free radicals in the heated basis samples. In the present studies, the active substance and the basis in the ointments were in contact during heating. The chemical bonds were probably thermally ruptured in

![Figure 3. The effect of storage temperature on the free radical concentrations ($N$) in the ointments containing: (a) fusidic acid and (b) neomycin](image-url)
the whole volume of the ointments, not only in fusidic acid and neomycin, but also in \textit{Vaselinum album}. It is highly probable that free radicals of fusidic acid and neomycin interact with free radicals of \textit{Vaselinum album}. These complex interactions may increase the free radical concentration in the ointments via chemical reactions of free radicals. The pairing of unpaired electrons of free radicals in the heated active substances and in the basis may decrease the free radical concentrations in the ointments. The results presented in Figure 3 concerned free radical concentration the total ointment samples. The obtained results are important, because of the contact of both the active substance and \textit{Vaselinum album} with the skin.

The specific spectroscopic correlations reflecting magnetic interactions in the ointment samples were proposed to examine the thermal stability of their chemical structures. Magnetic interactions affected spin-lattice relaxation processes in the samples and line broadening of their EPR spectra (14, 15). Microwave energy absorbed by unpaired electrons of free radicals returns during spin-lattice relaxation process to molecules forming structure of the sample. The time of spin-lattice relaxation processes remains unchanged for non thermally modified structure of the sample. Information about the spin-lattice relaxation was obtained from the correlation between amplitude (A) of the EPR line of the samples and microwave power (M/Mo). The fast spin-lattice relaxation processes reveal characteristic unsaturated EPR lines (14, 15). Amplitudes (A) of the unsaturated EPR lines increase with increasing of microwave power. For the slow spin-lattice relaxation processes the saturation effect appears for the low values of microwave power and it is accompanied by the characteristic decrease of amplitude (A) (14, 15). It was checked, whether the correlations between amplitudes (A) and microwave power (M/Mo) changed or not. The magnetic interactions are also responsible for the changes of the linewidth ($\Delta B_{pp}$) with microwave power (M/Mo) (14, 15). For homogeneous broadened EPR lines linewidth ($\Delta B_{pp}$) increases with increasing of microwave power (M/Mo). Linewidth ($\Delta B_{pp}$) of non-homogeneous broadened EPR lines does not change with microwave power (M/Mo). For thermally stable samples, the character of line broadening (homogeneous or nonhomogeneous) does not change. The detailed results about stability of magnetic interactions in the tested ointment samples are presented in Figures 4-7a-b.

The influence of microwave power on amplitudes (A) of the ointment containing fusidic acid and

<table>
<thead>
<tr>
<th>T [°C]</th>
<th>A [a. u.] [± 0.01 a. u.]</th>
<th>I [a. u.] [± 0.02 a. u.]</th>
<th>$\Delta B_{pp}$ [mT] [± 0.02 mT]</th>
</tr>
</thead>
<tbody>
<tr>
<td>35</td>
<td>0.1</td>
<td>0.70</td>
<td>0.46</td>
</tr>
<tr>
<td>38</td>
<td>0.14</td>
<td>0.74</td>
<td>0.46</td>
</tr>
<tr>
<td>40</td>
<td>0.14</td>
<td>0.75</td>
<td>0.51</td>
</tr>
<tr>
<td>45</td>
<td>0.21</td>
<td>2.52</td>
<td>0.65</td>
</tr>
<tr>
<td>50</td>
<td>0.22</td>
<td>3.30</td>
<td>0.67</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>T [°C]</th>
<th>A [a. u.] [± 0.01 a. u.]</th>
<th>I [a. u.] [± 0.02 a. u.]</th>
<th>$\Delta B_{pp}$ [mT] [± 0.02 mT]</th>
</tr>
</thead>
<tbody>
<tr>
<td>35</td>
<td>0.21</td>
<td>2.45</td>
<td>0.64</td>
</tr>
<tr>
<td>38</td>
<td>0.23</td>
<td>3.49</td>
<td>0.84</td>
</tr>
<tr>
<td>40</td>
<td>0.27</td>
<td>3.67</td>
<td>0.91</td>
</tr>
<tr>
<td>45</td>
<td>0.21</td>
<td>3.69</td>
<td>1.05</td>
</tr>
<tr>
<td>50</td>
<td>0.22</td>
<td>3.86</td>
<td>0.70</td>
</tr>
</tbody>
</table>

Table 1. The effect of storage temperature (T) on the parameters: amplitudes (A), integral intensities (I), and linewidths ($\Delta B_{pp}$), of the EPR spectra of the ointment containing fusidic acid. Data for the measurements with microwave power of 2.2 mW.
Figure 4. The changes of the amplitudes (A) of the ointment containing fusidic acid with microwave power (M/M₀) for the samples storage at: (a) 35°C and (b) 50°C. M – microwave power used during the measurement and M₀ – the total microwave power emitted by klystron (70 mW).

Figure 5. The changes of the amplitudes (A) of the ointment containing neomycin with microwave power (M/M₀) for the samples storage at (a) 38°C and (b) 50°C. M – microwave power used during the measurement and M₀ – the total microwave power emitted by klystron (70 mW).

Figure 6. The changes of the linewidths (ΔBpp) of the ointment containing fusidic acid with microwave power (M/M₀) for the samples storage at (a) 38°C and (b) 50°C. M – microwave power used during the measurement and M₀ – the total microwave power emitted by klystron (70 mW).

Figure 7. The changes of the linewidths (ΔBpp) of the ointment containing neomycin with microwave power (M/M₀) for the samples storage at (a) 38°C and (b) 50°C. M – microwave power used during the measurement and M₀ – the total microwave power emitted by klystron (70 mW).
neomycin for different storage temperatures is shown in Figures 4a-b and 5a-b, respectively. The amplitudes (A) of all the measured EPR lines increased with microwave power. It was characteristic for fast spin-lattice relaxation processes in the studied samples. Storage temperature did not effect relaxation processes in the examined antibacterial ointments.

The influence of microwave power on linewidths ($\Delta B_{pp}$) of the ointment containing fusidic acid and neomycin stored at temperatures: (a) 35, (b) 38, (c) 40, (d) 45 and (e) 50°C are shown in Figures 6a-b and 7a-b, respectively. The linewidths ($\Delta B_{pp}$) of all the EPR spectra increased with microwave power. Such correlation between linewidths ($\Delta B_{pp}$) and microwave power was characteristic for homogeneously broadened EPR lines (13).

The EPR spectroscopic analysis performed in this experimental work may be useful to chose the best temperatures for storage of the dermatological samples. The ointment contained fusidic acid and neomycin should be protect against temperatures which produce free radicals in their structures. Such elevated temperatures were found by measuring EPR spectra. The storage samples should not absorb microwaves in magnetic field. Electron paramagnetic resonance spectroscopy is recommended to optimize storage conditions of antibiotic ointments.

**CONCLUSION**

Spectroscopic analysis of microwave absorption at X-band (9.3 GHz) was the useful method to find thermal conditions of storage the dermatological antibiotic ointments containing fusidic acid and neomycin. Free radicals were not produced at low temperature 4°C during storage of these samples in refrigerator and at room temperature (23°C). The higher temperatures: 35-50°C resulted in thermal formation of free radicals ($\sim 10^9$ spin/g) in the examined ointments. The highest free radical concentration characterized the sample with fusidic acid and neomycin stored at 50°C. The lowest free radical concentration existed in the sample with fusidic acid stored at 35-40°C and the sample with neomycin storage at 35°C. Properties of free radicals in the antibiotics were not changed with storage temperature. All the measured EPR lines were homogeneously broadened. Fast spin-lattice relaxation processes existed in all the ointment samples independent on storage temperature. Electron paramagnetic resonance spectroscopy may be used to optimize thermal condition of storage of dermatological substances.

**Acknowledgments**

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**Conflict of interests**

The authors declare that there is no conflict of interests regarding the publication of this paper. They have no conflict of interests or no financial gains in mentioning the company names or trademarks.

**REFERENCES**


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Proper functioning of a human body requires that a balance is kept between the amount of energy provided to the body in the form of nutritional elements and the amount of energy which is used up. If surplus of energy exceeds the needs for a longer period of time, it begins to be stored in the form of fat tissue. Thus, long-term energy surplus contributes to the development of obesity.

In the last few years, the view on the significance of fat tissue has evolved (1, 2). The fat tissue has been recognized as an active endocrine organ, producing numerous protein substances, such as adiponectin, leptin or A-FABPs (3, 4). The presence of fat tissue of such hormones as leptin or ghrelin is of special importance in the context of obesity pathology, metabolic syndrome or type 2 diabetes.

It is also believed that the A-FABPs proteins play an important role in the pathogenesis of metabolic diseases. An interrelation between increased A-FABP concentration in the pericardial fat tissue and the heart dysfunction in obese patients has been observed. An increase of that protein has been also observed in patients with type 2 diabetes (5, 6).

Fructose contained in diet may present an adverse effect on lipid metabolism in both animals and people (7-10). An increased level of that mono-
saccharide may influence in the liver an acceleration of the synthesis of triglycerides (TG) coming from de novo lipogenesis (11-13). Carbohydrate and lipid metabolism is connected by the Randle cycle. The carbohydrate – lipid Randle cycle is controlled by insulin and glucagon, hormones regulating the concentration of glucose and fat acids in blood (14, 15).

Both an increased amount of carbohydrates as well as branched amino acids in diet stimulate the release of insulin from β cells of Langerhans islets to plasma of the blood. In those conditions intensified processes of glycolysis, glycogenogenesis as well as lipogenesis take place. According to Randle et al. (14), a mitochondrial PDH complex is the main enzyme for the control of the strength of carbohydrate and lipid metabolism.

Obese persons more often suffer from the carbohydrate metabolism disorders. The risk of diabetes increases with the increase of obesity. In this case insulin resistance, and to be more precise, peripheral insulin resistance is of strong significance. A few mechanisms participate in its creation, and first of all intensified lipolysis in fat tissue, forming fat deposits in insulin-sensitive tissues, activity of humoral factors produced by adipocytes, increased dimensions of adipocytes and effects of PPAR receptor stimulation (16).

Relation between the body fat and oxidative stress connected with the amount of reactive oxygen species (ROS) in adipocytes was also demonstrated (7). They are probably ROS that influence the development of inflammation and insulin resistance. To effectively protect tissue cells from oxidative stress, caused by adverse life style and environmental pollution, it would be commendable to introduce in everyday diet such supplements, which will constitute the most effective and safest source of antioxidants. Such supplements may be fruits and vegetables containing compounds decreasing the amount of ROS generated in a living organism (17-19).

Examples of such substances are flavonoids, commonly present in many plants. They significantly decrease the amount of ROS and therefore, they should be an element of daily diet. For most people, consuming each day the amount of fruit or vegetables sufficient to provide the body with 1 g of flavonoids is impossible. Therefore, supplementing the diet with preparations containing flavonoids is necessary (20). Consequently, there is a need to find such diet supplements and such forms, in which they can constitute a valuable addition.

Cornus mas (Cornelian cherry) represents a rich source of phenolic antioxidants. It was suggested that Cornelian cherry has very high antiradical activity based on studies of extracts from this plant. Cornelian cherry (Cornus mas L.) is one of the most popular plants of the Cornaceae family. Chemical studies showed that cornelian cherry contains a variety of amino acids, organic acids, polysaccharides and saponins, iridoids (21, 22).

Aronia (Aronia melanocarpa), which belongs to the rose family (Rosaceae), is one of the plants exhibiting considerable antioxidant potential (23-26). Aronia has been used as a dietary supplement in cases of cancer and as anti-inflammatory or antitumor drug. A positive impact of Aronia on animals with experimentally produced diabetes has also been tested. During the study, normalization of body weight and of biochemical parameters of diabetes (including a decrease of glucose level in blood and urine), along with reduction of thirst and amount of urine passed has been observed (27). Compounds present in Aronia juice and cornelian cherry fruits are a rich source of anthocyanins and polyphenolic substances, all reducing the amount of free radicals.

In this study, was examined the effect of addition of freeze-dried fruit of cornelian cherry or Aronia juice to basal diet; diet enriched in carbohydrates/ carbohydrates diet and enriched with fats/ fat diet.

Materials and methods

The experiments were performed in accordance with legal requirements, under a license granted by the Local Commission of Ethics in Kraków number 80/2009 17.09.2009. The experiment was conducted on the 3-month-old male Wistar rats, weighing 250 ± 15 g and caged in the temperature of 23°C, humidity 50-60%, and light dark cycle (12/12 h). Each group consisted of 6 animals.

Animals were fed a diet consisting of a base mix and starch, and with or without the addition of the tested substances. The base mix contained casein (20%), oil (5%), calcium carbonate (2.8%), calcium monohydrogen phosphate (2.9%), lecithin (1%), sodium chloride (0.3%) and minerals and vitamins mix (1%).

The animals in the control group (group CN) were fed the base mix (38%) with the addition of corn starch (62%). The FN group was fed glucose (30%) and corn starch (32%) apart from base mix (38%). The animals in the AN group were fed high-fat diet which consisted of base mix (38%), lard (31%) and corn starch (31%). The animals from the CD, FD and AD groups received on a daily basis an addition of the Cornus mas fruit lyophilisate in the form of powder in the amount equal to 10% of the daily base mix for the CN, FN and AN groups.
Fruits of this plant came from an experimental orchard of Agricultural University located in Garlica Murowana. From these fruits, stones were mechanically removed and the obtained part pressed to pulp. The processed material was lyophilized in the LIOGAM factory specializing in freeze drying of fruits and vegetables.

The animals in the CJ, FJ and AJ groups, on the other hand, received Aronia juice mixed with water to drink (mixed with water in a volume ratio of 3 : 1 (v/v)). The juice was produced by the Eko-Ar Company (it was 100% cold-pressed Aronia juice). Moreover, all animals had free access to feed and water.

After 5 weeks, they were euthanized by intraperitoneal injection of sodium thiopental 50 mg/kg. Blood of the animals was collected, from which plasma and fat tissue were extracted. The blood was centrifuged during 15 min (3000 r/min) and frozen until the analysis. Fat tissue was minced in 0.15 M phosphate buffer, pH = 7.4 to 10% final concentration using a basic ultraspeed tissue grinder, the Ultra Turrax T25 homogenizer (12000 r/min bursts). All procedures were performed on ice. Homogenized tissues were centrifuged at 3000 ◊ g for 15 min (0-4°C). The resulting supernatant was drawled and the pellet was discarded. The obtained samples of homogenate were frozen in -80°C until the time of the analysis.

Reagents
All chemicals, solvents, and standards of reagents used in experiments were produced by Sigma-Aldrich. Double-distilled deionized water (Milli-Q, Millipore 18.2 MW/cm 25°C) was used in all experiments.

Biochemical analysis
Biochemical analysis was made with the standard biochemical analyzer Alize B 3.0 with standard kits [total cholesterol (TCHOL), triacylglycerol (TG), uric acid (UA), urea and glucose] from Biomérieux, and it was controlled with Control Serum 1, ODC0003 and Control Serum 2, ODC0004 (OLYM.PUS). All the reagents were of analytical grade and were obtained from Sigma Aldrich Chemical Company (Steinheim, Germany).

Determination of total antioxidative activity with the FRAP method
The fat tissue homogenate was marked the FRAP method (Ferric Reducing – Antioxidant Power) described by Benzie and Strain (28). The absorbance in the tested samples was measured with the use of JASCO V-530 spectrophotometer at λ = 593 nm and the FRAP values in 30 min were calculated based on the standard curve.

Determination of glutathion concentration (GSH)
Marking the concentration of glutathion was performer according to Ellman method (29). The method uses the reduction of DTNB by thiole complexes (e.g., glutathione).

Determination of catalase activity (CAT)
The activity of catalase was determined according to the Aebi method (30), with the use of the JASCO V-530 spectrophotometer. The absorbance was measured for 3 min, at λ = 240 nm at 25°C. From the standard curve the value of CAT activity was calculated in commutation to protein [U/mg of protein]. One unit of CAT activity was defined as the amount of enzyme decomposing 1 µM of H2O2 per min.

Determination of superoxide dismutase (SOD) activity
The activity of superoxide dismutase was marked according to Flohe and Otting method (31). In this method the source of the superoxide anion is the reaction of xanthine oxidase, and the indicator reaction is the reduction of cytochrome C, the absorbance of which was recorded at 550 nm wavelength. Measurements were done in cuvettes to which the following solution was added: 950 µL of buffer for marking SOD containing: 50 µM of xanthine, 20 µM of cytochrome C, 50 mM of potassium-phosphate buffer pH 7.8 and 0.1 mM EDTANa3, and 30 µL of fat tissue homogenate. The measurement started by adding 20 µL of the xanthine oxidase solution and conducted in the temperature of 30°C. The SOD activity was marked based on the linear reading of changes of absorbance between the 15th s and the second min and 15th s at 550 nm wavelength, and it was expressed in the specific activity units (U), per 1 mg of protein (U/mg protein).

Statistics
Statistical analysis was conducted with STATISTICA PL v.7.1 (StatSoft, Tulsa, USA). The significance of statistical differences for biochemical parameters in different animal groups was checked on the basis of the non-parametric test ANOVA rank by Kruskal-Wallis. The value of p < 0.05 was adopted as a critical significance level. Calculations for the Spearman correlation were also made with the use of the same software.
RESULTS AND DISCUSSION

Dietary fructose consumption is one of the environmental factors contributing to the development of obesity (32-34). Treatment of obesity is currently based mainly on introducing physical activity and modifying the diet, and thus, non-pharmacological treatment. It is, therefore, important to determine and analyze the effect of dietary modifications on the fat tissue function, its biochemical parameters and on body mass reduction.

Obesity is now among frequently discussed topics, especially in the developed countries, where it poses the biggest problem. Namely, it entails a range of complications, among others an increased risk of metabolic and circulatory system diseases. The dynamics of progression of obesity and carbohydrate metabolism use disorder has caused a growing interest in the fat tissue function and the activity of antioxidative parameters present in this tissue (35, 36).

These days, in the era of obesity, in fact only a few publications are our source of knowledge on the activity of enzymes and hormones in adipocytes, on antioxidative potential or changes in fat tissues as a result of various types of diets. This situation is caused primarily by difficulties in isolating and marking enzymes, and by low concentration of

Table 1. Use of feed and energetic balance in the tested animal groups.

<table>
<thead>
<tr>
<th>Animal group</th>
<th>Average use of feed [g/5 weeks]</th>
<th>Caloric value of feed [kcal/g feed]</th>
<th>Average body mass growth [g/animal]</th>
<th>Energetic balance [kcal/g mass]</th>
</tr>
</thead>
<tbody>
<tr>
<td>CN</td>
<td>657.7</td>
<td>3.827</td>
<td>119.3 ± 18.5</td>
<td>21.09</td>
</tr>
<tr>
<td>CD</td>
<td>642.3</td>
<td>3.727</td>
<td>118.2 ± 13.1</td>
<td>20.25</td>
</tr>
<tr>
<td>CJ</td>
<td>593.8</td>
<td>3.727</td>
<td>113.8 ± 20.2</td>
<td>19.96</td>
</tr>
<tr>
<td>FN</td>
<td>624.69</td>
<td>3.827</td>
<td>128.5 ± 9.4</td>
<td>18.60</td>
</tr>
<tr>
<td>FD</td>
<td>652.32</td>
<td>3.727</td>
<td>108.5 ± 19.9</td>
<td>22.41</td>
</tr>
<tr>
<td>FJ</td>
<td>667.75</td>
<td>3.727</td>
<td>116.2 ± 36.7</td>
<td>21.99</td>
</tr>
<tr>
<td>AN</td>
<td>471.55</td>
<td>5.387</td>
<td>129.0 ± 21.2</td>
<td>19.69</td>
</tr>
<tr>
<td>AD</td>
<td>489.12</td>
<td>5.387</td>
<td>140.4 ± 28.8</td>
<td>18.42</td>
</tr>
<tr>
<td>AJ</td>
<td>416.28</td>
<td>5.387</td>
<td>93.2 ± 7.2</td>
<td>24.06</td>
</tr>
</tbody>
</table>

(CN) - control; (FN) - fructose; (AN) - high-fat; (CD) - cornelian cherry with control; (CJ) - chokeberry juice with control (FD) - cornelian cherry with fructose; (FJ) - chokeberry juice with fructose; (AD) - cornelian cherry with high-fat; (AJ) - chokeberry juice with high-fat.

Table 2. Activity of oxidative stress markers (FRAP, CAT, SOD, GSH) marked in fat tissue homogenates in Wistar rats.

<table>
<thead>
<tr>
<th>X1-diet</th>
<th>X2-supplement</th>
<th>FRAP [mM/mg protein]</th>
<th>CAT [U/mg protein]</th>
<th>GSH [nM/mg protein]</th>
<th>SOD [U/mg protein]</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>N</td>
<td>0.53 ± 0.18a</td>
<td>0.354 ± 0.127aa</td>
<td>0.058 ± 0.026a</td>
<td>4.97 ± 1.83abc</td>
</tr>
<tr>
<td>C</td>
<td>D</td>
<td>0.72 ± 0.1a</td>
<td>0.252 ± 0.128a</td>
<td>0.288 ± 0.263abc</td>
<td>2.26 ± 0.59ab</td>
</tr>
<tr>
<td>C</td>
<td>J</td>
<td>1.07 ± 0.52aa</td>
<td>0.181 ± 0.047a</td>
<td>0.172 ± 0.073a</td>
<td>2.11 ± 0.62a</td>
</tr>
<tr>
<td>F</td>
<td>N</td>
<td>0.68 ± 0.32a</td>
<td>0.557 ± 0.241a</td>
<td>0.063 ± 0.028a</td>
<td>2.7 ± 1.39aa</td>
</tr>
<tr>
<td>F</td>
<td>D</td>
<td>1.17 ± 0.50aa</td>
<td>0.34 ± 0.198aa</td>
<td>0.55 ± 0.374ac</td>
<td>5.74 ± 2.68ac</td>
</tr>
<tr>
<td>F</td>
<td>J</td>
<td>1.94 ± 0.89b</td>
<td>0.176 ± 0.085b</td>
<td>0.569 ± 0.208c</td>
<td>2.55 ± 1.24ab</td>
</tr>
<tr>
<td>A</td>
<td>N</td>
<td>0.97 ± 0.29a</td>
<td>0.243 ± 0.098a</td>
<td>0.279 ± 0.093abc</td>
<td>8.68 ± 2.77c</td>
</tr>
<tr>
<td>A</td>
<td>D</td>
<td>1.01 ± 0.46a</td>
<td>0.241 ± 0.037a</td>
<td>0.211 ± 0.053ab</td>
<td>7.38 ± 4.41c</td>
</tr>
<tr>
<td>A</td>
<td>J</td>
<td>0.47 ± 0.15c</td>
<td>0.256 ± 0.151c</td>
<td>0.116 ± 0.038c</td>
<td>7.01 ± 3.03bc</td>
</tr>
</tbody>
</table>

(CN) - control; (FN) - fructose; (AN) - high-fat; (CD) - cornelian cherry with control; (CJ) - chokeberry juice with control (FD) - cornelian cherry with fructose; (FJ) - chokeberry juice with fructose; (AD) - cornelian cherry with high-fat; (AJ) - chokeberry juice with high-fat. Data are presented as the means from independent measurements ± standard deviation (SD). Different letters in the same columns indicate significant differences according to Tukey’s test (p < 0.05).
those complexes in fat tissue. Many authors have described effects of diet on body mass. Kuroshima (37) found out that there was a lack of statistically significant changes in the body mass growth of rats fed with a diet with the addition of 32% sucrose solution in comparison to the control diet.

Based on the results obtained for our research model, combination of high-fat diet with Cornus mas fruit proved adverse, as the significant body mass increase occurred in the tested group. Supplementing that diet with Aronia juice caused the lowest body mass increase. Combination of Cornus mas or Aronia with the fructose diet also resulted in a significant decrease of the body mass growth in the tested animals.

Average use of feed per animal during the 5-weeks experiment as well as calorific value of the applied diet is presented in Table 1. Average body mass increase and the amount of calories needed for an increase of one gram of body mass depending on the applied diet is also shown. The lowest use of feed and the lowest body mass increase was observed in the animal group with high-fat diet and Aronia juice (AJ group). At the same time the animals in that group received the feed with the highest calorific value. In the FD group we observed

![Figure 1. Antioxidant parameters in fat tissue of rats in different types of diets (control, fructose, high-fat) without or with fruits of cornelian cherry or chokeberry juice (N – without supplement; D – cornelian cherry; J – chokeberry juice)
increased use of feed in comparison to the FN group but a decreased body mass growth, which may suggest the influence of Cornus mas on food absorption. In the rats fed with the fructose diet (FN) a higher body mass growth was observed in comparison to the control group.

Obesity and body mass growth cause increased generation of free radicals in fat tissue (38). This in turn causes an increase of the total antioxidative activity, marked as FRAP. The presence of polyphenols and anthocyanins is directly related to the antioxidative properties. It is possible that polyphenols are responsible for the FRAP value increase in the diets with the addition of Cornus mas or Aronia (24, 25, 39).

It was observed in the conducted research model that the high-fat diet increased the FRAP (Table 2, Fig. 1) value in a statistically significant way in comparison the control diet. Cornus mas – introduced to the feed only the fructose diet (FD group) caused a statistically significant increase of the FRAP value in comparison to the fructose diet. Additionally, it was observed that Aronia juice combined with the high-fat diet statistically significantly decreased the FRAP value in comparison with the pure high-fat diet.

Based on the conducted experiment one can draw a conclusion that both Cornus mas and Aronia served with an increased dose of fructose increase the antioxidant capacity of fat tissue. However, the combination of Aronia juice with the high-fat diet had an adverse effect on the value of that parameter, causing its statistically significant decrease.

The capacity of the complexes present in Cornus mas to level RFT was also demonstrated by the Ersoy et al. (39) research results. They compared 12 species of Cornus mas with regard to the antioxidative capacities of the methanol extract from fruits.

The first line of defense is preventing damages of biological particles by preventing the generation of the most reactive among RFT (e.g., radical hydroxyl). A special role in removing dangerous free radicals is played by enzymatic mechanisms (the activity of SOD and CAT), as well as by some non-enzymatic ones (GSH or UA).

Glutathione (GSH) plays the role of intracellular redox buffer of big capacity and decreases the pool of reactive electrophile compouds. It constitutes the main element of defense against antioxidative stress. The concentration of intracellular glutathione is particular for a given type of cells and it ranges from 5 to 10 mM (40). It is not constant and may undergo a significant decrease, among others in the organ cells of aging organisms as a result of oxidation to GSSG, increased degradation and decreased productions (41, 42).

The level of reduced GSH (Tab. 2, Fig. 1) in the high-fatty group (AN) was statistically significantly higher than in the control group (CN) or fructose group (FN). Adding Cornus mas and Aronia to the food rich in fructose caused a statistically significant increase of the GSH value in comparison to the F group. Cornus mas added to the control diet in comparison to the control diet itself influenced the statistically significant increase of glutathione.

The decrease of GSH concentration in the liver cells may be the result of oxidative stress induction. The decreased GSH value may be the cause of changes in the functioning of cell membranes due to the peroxidation of membrane lipids. In the conducted experiment an addition of Cornus or Aronia to the fructose diet was observed to cause a significant increase of the GSH level in the fat tissue.

Galinier showed that fat tissue of obese rats contained a higher concentration of hydrophilic antioxidants, such as glutathione or vitamin C (43, 44). The increase of glutathione concentration as a result of the diet is low and short-term, but still it may have enormous role in the detoxication of free radicals and in the defense of cells against oxidative stress. The addition of Cornus fruits to the control diet significantly increased the amount of glutathione in the fat tissue. Combining Cornus or Aronia with the fructose diet also caused a statistically significant increase of glutathione concentration in the tested tissue.

The enzyme catalase (CAT) plays a role in the protection of fat tissues from the toxic effects of \( \text{H}_2\text{O}_2 \) and partially reduced oxygen species. Catalase, iron-containing enzyme (oxidoreductase) which catalases the breakdown of \( \text{H}_2\text{O}_2 \) is a potentially destructive agent in cells. In the animal group with the feed enriched with fructose, the highest activity of CAT (Table 2, Fig. 1) was marked, it was statistically significantly higher than in the CN group and AN group. It was observed that the addition of Cornus mas or Aronia juice to the FN diet caused a statistically significant decrease of the CAT activity in comparison to the F group.

Galinier (43) based on the conducted research determined that obesity has no influence on the Mn-SOD and Cu/Zn-SOD activity but in the obese rats they recorded an increase of the catalase activity. In fat tissue marked the activity of basic antioxidative enzymes in fat, namely of CAT, glutathione peroxidase (GPx) and SOD activity (44). In obese rats there were significant linkages between obesity and
oxidative stress, and thus, also body defense mechanism, which are demonstrated by increased production and activity of antioxidants (43). In the conducted experiment, the SOD activity (Table 2, Fig. 1) was observed to be the highest and statistically significant in the animal group with high-fat diet in relation to the CN group and FN group, in which the lowest SOD activity value was observed. Adding *Cornus mas* to the fructose feed caused a statistically significant increase of the SOD activity in comparison to the FN group.

Adding *Cornus mas* or *Aronia* to the control feed in the animals in the CD group and the CJ group decreased the SOD activity in a statistically significant way in comparison to the CN group. In the fructose diet *Cornus mas* increased the SOD activity while *Aronia* (FJ group) did not influence the activity of the tested enzyme in comparison to the FN group.

The conducted experiment shows that the CAT and SOD enzyme activity in the fat tissue homogenate depended on the type of addition introduced to the base feed. Fructose caused increased CAT activity with a simultaneous decrease of SOD activity in comparison to the control group. Fat, on the other hand, worked in an opposite way; namely,
it decreased CAT activity while significantly increasing SOD activity. Adding *Cornus mas* or *Aronia* to the fructose diet caused a significant decrease of CAT activity. In the high-fat diet no influence of *Cornus mas* or *Aronia* on CAT activity was observed. As for the control diet, SOD activity decreased as a result of adding *Cornus* or *Aronia*.

Fructose is metabolized only in the liver where it enters the Embden–Meyerhof–Parnas (EMP) pathway after phosphorylation with the participation of fructokinase enzyme. Liver catabolism of that monosaccharide to fat acids includes the conversion of pyruvate synthetized in glycolyse pathway to acetyl-CoA with the participation of pyruvate dehydrogenase complex (PDH). A few mechanisms take part in its generation, and first of all intensified lipolyse in fat tissue, cumulating/fat deposits in the tissue dependent to insulin, the activity of humoral factors produced by adipocytes, increased size of adipocytes and the effects of PPAR receptor stimulation (16). Additionally, circulating levels of A-FABP are closely correlated with several key features of the metabolic syndrome, including adverse lipid profiles (increased serum triglyceride and LDL-cholesterol, and decreased HDL-cholesterol), insulin resistance, hyperglycemia and hypertension, independent of sex, age, and obesity. There is a significant increasing trend in A-FABP levels with the increasing number of components of the metabolic syndrome (45). As for biochemical analysis conducted in the obtained plasma, the level of lipids such as TCHOL, HDL and TG was marked (Table 3, Fig. 2). It was observed that the duration of the experiment did not cause significant changes in the cholesterol concentration in the FN and AN groups in comparison to the CN group. Besides, neither the tested additions of *Cornus mas* fruit nor *Aronia* juice had a statistically significant effect on the tested parameter value. The animal group with the high-fat diet with *Aronia* juice had a statistically significantly increased level of TCHOL in comparison to the CJ group or FJ group.

In the group of animals with high-fat diet with *Aronia* addition, there was a statistically significant increase of the HDL level in comparison to the AN group. Also *Aronia* introduced to the control feed had a similar effect on the HDL level in comparison to the CN group.

Busserolles (13) demonstrated that diet rich in fructose causes hypertriglycerydemia. The fructose content in the food used in the above mentioned research was similar (34%) to the model which was used in our experiment (30% of fructose). However, probably due to the duration of the experiment, no influence of the applied diets was observed on the cholesterol concentration in the blood plasma.

At the same time, changes in TG and HDL concentration were demonstrated. The level of TG marked in the FN group was statistically significantly higher than in the CN group and the AN group. The addition of *Cornus mas* decreased the level of TG in a statistically significant way only in the high-fat diet in comparison to the AN group. The TG value in the AD group was the lowest statistically significant only in comparison to the CD and FD groups. In the CD group a statistically significant TG increase was observed in comparison to the CN group. Adding *Aronia* to the fructose diet and high-

<table>
<thead>
<tr>
<th>X1-</th>
<th>X2-supplement</th>
<th>TCHOL [mM/L]</th>
<th>HDL [mg/dL]</th>
<th>TG [mM/L]</th>
</tr>
</thead>
<tbody>
<tr>
<td>CN</td>
<td>N</td>
<td>1.76 ± 0.04a</td>
<td>33.11 ± 6.63a</td>
<td>0.634 ± 0.196a</td>
</tr>
<tr>
<td>C</td>
<td>D</td>
<td>1.7 ± 0.17a</td>
<td>32.23 ± 0.87a</td>
<td>1.513 ± 0.27b</td>
</tr>
<tr>
<td>C</td>
<td>J</td>
<td>1.67 ± 0.17a</td>
<td>40.38 ± 6.28a</td>
<td>0.895 ± 0.206a</td>
</tr>
<tr>
<td>F</td>
<td>N</td>
<td>1.9 ± 0.31a</td>
<td>37.46 ± 5.93a</td>
<td>1.752 ± 0.235a</td>
</tr>
<tr>
<td>F</td>
<td>D</td>
<td>2.07 ± 0.43a</td>
<td>34.62 ± 7.61a</td>
<td>1.816 ± 0.342a</td>
</tr>
<tr>
<td>F</td>
<td>J</td>
<td>1.69 ± 0.26a</td>
<td>37.43 ± 5.11a</td>
<td>1.147 ± 0.175b</td>
</tr>
<tr>
<td>A</td>
<td>N</td>
<td>1.99 ± 0.48a</td>
<td>26.73 ± 8.46a</td>
<td>1.283 ± 0.544c</td>
</tr>
<tr>
<td>A</td>
<td>D</td>
<td>1.73 ± 0.08a</td>
<td>31.42 ± 1.62a</td>
<td>0.733 ± 0.272a</td>
</tr>
<tr>
<td>A</td>
<td>J</td>
<td>2.2 ± 0.33a</td>
<td>43.27 ± 11.05a</td>
<td>0.781 ± 0.121a</td>
</tr>
</tbody>
</table>

(CN) – control; (FN) – fructose; (AN) – high-fat; (CD) – cornelian cherry with control; (CJ) – chokeberry juice with control (FD) – cornelian cherry with fructose; (FJ) – chokeberry juice with fructose; (AD) – cornelian cherry with high-fat; (AJ) – chokeberry juice with high-fat. Data are presented as the means from independent measurements ± standard deviation (SD). Different letters in the same columns indicate significant differences according to Tukey’s test (p < 0.05).
A high-fat diet resulted in a decrease of the TG level in comparison to the groups FN and AN.

In the high-fat diet, *Aronia* juice (AJ group) significantly influenced the decrease of TG concentration and at the same time caused an increase of HDL concentration. Combining *Aronia* juice with the control diet (CJ group) caused a significant increase of the TG level. In the high-fat diet an opposite effect was observed: *Cornus mas* caused a significant TG decrease.

Obesity, hypertriglyceridemia, hypertension and insulin resistance especially strongly increase the production and retention of uric acid (UA) (46). Hayden et al. (46) demonstrated that hyperglycemia and decreased concentration of HDL fraction cholesterol also show a positive correlation with the UA concentration. Additionally, the works of Hikita (47) draw attention to the correlation between UA concentration and fat acids distribution. It was also demonstrated that UA concentration is influenced by leptin — a hormone produced by adipocytes. It is believed that it may be one of the factors conditioning the occurrence of hyperuricemia connected with adipose (48).

In the blood serum, the levels of UA, urea and glucose were also marked. Uric acid (Table 4, Fig. 1) generated in the blood serum of the animals with the fructose diet had a statistically significantly higher level than in the CN group and the AN group. In the CD group a statistically significant increase of UA was observed in comparison to the CN group. Adding *Cornus mas* to the other diets (FN and AN) did not cause statistically significant changes in the UA level. Introducing *Aronia* juice in the feeding of animals with the high-fat diet caused a statistically significant increase of the UA concentration in comparison to the AN group.

It was observed in the conducted research that an addition of aronia juice to the high-fat diet (AJ group) caused a significant increase of UA concentration. *Cornus* introduced to the control diet also caused a significant increase of that parameter in the CD group. Increased uric acid concentration is commonly observed in the persons with the so-called, lifestyle diseases: obesity, hypertension, diabetes, blood lipids disorders, arteriosclerosis.

The level of urea marked in the tested animal groups was influenced by *Aronia* juice (Table 4, Fig. 2). In the blood serum the highest level was marked in the CJ group and it was statistically significant in comparison to the CN group. In the AJ group a statistically significant decrease of the urea level in comparison to the AN group was observed. *Aronia* juice introduced to the control (CJ) and fructose diets caused a statistically significant increase of the measured parameter in comparison to the CN and FN diets.

The level of glucose (Table 4, Fig. 2) in the blood serum of the FN animal group was statistically significantly higher than in the CN groups. In the FD group there was a statistically significant decrease of the glucose value in comparison to the FN group. In the animals with the control feed with

<table>
<thead>
<tr>
<th>X1- diet</th>
<th>X2- supplement</th>
<th>UA [µM/L]</th>
<th>Urea [mM/L]</th>
<th>Glucose [mM/L]</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>N</td>
<td>130 ± 64&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.11 ± 2.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.27 ± 0.86&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>C</td>
<td>J</td>
<td>184 ± 74&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.95 ± 2.58&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.82 ± 0.79&lt;sup&gt;abc&lt;/sup&gt;</td>
</tr>
<tr>
<td>F</td>
<td>N</td>
<td>270 ± 43&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>8.46 ± 1.72&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.83 ± 1.17&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>F</td>
<td>D</td>
<td>276 ± 73&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>9.03 ± 1.26&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.91 ± 1.24&lt;sup&gt;ac&lt;/sup&gt;</td>
</tr>
<tr>
<td>F</td>
<td>J</td>
<td>296 ± 75&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>10.72 ± 1.52&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.29 ± 0.85&lt;sup&gt;ac&lt;/sup&gt;</td>
</tr>
<tr>
<td>A</td>
<td>N</td>
<td>117 ± 30&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.06 ± 1.82&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.85 ± 0.26&lt;sup&gt;ac&lt;/sup&gt;</td>
</tr>
<tr>
<td>A</td>
<td>D</td>
<td>116 ± 39&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.16 ± 0.28&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.4 ± 0.7&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>A</td>
<td>J</td>
<td>394 ± 124&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.9 ± 2.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.81 ± 0.96&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

(CN) – control; (FN) – fructose; (AN) – high-fat; (CD) – cornelian cherry with control; (CJ) – *chokeberry* juice with control (FD) – cornelian cherry with fructose; (FJ) – *chokeberry* juice with fructose; (AD) – cornelian cherry with high-fat; (AJ) – *chokeberry* juice with high-fat. Data are presented as the means from independent measurements ± standard deviation (SD). Different letters in the same columns indicate significant differences according to Tukey’s test (p < 0.05).
the addition of *Aronia* a statistically significant increase of the glucose concentration in comparison to the CN group was observed.

Dyslipidemia (including decreased concentration of HDL fraction cholesterol and increased plasma triglycerides levels) and uric acid metabolism disorders are the disorders characteristic of the insulin resistance syndrome (49). Introducing such supplements as *Cornus* fruit or aronia juice to everyday diet may have influence on the proper level of antioxidative balance in tissues.

**CONCLUSION**

The disruption of antioxidative balance of the body system is an important factor in development of many diseases, including type 2 diabetes or metabolic syndrome. *Cornus mas* and aronia juice contains many substances with antioxidiant properties. Fructose and fat diet encourages faster weight gain relative to the control diet.

**Conflict of interests**

The authors declare that there is no conflict of interests regarding the publication of this paper.

**Acknowledgments**

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Substituted isoxazolidines are best formed in 1,3-dipolar cycloadditions of nitrones to alkenes and a stereochemical outcome of these reactions has been widely studied. Consequently, numerous structurally diversified isoxazolidine-containing compounds were synthesized (1–3). Since various substituents can be incorporated into the isoxazolidine skeleton, including pharmacologically important fragments, a search for biologically active isoxazolidines is justified. Among the most active isoxazolidines, compounds having anticancer (4–12), antiviral (13–15), antibacterial (16), antifungal (17) and anti-inflammatory (18) properties have been identified. Various isoxazolidine analogues of nucleosides (19) and nucleotides (19) were also obtained and several examples of compounds exhibiting antiviral (15, 20) and/or anticancer (11, 12, 21) activities have been reported. Moreover, isoxazolidine-containing intercalators were synthesized based on the idea of incorporation of polyaromatic units such as pyrene, anthracene, phenanthrene (5, 22) or amonafide moieties (23) as substituents of the isoxazolidine ring. On the other hand, hybrids of isoxazolidines with carbapenem exhibited inhibitory activity toward *E. coli* better than that of imipenem used as a reference drug (24).

Similarly, a series of chromanyl-containing isoxazolidines displayed significant inhibitory activity against *Salmonella typhimurium* (MIC = 1.6 µg/mL) and against methicillin-resistant *Staphylococcus aureus* (MIC = 3.1 µg/mL) (25). Numerous phenolic compounds were designed and synthesized since antibacterial properties of phenols have been recognized many decades ago (26, 27). Among various conjugates of phenols, alkoxybenzenes and substituted aryls, bacteriostatic isoxazolidines (28–31) and isoxazolines (32–39) decorated with functionalized aromatic residues have also been obtained (Fig. 1). Having in mind our achievements in the synthesis of biologically-active 3-phosphonylated isoxazolidines (12, 40, 41) we designed a new series of compounds of a general structure 3 (Scheme 1) with the intention to study their antiviral, cytostatic and antibacterial properties.

**SYNTHESIS AND BIOLOGICAL ACTIVITY OF NOVEL PHENOL-CONJUGATES OF ISOXAZOLIDINES**

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**Abstract:** 1,3-Dipolar cycloadditions of the phosphonylnitrone with selected allylbenzenes produced mixtures of diastereoisomeric (3-diethoxyphosphoryl)isoxazolidines with good trans/cis diastereoselectivities (d.e. 80%) and good to excellent overall yields. No inhibitory activity against a broad panel of DNA and RNA viruses was detected for (3-diethoxyphosphoryl)isoxazolidines at 250 µM. Isoxazolidines trans: cis (95 : 5) slightly reduced human embryonic lung (HEL) cell viability (CC₅₀ = 45 µM). Four out of ten isoxazolidines inhibited the growth of *Staphylococcus epidermidis* ATCC 12228 (up to 40% for the most active compound). They were also inhibitory against *Staphylococcus aureus* ATCC 6538 although inhibition did not exceeded 25%. None of the isoxazolidines affected *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 15442 growth.

**Keywords:** phosphonates, isoxazolidines, 1,3-dipolar cycloaddition, anticancer activity, antibacterial activity

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EXPERIMENTAL

General

$^1$H, $^{13}$C and $^{31}$P NMR spectra were taken in CDCl₃ with TMS as internal standard on the Varian Mercury-300 machine at 300, 75.5 and 121 MHz, respectively. IR spectra were measured on an Infinity MI-60 FT-IR spectrometer. Melting points were determined on a Boetius apparatus and are uncorrected. Elemental analyses were performed by the Microanalytical Laboratory of this Faculty on Perkin-Elmer PE 2400 CHNS analyzer. The following adsorbents were used: column chromatography, Merck silica gel 60 (70–230 mesh); analytical TLC, Merck TLC plastic sheets silica gel 60 F₂₅₄.

General procedure for the synthesis of isoxazolidines trans-3 and cis-3

A solution of the nitrone 1 (1.0 mM) and allylbenzene 2a or its derivative 2b–2k (1.1 mM) in toluene (2 mL) was stirred at 65°C until the disappearance (TLC) of the starting nitrone. All volatiles were removed in vacuo and the crude products were subjected to chromatography on silica gel columns with a chloroform/methanol (100:1) mixture as eluent.

Diethyl (5-benzyl-2-methylisoxazolidin-3-yl)phosphonate (trans-3a)

Colorless oil. IR (KBr): ν [cm⁻¹]: 2981, 1454, 1442, 1239, 1054, 1024, 702. $^1$H NMR (CDCl₃, 300 MHz) δ [ppm]: 7.35–7.19 (m, 5H), 4.24 (dddd, $J = 7.8$ Hz, $J = 7.2$ Hz, $J = 6.6$ Hz, $J = 5.7$ Hz, 1H, HC₅), 4.23–4.09 (m, 4H, 2 ◊ POCH₂CH₃), 3.01 (dd, $J = 13.8$ Hz, $J = 5.7$ Hz, 1H, HaC₁), 2.90 (dd, $J = 10.2$ Hz, $J = 6.6$ Hz, $J = 1.5$ Hz, 1H, HC₃), 2.87 (d, $J = 1.2$ Hz, 3H), 2.77 (dd, $J = 13.8$ Hz, $J = 6.6$ Hz, 1H, HbC₁), 2.48 (ddd, $J = 19.2$ Hz, $J = 12.3$ Hz, $J = 7.2$ Hz, $J = 6.6$ Hz, 1H, HbC₄), 2.20 (ddd, $J = 12.3$ Hz, $J = 10.2$ Hz, $J = 7.8$ Hz, 1H, HbC₄),

Figure 1. Examples of bacteriostatic isoxazolidines and isoxazolines...
Diethyl (2-methyl-5-(3-methylbenzyl)isoxazolidin-3-yl)phosphonate (trans-3b)

Colorless oil. IR (KBr):  v [cm⁻¹]: 3475, 2981, 2910, 1609, 1487, 1442, 1295, 1163, 1054, 1024, 966 (signals of trans-3b were extracted from the spectra of a 95 : 5 mixture of trans-3 and cis-3b); ¹H NMR (CDCl₃, 300 MHz) δ [ppm]: 7.20–7.10 (m, 1H), 7.05–6.90 (m, 3H), 4.30–4.05 (m, 5H, H₅ and 2×POCH₂CH₃), 2.95 (ddd, J = 13.8 Hz, J = 6.0 Hz, 1H, HaC₁⁻), 2.92–2.80 (m, 1H, H₃C), 2.85 (d, J = 1.1 Hz, 3H), 2.72 (dd, J = 13.8 Hz, J = 7.2 Hz, 1H, HbC₁⁻), 2.45 (dddd, J = 18.3 Hz, J = 12.6 Hz, J = 7.2 Hz, J = 7.2 Hz, 1H, HaC₄), 2.32 (s, 3H), 2.18 (dddd, J = 12.6 Hz, J = 12.3 Hz, J = 10.2 Hz, J = 8.1 Hz, 1H, HbC₄), 1.31 (t, J = 6.9 Hz, 3H, POCH₂CH₃), 1.30 (t, J = 6.9 Hz, 3H, POCH₂CH₃), ¹³C NMR (CDCl₃, 75.5 MHz) δ [ppm]: 138.02, 137.35, 130.02, 128.36, 127.33, 126.25, 77.72 (d, J = 7.5 Hz, C₅), 64.29 (d, JPC = 168.7 Hz, C₃), 63.16 (d, J = 6.5 Hz, C-O-P), 62.52 (d, J = 6.9 Hz, C-O-P), 46.39 (d, J = 5.1 Hz CH₃-N), 39.82, 37.70 (d, J = 3.0 Hz), 21.66, 16.80 (d, J = 5.9 Hz), 16.74 (d, J = 5.6 Hz), ¹³P NMR (CDCl₃, 121.5 MHz) δ [ppm]: 23.87. Anal. calcd. for C₂₉H₂₂NO₃P: C, 58.70; H, 8.01; N, 4.28%; found: C, 58.95; H, 7.86; N, 4.41% (obtained on a 95 : 5 mixture of trans-3b and cis-3b).

Diethyl 5-(2-hydroxybenzyl)-2-methylisoxazolidin-3-ylphosphonate (trans-3e)

M.p. 90–91°C, colorless needles. IR (KBr):  v [cm⁻¹]: 3199, 2966, 2922, 2866, 1597, 1459, 1268, 1208, 1050, 1022, 766, 590, 566; ¹H NMR (CDCl₃, 300 MHz) δ [ppm]: 7.20–7.14 (m, 1H), 7.07–7.03 (m, 1H), 6.95 (brs, 1H, OH), 6.95–6.80 (m, 2H), 4.42 (dddd, J = 7.8 Hz, J = 7.2 Hz, J = 6.3 Hz, J = 3.3 Hz, 1H, H₅C₅), 4.22–4.10 (m, 4H, 2×POCH₂CH₃), 3.12 (dd, J = 15.3 Hz, J = 3.3 Hz, 1H, HbC¹⁻), 2.90 (s, 3H), 2.85 – 2.80 (brm, 1H, H₃C), 2.80 (dd, J = 15.3 Hz, J = 6.3 Hz, 1H, HbC¹⁻), 1.57 (t, J = 7.2 Hz, H₃C), 2.57 (dddd, J = 18.3 Hz, J = 12.3 Hz, J = 7.2 Hz, J = 7.2 Hz, 1H, HbC₄), 2.26 (dddd, J = 12.3 Hz, J = 11.4 Hz, J = 9.9 Hz, J = 7.8 Hz, 1H, HbC₄), 1.33 (t, J = 7.2 Hz, 3H, POCH₂CH₃), 1.32 (t, J = 7.2 Hz, 3H, POCH₂CH₃), ¹³C NMR (CDCl₃, 75.5 MHz) δ [ppm]: 155.25, 131.56, 128.31, 123.53, 120.09, 116.52, 77.46 (d, J = 5.4 Hz, C₅), 64.34 (d, JPC = 168.0 Hz, C₃), 63.34 (d, J = 6.6 Hz, C-O-P), 62.84 (d, J = 6.9 Hz, C-O-P), 46.38 (brs, CH₂-N), 36.55 (d, J = 2.3 Hz), 34.49, 16.72 (d, J = 5.7 Hz), 16.67 (d, J = 5.4 Hz), ¹³P NMR (CDCl₃, 121.5 MHz) δ [ppm]: 22.85. Anal. calcd. for C₂₉H₂₂NO₃P: C, 54.71; H, 7.35; N, 4.25%; found: C, 54.92; H, 7.20; N, 4.38%.

Diethyl 2-methyl-5-(2-methylbenzyl)isoxazolidin-3-ylphosphonate (trans-3d)

Colorless oil. IR (KBr):  v [cm⁻¹]: 3475, 2981, 2910, 1602, 1494, 1494, 1463, 1441, 1244, 1054, 1026, 966, 756; ¹H NMR (CDCl₃, 300 MHz) δ [ppm]: 7.25–7.15 (m, 2H), 6.90–6.80 (m, 2H), 4.30 (dddd, J = 10.2 Hz, J = 7.2 Hz, J = 6.4 Hz, J = 5.4 Hz, 1H, H₅C₅), 4.20–4.10 (m, 4H, 2×POCH₂CH₃), 3.82 (s, 3H), 3.03 (dd, J = 13.5 Hz, J = 5.7 Hz, 1H, HbC¹⁻), 2.99–2.90 (brm, 1H, H₃C), 2.88 (d, J = 0.9 Hz, 3H), 2.78(dd, J = 13.5 Hz, J = 7.2 Hz, 1H, HbC¹⁻), 2.44 (dddd, J = 19.2 Hz, J = 12.3 Hz, J = 6.9 Hz, J = 6.9 Hz, 1H, HbC₄), 2.17 (dddd, J = 12.6 Hz, J = 12.3 Hz, J = 10.2 Hz, J = 8.1 Hz, 1H, HbC₄), 1.33 (t, J = 7.2 Hz, 3H, POCH₂CH₃), ¹³C NMR (CDCl₃, 75.5 MHz) δ [ppm]: 157.47, 130.89, 127.89, 125.92, 120.49, 110.33, 76.74 (d, J = 10.0 Hz, C₅), 64.32 (d, JPC = 169.0 Hz, C₃), 63.13 (d, J = 6.5 Hz, C-O-P), 62.50 (d, J = 6.8 Hz, C-O-P), 55.39, 46.50 (d, J = 7.0 Hz, 3H), 37.66 (d, J = 3.9 Hz), 34.27, 16.77 (d, J = 5.9 Hz), 16.73 (d, J = 6.5 Hz). ¹³P NMR (CDCl₃, 121.5 MHz) δ [ppm]: 24.06. Anal. calcd. for C₂₉H₂₂NO₃P: C, 55.97; H, 7.63; N, 4.08%; found: C, 55.99; H, 7.89; N, 4.17%.
for C₆H₂NO₃P: C, 55.73; H, 7.90; N, 3.92%; found: C, 57.16; H, 8.01; N, 4.01%.

Diethyl (5-(4-hydroxy-3-methoxybenzyl)-2-methylisoxazolidin-3-yl)phosphonate (trans-3h)

M.p. 89–90°C, colorless needles. IR (KBr): v [cm⁻¹]: 3253, 2986, 2926, 2878, 1600, 1519, 1270, 1242, 1194, 1051, 1025, 964, 944. ¹H NMR (CDCl₃, 300 MHz) δ [ppm]: 6.84 (dd, J = 8.0 Hz, 1H), 6.74 (dd, J = 1.8 Hz, 1H), 6.70 (dd, J = 8.0 Hz, J = 1.8 Hz, 1H), 5.55 (s, 1H, OH), 4.28–4.10 (m, 5H, H₅C₅ and 2◊POCH₂CH₃), 3.88 (s, 3H), 2.91 (dd, J = 14.1 Hz, J = 6.0 Hz, 1H, H₅C₅), 2.89–2.80 (brm, 1H, H₅C₅), 2.86 (d, J = 1.0 Hz, 3H), 2.73 (dd, J = 14.1 Hz, J = 6.4 Hz, 1H, H₅C₅), 2.47 (d̄dd, J = 19.4 Hz, J = 12.8 Hz, J = 7.1 Hz, J = 7.1 Hz, 1H, H₅C₅), 2.18 (d̄dd, J = 12.8 Hz, J = 12.2 Hz, J = 10.2 Hz, J = 8.0 Hz, 1H, H₅C₅), 1.34 (t, J = 7.2 Hz, 3H, POCH₂CH₃), 1.31 (t, J = 7.2 Hz, 3H, POCH₂CH₃).

¹³C NMR (CDCl₃, 75.5 MHz) δ [ppm]: 146.40, 144.35, 129.36, 122.03, 113.32, 111.89, 77.89 (d, J = 7.0 Hz, C₅), 64.36 (d, Jₑₑ = 169.0 Hz, C₃), 63.19 (d, J = 6.5 Hz, C-O-P), 62.59 (d, J = 6.9 Hz, C-O-P), 56.10, 45.43, 39.48, 37.55, 16.84 (d, J = 6.4 Hz, 1H, H₅C₅), 16.80 (d, J = 5.9 Hz). ³¹P NMR (CDCl₃, 121.5 MHz) δ [ppm]: 23.83. Anal. calcld. for C₁₇H₂₈NO₅P: C, 53.48; H, 7.29; N, 3.90%; found: C, 53.53; H, 7.54; N, 3.95%.

Diethyl (5-(3,4-dimethoxybenzyl)-2-methylisoxazolidin-3-yl)phosphonate (trans-3i)

Colourless oil. IR (KBr): v [cm⁻¹]: 2981, 2986, 1516, 1464, 1261, 1238, 1158, 1054, 1028, 966 (signals of trans-3i were extracted from the spectra of a 93 : 7 mixture of trans-3i and cis-3i). ¹H NMR (CDCl₃, 300 MHz) δ [ppm]: 6.85–6.72 (m, 3H), 4.30–4.10 (m, 5H, H₅C₅ and 2◊POCH₂CH₃), 3.87 (s, 3H), 3.86 (s, 3H), 2.92 (dd, J = 14.1 Hz, J = 6.2 Hz, 1H, H₅C₅), 2.90–2.80 (brm, 1H, H₅C₅), 2.86 (dd, J = 1.0 Hz, 3H), 2.75 (dd, J = 14.1 Hz, J = 6.4 Hz, 1H, H₅C₅), 2.47 (d̄dd, J = 19.0 Hz, J = 12.5 Hz, J = 6.9 Hz, J = 6.9 Hz, 1H, H₅C₅), 2.18 (d̄dd, J = 12.5 Hz, J = 12.3 Hz, J = 10.2 Hz, J = 8.1 Hz, 1H, H₅C₅), 1.34 (t, J = 7.2 Hz, 3H, POCH₂CH₃), 1.31 (t, J = 7.2 Hz, 3H, POCH₂CH₃).

¹³C NMR (CDCl₃, 75.5 MHz) δ [ppm]: 148.65, 147.56, 129.89, 121.43, 113.87, 112.43, 111.06, 77.65 (d, J = 1.2 Hz, C₅), 64.15 (d, Jₑₑ = 167.5 Hz, C₃), 63.00 (d, J = 7.6 Hz, C-O-P), 62.40 (d, J = 7.1 Hz, C-O-P), 55.92, 55.86, 39.18, 37.84, 37.38 (d, J = 4.0 Hz), 16.65 (d, J = 5.9 Hz, 1H, H₅C₅), 16.60 (d, J = 6.0 Hz). ³¹P NMR (CDCl₃, 121.5 MHz) δ [ppm]: 23.84. Anal. calcld. for C₁₆H₂₆NO₅P: C, 54.69; H, 7.56; N, 3.75%; found: C, 54.63; H, 7.77; N, 3.84 (obtained on a 93 : 7 mixture of trans-3i and cis-3i).
Diethyl (5-(4-hydroxy-3,5-dimethoxybenzyl)-2-methylisoxazolidin-3-yl)phosphonate (trans-3k)

Colorless oil. IR (KBr): ν [cm⁻¹]: 2986, 2939, 2936, 1607, 1517, 1459, 1329, 1217, 1115, 1053, 810, 753 (signals of \( \nu = 3300, 2980, 2936, 1607, 1517, 1459, 1329, 1217, 1115, 1053, 968, 753 \) [ppm]: 146.83, 133.36, 129.07, 128.35, 105.87, 77.69 (d, \( J = 3.3 \) Hz, POCH₂CH₃), 3.86 (s, 6H), 2.92 (dd, \( J = 6.4 \) Hz, \( J = 6.6 \) Hz, 1H, \( \text{Hb}C₄ \)), 2.50 (dddd, \( J = 12.6 \) Hz, \( J = 11.7 \) Hz, \( J = 10.5 \) Hz, \( J = 8.4 \) Hz, \( J = 6.9 \) Hz, \( J = 6.6 \) Hz, 1H, \( \text{Hb}C₄ \)), 1.33 (t, \( J = 7.2 \) Hz, 3H, POCH₂CH₃). \(^1^C\) NMR (CDCl₃, 75.5 MHz) δ [ppm]: 152.84, 136.40, 133.80, 133.08, 106.01, 77.43 (d, \( J = 7.2 \) Hz, C₅), 64.03 (d, \( J = 64.03 \) Hz, C₅), 62.95 (d, \( J = 6.4 \) Hz, C-O-P), 62.44 (d, \( J = 6.9 \) Hz, C-O-P), 60.78, 56.03, 46.19 (d, \( J = 3.3 \) Hz, 3H), 39.89, 37.41 (d, \( J = 2.2 \) Hz, 1H, C-O-P), 16.58 (d, \( J = 5.7 \) Hz), 16.53 (d, \( J = 5.5 \) Hz). \(^3^P\) NMR (CDCl₃, 121.5 MHz) δ [ppm]: 23.07. Anal. calcd. for C₂₅H₂₅NO₇P: C, 52.44; H, 7.25; N, 3.60%; found: C, 52.64; H, 7.38; N, 3.63%.

Diethyl (2-methyl-5-(3,4,5-trimethoxybenzyl)isoxazolidin-3-yl)phosphonate (trans-3k)

Colorless oil. IR (KBr): ν [cm⁻¹]: 2986, 2939, 2936, 1607, 1517, 1459, 1422, 1239, 1128, 1054, 1024, 968, 753 (signals of trans-3k were extracted from the spectra of a 95 : 5 mixture of trans-3k and cis-3k).

Antiviral activity assay

The compounds were evaluated against different herpesviruses, including herpes simplex virus type 1 (HSV-1) strain KOS, thymidine kinase-deficient (TK-) HSV-1 KOS strain resistant to ACV (ACV), herpes simplex virus type 2 (HSV-2) strain G, vaccinia virus and vesicular stomatitis virus, varicella-zoster virus (VZV) TK- strains Oka, TK-VZV strains 07-1, human cytomegalovirus (HCMV) strains AD-169 and Davis, para-influenza-3 virus, reovirus-1, Sindbis virus, Coxsackie virus B4, Punta Toro virus, respiratory syncytial virus (RSV) and human immunodeficiency virus type 1 (HIV-1) (strain IIIb) and HIV-2 (strain ROD). The antiviral assays were based on inhibition of virus-induced cytopathicity or plaque formation in human embryonic lung (HEL) fibroblasts, African green monkey kidney cells (Vero), human epithelial cervix carcinoma cells (HeLa) or human CD4⁺ T-lymphocyte CEM cells. Confluent monolayer cell cultures or CEM cell (4 × 10⁴/200 µL well) cultures in microtiter 96-well plates were inoculated with 100 CCID₅₀ of virus (1 CCID₅₀ being the virus dose to infect 50% of the cell cultures) or with 20 plaque forming units (PFU) for VZV and the cell cultures were incubated in the presence of varying concentrations of the test compounds. Viral cytopathicity or plaque formation (VZV) was recorded as soon as it reached completion in the control virus-infected cell cultures that were not treated with the test compounds. Antiviral activity was expressed as the EC₅₀ or compound concentration required to reduce virus-induced cytopathicity by 50%.

Cytostatic and cytotoxicity assays

Cytostatic measurements were based on the inhibition of cell growth. HEL cells were seeded at a rate of 5 × 10⁴ cells/200 µL-well into 96-well microtiter plates and allowed to adhere and proliferate for 24 h. Then, medium-containing different concentrations of the test compounds were added. After 3 days of further incubation at 37°C, the cell number was determined with a Coulter counter. The murine leukemia L1210, human CD4⁺ T-lymphocytes and human cervix carcinoma (HeLa) cells were seeded in 96-well microtiter plates and exposed to different concentrations of the test compounds. Cells were allowed to proliferate for 48 h (L1210), 72 h (CEM) or 96 h (HeLa). The cytostatic concentration was calculated as the IC₅₀, or the compound concentration required to reduce cell proliferation by 50% relative to the number of cells in the untreated controls. The IC₅₀ values were estimated from graphic plots of the number of cells (percentage of control) as a function of the concentration of the test compounds. Alternatively, cytotoxicity of the test compounds was expressed as the minimum cytotoxic concentration (MCC) or the
compound concentration that caused a microscopically detectable alteration of (confluent) cell morphology.

**Antimicrobial activity assays**

Screening of antibacterial properties of the tested compounds was carried out using the broth microdilution method according to the recommendation of the European Committee for Antimicrobial Susceptibility Testing (EUCAST) EDIs5.1. (2003) with modifications. The method involved incubation of microorganisms on Mueller-Hinton broth with the isoxazolidines under investigation on microtiter plates and, after 24 h incubation, spectrometric measurement of cell density (630 nm). Antibacterial activity of the compounds was expressed as a percentage of growth compared to the control samples of bacteria which were incubated in the same medium without the tested compounds.

**RESULTS AND DISCUSSION**

**Chemistry**

Allylbenzene 2a and its derivatives 2b-k have already been described in the literature and among them compounds 2a-c, 2e, 2f, 2h and 2j are commercially available whereas 2d, 2g, 2i and 2k were obtained via standard O-alkylation of the respective allylphenols using CH$_3$I and Ag$_2$O (42).

Cycloadditions of the nitrone 1 with compounds 2a-k were carried out at 60°C in toluene and produced mixtures of diastereoisomeric (3-diethoxyphosphoryl)isoxazolidines trans-3 and cis-3 with good trans/cis diastereoselectivities (d.e. 80%) (Scheme 1, Table 1). In all cases good to excellent overall yields of final products were noticed (66 to 100%). The crude mixtures of the respective cycloadducts were subjected to chromatographic purification on silica gel columns to successfully isolate pure major trans isomers in most cases (Table 1).

![Scheme 1. 1,3-Dipolar cycloadditions of the phosphorylnitrone 1 to substituted allylbenzenes 2](attachment:image)

**Table 1. Isoxazolidines trans-3 and cis-3 produced via Scheme 1.**

<table>
<thead>
<tr>
<th>Entry</th>
<th>allyl aryl 2</th>
<th>Reaction time [h]</th>
<th>trans-3 : cis-3</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>C$_6$H$_5$</td>
<td>48</td>
<td>90 : 10</td>
<td>trans-3a (25) + trans-3a and cis-3a (60)$^a$</td>
</tr>
<tr>
<td>b</td>
<td>3-Me-C$_6$H$_5$</td>
<td>24</td>
<td>90 : 10</td>
<td>trans-3b and cis-3b (89)$^a$</td>
</tr>
<tr>
<td>c</td>
<td>2-HO-C$_6$H$_5$</td>
<td>48</td>
<td>90 : 10</td>
<td>trans-3c (50)$^a$ + trans-3c and cis-3c (35)$^a$</td>
</tr>
<tr>
<td>d</td>
<td>2-MeO-C$_6$H$_5$</td>
<td>24</td>
<td>90 : 10</td>
<td>trans-3d (37)$^a$ + trans-3d and cis-3d (63)$^a$</td>
</tr>
<tr>
<td>e</td>
<td>4-MeO-C$_6$H$_5$</td>
<td>48</td>
<td>90 : 10</td>
<td>trans-3e (19)$^a$ + trans-3e and cis-3e (77)$^a$</td>
</tr>
<tr>
<td>f</td>
<td>3-Me-2-HO-C$_6$H$_5$</td>
<td>48</td>
<td>86 : 14</td>
<td>trans-3f (45)$^a$ + trans-3f and cis-3f (42)$^a$</td>
</tr>
<tr>
<td>g</td>
<td>2-MeO-3-Me-C$_6$H$_5$</td>
<td>72</td>
<td>90 : 10</td>
<td>trans-3g (34)$^a$ + trans-3g and cis-3g (46)$^a$</td>
</tr>
<tr>
<td>h</td>
<td>3-MeO-4-HO-C$_6$H$_5$</td>
<td>48</td>
<td>90 : 10</td>
<td>trans-3h (45)$^a$ + trans-3h and cis-3h (42)$^a$</td>
</tr>
<tr>
<td>i</td>
<td>3,4-diMeO-C$_6$H$_5$</td>
<td>48</td>
<td>90 : 10</td>
<td>trans-3i and cis-3i (66)$^a$</td>
</tr>
<tr>
<td>j</td>
<td>3,5-diMeO-4-HO-C$_6$H$_5$</td>
<td>48</td>
<td>90 : 10</td>
<td>trans-3j (46)$^a$ + trans-3j and cis-3j (37)$^a$</td>
</tr>
<tr>
<td>k</td>
<td>3,4,5-triMeO-C$_6$H$_5$</td>
<td>72</td>
<td>90 : 10</td>
<td>trans-3k and cis-3k (81)$^a$</td>
</tr>
</tbody>
</table>

$^a$yield of pure isomer; $^b$yield of pure mixture of cis- and trans-isomers.
Table 2. Inhibitory effect of the tested compounds against the proliferation of murine leukemia (L1210), human T-lymphocyte (CEM) and human cervix carcinoma cells (HeLa).

<table>
<thead>
<tr>
<th>Entry</th>
<th>Isoxazolidine 3</th>
<th>IC_{50} (μM)\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ar</td>
<td>L1210</td>
</tr>
<tr>
<td>a</td>
<td>C\textsubscript{6}H\textsubscript{5}</td>
<td>trans-3a</td>
</tr>
<tr>
<td>b</td>
<td>3-Me-C\textsubscript{6}H\textsubscript{4}</td>
<td>trans-3b cis-3b (95:5)</td>
</tr>
<tr>
<td>c</td>
<td>2-HO-C\textsubscript{6}H\textsubscript{4}</td>
<td>trans-3c</td>
</tr>
<tr>
<td>d</td>
<td>2-MeO-C\textsubscript{6}H\textsubscript{4}</td>
<td>trans-3d</td>
</tr>
<tr>
<td>e</td>
<td>4-MeO-C\textsubscript{6}H\textsubscript{4}</td>
<td>trans-3e</td>
</tr>
<tr>
<td>f</td>
<td>3-Me-2-HO-C\textsubscript{6}H\textsubscript{4}</td>
<td>trans-3f</td>
</tr>
<tr>
<td>g</td>
<td>2-MeO-3-Me-C\textsubscript{6}H\textsubscript{4}</td>
<td>trans-3g</td>
</tr>
<tr>
<td>h</td>
<td>3-MeO-4-HO-C\textsubscript{6}H\textsubscript{4}</td>
<td>trans-3h</td>
</tr>
<tr>
<td>i</td>
<td>3,4-diMeO-C\textsubscript{6}H\textsubscript{4}</td>
<td>trans-3i cis-3i (93:7)</td>
</tr>
<tr>
<td>j</td>
<td>3,5-diMeO-4-HO-C\textsubscript{6}H\textsubscript{4}</td>
<td>trans-3j</td>
</tr>
<tr>
<td>k</td>
<td>3,4,5-triMeO-C\textsubscript{6}H\textsubscript{4}</td>
<td>trans-3k cis-3k (95:5)</td>
</tr>
</tbody>
</table>

\textsuperscript{a}50\% inhibitory concentration or compound concentration required to inhibit tumor cell proliferation by 50%.

The relative configurations at C3 and C5 in the trans- and cis-3 isoxazolidines were determined taking advantage of our previous studies on the stereochemical outcome of the cycloaddition of N-methylo-
C-(diethoxyphosphoryl)isoxazolidines 1 with various terminal alkenes (21, 40, 43, 44). In H NMR spectra of all trans-3 isoxazolidines the spectral patterns characteristic of the isoxazolidine fragment were almost identical and closely resembled those of the previously described trans-configured 5-substituted 3-
(dithyrophosphoryl)isoxazolidines obtained from the nitrone 1 and N-allylated nucleobases and their analogues (21).

Antiviral activity and cytostatic/cytotoxic evaluation

Pure trans- and cis-3 isoxazolidines were evaluated for their inhibitory activity against a wide variety of DNA and RNA viruses using following cell-based assays: (a) human embryonic lung (HEL) cell cultures: herpes simplex virus-1 (KOS), herpes simplex virus-2 (G), herpes simplex virus-1 (TK ACV KOS), vaccinia virus and vesicular stomatitis virus, cytomegalovirus (AD-169 strain and Davis strain), variella-zoster virus (TK VZV strain OKA and TK VZV strain 07-1); (b) vero cell cultures: para-influenza-3 virus, reovirus-1, Sindbis virus, Coxsackie virus B4, Punta Toro virus; (c) HeLa cell cultures: vesicular stomatitis virus, Coxsackie virus B4, and respiratory syncytial virus (RSV); (d) CEM cell cultures: human immunodeficiency virus-1 (HIV-1, strain IIIb and HIV-2 (strain ROD), Ganciclovir, cidofovir, acyclovir, brivudin, (S)-9-(2,3-dihydroxypropyl)adenine [(S)-DHPA], dextran sulfate (molecular weight 5000, DS-5000) and ribavirin were used as the reference compounds. The antiviral activities were expressed as their EC_{50}'s: the compound concentration required to reduce virus plaque formation (VZV) by 50% or to reduce virus-induced cytopathicity by 50% (other viruses).

The cytotoxicity of the tested compounds toward the uninfected host cells was defined as the minimum cytotoxic concentration (MCC) that causes a microscopically detectable alteration of normal cell morphology. The 50% cytotoxic concentration (CC_{50}), causing a 50% decrease in cell viability was determined using a colorimetric 3-(4,5-dimethylthi-
drazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sul-
phonphenyl)-2H-tetrazolium (MTS) assay system.

The cytostatic activity of the tested compounds was defined as the 50% cytostatic concentration (IC_{50}), causing a 50% reduction in cell proliferation and was determined against murine leukemia L1210, human lymphocyte CEM and human cervix carcinoma HeLa cells.

Unfortunately, no inhibitory activity against any of the tested viruses was assayed for the evaluated compounds at 250 µM. At the same time, none of the tested compounds affected cell morphology of...
HEL, HeLa and Vero cells at concentrations up to 100 µM. However, isoxazolidines trans-3k : cis-3k (95 : 5) reduced HEL cells viability (CC₀ = 45 µM). In addition, several compounds slightly inhibited L1210 and HeLa cell proliferation (IC₅₀ = 140–240 µM) (Table 2).

**Antibacterial activity**

Antibacterial activity of phenols as well as of several substituted isoxazolidines (28–31) prompted us to evaluate the antimicrobial properties of isoxazolidines trans-3a–k towards selected microorganisms. This idea is additionally substantiated by the observed lack of toxicity of these compounds toward normal, uninfected cells. In preliminary studies two Gram-positive bacteria (Staphylococcus aureus ATCC 6538, Staphylococcus epidermidis ATCC 12228) and two Gram-negative bacterial strains (Escherichia coli ATCC 25922 and Pseudomonas aeruginosa ATCC 15442) were selected.

*S. aureus* is a common human pathogen causing a wide variety of diseases, like skin or burns infection, pneumonia and endocarditis (45). *S. epidermidis* and *P. aeruginosa* are a common cause of nosocomial infections, especially in compromised patients (46, 47). Also, *E. coli* can cause diarrhea, infections of the urinary tract or neonatal meningitis (48). The treatment of the mentioned diseases is extremely difficult when infections are caused by antibiotic-resistant bacterial strains. Thus, a search for new and effective antimicrobial agents is justified.

Out of 10 isoxazolidines subjected to antimicrobial screening only compounds 3b, 3d, 3e and 3i inhibited the growth of the Gram-positive bacteria, while none of them affected the Gram-negative bacteria used in this study. The highest potency was found for *S. epidermidis* ATCC 12228 (Fig. 2). For isoxazolidines 3i the inhibition of growth increased steadily from ca. 20% to almost 40% over a concentration range of 5 to 200 mg/L, respectively. In
assays with trans-3e the growth of the strain oscillated around 70% in comparison to the control at the concentrations of 5–50 mg/L. In the presence of the compounds trans-3d and a 95 : 5 mixture of trans-3b and cis-3b the growth of S. epidermidis ATCC 12228 was also inhibited in both cases by ca. 30% at the highest (150 and 200 mg/L) concentrations.

A 25% inhibition of growth of S. aureus ATCC 6538 (Fig. 3) was noticed for isoxazolidines 3i at the concentration of 50 mg/L, while trans-3d and a 95 : 5 mixture of trans- and cis-3b were only slightly less active at concentrations of 25–200 mg/L than 3i.

None of isoxazolidines containing hydroxyl groups (3c, 3f, 3h, 3i, 3j) were endowed with antibacterial activity towards the tested strains. Nevertheless, some inhibitory activity against the evaluated Gram-positive bacteria was observed for compounds having methoxy groups, namely a 93 : 7 mixture of trans- and cis-3i (3,4-dimethoxyphenyl), trans-3e (4-methoxyphenyl) and trans-3d (2-methoxyphenyl). However, isoxazolidines trans-3k/cis-3k having a 3,4,5-trimethoxyphenyl residue appeared inactive.

CONCLUSIONS

Good trans/cis diastereoselectivities (d.e. 80%) and good to excellent overall yields of diastereoisomeric (3-diethoxyphosphoryl)isoxazolidines 3 were observed in 1,3-dipolar cycloadditions of the phosphonylnitrone 1 and selected allylbenzenes 2. The inhibitory activity of (3-diethoxyphosphoryl)isoxazolidines was assayed against a broad panel of DNA and RNA viruses but they appeared inactive at concentrations up to 250 µM. The isoxazolidines trans-3k : cis-3k (95 : 5) reduced HEL cell viability (CC_{50} = 45 µM). Four out of ten isoxazolidines inhibited the growth of Staphylococcus epidermidis ATCC 12228 by up to 40% for the most active compound 3i. They were also active against Staphylococcus aureus ATCC 6538 although inhibition did not exceed 25%. None of the isoxazolidines affected Escherichia coli ATCC 25922 and Pseudomonas aeruginosa ATCC 15442. Antimicrobial activity was discovered for isoxazolidines having methoxy groups (2- or 4-methoxy- and 3,4-dimethoxyphenyl, but not 3,4,5-trimethoxyphenyl) but not for phenolic compounds (3c, 3f, 3h and 3j). The influence of the methylene linker in the investigated isoxazolidines on their antimicrobial potency will be disclosed in due course since the respective analogues having aryl group substituted directly at C5 in the isoxazoline ring (C-nucleosides) showed no antiviral and cytotoxic activity (40).

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SYNTHESIS AND ANTIBACTERIAL ACTIVITY OF La(III), Ce(III), Pr(III), AND Nd(III) COMPLEXES OF CHRYSIN-4'-SULFONATE

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Abstract: New solid amorphous compounds of La(III), Ce(III), Pr(III), and Nd(III) with sodium salt chrysin-4'-sulfonic acid (1cNa) were obtained. Their composition and some physicochemical properties were studied by elementary analysis, thermogravimetric analysis as well as in solution by conductivity, UV/Vis and 'H NMR spectroscopies. Three water molecules are coordinated to central metal ion, while remaining nine molecules of water are weakly bonded in the solid Ln(1cNa)(H2O)9H2O complexes. The solution studies indicated that hydroxyl group or carbonyl oxygen of chrysin-4'-sulfonate were not involved in coordination to metal ion. Two of sulfonate groups are bridging, while the third one belongs to monodentate chsa-4' ligand, which dissociates easier than other two leading to a 1 : 1 electrolyte behavior of Ln(1cNa)(H2O)9H2O complexes. The antibacterial activity of synthesized compounds was determined by the cylinder-plate diffusion method and dilution method (determination of minimum inhibitory concentration – MIC).

Keywords: sodium salt of chrysin-4'-sulfonic acid, thermal analysis, lanthanide complexes, spectroscopic properties, chrysin

Flavons are easily available compounds, widely spread in cereals and herbs. The most abundant flavons are quercetin (3,5,7,3',4'-pentahydroxyflavon), morin (3,5,7,2',4'-pentahydroxyflavon), and chrysin (Fig. 1, 1a). Sulfonation of quercetin led to quercetin-5'-sulfonate, quercetin-8-sulfonate, and quercetin-8,5'-disulfonate, depending on reaction protocol (1, 2). The morin underwent monosulfonation at 5' position (3), while chrysin 1a in the same reaction conditions gave preferably 4'-sulfonate (Fig. 1, 1b) (3), although 8-sulfonate was also obtained and characterized structurally as barium salt (4). Although flavones indicated high antioxidant activity in vitro, their poor solubility in water is probably responsible for low food digestion uptake and rapid excretion (5). On the other hand, they are evidenced to be involved in cell signaling (6). Conversion of flavones into their sulfonate derivatives dramatically changes their physical and chemical properties. Sulfonates like 1b and its sodium salt

![Figure 1. Structure of chrysin and chrysin-4'-sulfonate derivatives](image-url)
as well as morin-5'-sulfonate (7) are water soluble. The antioxidant and pro-oxidant properties of flavonoids are influenced by metal ions (7, 8). Previously, we have demonstrated that 1a formed complexes with first transition metal ions and group XIII metal(III) ions in which C(4)=O; 5-OH oxygen donors were involved (9, 10). The same coordination mode was found for Ln(1a)₃•4H₂O complexes (where lanthanides(III) were Tb, Ho, Er, Yb) (11) for Ln(1a)₃ (where lanthanides(III) were La, Pr, Nd, Sm, Gd, Tb, Ho, Y) (12, 13) and for Ln(1a)₃(H₂O)₂Cl•2H₂O (where lanthanides(III) were Ce, Pr, Nd, Sm) (14). The paramagnetic metal ions induced remarkable large coordination shifts of anionic ligand 1a. Chrysin-4'-sulfonate, 1b and its sodium salt, 1cNa were then used to obtain first transition metal ion complexes with Co(II), Ni(II), Cu(II), Ti(IV), Mn(II), and Fe(III) (3, 15). The sparingly soluble complexes were obtained, in which the sulfonate group was not found to be involved in coordination; instead the C(4)=O; 5-OH chelation was suggested based upon considerable 30 cm⁻¹ shift of n(C=O) stretching vibration in IR spectra of complexes in comparison with that of 1c. Here we have characterized the complexes of 1c with early lanthanides: La(III), Ce(III), Pr(III), and Nd(III) both as solid compounds by elemental analysis, thermogravimetry and IR as well as in solution by conductivity, UV/Vis and ¹H NMR spectroscopies.

In the literature, there are many reports of antibacterial activity of flavonoids, but activity of sulfur derivatives and their complexes with metal ions are discussed only in papers (16-18).

The antibacterial activity of synthesized compounds was determined by the cylinder-plate diffusion method and dilution method to determine minimum inhibitory concentration – MIC.

**EXPERIMENTAL**

**Reagents**

Nachsa-4’ (1cNa) was obtained by the method described in paper (3). 0.2 M/L solutions of LaCl₃, PrCl₃, and NdCl₃ were obtained by hot dissolution of the appropriate amounts of La₂O₃, Pr₂O₃, and Nd₂O₃ oxides in concentrated hydrochloric acid (d = 1.19 g/mL). The excess of the acid was evaporated and the residue dissolved in redistilled water. 0.2 M/L solution of CeCl₃ were obtained dissolving the appropriate weight amount of the CeCl₃•7H₂O (99.9%, Sigma-Aldrich, USA) in redistilled water. Chrysin (> 98%, Sigma-Aldrich, USA), was used in this study.

All the other reagents: methanol, xylenol orange, arsenazo I, chloric(VII) acid, NaOH, dimethyloformamide (DMF), dimethyl sulfoxide (DMSO) were analytically pure.

**Syntheses of the compounds**

The complexes of 1c with La(II), Ce(III), Pr(III), and Nd(III) were obtained by the procedure described previously. Briefly, 18 mL of the hot aqueous solution of the appropriate metal chloride (3.6 mM) was added, with continuous stirring, into 135 mL of hot aqueous solution of 1cNa (10.8 mM). After cooling, the solution was kept at room tem-
perature for slow concentration. pH of the solution after cooling to room temperature was 1.5-2. The beige precipitates were formed, which were separated by centrifuging, washed with copious amount of water-methanol (1:1) solution, and dried in air at room temperature.

**Elemental analyses**

Elemental analyses for C, H and S were performed with an Elemental Analyzer EA 1108 apparatus (Carbo Erba, Italy). The content of Na was determined by an AAS Perkin Elmer 3100 spectrophotometer (Perkin-Elmer, USA). The metal content was determined spectrophotometrically with arsenazo I for La(III) and Ce(III) (19) and xylenol orange as indicator for Pr(III) and Nd(III) ions (20) in the samples dissolved in chloric(VII) acid. In separate experiments the metals were quantified by TG analysis. The final mass of the metal oxides obtained by heating the samples at 900°C was recal-
culated for metal ion percentage. The water content was determined by gravimetric method and derivatography. The complexes were isolated as hydrated compounds \( \text{Ln(C}_{15}\text{H}_{9}\text{O}_{7}\text{S})_3(\text{H}_2\text{O})_{12} \) (where \( \text{Ln} = \text{La, Ce, Pr, Nd} \)). The elemental analysis results are listed in Table 1.

**Thermogravimetric measurements**

The thermal stability of the prepared compounds was determined using TGA/DSC 1 thermogravimetric analyzer (Mettler-Toledo AG, Switzerland) in the temperature range 25-900°C in air with heating rate of 10°C/min. The thermogravimetric results of experiments for all studied compounds are collected in Table 2 and representative TG-TGA run for \( \text{Ce(C}_{15}\text{H}_{9}\text{O}_{7}\text{S})_3(\text{H}_2\text{O})_{12} \) is shown in Figure 4.

**Conductivity measurements**

Molar conductance at room temperature of the studied complexes was measured at 25°C in 10^{-3} M/L DMSO and DMF solution using a CPC-551 type conductivity meter. Physical properties of compounds are listed in Table 3.

**Spectroscopic analysis**

The UV/Vis and infrared spectra were taken with FT-IR NICOLET 6700 Thermo Scientific instrument. A UV/Vis spectra of obtained compounds and 1cNa were taken in methanol. The UV/Vis spectra of \( \text{Ln(1c)}_3(\text{H}_2\text{O})_{12} \) complexes and 1cNa are presented in Figure 2 and Table 4. The IR spectra of complexes were taken in KBr pellets and relevant fragments of the spectra are presented in Table 5 and in Figure 3.

\(^1\)H NMR spectra, in DMSO-d_6, were obtained on a Bruker Avance II 500 spectrometer at room temperature. The resonances were assigned as before (3).

\(^1\)H NMR (chemical shift [ppm], multiplicity, assignment, coupling constant):

\begin{align*}
\text{iNa} \text{ (Nachsa-4’):} & \quad 6.23 (1\text{H, d, 6-H, } J_{6,8} = 2.1 \text{ Hz}); 6.54 (1\text{H, d, 8-H}); 6.97 (1\text{H, s 3-H}); 7.78 (2\text{H, d, 3’}, 5’ – H, J_{2,3} = 8.3 \text{ Hz}); 8.06 (2\text{H, d, 2’}, 6’ – H); 10.94 (1\text{H, s, OH – 7}); 12.82 (1\text{H, s, OH – 5}); \\
\text{La(1c)}_3(\text{H}_2\text{O})_{12} & \quad 6.24 (1\text{H, d, 6-H, } J_{6,8} = 2.1 \text{ Hz}; 6.54 (1\text{H, d, 8-H}); 6.97 (1\text{H, s 3-H}); 7.78 (2\text{H, d, 3’}, 5’ – H, J_{2,3} = 8.3 \text{ Hz}); 8.06 (2\text{H, d, 2’}, 6’ – H); 10.97 (1\text{H, s, OH – 7}); 12.82 (1\text{H, s, OH – 5}); \\
\text{Ce(1c)}_3(\text{H}_2\text{O})_{12} & \quad 6.24 (1\text{H, d, 6-H, } J_{6,8} = 2.1 \text{ Hz}); 6.54 (1\text{H, d, 8-H}); 6.95 (1\text{H, s 3-H}); 7.71 (2\text{H, d, 3’}, 5’ – H, J_{2,3} = 8.3 \text{ Hz}); 8.04 (2\text{H, d, 2’}, 6’ – H); 11.00 (1\text{H, s, OH – 7}); 12.81 (1\text{H, s, OH – 5}); \\
\text{Pr(1c)}_3(\text{H}_2\text{O})_{12} & \quad 6.24 (1\text{H, s, 6-H}); 6.54 (1\text{H, d, 8-H}); 6.95 (1\text{H, s 3-H}); 7.67 (2\text{H, d, 3’}, 5’ – H, J_{2,3} = 8.3 \text{ Hz}); 8.03 (2\text{H, d, 2’}, 6’ – H); 11.00 (1\text{H, s, OH – 7}); 12.81 (1\text{H, s, OH – 5}); \\
\text{Nd(1c)}_3(\text{H}_2\text{O})_{12} & \quad 6.23 (1\text{H, s, 6-H}); 6.54 (1\text{H, d, 8-H}); 6.96 (1\text{H, s 3-H}); 7.71 (2\text{H, d, 3’}, 5’ – H, J_{2,3} = 8.3 \text{ Hz}); 8.03 (2\text{H, d, 2’}, 6’ – H); 11.00 (1\text{H, s, OH – 7}); 12.81 (1\text{H, s, OH – 5});
\end{align*}

![Figure 4. TG/DTG-DTA curves for Ce(C-HOS)(H-O)-9H complex](image-url)
Table 1. Results of elementary analysis of lanthanide ions complexes with chrysin-4'-sulfonate.

<table>
<thead>
<tr>
<th>Compound</th>
<th>M %</th>
<th>C %</th>
<th>S %</th>
<th>H %</th>
<th>Yeld</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Calc</td>
<td>Obt</td>
<td>Calc</td>
<td>Obt</td>
<td>Calc</td>
</tr>
<tr>
<td>La(1c)₃(H₂O)₃ ∑9H₂O</td>
<td>10.26</td>
<td>10.29</td>
<td>39.88</td>
<td>40.02</td>
<td>7.08</td>
</tr>
<tr>
<td>Ce(1c)₃(H₂O)₃ ∑9H₂O</td>
<td>10.32</td>
<td>10.82</td>
<td>39.85</td>
<td>39.95</td>
<td>7.08</td>
</tr>
<tr>
<td>Pr(1c)₃(H₂O)₃ ∑9H₂O</td>
<td>10.39</td>
<td>10.92</td>
<td>39.82</td>
<td>40.04</td>
<td>7.07</td>
</tr>
<tr>
<td>Nd(1c)₃(H₂O)₃ ∑9H₂O</td>
<td>10.46</td>
<td>10.48</td>
<td>39.79</td>
<td>39.95</td>
<td>7.07</td>
</tr>
</tbody>
</table>

Table 2. Temperature values of thermal decomposition of 1cNa and its complexes with lanthanide ions.

<table>
<thead>
<tr>
<th>Compound</th>
<th>ΔT₁</th>
<th>T</th>
<th>ΔT₂</th>
<th>n H₂O</th>
<th>H₂O, %</th>
<th>T₁</th>
<th>Residue mass, %</th>
<th>Final decomposition product</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>°C</td>
<td></td>
<td>°C</td>
<td></td>
<td></td>
<td>°C</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Calc</td>
<td>Obt</td>
<td>Calc</td>
<td>Obt</td>
<td></td>
<td>Calc</td>
<td>Obt</td>
<td></td>
</tr>
<tr>
<td>1cNa -4H₂O</td>
<td>40-125</td>
<td>90-190</td>
<td>415-950</td>
<td>3.5</td>
<td>14.72</td>
<td>14.5</td>
<td>950</td>
<td>16.82</td>
</tr>
<tr>
<td>La(1c)₃(H₂O)₃ ∑9H₂O</td>
<td>25-300</td>
<td>90</td>
<td>375-620</td>
<td>12</td>
<td>15.95</td>
<td>16.47</td>
<td>620</td>
<td>12.02</td>
</tr>
<tr>
<td></td>
<td>25-100</td>
<td>9</td>
<td>11.97</td>
<td>11.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ce(1c)₃(H₂O)₃ ∑9H₂O</td>
<td>25-300</td>
<td>85</td>
<td>370-720</td>
<td>12</td>
<td>15.94</td>
<td>19.62</td>
<td>720</td>
<td>12.69</td>
</tr>
<tr>
<td></td>
<td>25-100</td>
<td>9</td>
<td>11.96</td>
<td>11.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pr(1c)₃(H₂O)₃ ∑9H₂O</td>
<td>25-300</td>
<td>90</td>
<td>350-590</td>
<td>12</td>
<td>15.93</td>
<td>16.50</td>
<td>590</td>
<td>12.54</td>
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<tr>
<td></td>
<td>25-100</td>
<td>9</td>
<td>11.95</td>
<td>10.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nd(1c)₃(H₂O)₃ ∑9H₂O</td>
<td>25-300</td>
<td>85</td>
<td>350-615</td>
<td>12</td>
<td>15.89</td>
<td>16.54</td>
<td>615</td>
<td>12.36</td>
</tr>
<tr>
<td></td>
<td>25-100</td>
<td>9</td>
<td>11.95</td>
<td>10.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ΔT₁ (ΔT₂) – temperature ranges corresponding to dehydration endo effect of defined amount of water molecules (corresponding to decomposition of anhydrous compound Tᵣmin – temperature corresponding to minimum on DTG curve, T₁ – temperature of final product formation.)
8.3 Hz); 8.04 (2H, d, 2’, 6’ – H); 10.94 (1H, s, OH – 7); 12.82 (1H, s, OH – 5).

Microorganism tests
We have used ampicillin sensitive bacteria: *Escherichia coli* ATCC 10538 (Gram negative) and *Enterococcus hirae* ATCC 8043 (Gram positive) available from the Department of Biotechnology and Bioinformatics, Faculty of Chemistry, Rzeszow University of Technology. All reagents and bacterial cultures were prepared using Laminar Flow Cabinet ESCO Airstream. Lysogeny broth (LB) was used as bacterial medium. Second and/or third culture was used as working culture and incubated at 37°C (aerobic conditions) in INNOVA 40 Incubator Shaker with added refrigeration, till the turbidity of 0.5 McFarland’s standard was obtained (10⁸ Colony-Forming Units – CFU). The optical density measurements were carried out at 600 nm with a UV-160A SHIMADZU spectrophotometer.

Dilution method
To determine MICs of 1cNa and its complexes with lanthanide ions, broth micro dilution technique was used. Triplicate serial dilutions of test samples (in the range of 7.8 mg/mL to 1 mg/mL) were prepared in sterile microtiter plates in the LB broth. Working bacterial cultures were diluted to final density 10⁵ CFU and added to each well of prepared plate except to those used as media sterility. Bacterial broth cultures without tested compounds were used as negative control samples. Assignment of MIC was based on visual observation of turbidity of bacterial cultures, what was further confirmed by the measurement of optical density using BIO-RAD Microplate Reader model 550 at 630 nm.

Cylinder plate diffusion method
Working bacterial culture (0.5 McFarland’s standard turbidity) was used to flood the surface of solid Triptic Soy Agar (TBA) and drained dry. Glass cylinders sterilized in Prestige Medical 2100 classic autoclave (dimensions of outer diameter 8 mm × inner diameter 6 mm × height 10 mm) were then placed on Petri dishes with culture media. Test compounds (concentrations ranging from 0.125 mg/mL to 1 mg/mL) prepared in DMSO were thereafter used to fill the glass cylinders. DMSO and ampicillin (10 mg/mL) were used, respectively, as negative and positive control. For each set of concentrations of test compounds and control samples three-fold replication were applied. Plates were incubated at 37°C for 24 h in BIOMETRA OV3 Incubator. Zone of inhibition around glass cylinders (including the diameter of glass cylinder), measured in millimeters, were used as positive bioactivity.

RESULTS AND DISCUSSION
Early lanthanides form +3 cationic compounds, which are mostly ionic due to small ion radii (between 103 and 98 pm) similarly to Na⁺ (97 pm) (21). Still the coordination numbers of La³⁺, Ce³⁺, Pr³⁺, and Nd³⁺ are 8-9. We have obtained the compounds of chrysin-4’-sulfonate, 1c of the general stoichiometry Ln(1c)₃(H₂O)₁₂ as evidenced by exact match of elemental analytical data on C, H, S (Table 1) as well as metal percentage analysis and water content by TG analysis (Table 2). In synthetic protocol of compounds the sodium salt of 1b was added into acidic solution of lanthanide salt. At pH ca. 2 the products precipitated, then were filtered off and dried at room temperature.

Thermogravimetric analysis
The thermogravimetric measurements for all the studied complexes showed similar profile. The scheme of compounds decomposition consist of two general steps. The first one is related to dehydration, whereas the second stage is related with dehydration of ligands in complexes. Namely, the nine of water equivalents were released from Ln(1c)(H₂O)₁₂ at

<table>
<thead>
<tr>
<th>Compound</th>
<th>M, g/M</th>
<th>Conductivity, S-cm/M</th>
<th>Solubility in MeOH, M/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>La(1c)(H₂O)₃·9H₂O</td>
<td>1354.05</td>
<td>40.60</td>
<td>34.15</td>
</tr>
<tr>
<td>Ce(1c)(H₂O)₃·9H₂O</td>
<td>1355.05</td>
<td>38.80</td>
<td>34.55</td>
</tr>
<tr>
<td>Pr(1c)(H₂O)₃·9H₂O</td>
<td>1356.06</td>
<td>38.05</td>
<td>34.05</td>
</tr>
<tr>
<td>Nd(1c)(H₂O)₃·9H₂O</td>
<td>1357.05</td>
<td>37.65</td>
<td>34.40</td>
</tr>
</tbody>
</table>
Table 4. Values of absorption bands of 1cNa and its complexes with lanthanide ions in methanol ($c_{1cNa} = 3.15 \times 10^{-5}$ M/L, $c_{La-Na} = 1.50 \times 10^{-5}$ M/L, $c_{Ce-Na} = 1.48 \times 10^{-5}$ M/L, $c_{Pr-Na} = 1.47 \times 10^{-5}$ M/L, $c_{Nd-Na} = 1.47 \times 10^{-5}$ M/L, $l = 1$ cm).

<table>
<thead>
<tr>
<th></th>
<th>1cNa</th>
<th>La(1c),(H$_2$O)$_3$, 9H$_2$O</th>
<th>Ce(1c),(H$_2$O)$_3$, 9H$_2$O</th>
<th>Pr(1c),(H$_2$O)$_3$, 9H$_2$O</th>
<th>Nd(1c),(H$_2$O)$_3$, 9H$_2$O</th>
</tr>
</thead>
<tbody>
<tr>
<td>$v_0$ cm$^{-1}$</td>
<td>$\epsilon_{\text{max}}$ M$^{-1}$dm$^3$cm$^{-1}$</td>
<td>$v_0$ cm$^{-1}$</td>
<td>$\epsilon_{\text{max}}$ M$^{-1}$dm$^3$cm$^{-1}$</td>
<td>$v_0$ cm$^{-1}$</td>
<td>$\epsilon_{\text{max}}$ M$^{-1}$dm$^3$cm$^{-1}$</td>
</tr>
<tr>
<td>47169</td>
<td>38 152</td>
<td>46948</td>
<td>107 793</td>
<td>46948</td>
<td>96 419</td>
</tr>
<tr>
<td>40160</td>
<td>15 682</td>
<td>41494</td>
<td>404 20</td>
<td>41322</td>
<td>34 831</td>
</tr>
<tr>
<td>36764</td>
<td>32 682</td>
<td>36764</td>
<td>77 133</td>
<td>36764</td>
<td>70 392</td>
</tr>
</tbody>
</table>

$v_0$ _band center, $\epsilon_{\text{max}}$: molar absorption coefficient at band maximum.$10^{-3}$

Table 5. Frequencies of characteristic absorption bands in IR spectra (cm$^{-1}$) of 1a, 1b, 1cNa and complexes with lanthanide ions.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$v$(O-H), $v$(C=H)</th>
<th>$v$(C=C)</th>
<th>$v$(C=O)</th>
<th>$v$(C-OH)</th>
<th>$v$(C-O-C)</th>
<th>$v_\text{as}(SO_3^-)$</th>
<th>$v_\text{s}(SO_3^-)$</th>
<th>$v_\text{s}(SO_3^-)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a chrysin</td>
<td>3100-2500</td>
<td>1653</td>
<td>1612</td>
<td>1356</td>
<td>1168</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1b Hcbsa-4$^+$</td>
<td>3550-2500</td>
<td>1639</td>
<td>1609</td>
<td>1387</td>
<td>1164</td>
<td>1223</td>
<td>1131</td>
<td>1028</td>
</tr>
<tr>
<td>1cNa Nachsa-4$^+$</td>
<td>3550-2600</td>
<td>1658</td>
<td>1627</td>
<td>1373</td>
<td>1168</td>
<td>1228</td>
<td>1125</td>
<td>1027</td>
</tr>
<tr>
<td>La(1c),(H$_2$O)$_3$, 9H$_2$O</td>
<td>3550-2600</td>
<td>1657</td>
<td>1619</td>
<td>1359</td>
<td>1164</td>
<td>1214</td>
<td>1128</td>
<td>1028</td>
</tr>
<tr>
<td>Ce(1c),(H$_2$O)$_3$, 9H$_2$O</td>
<td>3550-2600</td>
<td>1658</td>
<td>1619</td>
<td>1361</td>
<td>1164</td>
<td>1217</td>
<td>1128</td>
<td>1029</td>
</tr>
<tr>
<td>Pr(1c),(H$_2$O)$_3$, 9H$_2$O</td>
<td>3550-2600</td>
<td>1657</td>
<td>1619</td>
<td>1360</td>
<td>1164</td>
<td>1213</td>
<td>1128</td>
<td>1028</td>
</tr>
<tr>
<td>Nd(1c),(H$_2$O)$_3$, 9H$_2$O</td>
<td>3550-2600</td>
<td>1657</td>
<td>1619</td>
<td>1359</td>
<td>1164</td>
<td>1213</td>
<td>1128</td>
<td>1027</td>
</tr>
</tbody>
</table>
temperatures below 100°C (representative TG-TGA run for Ce(1c)₃(H₂O)₁₂ is shown at Fig. 4); the maximum of endothermic peak for 9H₂O release was found at 85-95°C. The nine equivalents of water release was completed at ca 120°C in all cases. The total release of water was found at temperature as high as at 300°C, corresponding to ca 16% mass loss (Table 2). The total water release was overlapped slightly with thermal decomposition of ligand, which started already at ca 350°C. The anhydrous compounds were stable in the temperature 300-350°C (Table 2). The second stage of decomposition was accompanied by complex exothermic effect on DTA curve, and mass loss observed on TG curves in the temperature range 350-590°C. The total decomposition of ligands took place in this temperature range. The final products of the decomposition of the complexes in air were the lanthanide oxides at higher oxidation state than three in case of cerium and praseodymium. The oxidation of metal ions with oxygen was expected, because the temperature of the oxide formation: La₂O₃, CeO₂, Pr₂O₃, and Nd₂O₃, changes irregularly in the lanthanide series from 590 to 720°C. The final products of the decomposition of complexes in air are the lanthanide oxides like it was observed in case of MSA – Zn(II) complex, characterized structurally by X-ray crystallography (7). Clearly from thermogravimetric analysis it can be concluded that three water molecules are bound stronger in the solid Ln(1c)₃(H₂O)₁₂ complexes. Hence, the formula of complexes should be considered as Ln(1c)(H₂O)₃·9H₂O.

Table 6. The MIC (mg/mL) values of 1cNa and its complexes with lanthanide ions; dilution method.

<table>
<thead>
<tr>
<th>Compound</th>
<th>MIC, mg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>1cNa</td>
<td>0.25</td>
</tr>
<tr>
<td>La(1c)(H₂O)·9H₂O</td>
<td>0.25</td>
</tr>
<tr>
<td>Ce(1c)(H₂O)·9H₂O</td>
<td>0.50</td>
</tr>
<tr>
<td>Pr(1c)(H₂O)·9H₂O</td>
<td>NI</td>
</tr>
<tr>
<td>Nd(1c)(H₂O)·9H₂O</td>
<td>0.25</td>
</tr>
<tr>
<td>ampicilin</td>
<td>0.0098</td>
</tr>
</tbody>
</table>

NI – no inhibition in concentration range studied.

Table 7. Antibacterial test of 1cNa and its complexes with lanthanide ions against Escherichia coli and Enterococcus hirae; the cylinder – plate method.

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>Compound</th>
<th>Zone of inhibition, mm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 mg/mL</td>
<td>0.5 mg/mL</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>1cNa</td>
<td>11 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>La(1c)(H₂O)·9H₂O</td>
<td>12 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>Ce(1c)(H₂O)·9H₂O</td>
<td>10 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>Pr(1c)(H₂O)·9H₂O</td>
<td>6 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>Nd(1c)(H₂O)·9H₂O</td>
<td>12 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>DMSO</td>
<td>0 ± 0.0</td>
</tr>
<tr>
<td>Enterococcus hirae</td>
<td>1cNa</td>
<td>0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>La(1c)(H₂O)·9H₂O</td>
<td>0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>Ce(1c)(H₂O)·9H₂O</td>
<td>14 ± 2.3</td>
</tr>
<tr>
<td></td>
<td>Pr(1c)(H₂O)·9H₂O</td>
<td>13 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>Nd(1c)(H₂O)·9H₂O</td>
<td>0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>DMSO</td>
<td>0 ± 0.0</td>
</tr>
</tbody>
</table>
IR spectra of solid Ln(1c)₃(H₂O)₉H₂O

IR spectra of obtained complexes were taken in KBr pellets. Two regions of IR spectra were diagnostic for identification of coordination mode of anionic 1c to metal ions. The n(C=O) stretching vibration of 1a at 1653 cm⁻¹ undergoes hypsochromic shift into 1639 cm⁻¹ upon 4'-sulfonation to 1b (Fig. 3a). The n(C=O) band in anionic 1cNa occur at 1658 cm⁻¹. This band is also present at the same wavelength in all studied complexes Ln(1c)₃(H₂O)₉H₂O. Then it can be concluded the C(4)=O chromophore is not involved in metal binding in Ln(1c)₃(H₂O)₉H₂O complexes. On the other hand, the n(SO) stretching vibrations are considerably dependent on sulfonate group charge. There are two strong n(SO) bands observed in solid 1b (Fig. 3b) at 1223 and 1131 cm⁻¹ which are absent in the IR spectrum of 1a. These two bands are shifted considerably to 1228 and 1125 cm⁻¹, respectively upon deprotonation of sulfonate in 1cNa. In Ln(1c)₃(H₂O)₉H₂O complexes these two bands occur at 1221 and 1128 cm⁻¹; particularly the feature of n(SO)asym bands around 1125 cm⁻¹ are diagnostic for anionic sulfonate group presence in Ln(1c)₃(H₂O)₉H₂O complexes. Similar changes of n(SO)sym bands were observed within 1020-1040 cm⁻¹ region; there, the band at 1028 cm⁻¹ observed in 1b was split into two bands at 1027 and 1040 cm⁻¹ in 1cNa, and similarly at 1028 and 1042 cm⁻¹ in Ln(1c)₃(H₂O)₉H₂O complexes.

Studies of Ln(1c)₃(H₂O)₉H₂O in solutions

The Ln(1c)₃(H₂O)₉H₂O complexes are reasonably soluble in DMSO, DMF and in methanol. The conductivity measurements of their DMSO and DMF solutions indicated undoubtedly they behave as 1:1 electrolytes in both solvents (Table 2) (22). On the other hand the UV-Vis spectra of all the studied complexes in methanol showed unaltered UV ligand band in relation to 1cNa (Fig. 2) except tailoring of the band centered at 325 nm into Vis region, which is responsible for uncharacteristic beige color of the lanthanide complexes solutions.

The 1H NMR spectra of complexes were taken in DMSO-d₆ solvent. The position of all resonances of 1c ligand were unaltered in comparison with those of 1cNa. No paramagnetic shifted resonances were detected in the broad 200-100 ppm, neither the broadening of 1c resonances was observed in the spectra of the complexes despite the presence of unpaired f electrons in the complexes (f² in case of Nd²⁺ complex). This is in contrast with complexes of lanthanides with 1a (11), where large paramagnetic shifts were induced by C(4)=O; C(5)-O chelation to Tb³⁺, Ho³⁺, Er³⁺, and Yb³⁺.

Antibacterial activity

Lanthanides are known to possess good antibacterial activity and that they are used as antiseptic medicines. They are applied as complexes with inorganic ligands as well as with organic ones. The antibacterial action depends on the concentration of lanthanide ions (23). The antibacterial activities of 1cNa and its complexes with lanthanide ions were carried out against Escherichia coli (Gram negative) and Enterococcus hirae (Gram positive) using dilution method and cylinder plate diffusion method (Table 6, 7). From the above investigations it follows that Nachsa-4” is active against E. coli, MIC = 0.25 mg/mL but inactive against E. hirae. The complexes of La(III) and Nd(III) exhibited the same activity like 1cNa. However, the complex of Ce(III) shows inhibitory effect on the both tested strains, MIC = 0.5 mg/mL. Complex Pr(1c)₃(H₂O)₉H₂O also has MIC value of 0.5 mg/mL against E. hirae. The highest zone inhibition of 14 mm was recorded for complex of Ce(III) against E. hirae at concentration 1 mg/mL. It seems that the variation in the effectiveness of obtained complexes against bacterial tested strains arises from unique properties of the individual lanthanide ions.

The antimicrobial activity of flavonoids arises from their ability to affect three important biological processes that are essential for the survival bacterial cells. These include blocking of transcription and translation processes, inhibition of cytoplasm and membrane functions, and finally inhibition of energy metabolism. Similar mechanisms have been put forward to explain the antimicrobial activity exhibited by the flavonoid-metal ion complexes. The presence of metal ions in the complexes favors their binding to the enzymes in a covalent manner, thereby displaying better inhibitory activity than the parent flavonoid. Another important mode suggested for the inhibition of bacterial growth by flavonoid-metal ion complexes is their nonspecific intercalation with the DNA double helix, which in turn alters gene expression leading to arrest of cell division (24).

CONCLUSION

The series of Ln(1c)₃(H₂O)₉H₂O complexes were obtained from aqueous solutions at pH = 2 as solids. In these compounds the ligand provided three negative charges to neutralize Ln³⁺ central metal ion. Three molecules of water were strongly bound to metal ion as was deduced from thermal properties of the solids. The remaining nine molecules of water were weakly bonded in the solid Ln(1c)₃(H₂O)₉H₂O.
complexes. The solution studies indicated no hydroxyl group or carbonyl oxygen were involved in coordination to metal ion. Finally, the coordination sphere of those lanthanide(III) ions, which are typically 8 are presumably completed with bridging sulfonate oxygens, like it was observed in case of [Ba(chsa-8)]\(n\) (4) and [Eu(C\(\text{H}_8\text{O}_6\text{S})_2(\text{H}_2\text{O})_5]\(\infty\) (25) for which the X-ray structural data are available. According to the stoichiometry of the complexes described here, two sulfonate groups are bridging, while the third one belongs to monodentate chsa-4’ ligand, which dissociates easier than other two leading to 1 : 1 electrolyte behavior of Ln(1c)(H\(\text{O}_\text{3}\))\(9\)H\(2\text{O}\) complexes. Among compounds used in this investigation complex Ce(1c)(H\(\text{O}_\text{3}\))\(9\)H\(2\text{O}\) exhibits the highest antibacterial activity against tested strains presumably due to lanthanide contribution.

REFERENCES


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SYNTHESIS AND STUDY OF SUBSTITUTED AMIDES OF
ISOQUINOLINE-3- AND ISOQUINOLINE-1-CARBOXYLIC ACIDS
AS POTENTIAL ANTICONVULSANTS

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JAKUBOWICZ², PAULINA KLIMKIEWICZ¹, EWELINA LAL¹, MATEUSZ OCZKOWSKI¹,
EWA PRÓCHNIAK¹, ELŻBIETA PIRIANOWICZ-CHABER¹ and ALEKSANDER P. MAZUREK¹,²

¹Department of Drug Chemistry, Medical University of Warsaw, Banacha 1, 02-097 Warszawa, Poland
²National Medicines Institute, Chełmska 30/34, 00-725 Warszawa, Poland

Abstract: Previously obtained benzylamides of isoquinoline-3-carboxylic acids and isoquinoline-1-carboxylic
acids exhibited anticonvulsant activity. In search for more effective anticonvulsants, a series of amides of iso-
quinoine-3- and isoquinoline-1-carboxylic acids have been synthesized (1-9). The obtained compounds were
evaluated qualitatively for their anticonvulsant activity in the maximal electroshock seizure test (MES test),
imimal clonic seizure test (6Hz test) and subcutaneous metrazol seizure threshold test (sc MET test) as well
as in the rotorod neurotoxicity test (Tox test). One selected compound was tested quantitatively in 6Hz-test in
mice after intraperitoneal administration and showed activity ED₅₀ = 385.69 mg/kg and TD₅₀ > 600 mg/kg (1).

Keywords: anticonvulsants, substituted benzylamides of isoquinoline-1- and isoquinoline-3-carboxylic acids,
the minimal clonic seizure test, the rotorod neurotoxicity test

The therapy of epilepsy in the majority of cases
is based on pharmacological treatment. The fact that
there are many types of epilepsy makes the effec-
tiveness of treatment dependent on the choice of
appropriate medication based on adequate diagnosis
of the type of seizures and kind of epilepsy.
Antiepileptic drugs used currently may cause
numerous adverse side effects and are ineffective for
full control of epileptic fits for about 25-30% of the
patients with epilepsy (1-3). Therefore, there is a
need to design and synthesize new anticonvulsant
substances which would be more effective in the
therapy of epilepsy, especially in case of the drug-
resistant type, have a more advantageous pharma-
okinetic profile and cause fewer adverse side effects
as well as show a new mechanism of antiepileptic
action (4).

In our previous papers (5-10) we presented the
syntheses and results of the study of anticonvulsant
activity of a series of compounds with the structure
of aromatic amides derivative of heterocyclic and
isocyclic acids. Many of these compounds showed
anticonvulsant activity in in vivo tests for animal
types of epilepsy and relatively low neurotoxicity.
High activity of benzylamide of picolinic acid, for
which the value ED₅₀ was 17.8 mg/kg and therapeu-
index PI 28 in tests of maximal electroshock
(MES-test) conducted on rats after oral administra-
tion, was the reason why our attention was drawn to
the structure of this compound and why several
modifications were introduced to it (Fig. 1). The
results of the study of interdependencies between
the structure and the activity showed that the mutu-

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Figure 1. Structure of benzylamide of picolinic acid

MES ED₅₀=17.8 mg/kg (rats, p.o.)
TD₅₀ > 250 mg/kg

1111
al position of benzylamide group and the atom of nitrogen in heterocyclic ring of the acidic part is essential for anticonvulsant activity (6, 7). Moreover, it was observed that the increase of the distance between the heterocyclic ring of the acidic part and the amide group by introducing CH₂ group to the linear part of the molecule causes the reduction of anticonvulsant activity (6, 10). On the other hand, the introduction of an additional aromatic ring into the structure of heterocyclic ring of this compound allowed us to obtain derivatives of isoquinoline which were anticonvulsively active. The benzylamide of isoquinoline-3-carboxylic acid showed activity in mice (i.p.) in the maximal electroshock seizure test (MES) ED₅₀ = 117.77 mg/kg and in the rotord rod neurotoxicity test TD₅₀ > 500. Moreover, in the minimal clonic seizure test in mice (i.p.) both the benzylamide of isoquinoline-3-carboxylic acid and the benzylamide of isoquinoline-1-carboxylic acid showed activity with the dosage of 100 mg/kg (9). This indicates that the increase of the molecule in this part of the compound does not impair the anticonvulsant activity. The study conducted by Harold Kohn et al. showed that the substitution in the benzyl moiety significantly affects the anticonvulsant activity of lacosamide derivatives synthesized by the researchers (11-17). They observed that electron-donating substituents in its place (in the N-benzylamide group at the 4-site) may cause a significant rise of anticonvulsant activity (11, 13-17). On the other hand, our previous research indicated that substituting in the benzyl moiety by chloro-substituent led to compounds with diminished anticonvulsant profiles (10). Following these findings we designed and synthesized a series of analogues of benzylamide of isoquinoline-3-carboxylic acid and benzylamide of isoquinoline-1-carboxylic acid, into which we introduced electron-donating substituents or electron-withdrawing substituents into the N-benzyl group in the position 2 or 3 or 4 (Fig. 2). We synthesized fluoro-substituted (1, 2), methyl-substituted (3, 4, 5 and 6) and methoxy-substituted (7, 8) analogues. Compound 9 was synthesized in order to determine the influence of the molecule increase on the anticonvulsant activity also in its amide part by replacing the 6-membered aromatic ring with a bicyclic 6-membered aromatic ring. It has been observed that one of the elementary conditions responsible for the activity of these compounds is the appropriate lipophilicity of the molecule which ensures good solubility in body fluids and the penetration of biological barriers. The lipophilicity of a molecule may also influence the protein binding and the receptor affinity of the compound. Therefore, log P values of the partition coefficient between n-octanol and water of the compounds have been taken under consideration. As it was found previously, the optimal log P value of active benzylamide anticonvulsants ought to be > 0 and probably near to 3. However, it has been observed, that compounds with higher log P value can also show anticonvulsant activity (5-10). The goal of the undertaken research was to determine the influence of the changes on the anticonvulsant activity of the designed compounds, both in the case of the introduced changes into the size of the molecules and the electron effects and hydrophobic interactions.

**EXPERIMENTAL**

**Chemistry**

**General**

All used acids: isoquinoline-3-carboxylic acid and isoquinoline-1-carboxylic acid were purchased from Aldrich. Other reagents such as isobutyl chloroformate, N-methylmorpholine, 2-fluorobenzylamine, 4-fluorobenzylamine, 3-methylbenzylamine, 4-methylbenzylamine, 2-methoxybenzylamine and 4-methoxybenzylamine were supplied from Merck. DMF and THF were from POCH Gliwice. ¹H NMR spectra were recorded on a Varian VNMRS 300 MHz spectrometer. The chemical shifts were measured as δ units (ppm) relative to tetramethylsilane. Mass spectra were taken on Qualtra LC.LR spectrometer and
Table 1. Physical and analytical data of the synthesized compounds.

<table>
<thead>
<tr>
<th>Compound No.</th>
<th>Aryl site</th>
<th>Structure</th>
<th>R</th>
<th>Formula</th>
<th>M.w.</th>
<th>M.p. °C</th>
<th>log P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A</td>
<td>4</td>
<td>F</td>
<td>C₇H₁₃N₂OF</td>
<td>280,30</td>
<td>96-98</td>
<td>3.47</td>
</tr>
<tr>
<td>2</td>
<td>B</td>
<td>2</td>
<td>F</td>
<td>C₇H₁₃N₂OF</td>
<td>280,30</td>
<td>130-132</td>
<td>3.47</td>
</tr>
<tr>
<td>3</td>
<td>A</td>
<td>3</td>
<td>CH₃</td>
<td>C₆H₁₄N₂O</td>
<td>276,34</td>
<td>76-78</td>
<td>3.79</td>
</tr>
<tr>
<td>4</td>
<td>A</td>
<td>4</td>
<td>CH₃</td>
<td>C₆H₁₄N₂O</td>
<td>276,34</td>
<td>95-97</td>
<td>3.79</td>
</tr>
<tr>
<td>5</td>
<td>B</td>
<td>3</td>
<td>CH₃</td>
<td>C₆H₁₄N₂O</td>
<td>276,34</td>
<td>76-77</td>
<td>3.92</td>
</tr>
<tr>
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<td>B</td>
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<td>CH₃</td>
<td>C₆H₁₄N₂O</td>
<td>276,34</td>
<td>68-69</td>
<td>3.92</td>
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<tr>
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<td>OCH₃</td>
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<td>99-100</td>
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<td>A</td>
<td>4</td>
<td>OCH₃</td>
<td>C₆H₁₄N₂O₂</td>
<td>292,34</td>
<td>96-97</td>
<td>3.07</td>
</tr>
<tr>
<td>9</td>
<td>C</td>
<td>-</td>
<td>-</td>
<td>C₂₁H₁₆N₂O</td>
<td>312,38</td>
<td>148-149</td>
<td>3.99</td>
</tr>
</tbody>
</table>

*Lipophilicity of the compounds is expressed as log P value calculated by a computer method (19).

Table 2. 1H NMR spectra of the synthesized compounds.

<table>
<thead>
<tr>
<th>Compound No.</th>
<th>Chemical shift in δ (ppm) in CDCl₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.69 (d, J = 6.3 Hz, 2H, CH₂), 6.98-7.08 (m, 2H, 2xCH), 7.34-7.44 (m, 2H, 2xCH), 7.66-7.77 (m, 2H, 2xCH), 7.80-7.88 (m, 2H, 2xCH), 8.45 (d, J = 5.4 Hz, 1H, CH), 8.60 (s br, 1H, NH), 9.60-9.68 (m, 1H, CH)</td>
</tr>
<tr>
<td>2</td>
<td>4.82 (d, J = 6.3 Hz, 2H, CH₂), 7.06-7.18 (m, 2H, 2xCH), 7.24-7.34 (m, 1H, CH), 7.44-7.52 (m, 1H, CH), 7.68-7.84 (m, 2H, 2xCH), 7.98-8.08 (m, 2H, 2xCH), 8.64 (s br, 1H, NH), 8.67 (s, 1H, CH), 9.17 (s, 1H, CH)</td>
</tr>
<tr>
<td>3</td>
<td>2.35 (s, 3H, CH₃), 4.69 (d, J = 5.7 Hz, 2H, CH₂), 7.23-7.26 (m, 4H, 4xCH), 7.69-7.79 (m, 2H, 2xCH), 7.84-7.90 (m, 2H, 2xCH), 8.45 (d, J = 5.7 Hz, 1H, CH), 8.61 (s br, 1H, NH), 9.63 (d, J = 7.8 Hz, 1H, CH)</td>
</tr>
<tr>
<td>4</td>
<td>2.34 (s, 3H, CH₃), 4.69 (d, J = 5.7 Hz, 2H, CH₂), 7.15-7.19 (m, 2H, 2xCH), 7.31-7.36 (m, 2H, 2xCH), 7.71-7.83 (m, 2H, 2xCH), 7.86-7.92 (m, 2H, 2xCH), 8.45 (d, J = 5.7 Hz, 1H, CH), 8.69 (s br, 1H, NH), 9.58-9.64 (m, 1H, CH)</td>
</tr>
<tr>
<td>5</td>
<td>2.35 (s, 3H, CH₃), 4.70 (d, J = 6 Hz, 2H, CH₂), 7.10 (d, J = 6.9 Hz, 1H, CH), 7.18-7.28 (m, 3H, 3xCH), 7.67-7.80 (m, 2H, 2xCH), 8.01 (t, J = 7.5 Hz, 2H, 2xCH), 8.55 (s br, 1H, NH), 8.66 (s, 1H, CH), 9.14 (s, 1H, CH)</td>
</tr>
<tr>
<td>6</td>
<td>2.35 (s, 3H, CH₃), 4.69 (d, J = 6 Hz, 2H, CH₂), 7.15-7.19 (m, 2H, 2xCH), 7.31 (d, J = 8 Hz, 2H, 2xCH), 7.67-7.82 (m, 2H, 2xCH), 8.02 (t, J = 5 Hz, 2H, 2xCH), 8.55 (s br, 1H, NH), 8.66 (s, 1H, CH), 9.12 (s, 1H, CH)</td>
</tr>
<tr>
<td>7</td>
<td>3.90 (s, 3H, CH₃), 4.72 (d, J = 6 Hz, 2H, CH₂), 6.88-6.97 (m, 2H, 2xCH), 7.24-7.30 (m, 1H, CH), 7.38-7.43 (m, 1H, CH), 7.64-7.74 (m, 2H, 1H, CH), 7.76-7.80 (m, 1H, CH), 8.72-8.86 (m, 1H, CH), 8.43 (d, J = 5.7 Hz, 1H, CH), 8.60 (s br, 1H, NH), 9.59-9.65 (m, 1H, CH)</td>
</tr>
<tr>
<td>8</td>
<td>3.80 (s, 3H, CH₃), 4.66 (d, J = 5 Hz, 2H, CH₂), 6.87-6.92 (m, 2H, 2xCH), 7.33-7.38 (m, 2H, 2xCH), 7.68-7.78 (m, 2H, 2xCH), 7.80-7.88 (m, 2H, 2xCH), 8.44 (d, J = 5.4 Hz, 1H, CH), 8.55 (s br, 1H, NH), 9.61-9.66 (m, 1H, CH)</td>
</tr>
<tr>
<td>9</td>
<td>5.19 (d, J = 5.7 Hz, 2H, CH₂), 7.40-7.64 (m, 4H, 4xCH), 7.68-7.84 (m, 6H, 6xCH), 8.19 (d, J = 8.1 Hz, 1H, CH), 8.39 (d, J = 5.7 Hz, 1H, CH), 8.61 (s br, 1H, NH), 9.68 (d, J = 9.6 Hz, 1H, CH)</td>
</tr>
</tbody>
</table>

4000 QTRAP spectrometer. TLC was carried out on a 0.25 mm thickness silica gel plates (Merck Kieselgel 60 F-254). The spots were visualized in UV light or with 0.3% ninhydrin in EtOH/CH₃COOH (97 : 3, v/v). The solvent system used in TLC was CHCl₃/MeOH in different ratios. HPLC was performed on a Shimadzu chromatograph equipped with LC-10AT pump, SPD-10A UV spectrophotometer and a computer registrator/recorder (ChromaX 2010 POLLAB, Warsaw). The peaks were recorded at 271
Scheme 1. Synthesis of compounds 1-8

1. Isoquinoline-1-carboxylic acid 4-fluorobenzylamide
   \[R_1 = \text{structure}, \quad R_2 = H, \quad R_3 = H, \quad R_4 = F\]

2. Isoquinoline-3-carboxylic acid 2-fluorobenzylamide
   \[R_1 = \text{structure}, \quad R_2 = F, \quad R_3 = H, \quad R_4 = H\]

3. Isoquinoline-1-carboxylic acid 3-methylbenzylamide
   \[R_1 = \text{structure}, \quad R_2 = H, \quad R_3 = CH_3, \quad R_4 = H\]

4. Isoquinoline-1-carboxylic acid 4-methylbenzylamide
   \[R_1 = \text{structure}, \quad R_2 = H, \quad R_3 = H, \quad R_4 = CH_3\]

5. Isoquinoline-3-carboxylic acid 3-methylbenzylamide
   \[R_1 = \text{structure}, \quad R_2 = H, \quad R_3 = CH_3, \quad R_4 = H\]

6. Isoquinoline-3-carboxylic acid 4-methylbenzylamide
   \[R_1 = \text{structure}, \quad R_2 = H, \quad R_3 = H, \quad R_4 = CH_3\]

7. Isoquinoline-1-carboxylic acid 2-methoxybenzylamide
   \[R_1 = \text{structure}, \quad R_2 = \text{OCH}_3, \quad R_3 = H, \quad R_4 = H\]

8. Isoquinoline-1-carboxylic acid 4-methoxybenzylamide
   \[R_1 = \text{structure}, \quad R_2 = H, \quad R_3 = H, \quad R_4 = \text{OCH}_3\]
Synthesis of amides
The compounds 1-9 were synthesized using the mixed anhydrides method of peptide synthesis (18). The suitable acid (10 mM) was dissolved in DMF (15 mL) and THF (15 mL) was added. Next N-methylmorpholine (10 mM, 1.1 mL) was added and the mixture was stirred under nitrogen and chilled to -15°C. Isobutyl chloroformate (10 mM, 1.3 mL) was added dropwise to keep the temperature below -15°C. Isobutyl chloroformate (10 mM, 1.3 mL) was added dropwise to keep the temperature below -15°C. Then, the suitable amine: 2- or 4-fluorobenzylamine (2-F-BZA, 4-F-BZA); 2- or 4-methoxybenzylamine (2-OMe-BZA, 4-OMe-BZA); 3- or 4-methylbenzylamine (3-Me-BZA, 4-Me-BZA) or 1-naphthylmethylamine (10 mM) in THF was added in small portions and the reaction mixture was stirred at -15°C for 30 min and at room temperature for 1 h. The solution was concentrated in vacuo and the residue was dissolved in CHCl₃ (40 mL). This solution was washed with 20 mL portions of 1M HCl, saturated NaHCO₃ solution and saturated NaCl solution, then dried with anhydrous MgSO₄, filtered and concentrated in vacuo. The obtained compounds were purified by crystallization with EtOAc/hexane or MeOH/Et₂O. All stages of the synthesis were controlled by TLC. The purity and identity of the final compounds were determined by HPLC, elemental analyses, 1H NMR, MS. The elemental analyses were within ± 0.4% of the theoretical value. The analytical data confirmed that the purity of the products was ≥ 95%. The general procedure for the synthesis of the obtained compounds is shown in Schemes 1 and 2.

Computer calculations
The HyperChem 8.0 (Hypercube, Inc.) program was used. The semiempirical method PM 3 was applied for a single point calculation. Geometry
Table 4. Evaluation as anticonvulsants (ASP, mice i.p.) (20).

<table>
<thead>
<tr>
<th>Compound No.</th>
<th>6 Hz, 44 mA, 0.5 h</th>
<th>MES, 0.5 h</th>
<th>TOX, 0.5 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dose (mg/kg)</td>
<td>N/F</td>
<td>Dose (mg/kg)</td>
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<td>100</td>
<td>1/4</td>
<td>100</td>
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<tr>
<td></td>
<td>300</td>
<td>3/4 (3/4)</td>
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<td>2</td>
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<tr>
<td></td>
<td>300</td>
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</tbody>
</table>

6 Hz = minimal clonic seizure test model 44 mA (ASP), 44 mA (ASP), MES = maximal electroshock seizure test, TOX = neurological toxicity rotorod test (23), N/F = number of animals active or toxic over the number tested, after 2 h.

Table 5. Quantification (ASP, 6 Hz, 44 mA, TOX, mice i.p.) (21).

<table>
<thead>
<tr>
<th>Compound No.</th>
<th>Test</th>
<th>ED 50 (0.5 h) mg/kg</th>
<th>TD 50 (0.5 h) mg/kg</th>
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<tr>
<td>1</td>
<td>6 Hz</td>
<td>385.69</td>
<td>TOX &gt;600</td>
</tr>
<tr>
<td>Valproic acid</td>
<td>6 Hz</td>
<td>310 (126)</td>
<td>TOX 398</td>
</tr>
<tr>
<td>Lacosamide</td>
<td>6 Hz</td>
<td>10</td>
<td>TOX 27</td>
</tr>
</tbody>
</table>

6 Hz = minimal clonic seizure test model 44 mA (ASP), TOX = neurological toxicity (23).

Table 6. ED 50 biological response of 1 (ASP evaluation, mice i.p.) (22).

<table>
<thead>
<tr>
<th>Test</th>
<th>Time (h)</th>
<th>Dosage (mg/kg)</th>
<th>Protected/used</th>
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<tr>
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<td>250</td>
<td>1/8</td>
</tr>
<tr>
<td>6 Hz</td>
<td>0.50</td>
<td>325</td>
<td>2/8</td>
</tr>
<tr>
<td>6 Hz</td>
<td>0.50</td>
<td>400</td>
<td>6/8</td>
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<tr>
<td>6 Hz</td>
<td>0.50</td>
<td>550</td>
<td>5/8</td>
</tr>
<tr>
<td>6 Hz</td>
<td>0.50</td>
<td>650</td>
<td>8/8</td>
</tr>
<tr>
<td>TOX</td>
<td>0.50</td>
<td>400</td>
<td>0/4</td>
</tr>
<tr>
<td>TOX</td>
<td>0.50</td>
<td>600</td>
<td>0/8</td>
</tr>
</tbody>
</table>

6 Hz = minimal clonic seizure test model 44 mA (ASP), TOX = neurological toxicity (23).
optimization was performed by a Polak-Ribiere algorithm. Afterward the QSAR Properties module using atomic parameters derived by Ghose et al. (19) was applied to calculate log P values as a measure of the lipophilicity of the optimized structures of the compounds. RMS Fit module was used for overlaying the molecular structures of the compounds.

**Pharmacology**

The compounds (1-9) were evaluated qualitatively for anticonvulsant activity and neurotoxicity in anticonvulsant screening project (ASP) in the MES test, 6 Hz test and TOX tests. All the obtained compounds were administered intraperitoneally (i.p.) to mice. The tests of MES and TOX were performed according to the procedures described by Krall et al. (20). The maximal electroshock (MES) and the minimal clonic seizure tests (6Hz, 32 mA or 44 mA, ASP no. 31 test or no. 33 test, respectively) were used to assess a compound’s efficacy against electrically induced seizures by using a frequency 60 Hz and 0.2 s of the duration of stimulation in the first test as well as by using a lower frequency 6 Hz and longer duration time - 3 s in the second test. The MES test is appropriately accepted as a model for the generalized tonic-clonic seizures. On the other hand, the minimal clonic seizure (6 Hz) test is described as being similar to the partial complex seizures and is considered a model of resistance epilepsy. The results from these tests are expressed as N/F, where N equals the number of animals protected and F equals the number of animals tested (Table 4). For tests of toxicity (TOX), the result is presented as the number of animals displaying toxic effects out of the number of animals tested (Table 4). Compound 1 was examined quantitatively in the minimal clonic seizures (6Hz) in mice after i.p. administration (ASP no. 7 test) using the method described by Barton et al. (21). Therefore, the effective dose ED_{50} value and the median dose for neurological impairment TD_{50} were determined. The obtained results are given in Table 5. Moreover, the ED_{50} biological responses of compound 1 are given in Table 6 (22).

**RESULTS**

Physical and analytical data of the synthesized compounds are given in Tables 1, 2 and 3. The results of preliminary pharmacological tests are presented in Tables 4-6.

**DISCUSSION**

The results of previously conducted studies show that the substitution of the pyridine ring in highly active structures obtained earlier for the bicyclic, isoquinoline ring in the acidic part of the compounds does not cause the disappearance of anticonvulsant activity. However, in benzylamides derivative of isoquinoline obtained in this way we observed a significant decrease of activity as compared with the parent compound (9). It might indicate that the increase in the size of the molecule in this part of the compound may either hinder the binding with the binding site of the receptor or may change the permeability through biological membranes. It has been reported that the introduction of an electron-withdrawing substituent to N-benzylamide moiety may cause the increase in anticonvulsant activity (11, 13-17). This observation was confirmed by the results of our previous investigations (MES ED_{50} = 14.70 mg/kg for 2-fluorobenzylamide of picolinic acid and MES ED_{50} = 17.80 mg/kg for benzylamide of picolinic acid) (7). Therefore, it was decided to prepare two different series of substituted amides of isoquinoline-3- and isoquinoline-1-carboxylic acids in the present study. The first group of compounds contained an electron-withdrawing fluoro or a methoxy group at the 2 – or 4 – positions of the N-benzylamide moiety (1, 2, 7 and 8). Additionally, in order to determine the effect of the modification of the structural size of the N-benzyl moiety on anticonvulsant activity, there were synthesized methyl-substituted analogues (3, 4, 5 and 6) and the compound in which the benzyl group was replaced by the 1-naphthylmethyl group (9). All the obtained compounds were evaluated qualitatively for their anticonvulsant activity (MES-test, 6 Hz-test) and neurotoxicity (Tox test) (Table 4). Among them compounds 1 and 2 provided seizure protection in 6 Hz-test (respectively, 3/4 mice at a dose 300 mg/kg and 4/4 mice at the same dose). On the basis of the preliminary results compound 1 was put to further tests - quantitative 6 Hz test and neuroprotection evaluation, in which it showed activity ED_{50} = 385.69 mg/kg and toxicity TD_{50} > 600 (Table 5). Comparing the anticonvulsant activities in 6 Hz test for fluoro-substituted derivatives (1, 2) and the unsubstituted parent compound, we observed considerable decrease in activity. These findings show that electron-withdrawing substituents directly attached to the N-benzylamide moiety of isoquinoline derivatives do not improve anticonvulsant activity. In turn, the introduction of methoxy group, containing oxygen, into the same position let us obtain compounds (7, 8) which were not active in 6 Hz test in the highest administered doses (Table 4). Comparison of the anticonvulsant activities in 6Hz test (44 mA) for the compound 1 (ED_{50} = 385.69 mg/kg)
mg/kg) and the reference compounds: valproic acid (ED$_{50}$ = 310 mg/kg) and lacosamide (ED$_{50}$ = 10 mg/kg) demonstrated that compound 1 exhibited similar to valproic acid, but much lower than lacosamide seizure protection (Table 5). However, it has been reported that increasing the current intensity to 44 mA (6 Hz test) resulted in a marked reduction in potency for valproic acid (21, 24). In addition, we noticed a lack of seizure protection in MES-test for both the fluoro-substituted and the methoxy-substituted analogues (Table 4). This result was surprising because the previously obtained parent compound exhibited anticonvulsant activity in MES test in mice (ED$_{50}$ = 117.77 mg/kg; TD$_{50}$ > 500 mg/kg). However, it is difficult to determine whether it might be the result of the electron effect caused by these substituents or whether it is caused by the increase of the molecule in the amide part. Preliminary tests also show that compounds (3, 4, 5, and 6) without respect to the position of substitution are inactive and not toxic. These results confirmed the previous findings that the electron-donating substituents in benzyl site would yield derivatives of decreased activity or not active at all (11, 13, 14). The result of the pharmacological tests for compound 9 showed that increases in the size of the substituent of amide nitrogen atom would result in a loss in activity (Table 4). This observation seems to show a decrease of receptor affinity of such a structure.

Acknowledgement

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REFERENCES


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Iminothiazoline ring is present as a core in a number of biologically active heterocyclic compounds and natural products. Significant pharmaceutical and other important applications associated with thiazoline nucleus made it attractive candidate to be synthesized and explore for its versatile medicinal properties (1-3). Coumarin derivatives of iminothioazolines were reported to possess significant anticonvulsant activities against PTZ induced seizures (4). Sydnonyl-thiazoline derivatives like 2-[(3-arylsydnon-4-ylmethylene)hydrazono]-4-methyl-3-phenyl-2,3-dihydro-thiazole-5-carboxylic acid ethyl esters and 3,4-diphenyl-2-[(3-arylsydnon-4-ylmethylene)hydrazono]-2,3-dihydrothiazoles exhibit robust antioxidant activity (5). Chloro-thiazoline derivatives are potent, selective and less toxic antimicrobial agent (6) and were also known to exhibit good antimycobacterial activity (7).

Iminothiazoline derivatives inhibit indoleamine-N-methyl transferase enzyme, which catalyzes the biosynthetic steps of some psychomimetic agents (8), and are useful for the treatment of schizophrenia in humans. Pifithrin-α (PFT-α) is a reversible inhibitor of p53-mediated apoptosis and p53-dependent gene transcription and potent to improve therapeutic selectivity and higher doses of cytotoxic treatments on humans and also helps in the protection against many genotoxic agents (9). A wide variety of iminothiazoline analogues were found to be highly active against Schistosoma mansoni (10), they also possess an interesting anti-HIV and anticancer activities (11, 12). Acridinyl-thiazoline derivatives have been found to exhibit moderate CDK1 inhibitory activities against kinase CDK1 (13). Thiazoline derivative possesses significant kinase CDK1 inhibitory properties. 2-Phenylimino-1,3-thiazoline-4-acetamide possesses remarkable activity against Pyricularia oryzae fungus (14). 2-Imino-1,3-thiazoline derivative KHG22394 is skin whitening agent due to its ability to inhibit melanin production (15). Iminothiazoline containing heterocycles exhibit potent antihistaminic activity (16), serve as potent plant growth regulators (17), they exhibit good hypnotic activity (18) also possess significant anti-inflammatory and analgesic properties (19). Bis-thiazoline derivatives have shown remarkable antitumor activity against various human cell lines (20). We planned the synthesis of ethyl 4-[2-benzamido-4-methylthiazol-3(2H)-yl]benzoates (2a-j) starting from ethyl 4-(3-benzoylthiourea)benzoates (1a-j). (Scheme 1)

RESULTS AND DISCUSSION

Ethyl 4-[2-benzamido-4-methylthiazol-3(2H)-yl]benzoates (2a-j) were efficiently synthesized by
base-catalyzed direct cyclization of ethyl ester thioureas by treating with triethylamine and bromoacetone, produced in situ with bromine in dry acetone to get thiazoline derivatives (2a-j) in good yields (Scheme 1). The proposed structures of all the newly synthesized compounds were in full agreement with their spectral data FTIR, $^{1}H$ NMR, $^{13}C$ NMR and mass spectrometric analysis.

The FTIR spectral data of thiazoline derivatives showed the disappearance of characteristic N-H stretching peaks at 3298-3332 cm$^{-1}$ and appearance of new absorption peaks for imine moeity (C=N) observed at 1643-1654 cm$^{-1}$. (C-S) absorptions for thiazoline ring were appeared at 1147-1176 cm$^{-1}$ and the aromatic (C-C) absorption peaks were observed at 1574-1587 cm$^{-1}$. The $^{1}H$ NMR data exhibited a characteristic singlet for thiazoline proton at $\delta$ 6.41-6.48 ppm and the singlet of the CH$_3$ protons substituted on thiazoline ring was observed at $\delta$ 2.62-2.71 ppm. The $^{13}C$ NMR spectra showed the characteristic olefinic carbons of the thiazoline ring in the range of $\delta$ 101.2-101.9 ppm confirming the ring closure. Signal for the carbon of the methyl group was observed at $\delta$ 21.3-21.8 ppm and imino carbon signal appeared at $\delta$ 162.4-163.6 ppm. Two amidic and ester carbonyl carbon signals were observed at $\delta$ 168.2-168.9 ppm and $\delta$ 166.2-166.8 ppm. The EIMS spectra displayed the base peaks at $m/z$ 261 and further the structures of the title compounds were also confirmed by elemental analysis.

The antibacterial evaluation of ethyl 4-[2-benzamido-4-methylthiazol-3(2H)-yl]benzoates (2a-j) exhibited that most of the compounds were active against various Gram negative and Gram positive bacterial strains. It was found that halogen substituted thiazoline derivatives (2b), (2c), (2i) and (2j) showed good antibacterial activities against various bacterial strains as compared to other compounds of the series (Table 1).

**EXPERIMENTAL**

**Instruments**

$^{1}H$ NMR were recorder on a Brucker AM-300 spectrophotometer and chemical shifts of $^{1}H$ NMR were reported in parts per million (ppm). The melting points were determined on Stuart SMP3 melting point apparatus and uncorrected. FT-IR spectra were recorded using Shimadzu IR 460 spectrophotometer by Attenuated Total Reflectance (ATR) method. The elemental analysis was performed on leco CHNS-932 analyzer.

**General procedure for the synthesis of ethyl 4-[2-benzamido-4-methylthiazol-3(2H)-yl] benzoates (2a-j)**

To a stirred solution of ethyl 4-(3-benzoyl-thioureido)benzoates (1.0 g, 3 mM) dissolved in 15-20 mL of dry acetone, triethylamine (400 µL, 3 mM) was dropwise added through the rubber septum with the help of a syringe under inert atmosphere. Bromine solution (300 µL, 3 mM) in dry acetone (10 mL) was added dropwise and the reaction mixture was stirred for 3-4 h. Completion of reaction was monitored by TLC. Upon completion, the reaction mixture was filtered and the filtrate was concentrated on rotary under revaporator reduced pressure to get crude ethyl 4-[2-benzamido-4-methylthiazol-3(2H)-yl]benzoates (2a-j) which were then recrystallized from ethanol.

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Facile synthesis and antibacterial investigation of new ethyl... 1121

Ethyl 4-[2-benzamido-4-methylthiazol-3(2H)-yl]benzoates (2a)

Yield 68%; Rf 0.4; m.p. 108-110°C; IR (KBr) ν max: 3046 (C=C-H), 1724 (ester C=O), 1676 (amide C=O), 1643 (C=N), 1578 (C=C), 1147 (C-S)/cm -1; 1H NMR (CDCl3, δ, ppm): 7.86 (2H, d, J = 7.4 Hz, H-2í,H-6í), 7.79 (2H, d, J = 7.8 Hz, H-3, H-5), 7.62 (1H, d, J = 7.1 Hz, H-4í), 7.55 (2H, dd, J = 7.4, 7.1 Hz, H-3í,H-5í), 6.63 (2H, d, J = 7.8 Hz, H-2,H-6), 6.42 (1H, s, C=C-H), 4.31 (2H, q, J = 7.1 Hz, -CH2), 2.23 (3H, t, J = 5.6 Hz, -CH3), 2.64 (-CH3); 13C NMR (CDCl3, δ, ppm): 168.4 (amide C=O), 166.2 (ester C=O), 162.5 (N=C), 150.3 (N-C=), 145.5 (C-1), 136.6 (C-1í), 134.5 (C-4í), 132.2 (C-3,C-5), 131.2 (C-2í,C-6í), 128.3 (C-6í), 122.7 (C-4í), 101.6 (ester-CH3); MS (70 eV): m/z (%); [M+.] 366 (49), 261 (100%), 217 (26), 188 (53), 112 (18), 105 (21), 96 (31), 77 (20); Anal. Calcd. for C20H18N2O3S; C, 65.55; H, 4.94; N, 7.63; S, 8.75%; found: C, 65.46; H, 4.83; N, 7.48; S, 8.62%.

Ethyl 4-[2-(3-chlorobenzamido)-4-methylthiazol-3(2H)-yl]benzoate (2b)

Yield 70%; Rf 0.5; m.p. 87-88°C; IR (KBr) ν max: 3051 (C=C-H), 1734 (ester C=O), 1692 (amide C=O), 1657 (N=C), 1160 (C-S) cm -1; 1H NMR (CDCl3, δ, ppm): 7.93 (1H, d, J = 2.3 Hz, H-2í), 7.84 (2H, d, J = 7.8 Hz, H-3,H-5), 7.75 (1H, d, J = 7.2 Hz, H-6í), 7.66 (1H, dd, J = 7.1,2.2 Hz, H-4í), 7.47 (1H, dd, J = 7.2,7.1 Hz, H-5í), 6.72 (2H, d, J = 7.8 Hz, H-2,H-6), 6.46 (1H, s, C=C-H), 4.37 (2H, q, J = 7.1 Hz, -CH3), 2.27 (3H, t, J = 5.6 Hz, -CH3), 2.66 (-CH3); 13C NMR (CDCl3, δ, ppm): 168.6 (amide C=O), 166.5 (ester C=O), 163.2 (N=C), 150.6 (N-C=), 145.5 (C-1í), 137.6 (C-1), 153.5 (C-3í), 134.4 (C-4í), 132.6 (C-3,C-5), 131.4 (C-5í), 130.2 (C-2í), 128.3 (C-6í), 122.7 (C-4í), 118.2 (C-2,C-6í), 101.6 (ester-CH3); MS (70 eV): m/z (%); [M'] 400.5 (74), 402.5 (61), 261 (100%), 188 (30), 139.5 (36), 112 (23), 111.5 (27), 96 (18), 77 (34); Anal. Calcd. for C20H17N2O3SCl; C, 59.91; H, 4.26; N, 6.98; S, 7.98%; found: C, 59.83; H, 4.16; N, 6.87; S, 7.86%.

Ethyl 4-[2-(2,4-dichlorobenzamido)-4-methylthiazol-3(2H)-yl]benzoate (2c)

Yield 76%; Rf 0.5; m.p. 97-98°C; IR (KBr) ν max: 3058 (C=C-H), 1734 (ester C=O), 1692 (amide C=O), 1657 (N=C), 1160 (C-S) cm -1; 1H NMR (CDCl3, δ, ppm): 8.68 (2H, d, J = 7.8 Hz, H-3,H-5), 7.73 (1H, d, J = 7.1 Hz, H-6í), 7.57 (1H, s, H-3í), 7.48 (1H, d, J = 7.1 Hz, H-5í), 6.68 (2H, d, J = 7.8 Hz, H-2,H-6í), 6.48 (1H, s, C=C-H), 4.39 (2H, q, J = 7.1 Hz, -CH3), 2.31 (3H, t, J = 5.6 Hz, -CH3), 2.68 (-CH3); 13C NMR (CDCl3, δ, ppm): 168.7 (N-C=O), 166.7 (ester C=O), 163.4 (N=C), 150.7 (N-C=), 145.7 (C-1í), 140.6 (C-4í), 137.4 (C-2í), 136.5 (C-1), 133.4 (C-6í), 132.7 (C-3í), 131.4 (C-3í), 128.5 (C-5í), 122.6 (C-4í), 118.4 (C-2,C-6í), 101.8 (ester-CH3); MS (70 eV): m/z (%); [M'] 434 (56), 261 (100%), 188 (37), 173 (27), 145 (46), 112 (43), 111.5 (33), 77 (24); Anal. Calcd. for C20H16N2O3SCl2; C, 55.17; H, 3.69; N, 6.43; S, 6.37%; found: C, 55.11; H, 3.53; N, 6.34; S, 6.28%.
Ethyl 4-[4-methyl-2-(4-methylbenzamido)thiazol-3(2H)-yl]benzoate (2d)

Yield 74%; Rf 0.45; m.p. 118-119°C; IR (KBr)
v<sub>max</sub>: 3052 (C=C-H), 1725 (ester C=O), 1683 (amide C=O), 1646 (N=C), 1574 (C=N), 1147 (C-S) cm<sup>-1</sup>; ¹H NMR (CDCl₃, δ, ppm): 7.88 (2H, d, J = 7.8 Hz, H-3, H-5), 7.76 (2H, d, J = 7.3 Hz, H-2, H-6), 7.36 (2H, d, J = 7.3 Hz, H-3', H-5'), 6.65 (2H, d, J = 7.8 Hz, H-2', H-6'), 6.43 (1H, s, C=C-H), 4.34 (2H, q, J = 7.1 Hz, -CH₂), 2.26 (3H, t, J = 5.6 Hz, -CH₂), 2.75 (Ar-CH₃), 2.65 (-CH₃); ¹³C NMR (CDCl₃, δ, ppm): 168.5 (amide C=O), 166.4 (ester C=O), 162.4 (N=C), 150.3 (N=C=), 145.3 (C-1), 134.7 (C-4'), 133.5 (C-1'), 132.2 (C-3, C-5), 131.5 (C-2', C-6'), 130.6 (C-3', C-5'), 122.4 (C-4'), 117.2 (C-2, C-6), 101.4 (s=CH), 61.3 (-CH₂), 24.7 (Ar-CH₃), 15.4 (ester-CH₃); MS (70 eV): m/z (%); [M⁺] 380 (61), 261 (100%), 188 (34), 135 (53), 112 (29), 107 (38), 77 (20); Anal. calcd. for C₂₁H₂₀N₂O₃S; C, 63.61; H, 5.07; N, 7.07%; S, 8.09%; found: C, 63.53; H, 4.96; N, 7.95; S, 8.01%.

Ethyl 4-[4-methyl-2-(3-methylbenzamido)thiazol-3(2H)-yl]benzoate (2e)

Yield 77%; Rf 0.45; m.p. 112-113°C; IR (KBr)
v<sub>max</sub>: 3054 (C=C-H), 1726 (ester C=O), 1685 (amide C=O), 1451 (C≡N), 1583 (C≡C), 1153 (C-S) cm<sup>-1</sup>; ¹H NMR (CDCl₃, δ, ppm): 7.86 (2H, d, J = 7.8 Hz, H-3, H-5), 7.78 (1H, d, J = 2.4 Hz, H-2'), 7.73 (1H, d, J = 7.2 Hz, H-6'), 7.65 (1H, dd, J = 7.1, 2.4 Hz, H-4'), 7.58 (1H, dd, J = 7.2, 7.1 Hz, H-5'), 6.71 (2H, d, J = 7.8 Hz, H-2, H-6), 6.44 (1H, s, C=C-H), 4.36 (2H, q, J = 7.1 Hz, -CH₂), 2.25 (3H, t, J = 5.6 Hz, -CH₂), 2.73 (Ar-CH₃), 2.64 (-CH₃); ¹³C NMR (CDCl₃, δ, ppm): 168.6 (amide C=O), 166.3 (ester C=O), 162.6 (N=C), 150.6 (N=C=), 145.5 (C-1), 137.6 (C-3'), 136.3 (C-1'), 135.4 (C-4'), 132.2 (C-3, C-5), 131.3 (C-2'), 128.6 (C-1'), 124.8 (C-6'), 121.7 (C-4), 120.3 (C-2, C-6), 118.2 (C-5'), 117.5 (C-2'), 101.5 (s=CH), 61.2 (-CH₂), 56.4 (3'-OCH₃), 56.2 (4'-OCH₃), 21.3 (-CH₃), 15.2 (ester-CH₃); MS (70 eV): m/z (%); [M⁺] 426 (55), 261 (100%), 188 (39), 165 (52), 137 (41), 107 (38), 77 (19); Anal. calcd. for C₂₁H₂₁N₂O₃S; C, 61.95; H, 5.19; N, 6.56; S, 7.52%; found: C, 61.88; H, 5.12; N, 6.43; S, 7.44%.

Ethyl 4-[4-methyl-2-(2-bromobenzamido)thiazol-3(2H)-yl]benzoate (2f)

Yield 73%; Rf 0.5; m.p. 128-129°C; IR (KBr)
v<sub>max</sub>: 3045 (C=C-H), 1722 (ester C=O), 1675 (amide C=O), 1543 (C≡N), 1582 (C≡C), 1160 (C-S) cm<sup>-1</sup>; ¹H NMR (CDCl₃, δ, ppm): 7.89 (2H, d, J = 7.8 Hz, H-3, H-5), 7.81 (1H, d, J = 7.4 Hz, H-6), 7.77 (1H, dd, J = 7.2, 2.2 Hz, H-3'), 7.54 (1H, dd, J = 7.2, 7.1 Hz, H-4'), 7.46 (1H, dd, J = 7.3, 7.1 Hz, H-5'), 6.71 (2H, d, J = 7.8 Hz, H-2, H-6), 6.43 (1H, s, C=C-H), 4.31 (2H, q, J = 7.1 Hz, -CH₂), 2.23 (3H, t, J = 5.6 Hz, -CH₂), 2.67 (-CH₃); ¹³C NMR (CDCl₃, δ, ppm): 168.3 (amide C=O), 166.4 (ester C=O), 162.7 (N=C), 150.2 (N=C=), 145.6 (C-1), 140.3 (C-1'), 137.6 (C-4'), 133.5 (C-3'), 132.4 (C-6'), 131.8 (C-3, C-5), 129.6 (C-5'), 124.2 (C-2'), 122.5 (C-4), 118.2 (C-2, C-6), 101.8 (s=CH), 61.6 (-CH₂), 24.7 (Ar-CH₃), 21.4 (-CH₃), 15.1 (ester-CH₃); MS (70 eV): m/z (%); [M⁺] 396 (46), 261 (100%), 188 (34), 135 (53), 112 (29), 107 (38), 77 (20); Anal. calcd. for C₁₂H₁₀N₂O₃S; C, 63.61; H, 5.07; N, 7.07%; S, 8.09%; found: C, 63.53; H, 4.96; N, 7.95; S, 8.01%.
Facile synthesis and antibacterial investigation of new ethyl...

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Ethyl 4-[4-methyl-2-(3-methoxybenzamido)thiazol-3(2H)-yl]benzoate (2i)

Yield 65%; Rf 0.4; m.p. 101-103°C; IR (KBr) \(\nu_{\text{max}}\): 3063 (C=C-H), 1728 (ester C=O), 1688 (amide C=O), 1663 (ester C=O), 1654 (amide C=O), 1586 (C=C), 1176 (C-S) cm\(^{-1}\); \(^1\)H NMR (CDCl\(_3\), \(\delta\), ppm): 7.94 (1H, d, \(J = 7.1\) Hz, -CH\(_3\)), 6.64 (1H, s, C=C-H), 4.43 (2H, q, \(J = 7.1\) Hz, -CH\(_2\)), 2.71 (-CH\(_3\)); \(^13\)C NMR (CDCl\(_3\), \(\delta\), ppm): 168.8 (amide C=O), 166.8 (ester C=O), 163.6 (N=C), 160.5 (C-2), 150.6 (N=C), 145.7 (C-1), 137.5 (C-1’), 132.6 (C-3,C-5), 131.4 (C-5’), 124.6 (C-2’), 122.4 (C-4’), 121.6 (C-4), 118.7 (C-2,C-6), 116.5 (C-2’), 101.5 (=CH), 61.2 (=CH\(_2\)), 55.8 (=CH\(_3\)) 21.5 (-CH\(_3\)), 15.3 (ester-CH\(_3\)); MS (70 eV): \([M + .]^{+}\) 396 (63), 261 (100%), 188 (45), 135 (39), 112 (36), 97 (27); Anal. calcd. for C\(_{21}\)H\(_{17}\)N\(_2\)O\(_3\)S; C, 63.62; H, 5.06; N, 7.06; S, 8.25%; found: C, 63.82; H, 5.06; N, 7.20; S, 8.01%.

Ethyl 4-[4-methyl-2-(2-fluorobenzamido)thiazol-3(2H)-yl]benzoate (2j)

Yield 75%; Rf 0.4; m.p. 101-103°C; IR (KBr) \(\nu_{\text{max}}\): 3057 (C=C-H), 1724 (ester C=O), 1673 (amide C=O), 1646 (C=N), 1580 (C=C), 1163 (C-S) cm\(^{-1}\); \(^1\)H NMR (CDCl\(_3\), \(\delta\), ppm): 7.85 (2H, d, \(J = 7.8\) Hz, H-3,H-5), 7.41 (1H, d, \(J = 7.2\) Hz, H-6’), 7.33 (1H, d, \(J = 3.4\) Hz, H-2’), 6.94 (1H, dd, \(J = 7.2,7.4\) Hz, H-5’), 6.74 (1H, dd, \(J = 3.4,7.4\) Hz, H-4’), 6.67 (2H, d, \(J = 7.8\) Hz, H-2,H-6), 6.41 (1H, s, C=C-H), 4.27 (2H, q, \(J = 7.1\) Hz, -CH\(_2\)), 3.74 (3í-OCH\(_3\)), 2.24 (3H, t, \(J = 7.2\) Hz, -CH\(_3\)) 2.63 (-CH\(_3\)); \(^13\)C NMR (CDCl\(_3\), \(\delta\), ppm): 168.5 (amide C=O), 166.3 (ester C=O), 162.7 (N=C), 160.5 (C-3’), 150.2 (N=C), 145.3 (C-1), 137.5 (C-1’), 132.6 (C-3,C-5), 131.4 (C-5’), 124.6 (C-2’), 122.5 (C-4’), 121.6 (C-4), 118.7 (C-2,C-6), 116.5 (C-2’), 101.5 (=CH), 61.2 (=CH\(_2\)), 55.8 (=CH\(_3\)) 21.5 (-CH\(_3\)), 15.3 (ester-CH\(_3\)); MS (70 eV): \([M + .]^{+}\) 384 (72), 261 (100%), 188 (45), 135 (39), 112 (36), 97 (27); Anal. calcd. for C\(_{21}\)H\(_{17}\)N\(_2\)O\(_4\)S; C, 62.34; H, 4.36; N, 7.17; S, 8.25%; found: C, 62.35; H, 4.45; N, 7.28; S, 8.33%;

Antibacterial assay

In vitro investigation of antibacterial activity of iminothiazoline derivatives (2a-j), was performed by agar well diffusion assay technique against ten different Gram positive and Gram negative bacterial strains by using the Mueller Hinton Agar (MHA) method. The fresh inoculums of all bacteria were prepared and sterilized, normal saline was used for dissolution. The turbidity of all these cultures was adjusted by using 0.5 McFarland. A homogenous bacterial colony was developed by sterile cotton swabs. The inoculated plates were bored by 6 mm sized borer to make the wells. The sample dilutions were prepared by dissolving each sample (1.0 mg) in 1.0 mL of DMSO used as negative control in this bioassay. Standard drug levofloxacin was taken in the same concentration (1.0 mg/mL), a broad spectrum antibiotic (positive control) was prepared. All the plates were incubated at 37°C for 24 h. Antibacterial activities were determined by measuring the zone of inhibition (diameter = mm, ± standard deviation) and were presented by subtracting the activity of the negative control. All the newly synthesized thiazoline heterocycles are potentially bioactive compounds, which will lead to play an important role in drug discovery for the treatment of infectious diseases as they display significant to good antibacterial activities.

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SYNTHESIS AND DISCERNING OF ANTIMICROBIAL POTENTIAL OF NOVEL OXADIAZOLE DERIVATIVES OF CHLOROXYLENOL MOIETY

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Abstract: Chloroxylenol and oxadiazoles are the potent antibacterial and antifungal agents. 2-(4-Chloro-3,5-dimethylphenoxy)acetohydrazide (3), prepared from ethyl 2-(4-chloro-3,5-dimethylphenoxy)acetate (2), when cyclized with aromatic acids offered 2-{(4-chloro-3,5-dimethylphenoxy)methyl}-5-(aryl)-1,3,4-oxadiazole derivatives (4a-f). The novel compounds were characterized by elemental analysis, FTIR, ‘H-NMR, and mass spectral data. All the synthesized compounds were evaluated for antibacterial and antifungal potential. The compounds 4b and 4d incorporated with high electron withdrawing group, exhibited maximum antimicrobial potential, whereas other compounds also displayed antimicrobial activity to some extent.

Keywords: xylenol, oxadiazoles, ethylaryloxyacetate, hydrazides

RESULTS AND DISCUSSION

The esterification of 4-chloro-3,5-xylenol (1) with ethyl chloroacetate in anhydrous conditions resulted in formation of ethyl 2-(4-chloro-3,5-...
dimethylphenoxy)acetate (2). It was noticed that during reaction, ethyl acetate group was substituted over hydroxy group of 4-chloro-3,5-xylenol moiety and HCl molecule was eliminated. The formed ester (2) on hydrazination with hydrazine hydrate lead to formation of 2-(4-chloro-3,5-dimethylphenoxy)acetoxydrazide (3) and liberation of ethyl formate. The formed ester (2) on hydrazination with hydrazine hydrate lead to formation of 2-(4-chloro-3,5-dimethylphenoxy)acetoxydrazide (3) and liberation of ethyl formate. The hydrazide (3) intermediate when cyclized with different aromatic acids offered different 2-{(4-chloro-3,5-dimethylphenoxy)methyl}-5-(aryl)-1,3,4-oxadiazole derivatives (4a-f). The subjected synthetic route (Scheme 1) offered intermediaries and final compounds in good yield. The structures of all synthesized compounds were confirmed on the basis of single spot TLC pattern, elemental analysis and spectral data. The characteristic IR band at 1716 cm⁻¹ (C=O) and 1240 cm⁻¹ (C-O); 1H-NMR peak at 4.35 ppm (2H, q, J = 7.4 Hz, CH₂) and 6.84-6.92 ppm (2H, m, for Ar-H); Mass peak, at 242 (M⁺ peak) and 214 (base peak), confirmed the formation of ester compound (2). The distinctive IR band at 3238 cm⁻¹ (NH), 3305 cm⁻¹ (NH₂) and 1742 cm⁻¹ (C=O); 1H-NMR peak at 5.15 (2H, brs, for NH₂) and 9.46 ppm (1H, s, for NH); Mass peak, at 228 (M⁺ peak) and 155 (base peak), confirmed the formation of hydrazide compound (3). The typical IR band between 1522-1526

Table 1. Antimicrobial activity of compounds 2, 3, 4a-f

<table>
<thead>
<tr>
<th>Compound</th>
<th>Diameter of zone of inhibition in mm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S. aureus</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>11</td>
</tr>
<tr>
<td>4a</td>
<td>17</td>
</tr>
<tr>
<td>4b</td>
<td>22</td>
</tr>
<tr>
<td>4c</td>
<td>19</td>
</tr>
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<td>4d</td>
<td>23</td>
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<td>16</td>
</tr>
<tr>
<td>4f</td>
<td>15</td>
</tr>
<tr>
<td>Acetone</td>
<td>—</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>NT</td>
</tr>
</tbody>
</table>

‘—’ no inhibition; ‘NT’ – Not tested

![Scheme 1. Synthesis of oxadiazole derivatives of 4-chloro-3,5-xylenol moiety](image)
cm⁻¹ (C=\text{N}), 1152-1158 cm⁻¹ (C-O-C), and absence of C=O band; ¹H-NMR peak between 6.85-7.21 ppm (aromatic hydrogens), absence of NH and NH₂ signals confirmed the formation of all compounds 4a-f. The mass fragmentation pattern of compound 4a (Scheme 2), with m/z value at 314 (C_{17}H_{15}ClN_{2}O_{2}^+, M⁺ peak), 145 (C_{8}H_{5}N_{2}O^+, base peak), 169 (C_{9}H_{10}ClO^+), 155 (C_{8}H_{8}ClO^+), 139 (C_{8}H_{8}Cl^+), 77 (C_{6}H_{5}^+), 51 (C_{4}H_{3}^+), assisted in characterizing the mass fragmentation pattern for other compounds 4b-f. All the compounds structure and purity were further supported by their single TLC spot and elemental analysis.

All the synthesized compounds were subjected to antimicrobial activity against two bacterial and fungal strains using agar well diffusion method for zone of inhibition measurement based on standard literary reports (20-22). The antimicrobial results of all compounds revealed that compound 4b and 4d...
possess maximum antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*, and antifungal activity against *Candida albicans* and *Aspergillus flavus* that is near gentamicin and fluconazole range. Other compounds 2, 3, 4c, 4a, and 4e also showed antimicrobial activity to some extent such that zone of inhibition exhibited in the range between 10 to 22 mm against *S. aureus*, 9 to 18 mm against *E. coli*, 10 to 12 against *C. albicans* and 9 to 10 mm against *A. flavus*. Compounds 2 and 3 displayed no zone of inhibition against *C. albicans* and *A. flavus* (Table 1). The correlation of structure of all newly synthesized compounds and their resultant biological data revealed that compounds bearing high electron withdrawing groups -NO₂ and -Cl claps highest antibacterial and antifungal potential.

**EXPERIMENTAL**

The chemicals, solvents and reagents used in experiment were of LR grade and procured from various chemical firms like: Merck KGaA (Germany), Sigma-Aldrich Co. (USA), Ajax Finechem (Australia) and Qualigens, Hi-Media Laboratories Pvt. Ltd. (India). For vacuum filtration Ashless Whatman No. 1 filter paper was used. The melting points of newly synthesized compounds were determined in open glass capillaries using Stuart SMP11 melting point apparatus and are uncorrected. The proton magnetic resonance (1H-NMR) spectra were recorded on a Bruker 300 MHz instrument using CDCl₃, solvent, in δ value scale as downfield chemical shift in ppm against tetramethylsilane (TMS) the internal standard. The order 1H-NMR signals is stated as: multiplicity (s, single; d, doublet; t, triplet; m, multiplet), number of protons, and coupling constants (J value) in Hertz. The infrared (IR) spectra of compounds were recorded in KBr on a Shimadzu FT-IR 8300 instrument in a range of 400 to 4000 cm⁻¹. Mass spectra were recorded on KBr on a Shimadzu FT-IR 8300 instrument in a range of 400 to 4000 cm⁻¹. Mass spectra were recorded on a Shimadzu FT-IR 8300 instrument in a range of 400 to 4000 cm⁻¹. Mass spectra were recorded on a Shimadzu FT-IR 8300 instrument in a range of 400 to 4000 cm⁻¹.

### Synthesis of 2-(4-chloro-3,5-dimethylphenoxy)acetoxyacetohydrazide (3)

The mixture of compound 2 (0.02 M) and hydrazine hydrate (0.03 M) was refluxed for 6 h using ethanol as solvent, maintaining purely anhydrous conditions. Subsequently, the solvent was distilled off to remove excess of ethanol. The formed organic product was separated by filtration. Next, the filtered residue was dried at normal room temperature. Finally, the crude product was recrystallized from methanol to yield desired compound (19).

### General procedure for synthesis of 2-{(4-chloro-3,5-dimethylphenoxy)methyl}-5-phenyl-1,3,4-oxadiazole (4a-f)

Compound 2 (0.005 M) was treated with aromatic acid (0.001M) and refluxed for 6 h in the presence of phosphorus oxychloride. The refluxed mixture was cooled and poured into crushed ice, and neutralized with sodium carbonate that offered a crude solid mass formed in solvent. The crude solid mass was filtered and recrystallized from methanol to yield desired compounds (19).
1522 (C=N), 1156 (C-O-C); 1H-NMR (CDCl₃-d₆) δ, ppm: 2.65 (6H, s, Ar-CH₃), 4.35 (2H, q, J = 7.2 Hz, CH₂), 6.86-7.12 (7H, m, Ar-H); EI-MS (m/z): 314 (parent ion), 316 (M+2), 145 (base peak), 171, 169, 157, 155, 141, 77, 51; Anal. calcd. for C₁₇H₁₅ClN₂O₂; C (64.87), H (4.80), N (8.9)%; found: C (64.91), H (4.84) and N (8.94)%.

2-{(4-chloro-3,5-dimethylphenoxy)methyl}-5-(4-nitrophenyl)-1,3,4-oxadiazole (4b)
Pale yellow crystals (Yield 78%, m.p. 204-206°C), IR (KBr, cm⁻¹): 3045 (aromatic C-H), 2924 (C-H), 1526 (C=N), 1158 (C-O-C); 1H-NMR (CDCl₃-d₆) δ, ppm: 2.65 (6H, s, Ar-CH₃), 4.36 (2H, q, J = 7.2 Hz, CH₂), 6.92-7.18 (6H, m, for Ar-H); EI-MS (m/z): 359 (parent ion), 361 (M+2),190 (base peak), 171, 169, 157, 155, 141, 139; Anal. calcd. for C₁₇H₁₄ClN₃O₄; C (56.75), H (3.92), N (11.68)%; found: C (56.82), H (3.94) and N (11.71)%.

4-[5-{(4-chloro-3,5-dimethylphenoxy)methyl}-1,3,4-oxadiazol-2-yl]benzenamine (4c)
White crystals (Yield 72%, m.p. 212-214°C), IR (KBr, cm⁻¹): 3048 (aromatic C-H), 2925 (C-H), 1524 (C=N), 1156 (C-O-C); 1H-NMR (CDCl₃-d₆) δ, ppm: 2.66 (6H, s, for Ar-CH₃), 4.34 (2H, s, for CH₂), 5.26 (2H, brs, for NH₂), 6.96-7.19 (6H, m, for Ar-H); EI-MS (m/z): 329 (parent ion), 331 (M+2),160 (base peak), 171, 169, 157, 155, 141, 139; Anal. calcd. for C₁₇H₁₆ClN₃O₂; C (61.91), H (4.89), N (12.74)%; found: C (61.97), H (4.92) and N (12.82)%.

2-{(4-chloro-3,5-dimethylphenoxy)methyl}-5-(4-chlorophenyl)-1,3,4-oxadiazole (4d)
White crystals (Yield 80%, m.p. 196-198°C), IR (KBr, cm⁻¹): 3042 (aromatic C-H), 2924 (C-H), 1526 (C=N), 1158 (C-O-C); 1H-NMR (CDCl₃-d₆) δ, ppm: 2.65 (6H, s, for Ar-CH₃), 4.34 (2H, s, for CH₂), 6.96-7.12 (6H, m, for Ar-H); EI-MS (m/z): 348 (parent ion), 350 (M+2), 179 (base peak), 171, 169, 157, 155, 141, 139; Anal. calcd. for C₁₇H₁₆ClN₃O₂; C (58.47), H (4.04), N (8.02)%; found: C (58.52), H (4.07) and N (8.08)%.

2-{(4-chloro-3,5-dimethylphenoxy)methyl}-5-(4-methoxyphenyl)-1,3,4-oxadiazole (4e)
White crystals (Yield 65%, m.p. 187-189°C), IR (KBr, cm⁻¹): 3048 (aromatic C-H), 2927 (C-H), 1526 (C=N), 1158 (C-O-C); 1H-NMR (CDCl₃-d₆) δ, ppm: 2.65 (6H, s, for Ar-CH₃), 3.86 (3H, s, for OCH₃), 4.36 (2H, s, for CH₂), 6.94-7.21 (6H, m, for Ar-H); EI-MS (m/z): 344 (parent ion), 346 (M+2),175 (base peak), 171, 169, 157, 155, 141, 139; Anal. calcd. for C₁₇H₁₆ClN₃O₂; C (62.70), H (4.97), N (8.12)%; found: C (62.74), H (4.92) and N (8.16)%.

Biological evaluation
The newly synthesized molecules were evaluated for their antimicrobial potential using Gram positive bacteria, namely E. coli (NCIM 269) and S. aureus (NCIM 2901) and fungi namely C. albicans (MTCC 227) and A. flavus (MTCC 2206), obtained from University Science Malaysia, Penang, Malaysia and Department of Biotechnology, AIMST University, Malaysia.

Antibacterial activity
All the novel compounds 2, 3, and 4a-f were screened for their antibacterial potential by employing in vitro bioassay using agar well diffusion method. Experiment was done over Mueller-Hinton plates. In the study, newer compounds were dissolved in 1 mL of acetone. During the study, bacterial strains were grown to log phase overnight at 37°C with regular shaking. The bacterial cultures were spread over plates on which wells were created using cork borer (4 mm). The wells were loaded with 0.1 mg/mL solution of compounds in acetone, 0.5 mg/mL of gentamicin (positive standard) and acetone (negative standard) using a micropipette. The plates were incubated for 24 h at 37°C, and the same procedure was repeated three times. Lastly, after 24 h incubation, the zones of inhibition were observed on mm scale (20-23).

Antifungal activity
The newly synthesized compounds 2, 3, and 4a-f were assessed for their antifungal potential based on previously published report (20-23), by employing agar well diffusion method using Mueller-Hinton plates. During the study, compounds were dissolved in 1 mL of acetone, and plates wells were loaded by 1 mg/mL test compounds solution, 0.5 mg/mL of fluconazole (positive
standard) and acetone (negative standard). The plates were incubated for 24 h at 28°C. The experiment was repeated in triplicate, and the zones of inhibition were measured in mm scale.

CONCLUSION

The successful synthesis of novel molecules was confirmed by experimental, spectral, and elemental analysis reports. The in vitro antimicrobial studies results of novel synthesized compounds supported their high antibacterial and antifungal potential. The correlation of structures and biological activity of novel molecules revealed that among all derivatives of 4-chloro-3,5-xylenol, the oxadiazoles possessing highly electronegative groups displays higher antibacterial and antifungal potential. Therefore, present study concludes that novel 4-chloro-3,5-xylenol derived oxadiazoles possess high antimicrobial potential and needs furthermore studies to be developed as prospective antimicrobials.

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Panax ginseng C. A. Meyer (Araliaceae) is a perennial medicinal plant with fleshy roots distributed in eastern Asia. It is used as an aphrodisiac, sexual rejuvenator and a panacea for longevity and to treat to relieve headache, fatigue, diabetes, dizziness, nausea, asthma, Alzheimer’s disease, hemorrhage and impotence. Phytochemical investigation of methanolic extract of the roots of P. ginseng resulted in the isolation of new dammarane-type compounds characterized as dammar-1-en-3-on-7β,19,11β,21-tetradecanoyloxy-18,22β-diol (1), dammar-24-en-3β-ol-20β-isoprenoyloxy-3β-O-D-galactopyranoside (3), dammar-12,24-dien-3α,6β,15α-triol-3-O-α-L-arabinopyranosyl-β-D-arabinopyranoside (4), dammar-24-en-3α,6β,16α,20β-tetraol-3-O-α-D-xylpyranosyl-6-O-β-D-xylpyranoside (5) and dammar-12,24-dien-3α,6β-diol-3-O-β-D-arabinopyranosyl-(2→1′)-O-L-arabinopyranosyl-6-O-β-L-rabonopyranoside (6) along with the known steroidal glucoside β-sitosterol 3-β-D-glucopyranoside. The structures of all the isolated phytoconstituents have been established on the basis of spectral data analysis and chemical reactions.

Keywords: Panax ginseng, polygonaceae, roots, dammarane triterpenoids, characterization

EXPERIMENTAL

General

Melting points were determined on a Perfit melting apparatus (Ambala, Haryana, India) and are uncorrected. UV spectra were measured with a Lambda Bio 20 spectrophotometer (Perkin-Elmer-Rotkreuz, Switzerland) in methanol. Infrared spectra were recorded on a Bio-Rad FTIR 5000 (FTS 135, Kawloon, Hong Kong) spectrophotometer using KBr pellets. 1H and 13C NMR spectra were scanned on Advance DRX Bruker spectropsin 400 and 100 MHz, respectively, instruments (Karlsruhe, Germany) using TMS as an internal standard. Mass spectra were obtained by effecting FAB ionization at 70 eV on a JEOL-JMS DX 303 spectrometer (Japan) equipped with direct inlet probe system. Column chromatography was performed on a silica gel (60-120 mesh, Qualigen, Mumbai, India), column. TLC was run on silica gel G (Qualigen) coated plates. Spots were visualized by exposing to
iodine vapers, UV radiation and spraying with ceric sulfate solution.

**Plant material**

The roots of *P. ginseng* were procured from the Khari Baoli market of Delhi and identified by Prof. M.P. Sharma, Department of Botany, Jamia Hamdard, New Delhi. A voucher specimen is deposited in the herbarium of the Phytochemical Research Laboratory, Faculty of Pharmacy.

**Extraction and isolation**

The air-dried roots (2.0 kg) were coarsely powdered, defatted with petroleum ether and extracted with methanol exhaustively in a Soxhlet apparatus. The combined extracts were filtered and extracted with methanol in increasing order of polarity in various combinations of chloroform, chloroform-methanol (19.9 : 1, 99 : 1, 97 : 3, 19 : 1, 93 : 7, 9 : 1, 17 : 3, 3 : 1, 3 : 2, 2 : 3, 4 : 1) and methanol. The fractions were collected separately and matched by TLC to check homogeneity. Similar fractions having the same Rf values were combined and crystallized. The isolated compounds were recrystallized to get pure compounds. The following compounds were isolated from the methanolic extract of the roots of *P. ginseng*:

**Dammar-1-en-3-one-18,22α-diol-7,11,19,21-tetracaprate (1)**

Elution of the column with chloroform-methanol (19 : 1) afforded light yellow gummy mass of 1. UV λ_{max} (MeOH): 221 nm (log ε 3.9); IR (KBr, cm⁻¹): 3312, 2928, 2837, 1729, 1721, 1692, 1652, 1457, 1372, 1265, 1160, 1072; ¹H NMR (CDCl₃, δ, ppm): 7.02 (1H, d, J = 8.7 Hz, H-1), 6.89 (1H, d, J = 8.7 Hz, H-2), 4.23 (1H, ddd, J = 4.6, 4.2, 8.5 Hz, H-11α), 4.11 (1H, dd, J = 5.5, 8.6 Hz, H-7α), 4.08 (2H, d, J = 6.6 Hz, H-21), 3.57 (1H, ddd, J = 6.9, 7.7, 9.8 Hz, H-22α), 3.99 (2H, brs, H-19), 3.60 (2H, brs, H-18), 2.75-1.30 (34H, m, 4 × CH₁₂, 6 × CH₂), 1.25 (48H, brs, 24 × CH₃), 1.02 (3H, d, J = 6.7 Hz, Me-26), 0.97 (3H, d, J = 6.5 Hz, Me-27), 0.85 (3H, brs, Me-29), 0.82 (3H, brs, Me-28), 0.66 (3H, brs, Me-30), 0.93 (3H, t, J = 6.7 Hz, Me-10′′), 0.90 (3H, t, J = 6.6 Hz, Me-10″), 0.79 (3H, t, J = 6.8 Hz, Me-10″″); ¹³C NMR (CDCl₃, δ, ppm): 131.07 (C-1), 116.36 (C-2), 190.95 (C-3), 45.41 (C-4), 45.58 (C-5), 19.03 (C-6), 70.55 (C-7), 38.62 (C-8), 45.96 (C-9), 36.07 (C-10), 63.09 (C-11), 28.11 (C-12), 45.28 (C-13), 50.22 (C-14), 44.05 (C-15), 28.36 (C-16), 53.45 (C-17), 60.59 (C-18), 60.54 (C-19), 33.72 (C-20), 60.45 (C-21), 27.72 (C-23), 31.92 (C-24), 35.29 (C-25), 24.71 (C-26), 24.85 (C-27), 29.18 (C-28), 28.96 (C-29), 22.65 (C-30), 170.35 (C-1′), 169.58 (C-1″), 167.22 (C-1‴), 167.06 (C-1″″), 14.15 (C-10′′′), 15.89 (C-10″″′), 16.07 (C-10″″′″), 16.23 (C-10″″′″″), 34.03 (4 × CH₂), 34.18 (4 × CH₃), 32.51 (4 × CH₂), 29.38 (8 × CH₂), 29.21 (12 × CH₂); FAB MS m/z (rel. int.): 1139 [M + H⁺] (C₇₀H₁₂₃O₁₁) (1.8), 983 (2.7), 518 (11.3), 171 (3.6), 155 (47.2).

**β-Sitosterol 3-β-D-glucopyranoside (2)**

Further elution of the column with chloroform-methanol (19 : 1) yielded colorless amorphous powder of 2, recrystallized from methanol, yield: 200 mg; Rf 0.35 (chloroform : methanol : 9 : 1); m. p. 265-267°C; UV λ_{max} (MeOH): 241 nm (log ε 2.9); IR (KBr, cm⁻¹): 3401, 2918, 2849, 1654, 1377, 1261, 1172, 1082; ¹H NMR (CDCl₃, δ, ppm): 3.14 (1H, m, H-1, H-6), 5.11 (1H, d, J = 7.2 Hz, H-1′), 4.37 (1H, m, H-5′), 4.31 (1H, m, H-4′), 4.16 (1H, m, H-3′), 3.91 (1H, m, H-2′), 3.54 (1H, brs, w = 18.5 Hz, H-3), 3.37 (2H, brs, H₂-6′), 0.99 (3H, brs, M-19′), 0.92 (3H, d, J = 6.2 Hz, Me-21′), 0.87 (3H, brs, Me-27), 0.84 (3H, brs, Me-26), 0.82 (3H, brs, Me-29), 0.67 (3H, brs, Me-18); ¹³C NMR (CDCl₃, δ, ppm): 37.28 (C-1′), 31.93 (C-2′), 70.62 (C-3′), 42.33 (C-4′), 140.31 (C-5), 122.10 (C-6′), 29.33 (C-7), 34.23 (C-8), 50.21 (C-9), 36.14 (C-10′), 22.66 (C-11′), 38.89 (C-12), 39.78 (C-13′), 56.18 (C-14′), 27.21 (C-15), 28.22 (C-16′), 56.12 (C-17′), 11.85 (C-18′), 19.33 (C-19′), 36.73 (C-20′), 19.03 (C-21′), 33.98 (C-22′), 26.18 (C-23′), 45.68 (C-24′), 29.68 (C-25′), 21.07 (C-26′), 19.78 (C-27′), 24.94 (C-28′), 14.07 (C-29′), 101.20 (C-1′′), 73.92 (C-2′′), 68.61 (C-3′′), 73.62 (C-4′′), 78.58 (C-5′′), 62.11 (C-6′′); +ve ion FAB MS m/z (rel. int.): 576 [M⁺] (C₅₀H₄₅O₁₁) (11.3), 413 [M − C₆H₁₀O₅] (10.1), 398 (100).

**Dammaren-3-0-β-galactosyloxy-20-isoprenoate (3)**

Elution of the column with chloroform-methanol (93 : 7) afforded colorless crystals of 3, m. p. 215-216°C; UV λ_{max} (MeOH): 215 nm; IR (KBr, cm⁻¹): 3365, 3271, 2928, 2841, 1723, 1645, 1456, 1375, 1559, 1072, 1043, 901 cm⁻¹; ¹H NMR (CDCl₃, δ, ppm): 5.17 (1H, t, J = 6.9 Hz, H-24), 3.41 (1H, dd,
Elution of the column with chloroform-methanol (17:1) furnished colorless crystals of 5, m.p. 214-215°C, UV $\lambda_{\text{max}}$ (MeOH); 216 nm (log ε 3.8); IR (KBr, cm$^{-1}$): 3392, 3266, 2931, 2838, 1645, 1468, 1261, 1051; $^1$H NMR (DMSO-d$_6$, δ, ppm): 5.13 (1H, dd, δ = 5.3, 5.9 Hz, H-3β), 3.38 (1H, dd, δ = 7.9, 4.2, 4.3 Hz, H-6δ), 3.31 (1H, ddd, δ = 4.2, 6.5, 4.7 Hz, H-16β), 1.65 (3H, brs, Me-26), 1.56 (3H, brs, Me-27), 1.23 (3H, brs, Me-21), 0.98 (3H, brs, Me-19), 0.91 (3H, brs, Me-28), 0.83 (3H, brs, Me-29), 0.79 (3H, brs, Me-30), 0.74 (3H, brs, Me-18), 2.38-1.12 (20H, m, 8 ◊ CH$_2$, 4 ◊ CH), 5.02 (1H, d, δ = 7.7 Hz, H-1″), 3.42 (1H, m, H-2′′), 3.18 (1H, m, H-3′′), 3.10 (1H, m, H-4′′), 3.65 (2H, d, δ = 9.5 Hz, H-5′′), 4.90 (1H, d, δ = 7.2 Hz, H-1″), 3.45 (1H, m, H-2″), 3.16 (1H, m, H-3″), 3.09 (1H, m, H-4″), 3.48 (2H, d, δ = 9.7 Hz, H-5″); $^1$C NMR (DMSO-d$_6$, δ, ppm): 38.79 (C-1), 28.71 (C-2), 81.29 (C-3), 38.62 (C-4), 55.56 (C-5), 72.41 (C-6), 34.43 (C-7), 40.11 (C-8), 50.93 (C-9), 42.14 (C-10), 21.15 (C-11), 38.62 (C-4), 55.56 (C-5), 72.41 (C-6), 34.43 (C-7), 40.11 (C-8), 50.93 (C-9), 36.21 (C-10), 21.45 (C-11), 25.62 (C-12), 42.14 (C-13), 49.46 (C-14), 31.09 (C-15), 68.95 (C-16), 48.01 (C-17), 15.31 (C-18), 22.11 (C-19), 71.99 (C-20), 28.92 (C-21), 30.63 (C-22), 25.71 (C-23), 125.26 (C-24), 130.11 (C-25), 17.51 (C-26), 25.52 (C-27), 17.65 (C-28), 15.98 (C-29), 16.76 (C-30), 103.86 (C-1″), 78.61 (C-2′″), 76.11 (C-3′″), 69.72 (C-4′′), 61.03 (C-5′″), 103.61 (C-1″), 76.52 (C-2″), 75.25 (C-3″), 69.96 (C-4″), 60.82 (C-5″); +ve FAB MS m/z (rel. int.): 741 [M + H]$^+$ (C$_{40}$H$_{69}$O$_{12}$) (5.5), 607 (7.2), 474 (11.6), 149 (23.7), 133 (20.2).

**Dammarene-3,6,15-triol diarabinoside (4)**

Further elution of the column with chloroform-methanol (17:1) yielded colorless crystals of 6, m.p. 222-223°C, UV $\lambda_{\text{max}}$ (MeOH); 216 nm (log ε 4.2); IR (KBr, cm$^{-1}$): 3415, 3382, 3266, 2927, 2841, 1645, 1463, 1265, 1052; $^1$H NMR (DMSO-d$_6$, δ, ppm): 5.15 (1H, dd, δ = 7.2, 6.5 Hz, H-12), 5.09 (1H, t, δ = 7.2 Hz, H-24), 3.58 (1H, dd, δ = 4.5, 5.8 Hz, H-3β), 3.19 (1H, ddd, δ = 5.6, 7.9, 3.7 Hz, H-6δ), 1.66 (3H, brs, Me-26), 1.60 (3H, brs, Me-27), 1.07 (3H, brs, Me-19), 1.04 (3H, d, δ = 6.1 Hz, Me-21), 1.01 (3H, brs, Me-28), 0.92 (3H, brs, Me-29), 0.89 (3H, brs, Me-30), 0.85 (3H, brs, Me-18), 2.02-1.23 (20H, m, 8 ◊ CH$_2$, 4 ◊ CH), 5.02 (1H, d, δ = 7.8 Hz, H-1″α), 3.81 (2H, dd, δ = 9.5, 9.7 Hz, H-5″), 3.29 (1H, m, H-4″), 3.24 (1H, m, H-4″), 3.02 (1H, m, H-4″), 4.86 (1H, d, δ = 6.5 Hz, H-1″β), 3.63 (2H, dd, δ = 6.3, 5.9 Hz, H-5″), 3.31 (1H, m, H-4″), 3.22 (1H, m, H-3″), 3.17 (1H, m, H-3″); $^1$C NMR (DMSO-d$_6$, δ, ppm): 38.43 (C-1), 29.24 (C-2), 80.28 (C-3), 78.81 (C-4), 50.22 (C-5), 103.86 (C-1″), 78.21 (C-2′′), 72.13 (C-3′′), 63.36 (C-5″); +ve FAB MS m/z (rel. int.): 723 [M + H]$^+$ (C$_{41}$H$_{71}$O$_{11}$) (2.7), 572 (9.1), 439 (8.8), 149 (12.6), 133 (22.5).
New dammarane-type triterpenoids from the roots of *Panax ginseng* C. A. Meyer

Figure 1. Structural formulae of compounds 1 and 3-6

2H, J = 10.8 Hz, H-5'), 4.88 (1H, d, J = 6.6 Hz, H-1''), 3.50 (1H, m, H-2''). 3.33 (1H, m, H-3''), 3.25 (1H, m, H-4''), 3.83 (2H, d, J = 7.2 Hz, H-5''); 13C NMR (DMSO-d6, δ, ppm): 35.12 (C-1), 28.16 (C-2), 79.95 (C-3), 40.79 (C-4), 57.73 (C-5), 71.35 (C-6), 36.14 (C-7), 38.21 (C-8), 53.22 (C-9), 36.31 (C-10), 23.45 (C-11), 124.93 (C-12), 132.15 (C-13), 52.11 (C-14), 33.53 (C-15), 32.21 (C-16), 51.63 (C-17), 16.37 (C-18), 19.44 (C-19), 26.15 (C-20), 28.62 (C-21), 32.83 (C-22), 27.97 (C-23), 124.81 (C-24), 132.35 (C-25), 27.42 (C-26), 17.96 (C-27), 17.42 (C-28), 16.93 (C-29), 17.16 (C-30), 105.56 (C-1''), 79.03 (C-2''), 77.91 (C-3''), 71.73 (C-4''), 63.22 (C-5''), 104.71 (C-1'''), 78.38 (C-2'''), 77.72 (C-3'''), 71.41 (C-4'''), 63.25 (C-5'''), 106.85 (C-1''''), 76.44 (C-2''''), 74.52 (C-3''''), 74.11 (C-4''''), 62.95 (C-5''''); +ve FAB MS m/z (rel. int.): 839 [M + H]+ (C45H75O14) (6.5), 569 (7.1), 553 (6.8), 420 (10.3), 149 (12.8), 133 (18.5).
RESULTS AND DISCUSSION

Compound 1 showed IR absorption bands for hydroxyl groups (3312 cm⁻¹), ester functions (1729, 1721, 1718 cm⁻¹), carbonyl group (1692 cm⁻¹) and unsaturation (1652 cm⁻¹). On the basis of FAB mass and ¹³C NMR spectra, the molecular ion peak of 1 was determined at m/z 1139 [M+H]^+ consisting with a molecular formula of a tetra-acyl triterpenoid, C₇₀H₁₂₃O₁₁. The ion peaks generating at m/z 983 [M-CH₃(CH₂)₈ COO]^+, 518 [M – 4 x CH₃(CH₂)₈ COO]^+, 171 [CH₃(CH₂)₈ COO]^+ and 155 [CH₃(CH₂)₈ CO]^+ suggested that the triterpenoid was esterified with four units of capric acid. The ¹H NMR spectrum of 1 exhibited two one-proton doublets at δ 7.02 (J = 8.7 Hz) and 6.89 (J = 8.7 Hz) ppm assigned to cis-oriented vinylic H-1 and H-2, respectively, nearby to the C-3 carbonyl function. A one-proton double
doublet at $\delta$ 4.11 ppm ($J = 5.5, 8.6$ Hz), two one-proton triple doublets at $\delta$ 4.23 ($J = 4.6, 4.2, 8.5$ Hz) and $3.57$ ($J = 6.9, 7.7, 9.8$ Hz) ppm were ascribed to oxygenated methylene H-7$\alpha$, and H-11$\alpha$ and carbinol H-22$\alpha$ protons, respectively. Two broad singlets at $\delta$ 3.60 ppm and 3.99 ppm and a doublet at $\delta$ 4.08 ppm ($J = 6.6$ Hz), all integrated for two protons each, were attributed to oxygenated methylene H$_2$-18, H$_2$-19 and H$_2$-21 protons, respectively. Two three-proton doublets at $\delta$ 1.02 ppm ($J = 6.7$ Hz) and 0.97 ppm ($J = 6.5$ Hz) and three-three proton broad singlets at $\delta$ 0.85, 0.82 and 0.66 ppm were associated with the secondary methyl Me-26 and Me-27 and tertiary methyl Me-29, Me-28 and Me-30 protons, respectively. Four three-proton triplets at $\delta$ 0.93 ppm ($J = 6.7$ Hz), 0.90 ppm ($J = 6.6$ Hz), 0.87 ppm ($J = 6.3$ Hz) and 0.79 ppm ($J = 6.8$ Hz) were accounted to primary methyl Me-10$'$, Me-10$''$, Me-10$'''$, and Me-10$''''$ protons, respectively. The remaining methylene and methine protons appeared from $\delta$ 2.75 to 1.25 ppm. The $^1$C NMR spectrum of 1 exhibited signals for the carbonyl carbon at $\delta$ 190.95 ppm (C-3), ester carbons between $\delta$ 170.35-167.06 ppm, vinyl carbons at $\delta$ 130.07 ppm (C-1) and 116.36 ppm (C-2), oxygenated methine carbons from $\delta$ 70.55 to 63.09 ppm, oxygenated methylene carbons $\delta$ 60.59 ppm (C-18), 60.54 ppm (C-19) and 60.45 ppm (C-21) and methyl carbons between $\delta$ 29.18-14.15 ppm. The $^1$H/$^1$H COSY spectrum of 1 showed correlations of H-1 with H-2 and H$_2$-19; H-7 with H-5, H$_2$-6 and H$_2$-18; H-11 with H$_2$-12, H-9 and H-13; H-22 with H-20, H$_2$-21 and H$_2$-23; and Me-26 with H-25 and Me-27. The HMBC spectrum of 1 exhibited interactions of H-2, H-1 and Me-29 with C-3; H$_2$-6 and H$_2$-18 with C-7; H-9, H$_2$-12 and H-13 with C-11; H-22 and H$_2$-21 with C-20; H-7 and H$_2$-2$'$ with C-1$'$; H$_2$-19 with C-1$'''$; H-2$'''$ and H-11 with C-1$''''$ and H$_2$-21 with C-1$''''$$''$. The $^1$H and $^1$C NMR spectra of triterpenic nucleus of 1 were compared with the dammarene-type triterpenoids (19-21). On the basis of these evidences, the structure of 1 had been elucidated as dammar-1-ene-3-on-7[$\beta$,19,11[$\beta$,21-tetradecanoyloxy-18,22$\beta$]-diol. This is a new tetracyclic dammarenone.

Compound 2 was the known steroidal constituent characterized as $\beta$-sitosterol 3-$\beta$-D-glucopyranoside.

Compound 3 had IR absorption bands for hydroxyl groups (3365, 3271 cm$^{-1}$), unsaturation (1645 cm$^{-1}$) and ester function (1723 cm$^{-1}$). On the basis of FAB mass and $^1$C NMR spectra, the molecular ion peak of 3 was established at $m/z$ 691 [M+H]$^+$ consistent with a molecular formula of isopentanoyloxytriterpenic glycoside, C$_{41}$H$_{71}$O$_8$. The ion fragments arising at $m/z$ 589 [M – Me$_2$CHCH$_2$COO]$^+$, 527 [M –CH$_2$O$_2$]$^+$, 179 [C$_{18}$H$_{20}$O$_4$]$^{-}$ and 163 [C$_{18}$H$_{18}$O$_6$]$^{-}$ suggested that the triterpenic unit was linked with an isoprenol and a hexose moiety. The $^1$H NMR spectrum of 3 showed a one-proton triplet at $\delta$ 5.17 ppm ($J = 6.9$ Hz) and a one-proton doublet at $\delta$ 5.01 ppm ($J = 7.5$ Hz) assigned to vinylic H-24 and anomic H-1’ protons, respectively. The other sugar protons appeared between $\delta$ 4.27- 3.08 ppm. A one-proton doublet at $\delta$ 3.41 ppm with coupling interactions of $5.7$ and $9.3$ Hz was ascribed to oxygenated methine H-3$\alpha$ proton. Two three-protons broad singlets at $\delta$ 1.61 and 1.56 ppm were attributed to C-26 and C-27 methyl protons located on the C-25 vinylic carbon. The other tertiary methyl protons appeared as three-proton singlets from $\delta$ 1.22 to 0.73 ppm. Two three-proton doublets at $\delta$ 0.81 ($J = 6.6$ Hz) and 0.78 ppm ($J = 6.5$ Hz) were due to secondary C-4$''$ and C-5$''$ methyl protons, respectively. The $^1$C NMR spectrum of 3 exhibited signals for the vinylic carbons at $\delta$ 124.91 (C-24) and 131.33 ppm (C-25), oxygenated methine carbon at $\delta$ 76.31 ppm (C-3), anomic carbon at $\delta$ 105.09 ppm (C-1’), other sugar carbons between $\delta$ 75.41-61.93 ppm, ester carbon at $\delta$ 173.25 ppm (C-1’’’), methyl carbons from $\delta$ 31.79 ppm to 15.53 and oxygenated quaternary carbon at $\delta$ 73.26 ppm (C-20). The $^1$H/$^1$H COSY spectrum of 3 showed correlations of H-1’ with H-3, H-2’ and H-3’; H-3 with H-1’, H-2; and Me- 28; H-24 with Me-26, Me-27 and H$_2$-23; and H- 3’’ with H$_2$-2’’, H-4’’ and H-5’’. The HMBC spectrum of 3 exhibited interactions of H$_2$-2, Me-28 and H-1’ with C-3; Me- 21, H-17, H$_2$-22 and H$_2$-23 with C-20; Me- 26, Me- 27, H-24 and H$_2$-23 with C-25; and H$_2$- 2’’ and H-3’’ with C-1’’’. The interactions of important carbon signals with corresponding protons were determined by HSQC spectrum. The $^1$H and $^1$C NMR spectra of triterpenic nucleus of 3 were compared with the dammarene-type triterpenoids (19-21). Acid hydrolysis of 3 yielded D-galactose, TLC comparable ($(R_f = 0.484, n$ butanol-ethanol-water, 4:1:2.2; [\alpha]$_{D}^{20}$ = +80.2$^0$, conc. 10 in H$_2$O). On the basis of the foregoing account, the structure of 3 was determined as dammar-24-en-3[$\beta$-isoprenoylloxy-3$\beta$-O-D-galactopyranoside. It is a new dammarene-type triterpenic galactoside.

Compound 4 gave positive tests for glycosides and had IR distinctive absorption bands for hydroxyl groups (3412, 3341, 3286/cm$^{-1}$) and unsaturation (1648/cm$^{-1}$). Combination of FAB mass and $^1$C NMR spectra established its molecular ion peak at $m/z$ 723 [M+H]$^+$ corresponding to the molecular formula of a triterpenic diglycoside C$_{40}$H$_{67}$O$_{11}$. The ion
peaks arising at m/z 572 [M-C₇H₆O₅]+, 149 [C₇H₆O₃]+, 133 [C₈H₇O₄]⁺ and 439 [572 – C₇H₆O₅]+ indicated that two C₆ sugar units were present in the molecule. The ¹H NMR spectrum of 4 showed two one-proton triplets at δ 5.33 ppm (J = 7.1 Hz) and 5.12 ppm (J = 6.8 Hz) assigned to vinyl H-12 and H-24 protons, respectively. Two one-proton double doublets at δ 3.54 ppm (J = 5.5, 5.9 Hz) and 3.50 ppm (J = 5.5, 5.8 Hz) and a one-proton triple doublet at δ 3.34 ppm (J = 5.8, 7.7, 9.6 Hz) assigned correspondingly to oxygenated methine H-3β, H-1β and H-6α protons. Two one-proton doublets at δ 5.01 ppm (J = 7.5 Hz) and 4.86 ppm (J = 6.5 Hz) interacting with δ 105.11 and 105.91 ppm in the HSQC spectrum were attributed to anomeric H-1’’ and H-1’ protons, respectively, attached on separate carbons. The other sugar protons appeared between δ 3.81-3.17 ppm. Two three-proton broad singlets at δ 1.67 and 1.55 ppm and five three-proton broad singlets at δ 1.08-0.83 ppm were ascribed to C-26 and C-27 methyl protons located on the vinylcarbinyl carbon C-25 and other tertiary methyl protons. A three-proton doublet at δ 1.03 ppm (J = 5.8 Hz) was attributed to C-21 secondary methyl protons suggesting dammarene-type triterpenoid. The ¹³C NMR spectrum of 4 exhibited signals for vinyl carbons at δ 126.52 ppm (C-12), 132.74 ppm (C-13), 126.31 ppm (C-24) and 132.57 ppm (C-25), oxygenated methine carbons at δ 80.28 ppm (C-3), 71.75 ppm (C-6) and 76.85 ppm (C-15), anomeric carbons at δ 105.91 ppm (C-1’’) and 105.11 ppm (C-1’), other sugar carbons between δ 78.92-63.36 ppm and methyl carbons from δ 29.61 to 16.72 ppm. The HSQC correlation spectrum of 4 established important interactions of carbon atoms with the respective protons. The ‘‘H-¹³C COSY spectrum of 4 exhibited correlations of H-3 with H₂-2, H₂-29, H-5 and H-1’’; H-6 with H-5, H-7 and H-1’’; H-15 with Me-30, H₂-16 and H-17; H-12 with H-9, H-21 and H-17; and H-24 with H₂-23, Me-27 and Me-26. The HMBC spectrum of 4 showed interactions of H-2’, H₂-5’ and H-3 with C-1’’; H-2’’, H₂-5’’ and H-6 with C-1’’; H₂-16 and H-17 with C-15; H-11, H-12, Me-30, H-17 and H-20 with C-13; and Me-26, Me-27, H₂-23 and H-24 with C-25. Acid hydrolysis of 4 yielded D-arabinose (Rₚ = 0.54, n-butanol-ethanol-water, 4:1:2.2; [α]D = +105⁰, conc 10 in H₂O). The ‘‘H and ¹³C NMR spectra of triterpenic nucleus of 4 were compared with the dammarene-type triterpenoids (19-21). On the basis of the above discussion the structure of 4 has been elucidated as dammar-12,24-dien-3α,6β,15α-triol-3-O-α-L-arabinopyranosyl-6β-D-arabinopyranoside. This is a new dammarene-type triterpenic diarabinoside.

Compound 5 gave positive tests for triterpenic glycosides and showed characteristic IR absorption bands for hydroxyl functions (3392, 3266 cm⁻¹) and unsaturation (1645 cm⁻¹). Its molecular ion peak was determined at m/z 741 [M+ H⁺]⁺ on the basis of FAB mass and ¹³C NMR spectra consistent with the molecular formula of a triterpenic diglycoside C₄₀H₆₉O₁₂. The ion peaks generating at m/z 607 [M-C₇H₆O₅]⁺, 474 [607 – C₇H₆O₅]⁺, 149 [C₇H₆O₃]⁺ and 133 [C₈H₇O₄]⁺ suggested that two pentose units were linked to the molecule. The ‘‘H NMR spectrum of 5 showed three one-proton signals as double doublet at δ 5.13 ppm (J = 7.2, 6.5 Hz) and as doublets at δ 5.02 ppm (J = 7.7 Hz) and 4.90 ppm (J = 7.2 Hz) assigned to vinylic H-24 and anomeric H-1’’ and H-1’ protons, respectively. Three one-proton signals as a double doublet at δ 3.60 ppm (J = 5.3, 5.9 Hz) and as a triple doublet at δ 3.38 ppm (J = 4.2, 7.9, 4.3 Hz) and δ 3.31 ppm (J = 4.2, 6.5, 4.7 Hz), all interacting with the nearby carbons of the triterpenoid in HMBC spectrum were attributed to oxygenated methine H-3β, H-6α and H-16β protons, respectively. The other sugar protons resonated between δ 3.65-3.09 ppm. Eight three-proton broad signals from δ 1.65 to 0.74 ppm were ascribed to C-26 and C-27 methyl protons attached to the vinylic carbon C-25 and to six tertiary methyl protons. The presence of Me-21 proton singlet at δ 1.23 ppm suggested the existence of one of the hydroxyl group at C-20. The ‘‘C NMR spectrum of 5 exhibited signals for vinylic carbons at δ 125.26 ppm (C-24) and 130.11 ppm (C-25), oxygenated aglycone methine carbons at δ 81.29 ppm (C-3), 72.41 ppm (C-6) and 69.85 ppm (C-16), hydroxylated quaternary carbon at δ 71.99 ppm (C-20), anomeric carbons at δ 103.86 ppm (C-1’’) and 103.61 located on different carbons of the triterpenoids, and the other sugar carbons between δ 6.83-60.84 ppm. The ‘‘H-¹³C COSY spectrum of 5 showed interactions of H-3 with H-1, H₂-2, Me-29 and H-1’’; H-16 with H-5, H-7, Me-28 and H-1’’; H-12 with H-9, H-11 and H-17; and H-24 with H₂-23, Me-27 and Me-26. The HMBC spectrum of 4 showed interactions of H-2’, H₂-5’ and H-3 with C-1’’; H-2’’, H₂-5’’ and H-6 with C-1’’; H₂-16 and H-17 with C-15; H-11, H-12, Me-30, H-17 and H-20 with C-13; and Me-26, Me-27, H₂-23 and H-24 with C-25. Acid hydrolysis of 4 yielded D-arabinose (Rₚ = 0.54, n-butanol-ethanol-water, 4:1:2.2; [α]D = +105⁰, conc 10 in H₂O).
δ 103.61 ppm. The 'H and 13C NMR spectral data of the triterpenic nucleus of 5 were compared with the respective spectral values of dammarene-type triterpenoids (19-21). Acid hydrolysis of 5 yielded D-xylose as a glycone unit ([Rr] = 0.67, n-butanol-ethanol-water, 4 : 1 : 2.2; [α]D20 = + 18.8º, conc. 10 in H2O). On the basis of the foregoing discussion the structure of 5 was determined as dammar-24-en-3α,6β,16α,20β-tetraol-3-O-α-D-xylopyranosyl-6-O-β-D-xylopyranoside. This is a new dammarene-type triterpenic dixyloside.

Compound 6 gave positive tests for triterpenic glycosides and exhibited characteristic absorption bands for hydroxyl functions (3415, 3282 cm⁻¹) and unsaturation (1645 cm⁻¹). It had a molecular ion peak at m/z: 839 [M+ H]+ determined by combination of FAB mass and 13C NMR spectra which corresponded to the molecular formula of a triterpenic diglycoside C45H75O14. The ion peaks generating at m/z 569 [M – (C5H8O4)]−, 553 [M – (C5H8O4)2]− and 420 [M – (C5H8O4)3]− indicated that three glycosidic units were attached to the aglycone moiety. The 'H NMR spectrum of 6 exhibited two one-proton signals as a double doublet at δ 5.15 ppm (J = 6.5, 7.5 Hz) and as a triplet at δ 5.09 ppm (J = 7.2 Hz) assigned to vinylic H-12 and H-24 protons, respectively. Two one-proton signals as a double doublets at δ 3.58 ppm (J = 4.5, 5.8 Hz) and as a triple doublets at δ 3.19 ppm (J = 5.6, 7.9, 3.7 Hz) both interacting with the adjacent methylene, methine and methyl signals in 'H-'H COSY and HMBC spectra, were ascribed to oxygenated methine H-1’α, H-1’β and H-6’α protons, respectively. The other protons resonated between δ 3.88 – 3.23 ppm. Two three-proton broad singlets at 1.66 and 1.60 ppm, a three-proton doublet at δ 1.04 ppm (J = 6.1 Hz) and five three-proton broad singlets at δ 0.85, 1.07, 1.01, 0.92 and 0.89 ppm were associated correspondingly with the C-26 and C-27 methyl protons located on the vinylic carbon C-25, secondary C-21 methyl protons and tertiary C-18, C-19, C-28, C-29 and C-30 methyl protons, respectively. The 13C NMR spectrum of 6 displayed signals for vinylic carbons at δ 124.93 ppm (C-12), 132.15 ppm (C-13), 124.81 ppm (C-24) and 132.35 ppm (C-25), oxygenated methine carbons at δ 79.95 ppm (C-3) and 71.35 ppm (C-6), anomicer carbons at δ 105.56 ppm (C-1’), 104.71 ppm (C-1’’), 106.85 ppm (C-1’’’), other sugar carbons appeared between δ 79.03-62.95 ppm. The presence of C-2’ carbon signal in the deshielded region at δ 79.03 ppm suggested location one of the sugar unit at C-2’. The other triterpenic carbons, compared with the reported values, resonated from δ 57.73 to 16.37 ppm. The 'H-'H COSY spectrum of 6 showed correlations of H-3 with H2-2, Me-29, H-5 and H-1’; H-6 with H-5, H-7, Me-28 and H-1’’; H-2’ with H-1’, H-3’ and H-1’’’; H-12 with H-9, H-11, and H-17; and H-24 with H2-23, Me-26 and Me-27. The HMBC spectrum of 6 exhibited interactions of H-2’, H-5’ and H-3 with C-1’; H-2’’, H-5’’ and H-2’ with C-1’’; H-2’’’, H-5’’’ and H-6 with C-1’’’; H-12, H-11, C-17 and Me-30 with C-13; and H-24, H2-23, H-24, Me-26 and Me-27 with C-25. The important proton signals of 6 correlated with the respective carbon signals in the HSQC spectrum. The 'H and 13C NMR spectra of triterpenic nucleus of 6 were compared with the dammarene-type triterpenoids (19-21). Acid hydrolysis of 6 yielded DL-arabinose (co-TLC comparable, Rr = 0.54, n-butanol-ethanol-water, 4 : 1 : 2.2; [α]D20 = + 92º, conc. 10 in H2O). On the basis of these informations the structure of 6 was elucidated as dammar-12,24-dien-3α, 6β-diol-3-O-β-D-arabinopyranosyl(2→1’)-O-L-arabinopyranosyl-6-O-β-L-arabinopyranoside. This is a new dammariene triarabinoside.

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New dammarane-type triterpenoids from the roots of *Panax ginseng* C. A. Meyer


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**ANTIFUNGAL ACTIVITY OF THE ROOT EXTRACTS OF *PULSATILLA PATENS* AGAINST *CANDIDA GLABRATA***

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**Abstract:** Plants from the genus *Pulsatilla* produce a variety of secondary metabolites with biological activity. These species play a special role in herbal medicine and are used in traditional folk medicine to treat many diseases and ailments. Due to its numerous medicinal properties, they are now also widely used as dietary supplements. In the present study, the antifungal activity of crude extracts of the root of *Pulsatilla patens* (L.) Mill. against the yeast *Candida glabrata* with an IC₅₀ of 9.37 µg/mL is reported.

**Keywords:** *Pulsatilla patens* subsp. *patens*, root and leave extracts, biological activity, microbiological assays

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*Pulsatilla patens* (L.) Mill. (syn. *Anemone patens* L., family Ranunculaceae) is a plant species known under the common name of Eastern Pasqueflower and is native to Europe, Russia, Mongolia, China, Canada and United States. There are three distinguishing subspecies of *P. patens*, *P. patens* subsp. *patens* (L.) Mill., subsp. *multifida* (G. A. Pritzel) Zämélis and subsp. *flavescens* (Zucc.) Zämélis.

*P. patens* is an endangered plant and is considered critically threatened in the Czech Republic (1), included in the Red Data Books of Germany as an endangered plant (2), vulnerable in Sweden (3), relatively restored in Lithuania, and in decreasing number in St. Petersburg and Kaliningrad (4) region of Russia, Latvia (5), and Slovakia (6). In Finland and Estonia, the populations of *P. patens* are considered to be relics (7, 8). In Poland, *P. patens* has been a strictly protected since 1958 (9). The reason for the current status of *P. patens* in North America lies mostly in the reduced area of prairies used for cattle grazing or mowing (10). In Europe the reasons for increased threatened status include destruction and disappearance of natural habitats, cessation of grazing, forest fires, and the habitat eutrophication (11, 12). *P. patens* has been used for centuries in traditional Chinese medicine (13) for the treatment of homeopathic eye ailments, earache, stress, anxiety, tension, skin eruptions, rheumatism, bronchitis, coughs and asthma. Previous phytochemical studies of the roots of *Pulsatilla* species revealed the presence of a high diversity of secondary metabolites (14). The triterpene saponins were isolated from *P. patens* ssp. *multifida* (G.A. Pritzel) Zämélis (15), *P. chinensis* (Bunge) Regel (16-18), *P. koreana* Nakai (19-21), *P. cernua* (Thunb.) Bercht. et Opiz. (22, 23), *P. dahurica* (Fisch. ex DC.) Spreng. (24), *P. turczaninovii* Kryl. et Serg. (25), *P. nigricans* Storck (26), and *P. pratensis* (L.) Mill. (27). Polyphenolic compounds such as flavonoids and anthocyanidins are produced by *P. montana* ssp. *balcana* (Velen.) Zämélis & Paegle, *P. halleri* ssp. *rhodopaea* (Stoj. et Stef.) K. Krause and *P. slaviankae* (Zimmer.) Jordanov & Kožuharov (28). Saponins from *Pulsatilla* spp. have demonstrated multiple biological properties including antitumor (29-31), cognition-enhancing (32, 33), neuroactive (34), neuroprotective (35), immunomodulatory (36), antioxidant

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(37), antimicrobial (38) and cytotoxic (39) activities. In the course of our studies on medicinal plants we evaluated the extracts of leaves and roots of P. patens for their antifungal and antimicrobial activities.

**MATERIAL AND METHODS**

**Plant material**
The leaves and roots of P. patens were collected from Knyszynska Forest, Podlaskie Province, in North-Eastern Poland in May 2013 and identified by Prof. Grażyna Łaska from the Białystok University of Technology, Faculty of Civil and Environmental Engineering, Poland. A voucher specimen has been deposited at the Herbarium “The Herbal Corner”, Podlaskie Province.

**General experimental procedures**
The plant material in the form of fresh roots (23.6 g) and leaves (32.3 g) was extracted by accelerated solvent extraction (ASE) method (Büchi E-916) with 80% methanol and evaporated under reduced pressure. The crude extracts of roots (1.5 g) and leaves (2 g) were analyzed for their antimicrobial, antileishmanial and antimalarial activities.

**Antimicrobial assay**
All organisms are obtained from the American Type Culture Collection (Manassas, VA) and include the fungi Candida albicans ATCC 90028, C. glabrata ATCC 90030, C. krusei ATCC 6258, Cryptococcus neoformans ATCC 90113, and Aspergillus fumigatus ATCC 204305 and the bacteria Staphylococcus aureus ATCC 29213, methicillin-resistant S. aureus ATCC 33591 (MRS), Escherichia coli ATCC 35218, Pseudomonas aeruginosa ATCC 27853, and Mycobacterium intracellulare ATCC 23068. Susceptibility testing is performed using a modified version of the CLSI (formerly NCCLS) methods (40-43). M. intracellulare is tested using a modified method of Franzblau et al. (44). Samples are serially-diluted in 20% DMSO/saline and transferred in duplicate to 96-well flat bottom microplates. Microbial inocula are prepared by correcting the OD630 of microbe suspensions in incubation broth to afford final target inocula. Drug controls [Ciprofloxacin (ICN Biomedicals, Ohio) for bacteria and Amphotericin B (ICN Biomedicals, Ohio) for fungi] are included in each assay. All organisms are read at either 530 nm using the Biotek Powerwave XS plate reader (Bio-Tek Instruments, Vermont) or 544 ex/590 em, (M. intracellulare, A. fumigatus) using the Polarstar Galaxy Plate Reader (BMG LabTechnologies, Germany) prior to and after incubation. Minimum fungicidal or bactericidal concentrations are determined by removing 5 µL from each clear well, transferring to agar and incubating. The MFC/MBC is defined as the lowest test concentration that kills the organism (allows no growth on agar).

**Assay for screening antimalarial activity and cytotoxicity**
The antimalarial activity is determined against chloroquine sensitive (D6) and chloroquine resistant (W2) strains of Plasmodium falciparum by measuring plasmodial LDH activity according to the procedure of Makler and Hinrichs (45). A suspension of red blood cells infected with D6 or W2 strain of P. falciparum (200 µL, with 2% parasitemia and 2% hematocrit in RPMI 1640 medium supplemented with 10% human serum and 60 µg/mL Amikacin) is added to the wells of a 96-well plate containing 10 µL of serially diluted samples (plant extracts, column fractions or pure compounds). The plate is incubated at 37°C, for 72 h in a modular incubation chamber with 90% N2, 5% O2, and 5% CO2. Parasitic LDH activity is determined by mixing 20 µL of the incubation mixture with 100 µL of the MalstatTM reagent (Flow Inc., Portland, OR) and incubating at room temperature for 30 min. Twenty microliters of a 1 : 1 mixture of NBT/PES (Sigma, St. Louis, MO) is then added and the plate is further incubated in the dark for 1 h. The reaction is then stopped by adding 100 µL of a 5% acetic acid solution and the absorbance is read at 650 nm. Artemisinin and chloroquine are included as the drug controls. IC50 values are computed from the dose response curves of growth inhibition using XLfit 4.2. The in vitro cytotoxicity of samples to mammalian cells was also tested in order to determine the selectivity index of the antimalarial activity. The assay is performed in 96-well tissue culture-treated plates. Vero cells (monkey kidney fibroblasts) are seeded to the wells of 96-well plate at a density of 25,000 cells/well and grown for 24 h. Samples at different concentrations are added and cells are further incubated for 48 h. Cell viability is determined by Neutral Red method (46). IC50 values are obtained from dose response curves. Doxorubicin is included as drug control.

**RESULTS**
Microbiological assays of the root extracts of P. patens showed activity against fungal pathogen Candida glabrata with an IC50 value of 9.37 µg/mL.
The results of all antimicrobial activity tests are shown in Tables 1 and 2.

*C. glabrata* is the second most common pathogenic species after *Candida albicans*. *C. glabrata* is problematic in that it is inherently resistant to theazole drugs, which are the most commonly prescribed antifungal medications. Antifungal drugs, like fluconazole and ketoconazole, are not effective in 15-20% of cases against *C. glabrata* (47). *C. glabrata* is still susceptible to polyene drugs such as amphotericin B and nystatin, along with variable vulnerability to fluconazole and caspofungin. However, high renal toxicity and other side effects of amphotericin B contained drugs makes the use of such therapy the last resort approach. In the light of the limitation of existing antifungal therapy against *C. glabrata* the search for new safer drugs in this number natural products-derived agents or herbal preparations is highly desirable.

The extracts from the roots and leaves of *P. patens* showed decreased ability to inhibit the growth of the other four bacteria (*Staphylococcus aureus*, MRSA, *Escherichia coli*, *Pseudomonas aeruginosa*, *Mycobacterium intracellulare*), and four different fungi (*Candida albicans*, *Candida krusei*, *Aspergillus fumigatus*, *Cryptococcus neoformans*) pathogenic to humans (Table 1, 2).

Antimalarial assays of the extracts from the roots of *P. patens* showed low activity (27% of inhibition) against the protozoan, when the antimalarial drug chloroquine (positive control) showed 94-98% of inhibition (Tab. 3).

**DISCUSSION AND CONCLUSION**

The genus *Pulsatilla* comprises about 30 species, but the predominant subspecies in Poland is *Pulsatilla patens subsp. patens* which is diploid (2n

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**Table 1. The test results of the antimicrobial activity of roots and leaves extracts of Pulsatilla patens (primary screen).**

<table>
<thead>
<tr>
<th>Tested strain</th>
<th>Extracts from roots (50 µg/mL)</th>
<th>Extracts from leaves (50 µg/mL)</th>
<th>Amphotericin B (5 µg/mL)</th>
<th>Ciprofloxacin (1 µg/mL)</th>
</tr>
</thead>
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<tr>
<td><em>C. albicans</em></td>
<td>27</td>
<td>2</td>
<td>100</td>
<td>ND</td>
</tr>
<tr>
<td><em>C. glabrata</em></td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>ND</td>
</tr>
<tr>
<td><em>C. krusei</em></td>
<td>17</td>
<td>0</td>
<td>100</td>
<td>ND</td>
</tr>
<tr>
<td><em>A. fumigatus</em></td>
<td>3</td>
<td>7</td>
<td>99</td>
<td>ND</td>
</tr>
<tr>
<td><em>C. neoformans</em></td>
<td>22</td>
<td>0</td>
<td>82</td>
<td>ND</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>8</td>
<td>0</td>
<td>ND</td>
<td>89</td>
</tr>
<tr>
<td>MRSA</td>
<td>10</td>
<td>0</td>
<td>ND</td>
<td>94</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>11</td>
<td>5</td>
<td>ND</td>
<td>96</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>4</td>
<td>4</td>
<td>ND</td>
<td>100</td>
</tr>
<tr>
<td><em>M. intracellulare</em></td>
<td>0</td>
<td>0</td>
<td>ND</td>
<td>72</td>
</tr>
</tbody>
</table>

* The results in %, ND - not determined

**Table 2. Dose reponse (IC50 in µg/mL) results of the of the root extracts of Pulsatilla patens (L.) Mill.**

<table>
<thead>
<tr>
<th>Test strain</th>
<th>Extracts from roots</th>
<th>Amphotericin B</th>
<th>Ciprofloxacin</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. albicans</em></td>
<td>-</td>
<td>0.130</td>
<td>ND</td>
</tr>
<tr>
<td><em>C. glabrata</em></td>
<td>9.37</td>
<td>0.342</td>
<td>ND</td>
</tr>
<tr>
<td><em>C. krusei</em></td>
<td>-</td>
<td>0.669</td>
<td>ND</td>
</tr>
<tr>
<td><em>A. fumigatus</em></td>
<td>-</td>
<td>1.341</td>
<td>ND</td>
</tr>
<tr>
<td><em>C. neoformans</em></td>
<td>42.27</td>
<td>0.372</td>
<td>ND</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>-</td>
<td>ND</td>
<td>0.117</td>
</tr>
<tr>
<td>MRSA</td>
<td>-</td>
<td>ND</td>
<td>0.134</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>-</td>
<td>ND</td>
<td>0.005</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>-</td>
<td>ND</td>
<td>0.096</td>
</tr>
<tr>
<td><em>M. intracellulare</em></td>
<td>-</td>
<td>ND</td>
<td>0.426</td>
</tr>
</tbody>
</table>

* The results in IC50, ND - not determined
Table 3. The test results of the antimalarial activity of roots and leaves extracts of *Pulsatilla patens* (L.) Mill.

<table>
<thead>
<tr>
<th>Tested strain</th>
<th><em>Plasmodium falciparum</em> (D6 % Inh.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroquine</td>
<td>97</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>94</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>98</td>
</tr>
<tr>
<td>Extracts from roots</td>
<td>27</td>
</tr>
<tr>
<td>Extracts from leaves</td>
<td>0</td>
</tr>
</tbody>
</table>

"P. patens" is a monoeccious, long-lived perennial species (up to tens of years) with a vertically branching rhizome which can form several shoots and makes older plants form clumps. It is a hemicryptophyte which definitely exclude capacity for vegetative reproduction (49). *P. patens* tend to flower between March and May.

*P. patens* is a lowland species of Boreo-meridional-continental and circumpolar distribution in the area between 44°30′ and 63°30′ north latitude and 12° and 70° east longitude (50). In Poland, *P. patens* reaches the western border of its range and the number of its sites clearly decreases towards the west and south. In north-eastern part of Poland, there are about 80% of sites of *P. patens* in the country which is suitable for successful conservation and management.

The effectiveness and health benefits of the use of biologically active plant extracts from *P. patens* was verified in numerous clinical research (51-53). This species was used for centuries to treat many diseases and ailments. Numerous applications of *P. patens* in traditional medicine is one of the reasons for reducing the abundance of this species. Currently *P. patens* is a threatened plant species in Europe, listed in the Annex II of European Union Habitats Directive (92/43/ETY) (54) and in Appendix I of the Bern Convention (55).

In Poland, *P. patens* requires active protection (9). It has been classified to be protected within the programme Natura 2000 (56). In the “Red List of Vascular Plants in Poland” it is classified as critically endangered (E category) (57), in “Red Data Book of the Polish Carpathians” as endangered (CR category) (58) and in the “Polish Red Data Book of Plants”, is listed as of low risk (LR) species (59).

Collection of plant material for testing was done in accordance with Decision No. WPN.6400. 29.02.2013 issued by the Regional Directorate of Environmental Protection in Białystok. In order to obtain larger amount of plant material for future use in the production of antifungal drugs, a cooperation agreement was signed between the Białystok University of Technology and Botanical Garden “Herbal Corner”. Within this collaboration whole plant species were transferred from natural environment to the Botanical Garden for their propagation.

In 2015, the application for an invention patent titled „The use of *Pulsatilla patens* (L.) Mill. in the treatment of fungal diseases” was submitted to the Polish Patent Office by the authors of this publication. Pharmaceutical application of others *Pulsatilla* species were patented by Chinese and Korean authors (60, 61). The first invention provides a pharmaceutical composition comprising an aqueous extract of root of *Pulsatilla chinensis* and its use in medicine. Chinese root extract shows very low toxicity and minor side effects. The application of this extract increases the effectiveness of the treatment of anemia in patients. The second invention relates to the use of *Pulsatilla koreana* extract as an active ingredient inhibiting the proliferation of the immunocytes and preventing acute and chronic inflammation.

Phytochemical research confirms the high content of a variety of secondary metabolites derived from the *Pulsatilla* species. The compound isolated from the methanol extract of the root of *P. patens* subsp. *multifida* inhibits the growth of skin cancer and is used to treat diseases of the eye, ear homeopathic pain, skin eruptions, rheumatism, bronchitis, cough, asthma and in the alleviation of stress, anxiety and tension (15). *P. chinensis* provided triterpene acid and triterpenoid glycosides (saponins *Pulsatilla* A and B) (62). The triterpene acid obtained from the methanol extract of the root of this species exhibits high cytotoxic activity against malignant lung cells. The triterpene saponins from plant material of *P. chinensis* are currently used in the treatment of inflammatory diseases (63) and have highly antiprotozoal (64), antibacterial (65), antiparasitic (66), antifungal (67) and molluscicidal (68) activity. Pharmacological studies of secondary metabolites from *P. koreana* showed that protoanemonin possess antifungal activity and act as an antibiotic. Additionally, the biologically active components isolated from the root of *P. koreana* have antitumor (69), anti-inflammatory (70), antiparasitic (71) and antibacterial (72) activity. Natural pigments (anthocyanins) isolated from *P. cernua* have anti-allergic, antifungal, antitumor and anti-ulcer activity (73).

Numerous plant species were tested for their antifungal activity. The mixture of saponins isolated from *Maesa lanceolata* inhibited the growth of *Candida albicans* and *Microsporum canis* at the concentration of 16 (48). *P. patens* (L.) Mill. is a monoeccious, long-lived perennial species (up to tens of years) with a vertically branching rhizome which can form several shoots and makes older plants form clumps. It is a hemicryptophyte which definitely exclude capacity for vegetative reproduction (49). *P. patens* tend to flower between March and May.

*P. patens* is a lowland species of Boreo-meridional-continental and circumpolar distribution in the area between 44°30′ and 63°30′ north latitude and 12° and 70° east longitude (50). In Poland, *P. patens* reaches the western border of its range and the number of its sites clearly decreases towards the west and south. In north-eastern part of Poland, there are about 80% of sites of *P. patens* in the country which is suitable for successful conservation and management.
100 mg/mL (74). Secondary metabolites isolated from
Colubrina retusa L. (Rhamnaceae) showed antifungal
activity against Candida albicans, Cryptococcus neoform-
ans and Aspergillus fumigatus (75). Bader (76)
reported antifungal activity of some saponins against
Candida albicans. Steroidal saponins from Yucca
schidigera Roezl. ex Ortgies display effective growth-
inhibitory activities against food-deteriorating and
film-forming yeasts, as well as dermatophytic yeasts
and fungi (77). Various species of the genus
Phytolacca (Phytolaccaceae) also show antifungal
activity (78-79). Mixture of saponins from the seeds of
Chenopodium quinoa Willd. (Chenopodiaceae) inhib-
ited the growth of Candida albicans at the concentra-
tion of 50 mg/mL (80). Many plant species inhibited
the growth of Candida albicans (81-82) but has not
been tested against Candida glabrata.

The wide chemical and biological diversity of
plants secondary metabolites accelerated an intense
search for new natural-product derived drugs, espe-
cially new antibiotics and antifungal agents.
Relatively high and selective activity of the roots
extracts of Pulsatilla patens against Candida
glabrata discovered in our studies prompts further
research on isolation and identification of active
components from this plant.

Acknowledgments

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Service Specific Cooperative Agreement of the
United States.

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Antifungal activity of the root extracts of *Pulsatilla patens* against...


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Pakistan has been described as one of the countries that have the richest floral biodiversity worldwide. Indeed, this is due to its unique geographical location, climatic conditions, and geomorphological characteristics. Herbal medicinal systems, knowledge, and practices have been transmitted through the ages. For centuries, medicinal plants were the only resources available for the treatment of several diseases which plagued humanity used for amelioration, mitigation and treatment of different diseases. In fact, many of today’s drugs have been derived from medicinal plants (1). Additionally, the World Health Organization has reported that 80% of the world’s population relies on herbal medicine for primary health care (2). However, to the best of our knowledge, several medicinal plants used as folk medicine have not received scientific attention yet. C. sophera is one of them, which require scientific attention. Detailed literature survey showed that the whole plant extract is used traditionally for treatment of rheumatic disorders, joint pain, inflammatory fevers, diabetes, dyspnœa, aposita itching, syphilitic sores and liver diseases (3). According to published material on C. sophera, hepatoprotective activity (3) and hypoglycemic activity (4) have been reported. The constituents isolated from C. sophera include cyclosophoside A; 1,8-dihydroxy-2-methylanthraquinone 3-neohesperidoside; chrysophanol; physcion; 1,2,7-trihydroxy-6,8-dimethoxy-3-methylanthraquinone; 1,2,6-trihydroxy-7,8-dimethoxy-3-methylanthraquinones; 1,8-dihydroxy-3,6-dimethoxy-2-methyl-7-vinylanthraquinone; 1,3-dihydroxy-5,7,8-trimethoxy-2-methylanthraquinone; emodin and 5,7,3’,4’-tetrahydro-3-methoxyflavone-5-O-Rha-7-O-Glc-Xyl (5).

In the present investigation, dichloromethane and methanol extracts of C. sophera were evaluated for their possible antioxidant activities and inhibitory action against key enzymes.
MATERIALS AND METHODS

Plant material and extraction

The plant material (leaves and roots of *C. sophera*) was collected from the periphery of Sargodha city, Pakistan. The specimens for identification of the plant material were prepared and the plant was identified as *C. sophera* by Professor Dr. Altaf Ahmad Dasti, Director, Institute of Pure and Applied Biology, Bahauddin Zakariya University, Multan. The voucher specimen was deposited in the herbarium of the department. The maceration of powdered plant material (500 g) was done in dichloromethane separately for a period of 24 h and then filtered. The procedure was repeated thrice using 1.5 L dichloromethane in each step. The filtrates from each step were combined separately and concentrated *in vacuo* at 35°C. The same process was adopted for extraction of plant material using methanol as solvent successively.

Quantification of phenolic compounds

**Determination of total phenol content**

The total phenol content was determined as described by Slinkard and Singleton (6) with slight modifications. Briefly, 0.25 mL plant extract (0.5 mg/mL) was mixed with a tenfold diluted Folin-Ciocalteu reagent solution (Sigma-Aldrich) and the mixture was shaken vigorously. After 3 min, 0.75 mL of 75 g/L sodium carbonate solution (1% in water) was added to the mixture and was allowed to react for 2 h at room temperature. The absorbance was then read at 760 nm. The total phenol content was expressed as mg gallic acid equivalents (GAE) per g crude extract using a gallic acid standard curve.

**Determination of total flavonoid content**

The total flavonoid content was determined as described by Berk et al. (7) with slight modifications. Briefly, 1 mL anhydrous aluminium trichloride (2%) solution in methanol was added to 1 mL plant extract. The absorbance of the mixture was shaken vigorously. After 3 min, 0.75 mL of 75 g/L sodium carbonate solution (1% in water) was added to the mixture and was allowed to react for 2 h at room temperature. The absorbance was then read at 415 nm. The total flavonoid content was expressed as mg rutin equivalents (RE) per g crude extract using a rutin standard curve.

**Determination of antioxidant activities**

*1,1-Diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay*

The effect of the plant extracts on DPPH radical was assessed according to the method described by Sarikurkcu (8). Briefly, 1 mL of plant extract was added to 4 mL DPPH solution (0.004%) in methanol. The absorbance was measured at 517 nm after 30 min incubation at room temperature in the dark. The percentage inhibition was calculated by the formula:

\[
\text{\% Inhibition} = 100 - \frac{\text{Absorbance of test}}{\text{Absorbance of control}} \times 100
\]

Gallic acid and BHT were used as a positive control and IC_{50} was then determined.

*In vitro superoxide anion scavenging assay*

The assay is performed by the method of Beauchamp and Fridovich (9). To 50 mM phosphate buffer maintained at pH of 7.6 were added sequentially: riboflavin (20 µg), 12 mM EDTA and nitro blue tetrazolium (NBT) 0.1 mg/3 mL. The test extract (0.5 mg/mL) was then added to this reaction mixture and illuminated, for 150 s. The absorbance was recorded at 590 nm immediately after illumination. Methanol was used as blank. Quercetin was used as reference standard. Each experiment was carried out at least three times and the data presented as an average of three independent determinations.

**Determination of cholinesterase and \(\alpha\)-glucosidase activity**

*Cholinesterase inhibition assay*

Cholinesterase inhibitory activity was measured using Ellman’s method as previously reported by Aktumsek et al. (10) with slight modifications. The plant extract (50 µL) was mixed with dithiobis-nitrobenzoate (DTNB) (125 µL) and cholinesterase solution (25 µL) in Tris-HCl buffer (pH 8.0) in a 96-well microplate. The reaction was initiated by the addition of 25 µL of acetylthiocholine iodide or butyrylthiocholine chloride. The absorbance was read at 405 nm after 10 min incubation at room temperature. The percentage inhibition was calculated by formula given below.

\[
\text{\% Inhibition} = 100 - \frac{\text{Absorbance of test}}{\text{Absorbance of control}} \times 100
\]

Galantamine is used as a positive control and IC_{50} value was determined.

*\(\alpha\)-Glucosidase inhibition assay*

\(\alpha\)-Glucosidase inhibitory activity was performed following the previous method described by Palanisamy et al. (11) with some modifications. Plant extract (50 µL) was mixed with glutathione (50 µL), \(\alpha\)-glucosidase solution (50 µL) in phosphate buffer (pH 6.8) and PNPG (50 µL) in a 96-well microplate and incubated for 15 min at 37°C. The reaction was stopped by addition of 0.2 M sodi-
um carbonate (50 μL) and the absorbance was read at 400 nm. The percentage inhibition of α-glucosidase was calculated as follows:

\[
\text{% Inhibition} = 100 - \left( \frac{\text{Absorbance of test}}{\text{Absorbance of control}} \right) \times 100
\]

Acarbose was used as a positive control and IC\text{50} was determined.

**Statistical analysis**

The experiments were carried out in triplicate. The results are expressed as the mean ± standard deviation (SD). The differences between the different extracts were analyzed using one-way analysis of variance (ANOVA) followed by Tukey’s honestly significant difference post hoc test with using SPSS v. 14.0.

**RESULTS**

**Extraction**

The extraction of leaves of *C. sophera* with methanol afforded maximum yield of extract weighing 40.5 g followed by methanol extract of roots with weight of 25.3 g. However, the yield of the dichloromethane extracts of both the plant parts was lower than their respective methanol extracts. The results of extraction of dried leaves and roots of *C. sophera* are shown in the Table 1.

**Quantification of phenolic content**

The total phenol and flavonoid content of the plant extracts are summarized in Table 2. Dichloromethane extract of root (CSRD) and leaves (CSLD) of *C. sophera* has higher phenolic content than methanol extract of root (CSRM) and leaves (CSLM) of *C. sophera*. On the other hand, it was observed that the flavonoid content of the methanol extract of root (CSRM) and leaves (CSLM) of *C. sophera* showed the highest flavonoid content.

**Determination of antioxidant activities**

Table 3 summarizes the reducing power and radical scavenging of *C. sophera*. It was found that the plant extracts showed variable radical scavenging capabilities on DPPH. Additionally, it was noted that the plant extracts exhibited variable reducing potential. However, as shown in Table 2, none of the plant extracts exhibited radical scavenging capabilities on DPPH an reducing activity which was significantly (p < 0.05) lower than gallic acid, BHT and quercetin respectively.

**In vitro enzyme inhibition**

It was observed that the plant extracts exhibited variable inhibitory effects on acetyl cholinesterase. The plant extracts were significantly (p < 0.05) less active than the positive control eserine against cholinesterases. However, both leaves and root methanol extracts of *C. sophera* significantly (p < 0.05) inhibited α-glucosidase (IC\text{50}: 11.9 mg/mL; IC\text{50}: 12.3 mg/mL, resp.) as compared to acarbose (IC\text{50}: 38.3 mg/mL), respectively (Table 4).

**DISCUSSION**

The use of plant-based products for the management and treatment of diseases is gaining much momentum from both scientific and consumer per-

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**Table 1:** Results of extraction of leaves and roots of *C. sophera*.

<table>
<thead>
<tr>
<th>Part used</th>
<th>Weight of dried material (g)</th>
<th>Solvent</th>
<th>Name of extract</th>
<th>Extract weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaves</td>
<td>500</td>
<td>Dichloromethane (3 x 1.5 L)</td>
<td>CSLD</td>
<td>07.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methanol (3 x 1.5 L)</td>
<td>CSLM</td>
<td>40.5</td>
</tr>
<tr>
<td>Roots</td>
<td>500</td>
<td>Dichloromethane (3 x 1.5 L)</td>
<td>CSRD</td>
<td>02.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methanol (3 x 1.5 L)</td>
<td>CSRM</td>
<td>25.3</td>
</tr>
</tbody>
</table>

**Table 2:** Total phenol and flavonoid content of the plant extracts.

<table>
<thead>
<tr>
<th>Plant extracts</th>
<th>Total phenol content (mg)</th>
<th>Total flavonoid content (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSLD</td>
<td>30.56 ± 1.20</td>
<td>29.53 ± 1.003</td>
</tr>
<tr>
<td>CSLM</td>
<td>32.10 ± 1.179</td>
<td>30.61 ± 0.042</td>
</tr>
<tr>
<td>CSRD</td>
<td>33.40 ± 1.179</td>
<td>32.24 ± 0.092</td>
</tr>
<tr>
<td>CSRM</td>
<td>39.11 ± 1.179</td>
<td>34.61 ± 0.072</td>
</tr>
</tbody>
</table>

CSLD: *C. sophera* leaves dichloromethane extract; CSLM: *C. sophera* leaves methanol extract; CSRD: *C. sophera* root dichloromethane extract; CSRM: *C. sophera* root methanol extract.
spectives. Indeed, herbal therapies have been used for curative purposes since the dawn of civilization. The relentless efforts for wellbeing and to combat diseases have guided scientists as well as health care providers towards safer and natural alternatives such as medicinal plants. Currently, there is a renewed interest in natural inhibitors from plant-based medicines to modulate physiological effects of enzymes linked to several pathologies such as diabetes, obesity, neurodegenerative diseases, and inflammation, amongst others. The present study has endeavored to investigate the possible inhibitory effects of C. sophera to modulate key enzyme involved in diabetes (α-glucosidase) and neurodegenerative disorders (acetylcholinesterase).

Diabetes is a chronic disease characterized by elevated blood sugar level which leads to the onset of serious health complications such as cardiovascular problems, nephropathy, and neuropathy (12). The inhibition of α-glucosidase which is involved in the hydrolysis of sugars in vivo has been an important strategy for the management of diabetes thereby lowering postprandial glucose level. Inhibitors of α-glucosidase delay the breaking down of carbohydrate in the gut and decrease postprandial blood glucose peak in diabetic patients. Synthetic oral hypoglycaemic agents such as acarbose, miglitol, and voglibose are currently used for the treatment of diabetes (13). However, their side effects, such as abdominal discomforts and flatulence, have guided research towards safer and more effective alternatives notably from natural sources (14). In the present study, the plant extracts showed inhibition against α-glucosidase. Additionally, it was noted that the plant extracts were potent inhibitors of α-glucosidase, dichloromethane and methanol extracts of C. sophera showed significantly lower IC50 values than acarbose and therefore can be potentially useful as an effective therapy for postprandial hyperglycemia with minimal side effects. This is in line with the results of Table 3 and Table 4.

### Table 3: Antioxidant activities of dichloromethane and methanol extracts of leaves and roots of C. sophera observed in DPPH radical scavenging and superoxide anion scavenging.

<table>
<thead>
<tr>
<th>Extract/ Standard</th>
<th>Conc. (mg/mL)</th>
<th>DPPH radical scavenging assay %RSA (IC50)</th>
<th>Superoxide anion scavenging assay %RSA (IC50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSLD</td>
<td>0.5</td>
<td>-2.11 (-)</td>
<td>16 (-)</td>
</tr>
<tr>
<td>CSLM</td>
<td>0.5</td>
<td>10.53 (-)</td>
<td>10 (-)</td>
</tr>
<tr>
<td>CSRD</td>
<td>0.5</td>
<td>38.37 (-)</td>
<td>46 (-)</td>
</tr>
<tr>
<td>CSRM</td>
<td>0.5</td>
<td>80.30 (230 ± 2.9)</td>
<td>53 (280 ± 1)</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>0.5</td>
<td>96.97 (22.6 ± 0.4)</td>
<td>-</td>
</tr>
<tr>
<td>BHT</td>
<td>0.5</td>
<td>85.87 (128.8 ± 2.1)</td>
<td>-</td>
</tr>
<tr>
<td>Quercetin</td>
<td>0.5</td>
<td>-</td>
<td>99 (94.1 ± 1.1)</td>
</tr>
</tbody>
</table>

CSLD: C. sophera leaves dichloromethane extract; CSLM: C. sophera leaves methanol extract; CSRD: C. sophera root dichloromethane extract; CSRM: C. sophera root methanol extract.

### Table 4: Enzyme inhibition activities of dichloromethane and methanol extracts of C. sophera leaves and roots against α-glucosidase and acetylcholinesterase.

<table>
<thead>
<tr>
<th>Extract/ Standard</th>
<th>α-Glucosidase</th>
<th>Acetylcholinesterase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inhibition at conc. of 0.5 mg/mL (%)</td>
<td>IC50 (µg/mL)</td>
</tr>
<tr>
<td>CSLD</td>
<td>68.4 ± 1.7</td>
<td>101.1 ± 0.7</td>
</tr>
<tr>
<td>CSLM</td>
<td>95.2 ± 1.8</td>
<td>119.0 ± 0.2</td>
</tr>
<tr>
<td>CSRD</td>
<td>82.5 ± 1.3</td>
<td>74.1 ± 1.0</td>
</tr>
<tr>
<td>CSRM</td>
<td>96.9 ± 1.5</td>
<td>12.3 ± 0.5</td>
</tr>
<tr>
<td>Acarbose</td>
<td>92.2 ± 0.1</td>
<td>38.3 ± 0.1</td>
</tr>
<tr>
<td>Eserine</td>
<td>-</td>
<td>92.1 ± 1.6</td>
</tr>
</tbody>
</table>

CSLD: C. sophera leaves dichloromethane extract; CSLM: C. sophera leaves methanol extract; CSRD: C. sophera root dichloromethane extract; CSRM: C. sophera root methanol extract.
with report of Picot (15) who reported natural α-glucosidase inhibitors from plants to have strong inhibition towards the activity of the enzyme compared to acarbose.

Plant extracts from the present study were found to inhibit acetylcholinesterase although their inhibitory action was less potent than the known inhibitor eserine. The inhibition of cholinesterases leads to an increase in the concentration of acetylcholine in the brain which subsequently results in an increase in communication between the brain nerve cells (12, 16). Indeed, both acetylcholinesterase and butyrylcholinesterase inhibitors have been key targets for the treatment of neurodegenerative disorders such as Alzheimer’s disease (17, 18). Cholinesterase inhibitors constitute, to date, the most effective approach to treat the cognitive symptoms of neurological disorders. Hence, plants studied in the present study can be of therapeutic utility both on cognitive performances and on the quality of life in these patients.

The variation in activity of the plant extracts against these enzymes might be explained based on the complex composition and potential synergistic effect(s) of individual phytochemicals present in each sample. Interestingly, we found varying concentration of phenolics and flavonoids in extracts of these plants. Previously, it has been reported that inhibitory activity of plant extracts might be due to the presence of several phytochemicals such as flavonoids, saponins, and tannins. Additionally, studies on α-glucosidase inhibitors isolated from medicinal plants suggest that several potential inhibitors belong to flavonoid class which has features of inhibiting metabolic enzymes. Recently, it has been shown that phenolics play a role in mediating amylose inhibition and therefore have potential to contribute to the management of type 2 diabetes (19). Wakhande (20) studied the ethanol extract of *C. sophera* for hepatoprotective activity in rats. The plant extract (500 mg/kg, p.o.) showed a remarkable hepatoprotective activity against paracetamol induced hepatotoxicity as judged from the serum markers for liver damage. Paracetamol induced a significant rise in aspartate amino transferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin and decrease in total protein. Treatment of rats with ethanol extract (500 mg/kg) significantly altered serum marker enzymes levels to near normal against paracetamol treated rats. The activity of the extract was comparable to the standard drug Liv-52 (5 mL/kg, p.o.). The significant hepatoprotective activity demonstrated by the ethanolic extract of *C. sophera* was correlated to the higher content of flavonoids. The anti-inflammatory activity of *C. sophera* was studied using the carrageenan, dextran induced rat paw edema, and cotton pellet induced granuloma in rats. The ethanol extract was administered at the concentrations of 200 and 400 mg/kg body weight whereas rhamnetin (RN) was administered at a dose of 10 and 15 mg/kg, b.w. Indomethacin was used as standard drug. The ethanol extracts at 400 mg/kg, showed maximum inhibition of inflammation induced by carrageenan (44%), dextran (40%), cotton pellets (37.47%). On the other hand, rhamnetin (15 mg/kg) exhibited maximum anti-inflammatory effect, which is 79 and 33% at the end of 3h with carrageenin, and dextran-induced rat paw edema, respectively. In a chronic test, rhamnetin (15 mg/kg) showed 43.32% reduction in granuloma weight (21).

Free radicals are known to play a pivotal role in the onset and exacerbation of several pathologies (22). By counteracting these free radicals, antioxidants help in preserving good health. Indeed, phytochemicals have received much interest owing to their molecular structure which consists of hydroxyl groups on aromatic rings and this has been associated with their functionality as oxidant scavengers (23). The methanolic extracts of leaves of *C. grandis*, *C. didymobryta*, *C. glauca*, *C. fistula*, *C. occidentalis*, *C. nodosa* and *C. sophera* were examined for total antioxidant capacity using phosphomolybdate method and DPPH radical scavenging. The *C. glauca* leaves methanol extract showed highest antioxidant capacity and most potent DPPH free radical scavenging with values of 533.85 mg ascorbic acid equivalent (AAE/g extract) and 18.53 µg/mL, respectively. The SC50 (concentration required to scavenge 100 µg/mL DPPH radical by 50%) exhibited by the remaining extracts was in the order of *C. grandis* (50.37 µg/mL), *C. nodosa* (75.15 µg/mL), *C. fistula* (128.86 µg/mL), *C. didymobryta* (202.75 µg/mL), *C. occidentalis* (213.48 µg/mL) and *C. sophera* (225.88 µg/mL). The descending order of total antioxidant capacity was *C. grandis* (240 mg AAE/g extract), *C. nodosa* (210.51 mg AAE/g extract), *C. fistula* (198.55 mg AAE/g extract), *C. didymobryta* (184.01 mg AAE/g extract), *C. sophera* (136.53 mg AAE/g extract) and *C. occidentalis* (134.50 mg AAE/g extract) (24). In another study, the ethanolic extract of *C. sophera* leaves and its chloroform, ethylacetate and ethanol soluble fractions were evaluated for their antioxidant and anti-inflammatory potential. The test fractions demonstrated in vitro scavenging of hydroxyl radical in range of 24 to 95% and DPPH radicals scavenging with IC50 values ranging in between 0.9-0.2
mg/mL. However, only ethanolic fraction exhibited maximum anti-inflammatory activity by a reduction in carrageenan induced paw edema at dose of 750 mg/kg p.o. (69.7% at 4 h interval). Similarly, ethanolic fraction showed greater protection (56.7%) than other fractions against acetic acid induced writhing. It was suggested that flavonoids present in C. sophera might have caused these activities (25).

Phytochemicals act by inhibiting oxidative chain reactions at cellular level thereby increasing their therapeutic efficacy (26). In the present study, the phenolic content of the plant extracts was estimated using the Folin-Ciocalteau method. This method is rapid and simple but also measures various interfering nonphenolic compounds such as ascorbic acid, thiol, and nitrogen containing compounds (27). Flavonoids are the major class of phenolic compounds and are known to exhibit strong antioxidant activities (28, 29). Interestingly, in the present study, it was observed that Oh extract showed high phenolic and flavonoid content.

The reducing power of plant extracts is regarded as an indication of their antioxidant capacities (30). Results from the present study have demonstrated that the plant extracts showed good abilities to quench DPPH. The ability of the plant extracts to quench was related to the observed high phenol content.

CONCLUSION

Data gathered from the present investigation demonstrated that methanol and dichloromethane extract possessed antioxidant capabilities and exhibited inhibitory potential against cholinesterase and α-glucosidase in vitro. Furthermore, to date, no such scientific information on this plant has been gathered. However, it was observed that the antioxidant capacities and cholinesterase inhibitory activities of the plant extracts were less potent than the controls. Further works related to the isolation of the active constituents through bioassay-directed fractionation are in progress in our laboratory. To mark those compounds that can be exploited for novel drug development and to be used as inhibitors of enzymes of therapeutic importance. Superoxide anion scavenging activity, it could be presumed that this extract is able to prevent lipid peroxidation and further suggest that the extract is a potential therapeutic agent for the control of oxidative and non-oxidative damage caused by reactive oxygen and nitrogen species. The results have demonstrated that the root possesses antioxidant and superoxide anion scavenging abilities, which indicates that the plant contains certain compounds which are potential antioxidants. Since reactive oxygen species are thought to be associated with food deterioration and the pathogenesis of chronic infections, and inflammatory diseases, the observed inhibitory potential may partially explain the helpful effects of C. sophera in treating different disease conditions. The antioxidants present in this plant extract may function by combining with oxygen or preventing oxygen from reacting with components of the food. It is however, worthwhile to further investigate the in vivo potentials of this plant and also isolate the active components which could ultimately lead to their application in the food industry as an antioxidant flavour or in pharmaceutical formulations.

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Hedera helix (English ivy, Common ivy) is an evergreen climbing plant of Araliaceae family, with deep green, glossy, coriaceous leaves, whose shape depends on the type of a branch (flowering or non-flowering) and the location on stems. English ivy naturally grows in Western, Central and Southern Europe and was also introduced in North America and Asia.

The biologically active compounds, mainly responsible for the medicinal application of the H. helix extracts, in medicine are triterpene saponins, particularly hederacoside C (not less than 3.0% in dried herbal substance, according to European Pharmacopoeia (1)) with a small amount of α-hederin, hederagenin (which can be developed by hydrolysis during the drying process). Phenolics (flavonoids, coumarins and phenolic acids), steroids, volatile oil, and polyacetylenes are also present in leaves (2).

The Hedera leaves extracts exhibit spasmolytic/antispasmodic, anti-inflammatory, antimicrobial, analgesic, antimitogenic and antioxidant activities, and are used as an expectorant in case of a productive cough, for catarrhs of the respiratory tract, and in case of the symptoms of chronic inflammatory bronchial conditions (2-7).
The aim of the study was to determine the hederacoside C content in three different extracts from the leaves of H. helix, to evaluate the antimicrobial activity in vitro, and the anti-inflammatory activity in the carrageenan-induced paw edema test as well as the effects on bronchial and lung tissue of the most potent H70 extract.

MATERIALS AND METHODS

Plant material

The leaves of H. helix were collected in the autumn in the Botanical Garden of Danylo Halytsky Lviv National Medical University, Lviv, Ukraine. The plant was authenticated by dr. O.Cherpak (Department of Pharmacognosy and Botany of the same University). A voucher specimen No. LVMU 13209/6 was deposited in the herbarium of the same department.

Preparation of the extracts

The air-dried leaves of H. helix were powdered, then extracted with water, 30% ethanol and 70% ethanol separately (6 × 30 min) in a water bath under reflux. The extracts were combined and evaporated to the soft residue under reduced pressure and re-dissolved in water, then lyophilized to attain dry amorphous yellowish to yellow-green powders with a light aromatic odor and a specific bitter taste, and marked as H, H30 and H70. The procedure of preparation of the extracts according to the scheme was repeated three times and yield (mean ± SEM) was calculated as a percentage regarding the plant material.

TLC analysis of the extracts

10.0 mg of each extract (H, H30 and H70) was dissolved in 1 mL of 70% ethanol and analyzed by TLC chromatography applying specific spray reagents to detect the presence of the selected saponins, flavonoids and phenolic acids.

Phenolic acids and flavonoids were analyzed by TLC on cellulose plates with the mobile phase: ethyl acetate – anhydrous formic acid – glacial acetic acid – water (100 : 11 : 11 : 26). The plates were viewed under UV-254 and 365 nm light and sprayed before and after with Naturstoffreagenz A (β-aminoethyl ester of diphenylboric acid) (NA) (0.1% in ethanol). The analysis of saponins was performed by TLC on silica gel plates in the mobile phase: anhydrous formic acid – ethanol – acetone – ethyl acetate (4 : 20 : 20 : 30) and after that sprayed with an alcoholic solution of sulfuric acid, heated for 10 min at 110°C, and observed at daylight and UV-365 nm (8).

Quantification of triterpene saponins (hederacoside C) by HPLC

An assay of hederacoside C in the plant material and the extracts was carried out using HPLC-method according to European Pharmacopoeia (1) for Ivy leaf.

Pharmacological tests

Animals

Adult (3-4 month old) Swiss albino mice (25 – 30 g) and Wistar albino rats (180 – 280 g) of both sexes were obtained from the animal house of the Department of Pharmacology, Danylo Halytsky Lviv National Medical University, Lviv, Ukraine. The animals were allowed to acclimatize for 7 days. They were maintained in steel cages under standard laboratory conditions (temperature 25 ± 2°C in a natural light-dark cycle). Standard laboratory chow and water were supplied ad libitum. The ethical guidelines for investigations using conscious animals were obeyed; the procedures were approved by the Danylo Halytsky Lviv National Medical University’s Ethics Committee and complied with international guidelines.

Acute toxicity test

The acute toxicity (LD₅₀) of the extracts (H, H30 and H70) was determined in Swiss albino mice according to the procedures outlined by the Organization for Economic Co-operation and Development (OECD) (9, 10).

The animals were randomly divided into 10 groups, each of them with 6 male mice. Groups 1 – 5 were intraperitoneally administered the aqueous solutions of the dry extracts at a dose of 0.5, 1.0, 1.5, 2.0, and 2.5 g/kg body weight, respectively; groups 7 – 9 were intragastrically administered the extracts at a dose of 5.0, 7.5 and 10.0 g/kg, respectively. The animals of group 6 received water intraperitoneally and group 10 – water intragastrically (control groups). The animals were followed for any signs of toxicity and/or death within 24 h and then observed for 14 days.

Antimicrobial activity

The antimicrobial activity of the H, H30 and H70 extracts was tested by the disc diffusion method against standard strains: Gram-positive bacteria: Staphylococcus aureus ATCC 25923, Staphylococcus epidermidis ATCC 12228; Gram-negative bacteria: Escherichia coli ATCC 25922, Pseudomonas aeruginosa ATCC 9027, Proteus vulgaris ATCC 4636 and yeast: Candida albicans ATCC 885653, by the procedure according to European Pharmacopoeia (1).
The surface of a culture medium (sterile nutrient agar for bacteria and Sabouraud agar for yeast) was inoculated with 1 mL of suspension cultures adjusted to a microorganism concentration of $1 \times 10^9$ colony forming units (CFU)/mL. After the incubation, known amounts (0.5, 1.0 and 1.5 mg) of each dry extract were dissolved in distilled water (50 µL), applied on sterile paper discs (6 mm in diameter, paper chromatography Whatman No.1), and allowed to dry at room temperature. The discs were applied to the surface of the culture medium with inoculated strains and incubated at 37°C for 24 and 72 hours for bacteria and yeast, respectively. The antimicrobial activity was determined through the zone of the microorganisms growth inhibition around the disk in mm. The mean values were calculated for five parallel experiments.

**Anti-inflammatory activity**

The anti-inflammatory activity of the H, H30 and H70 extracts was investigated in an aseptic edema model, which was induced by carrageenan (7, 9, 11). Rats (250-280 g) of both sexes were divided into five groups of six animals each. The extracts dissolved in water were administrated intragastrically at a dose of 200 mg/kg once a day for four days, while sodium diclofenac as a reference drug was given at a dose of 8 mg/kg in water solution. The control group (negative control) received only vehicle (1 mL of distilled water by the same scheme). On the fifth day of the experiment, 0.1 mL (2%, w/v) of carrageenan solution in water was subcutaneously injected into the plantar surface of the right hind paws of all the animals. The paw volume was measured with a plethysmometer two times: once before the injection of carrageenan and then after 3 h following the carrageenan administration.

The ratio of the anti-inflammatory effects of the *Hedera* extracts was calculated by the following expression: the anti-inflammatory activity (%) = (1 - D/C) × 100, where D represents the percentage difference in paw volume after carrageenan was administered to the rats, and C – the percentage difference of volume in the control group.

**Influence on the lungs and bronchus**

The animals (rats, 180-220 g) were divided into three groups, each of them with 6 rats: 1 – the intact animals; 2 – the control group, with intranasally administered Sephadex G-50 suspension (50 mg/mL) at a dose of 2.0 mL/kg of body weight once a day for 5 days; 3 – the animals which were intragastrically administered an aqueous solution of the *Hedera* leaves dry extract (H70 at a dose of 200 mg/kg twice a day) and sephadex for 5 days (12, 13).

The body weight of each rat was assessed by ether on the sixth day of the experiment and bronchus and lung tissue fixed in 10% formalin solution was evaluated by histopathological examination; sections of tissue were stained by hematoxylin and eosin (HE stain) (14).

**Statistical analysis**

The results were expressed as a mean ± standard error of a mean (SEM). Student’s *t*-test was used to analyze the data and *p* < 0.05 was considered to be statistically significant.

**RESULTS AND DISCUSSION**

**Phytochemical analyses**

The H, H30 and H70 extracts were obtained (with yield 23.0 ± 0.06; 24.5 ± 0.07 and 24.6 ± 0.07%, respectively) from the *Hedera helix* leaves. Phytochemical screening of the extracts by TLC showed the presence of saponins (α-hederin and hederacoside C), flavonoids (rutin), and phenolic acids (isochlorogenic, chlorogenic and rosmarinic acid).

The hederacoside C content in the extracts H, H30 and H70, determinated by HPLC analysis according to European Pharmacopoeia (1) was 1.71 ± 0.04; 8.95 ± 0.05; 13.95 ± 0.06%, respectively.

**The acute toxicity (LD₅₀)** of all the extracts was determined in mice according to the procedures outlined by the Organization for Economic Co-operation and Development (OECD) (9, 10). The LD₅₀ of the intraperitoneally administered H, H70 and H30 extracts was 1.0, 1.5 and 2.5 g/kg respectively; the LD₅₀ of the intragastrically administered extracts was more than 10 g/kg. At a single intragastric dose of 10 g/kg and up to 1.0 g/kg of the intraperitoneally administered extracts of *Hedera* leaves no signs of toxicity or death in mice within the first 24 h and during the 14-day observation period were observed when compared with the control group. Thus, the extracts considered to be non-toxic (9).

**Antimicrobial activity**

The *Hedera* leaves dry extracts demonstrated dose-dependent antimicrobial activity against all the
studied standard strains. The obtained results are presented in Table 1. The H70 extract at a concentration of 1.0 and 1.5 mg/disk against Pseudomonas aeruginosa with 22 mm of the growth inhibition zone showed the highest antibacterial activity. The H and H30 extracts at all the analyzed concentrations and the H70 extract at a concentration of 0.5 mg/disk showed insignificant antimicrobial activity which rose slightly with increasing concentration of the extracts in the samples.

Antimicrobial activity of saponin-containing extracts of H. helix leaves were previously observed against different strains of bacteria and yeast (15-19). The in-vitro experiments were carried out for extracts by different methods. Water, ethanol, methanol, hexane, chloroform, and ethyl acetate were used as solvents to prepare the extracts (2). The antibacterial activity of saponins from Hedera helix were tested against 22 strains of bacteria and one against yeast species (Candida albicans). At 10 and 5 mg/mL concentration of the saponin, the solution was bactericidal against all the 23 tested strains. Generally, the saponins were more active against the Gram-positive (MIC was 0.312 to 1.250 mg/mL) than against the Gram-negative bacteria (MIC was 1.25 to 5.0 mg/mL). An ethanolic extract of Hedera leaves completely inhibited the growth of Staphylococcus aureus and Pseudomonas aeruginosa and partially inhibited the growth of Escherichia coli (16). Hederagenin derivatives inhibited yeast species (Candida albicans, C. krusei, C. tropicalis, C. pseudotropicalis and C. glabrata) at 50 µg/mL or less. The MIC for the dermatophytes were within the range 5-100 µg/mL (17). Monodesmosidic hederagenin derivatives were shown to exhibit a broad spectrum of activity against yeast as well as dermatophyte species in-vitro by the agar diffusion assay. α-Hederin was the most active compound and Candida glabrata was the most susceptible strain (MIC 6.7 µM) (18). The ethyl acetate and methanol extracts of Hedera helix showed the antibacterial activity against three Gram-positive and two Gram-negative selected bacteria strains at a concentration of 22 mg/mL (19).

### Anti-inflammatory activity
The results of the anti-inflammatory activity of the Hedera extracts in the carrageenan-induced paw edema test in rats are presented in Table 2. The extracts H, H30 and H70 at a dose of 200 mg/kg (corresponding to 3.42, 17.9 and 27.9 mg hederacoside C, respectively) suppressed the development of inflammation. The H70 extract was the most effective with 20.61% of the anti-inflammatory activity, but less than the reference substance (sodium diclofenac) with 53.76% activity.

As H70 with the highest content of hederacoside C showed the highest anti-inflammatory activi-

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**Table 1. Determination of antimicrobial activity* of Hedera leaves extracts.**

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Exacts (mg / disc)</th>
<th>H</th>
<th>H30</th>
<th>H70</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>0.5</td>
<td>1.0</td>
<td>1.5</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td></td>
<td>0</td>
<td>8</td>
<td>9</td>
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<td></td>
<td></td>
<td>0.5</td>
<td>1.0</td>
<td>1.5</td>
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<td></td>
<td></td>
<td>0</td>
<td>10</td>
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<td></td>
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<td>15</td>
<td>16</td>
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</tbody>
</table>

* - mean diameter of five replicates.

**Table 2. Anti-inflammatory activity of the Hedera extracts in carrageenan-induced acute paw edema.**

<table>
<thead>
<tr>
<th>Biological tests</th>
<th>Control group water 1 mL</th>
<th>H 200 mg/kg</th>
<th>H30 200 mg/kg</th>
<th>H70 200 mg/kg</th>
<th>Sodium diclofenac 8 mg/kg</th>
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<tbody>
<tr>
<td>Edema rate (%)</td>
<td>131.5 ± 6.22</td>
<td>116.5 ± 2.13*</td>
<td>109.87 ± 3.90*</td>
<td>104.40 ± 2.77*</td>
<td>60.81 ± 5.69*</td>
</tr>
<tr>
<td>Anti-inflammatory activity (%)</td>
<td>–</td>
<td>11.41 ± 2.80</td>
<td>16.45 ± 2.96</td>
<td>20.61 ± 2.11</td>
<td>53.76 ± 4.33</td>
</tr>
</tbody>
</table>

*p < 0.05 significantly differed in comparison with the control group, n = 6.
Anti-inflammatory, antimicrobial activity and influence... 1163

...ty, some relationship between level of this saponin and activity can be suggested.

Other authors previously reported the anti-inflammatory activity of H. helix extracts and its individual compounds.

The anti-inflammatory effects of a crude saponin extract and saponin’s purified extracts of Hedera helix in carrageenan- and cotton-pellet-induced acute and chronic inflammation models in rats were investigated by Söyleman. In carrageenan-induced acute inflammation model in rats, the crude saponin extract administered orally at 100 and 200 mg/kg body weight was the most potent extract with 77% anti-inflammatory effects, but less active than indomethacin (89.2%). The saponin’s purified extract of H. helix was more potent than the crude saponin extract in its chronic anti-inflammatory effect (60% and 49%, respectively), whereas indomethacin was more active (66%) (6).

α-Hederin and hederacoside C isolated from Hedera leaves administrated orally at concentrations of 0.02 mg/kg body weight were ineffective in the first phase of inflammation in carrageenan-induced acute paw oedema in rats, whereas hederacoside C showed the anti-inflammatory effect in the second phase of acute inflammation. Hederacoside C may exert its anti-inflammatory activity by blocking bradykinin or other inflammation mediators (7).

The ethanol extract from fresh H. helix leaves was tested for its anti-inflammatory properties in formalin-induced paw edema in mice (20). Intraperitoneal injections of 7.5 mL/kg body weight of ethanol extract showed anti-inflammatory activity with 88.89% inhibition, compared to diclofenac, as a reference drug, which showed 94.44% inhibition.

The methanol extract of the leaves of H. helix showed significant dose-depended analgesic and anti-inflammatory activities in the tail flick/hot-plate test and egg albumen-induced rat paw edema tests that were comparable to the test drugs (morphine 20 mg/kg and indomethacin 50 mg/kg respectively (21). The extract administered orally at a dose of 500 mg/kg showed the highest percentage of the inhibition of oedema (44.50%) at 90 minutes and was more potent than a reference drug - indomethacin (50 mg/kg) with 34.65% inhibition.

The previous results of investigations showed that anti-inflammatory activity of H. helix depends on the methods of preparing extracts (raw or dried plant material, method of extractions, etc.), so Hedera extracts may indeed vary with respect to their effectiveness.

Influence on the lungs and bronchus

In the following study of anti-inflammatory activity, it was shown that the H70 extract was twofold/twice as effective as an aqueous extract and exhibited stronger activity than the H30 extract, so the H70 extract was selected for further study of the influence on the lungs and airways. An experimental model of bronchiolitis was induced by an intranasal administration of Sephadex 50, which, in contact with the airways, causes proliferation of bronchus epithelium, increasing its reactivity and interstitial edema (12, 13). This model of lung injury by instillation of xenobiotic particles is responsible for bronchiolitis in humans and has been used previously to demonstrate the anti-inflammatory properties of some compounds based upon their ability to modulate lung edema (13).

The variable changes in the body weight of rats in all the groups were observed during the experiment. The intact rats gained weight throughout the duration of the experiment, whereas weight loss was...
observed in rats of the control group. The group administered Sephadex 50 and treated with the H70 extract at a dose of 200 mg/kg showed no significant changes in animals’ weight.

The histological examination of bronchus and lung tissue of the intact animals (Fig. 1), bronchus and lung tissue in the control group of the animals (Fig. 2) and those treated with the Hedera extract (Fig. 3) were evaluated.

The microscopic examination in the animals’ lung tissue of the control group (Fig. 2) in comparison with the intact animals (Fig. 1) showed the complexity of changes such as dystrophy, hemodynamic disorders, and the reaction of peribronchial lymphoid tissue in various degrees of severity. There were mild focal degenerative changes – vacuolization of the epithelium of the bronchi and endothelium of small vessels. Hemodynamic disorders were manifested by unevenly pronounced plethora of the microvasculature, interstitial edema of interalveolar membranes, and focal inside alveolar hemorrhages. Pronounced changes were observed in peribronchial lymphoid tissue such as hyperplasia of T- and B-dependent areas, sometimes with the formation of secondary lymphoid follicles with germinal centers. Proliferation of lymphoid elements led to bronchoconstriction and a decrease in vessels in diameter, sometimes at 1/3 – 2/3 of lumen (Fig. 2).

In the group of the animals treated with the dry extract of Hedera leaves (H70), vacuoles in epithelial cells of some bronchi and bronchioles were detected in the tested material; it may be a manifestation of dystrophy or an increase in secretory activity of cells (moderate severity) (Fig. 3a). Stenosis of the lumen of the bronchi was insignificant. Hemodynamic disorders were observed in some areas of lung tissue only. Peribronchial lymphoid proliferates were more significant than in the intact animals, but less pronounced than in the control group (Fig. 3b).

As a result of the investigation of the Hedera extracts action, the reduction of dystrophy, correction of hemodynamic disorders, such as edema and hemorrhage, and a decrease in reaction of bronchus-

![Figure 2](image1.png)

**Figure 2.** Cross sectional cut of animals’ lung tissue in control group, HE stain (×100): a – pronounced lymphoid hyperplasia in peribronchial stroma with bronchoconstriction; b – respiratory zone of lung tissue with characters of pronounced plethora: 1 – lymphoid tissue, 2 – lumen of the bronchus, 3 – mucus, 4 – hemorrhages

![Figure 3](image2.png)

**Figure 3.** Cross sectional cut of animals’ lung tissue in group, treated with the 70, HE stain (×100): a – pronounced lymphoid hyperplasia in peribronchial stroma with formation of secondary lymphoid follicles; b – respiratory zone of lung tissue with signs of uneven plethora microvasculature: 1 – lymphoid tissue, 2 – lumen of the bronchus, 3 – mucus, 4 – blood.
associated lymphoid tissue were observed. It can be explained by the decreasing immunoreactivity of macrophage cells. The mucolytic effect of the extract can be explained by an increase in the bronchial secretory activity as well as an increase in exudation with the release of fluid and erythrocytes from the microvasculature. The mucolysis can be caused by reducing the surface tension of the mucus thanks to the surface-active effect of the saponins. The inhibition of reactions in hyperplastic lymphoid tissue may have an indirect effect, because it suppresses the bronchial spasm and results in the absence of mucus retention in bronchus. Therapeutic effects of the H70 extract on the bronchi and lung tissue are supported by the antibacterial and anti-inflammatory activity.

Herbal preparations containing extracts from the *Hedera* leaves are popular in many European countries. The effectiveness of the treatment of a productive acute cough in the upper respiratory tract infections, bronchial asthma or a chronic obstructive pulmonary disease was established in clinical studies (2, 22-25).

The results of the research using an experimental model of bronchiolitis suggest that the H70 extract with the highest content of hederacoside C and with the strongest antimicrobial and anti-inflammatory activity in comparison with H and the H30 extracts should be recommended for making effective expectorant preparations.

**CONCLUSIONS**

The H70 extract obtained of *H. helix* leaves with the highest hederacoside C content showed the most potent anti-inflammatory and antibacterial activity, in comparison with the H and H30 extracts. It can be concluded, that there is some relation between the hederacoside C content and investigated activity. In the literature there is a lack of data comparing biological activity of the extracts prepared in this manner.

The reduction of dystrophy, correction of hemodynamic disorders, and the decrease in reactivity of bronchus-associated lymphoid tissue of the lung by the H70 extract were proved histopathologically.

The results of the research have provided additional evidence considering the effectiveness of *H. helix* leaves in the lung and bronchus infections. Concluding, the H70 extract is considered to be promising in the bronchopulmonary diseases treatment.

**Conflict of interest**

The authors have declared no conflict of interest.

**REFERENCES**


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The most effective method of protection against infectious diseases, both in human and animals is through vaccination. *Pasteurella multocida* is a very common microorganism and cause a lot of damage to poultry and farmed animals such as *pasteurellosis* in poultry that has a great economic impact on commercial and backyard poultry production (1-4).

Development of vaccine is one of the most successful public health interventions up till now. Historically, vaccine development is based on Louis Pasteur’s pattern, i.e., “isolate, inactivate, inject”. By the use of modern technique and technology in developing and formulating the vaccine on modern concepts of rational design causes an increase in number of the vaccine candidates (5-7). The ultimate target of vaccination is to produce a strong immune response for providing long term protection against infectious diseases (8). For an effective vaccine a suitable antigen delivery system is mandatory along with appropriate antigen (9). Generally, vaccines developed from killed whole organism or subunit needs the addition of an adjuvant to be more effective unlikely attenuated live vaccines. Mamo and Poland also suggest that most candidate vaccines represent “minimalist” compositions (10), which exhibit low immunogenicity. Adjuvant and novel delivery systems that boost immunogenicity are increasingly demanded as we go toward the era of modern vaccines. Adjuvants are compounds that enhance the immunogenicity of co-inoculated antigens. The word adjuvant comes from the Latin word *adjuvare*, which means to help or to enhance (11). Adjuvants are used for different purposes: to enhance the immunogenicity; to improve the efficacy of vaccines; to reduce the antigen amount or the number of immunization doses required for sufficient protective immunity and as antigen delivery.
systems for the uptake of antigens by the mucosa. An abscess at the inoculation site assisted the generation of higher specific antibody titers arise the concept of adjuvants (12, 13). The antigen specific antibody response is increased even by an abscess generated by the injection of unrelated substances (8, 13, 14).

Three general types of immunostimulatory compounds were proposed by Edelman and Tackett, namely: adjuvants per se; carriers; and vehicles. Aluminum salts, saponin, monophosphoryl lipid A, muramyl di- and tripeptides, cytokines, Bordetella pertussis, and others are included in the adjuvants per se category. In the carriers bacterial toxoids, fatty acids, and living vectors are included which mainly provide T cell help. Mineral oil emulsions e.g., incomplete Freunds adjuvant, biodegradable oil emulsions e.g., emulsions containing peanut oil, squalene, or squalane, non-ionic block copolymer surfactants, liposomes, and biodegradable polymer microspheres are included in the vehicle category.

A more recent categorization of adjuvants or immunoadjuvants has been proposed by Cox and Coulter which based on five potential modes of action. These are immunomodulation (modification of cytokine networks), presentation (maintenance of antigen conformation), cytotoxic T lymphocytes (CTL) induction, targeting and depot generation (15-17). Three main sources of immunoadjuvants compounds have been identified by Audibert and Lise (18); (1) bacterial such as monophosphoryl lipid A, trehalose dymicolate, choleric toxin or lipopolysaccharides and their derivatives; (2) vegetal, such as saponin or glucan extract, and (3) chemicals such as aluminium hydroxide, surfactants, emulsions, or micro- and nanoparticles. Cytokines like IFNγ or GM-CSF and hormones like dihydroyepiandrosterone (DHEA) can also be defined as a fourth group (18).

Nanoparticles of different size, form, surface properties and varying in composition, can be designed and developed by using nanotechnology for application in the area of medicine (19). Due to the size similarity of nanoparticles to cellular components, nanoparticles can enter living cells using the cellular endocytosis mechanism, in particular pinocytosis. In the past decade, the role of nanotechnology in vaccine development has been increased exponentially resulting in the birth of “nanovaccinology” (7). Nanoparticles are used as either a delivery system to enhance antigen processing and/or as an immunostimulant adjuvant to activate or enhance immunity in both prophylactic and therapeutic approaches.

The emulsion concept was developed by Freund (20). The initial emulsion was very unstable and viscous, and caused a strong local reaction. The weaker stability of vaccines was the main problem (1, 21), a change in the storage temperature or freezing might be damaging to vaccines (22). With the discovery of new generations of oils and surfactants, it is now possible to develop stable, safe and fluid emulsions (23).

Currently, oil-in-water (o/w) emulsions are safely employed and effectively used as vaccine adjuvants in various vaccine products (some of which are already approved for human use and/or under clinical trials) (24). Edelman (17) in the early eighties listed a range of criteria such as toxicity, hypersensitivity reactions, carcinogenicity and teratogenicity as important in the choice of adjuvants in vaccine development. Therefore, the ideal adjuvant would be non-toxic, biodegradable, cheap and non-immunogenic by itself and must not have any interaction with the antigen (17). Palm oil is biodegradable vegetal oil and considered safe as no hypersensitivity or anaphylactic reaction has been reported.

The stability and quality of emulsions are important parameters and has a direct impact on the efficacy and the safety of adjuvants. Currently, the characterization of stability is based on physico-chemical analysis. Fast analytical tools could contribute to better characterization of physical and chemical properties of emulsion used as adjuvants for vaccines. Various parameters such as droplet test, conductivity, viscosity, particle size and stability at various temperatures can be used for the physicochemical characterization of an emulsion.

In this study, we formulated the nanovaccine using a mixture of palm oil (containing long chain triglycerides, LCT) and coconut oil (containing medium chain triglycerides, MCT) as adjuvant for duck pasteurellosis and measure its stability under different stress conditions under storage at 4°C, 25°C, and 40°C. The stability parameters that are unique to injectable emulsions include oxidation and hydrolysis of the oil and/or emulsifier, change in emulsion pH resulting from an increase in free fatty acid content, and rancidity of the oil, viscosity and particle size. Any physical or chemical interaction can limit the shelf-life of the preparations.

EXPERIMENTAL

Materials

Palm oil (LCT) was obtained from Felda, Delima Oil Products Sdn. Bhd, Kuala Lumpur, Malaysia. Medium-chain triglycerides (MCT) from
coconut oil (trade name, ENERSOS) was purchased from Valens USA distributed in Malaysia by Pharmd Sdn. Bhd PJ, Malaysia. Tween 80 was obtained from Sigma Aldrich, USA. Xanthan gum (*Xanthomonas campestris*) was purchased from Sigma Aldrich, USA. Glycerol was purchased from HmbG Chemicals, Germany. All other chemicals used were of analytical grade. Deionized water was used for all preparations.

**Antigen**

Purified whole cell inactivated *Pasteurella multocida*, capsular serogroup A strains, was kindly provided by Onebiotech Sdn. Bhd. Malaysia. The antigen was inactivated with formalin.

**Adjuvant**

A 50 : 50 v/v mixture of palm oil (LCT) and coconut oil (MCT), was used as a novel adjuvant in the formulation of *Pasteurella multocida* vaccine.

### Methods

#### Formulation of vaccine

Palm oil (LCT) with coconut oil (MCT) was mixed along with 2.75% Tween 80 and 0.2% xanthan gum in oil and 2.5% glycerol as wetting agent was added in aqueous phase which also contained the antigen (Table 1). Mixture was prepared by using APV 2000 (Denmark) with low shear mixture and IKA-Eurostar (Germany) 6000 high shear mixture for 3 min. Wetting agent was added in aqueous phase and mixed with a low shear mixture for 2 min. The ratio of aqueous antigen to the oil adjuvant was 50 : 50 (25). The aqueous phase was added to the oil phase with continuous mixing and afterward with high shear mixture. This coarse emulsion was passed through high pressure APV 2000 (Denmark) for nano sizing at 600 bar pressure for seven cycles (schematic workflow is shown in Fig. 1). For physicochemical stability analysis, the prepared nanovaccine was stored at

<table>
<thead>
<tr>
<th>LCT Palm oil</th>
<th>MCT coconut oil</th>
<th>Xanthan gum</th>
<th>Tween 80</th>
<th>Glycerol</th>
<th>Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>22.2 mL</td>
<td>22.2 mL</td>
<td>0.1 g</td>
<td>3 g</td>
<td>2.5 g</td>
<td>50 mL</td>
</tr>
</tbody>
</table>

Figure 1. Workflow of vaccine formulation
4°C, 25°C and 40°C for six months and observed periodically.

**pH**

The pH value of formulated nanovaccine was determined by using the Seven Easy pH Meter S20 (Mettler Toledo, Switzerland). The pH meter was standardized with standard buffers at pH 4.01, 7.0 and 9.21.

**Viscosity**

The dynamic viscosities of the formulated nanovaccine were determined by Brookfield DV-III Ultra Programmable Rheometer (Middlesboro, MA, USA) using SC418 spindle. Vaccine samples (7.0 mL) from each storage temperature (4°C, 25°C and 40°C) were allowed to equilibrate to room temperature and were then loaded in the viscometer. All measurements were taken after 5 min of equilibration.

**Particle size**

Particle size and polydispersity of the nanovaccine was determined by dynamic light scattering (DLS) with the Malvern Instruments Zetasizer Nano-ZS (Worcestershire, UK). DLS measurements were carried out by measuring the hydrodynamic diameter. The diameter obtained with this
technique is that of a sphere with the same translational diffusion coefficient of the particle being measured. The particle size of the nano vaccine formulations was measured on the day of manufacturing and at periodic intervals for 6 months. Standard viscosity and refractive index values of water as the bulk phase were employed in the software analysis. The absorption and refractive index values of palm oil and coconut oil were used to approximate those of the emulsion droplets. A total of 15 measurements for each time point of all DLS measurements were performed in triplicate on each of three separate aliquots. The visual inspection of formulations for phase separation and agglomeration was done prior to light scattering analysis.

Zeta potential

Zetasizer Nano-ZS from Malvern Instruments was used to measure zeta potential of the formulated vaccine. Standard values for the viscosity and refractive index of water supplied by the instrument software were used. Smoluchowski limit of the Henry function assumption was used for data analysis. Fifteen measurements were made at 25°C using an automatic software determination of the measurement duration between 15-30 runs. The applied voltage and laser attenuation settings were automatically optimized by the software.

Transitional electron microscopy

Transmission electron microscopy (TEM) study was performed at ambient temperature using a Philips CM100, at 100 kV. Digital images were recorded using an Optronics 1824 x 1824 pixel CCD camera with an AMT40 version 5.42 image capture engine, supplied by Deben UK. Cryoelectron microscopy was performed using a Tecnai 12 BioTwin, with a Tvisps TemCam F416 (4 k x 4 k) CCD camera.

Fourier transform infrared spectroscopy (FTIR)

For the purpose of characterization of vaccine composition ATR-FTIR technique was employed by using ATR-FTIR spectrophotometer (Perkin-Elmer, Fremont, USA) and the samples (Pasteurella multocida, antigen, adjuvant, coconut oil, palm oil and vaccine) were scanned from the range 4000 to 500 cm⁻¹.

Gas chromatography (GC) analysis

A gas chromatography (GC) 2010 instrument (Shimadzu Corp., Kyoto, Japan) with a flame ionization detector (FID), was used for the analysis of fatty acid. Temperature condition at 250°C and 275°C were maintained for injector and detector, respectively. Initially, the temperature of the column was kept at 180°C for 2 min and then slowly increased to 240°C at a rate of 4°C/min. Nitrogen gas used as a carrier, has flown through at the rate of 60 cm/s. To determine the quantity of total fatty acid [palmitic acid (C16:0) and oleic acid (C18:1)] in nanovaccine samples were treated to prepare fatty acid methyl esters (FAMEs). 0.5 mL of 0.5 M sodium methoxide solution (prepared by mixing sodium methoxide powder in anhydrous methanol) was added to a solution of 50 mg of sample in 1 mL of hexane in a 2 mL screw-capped vial. The vial was capped and screwed on tightly. The solution in the vial was homogenized using an Autovortex SA6
(Stuart Scientific, UK) for 1 min and allowed to stand until the upper layer became clear (5 min). The distinct upper layer of methyl ester was separated carefully in a capped vial. Aliquot of the 1 µL FAME hexane solution was injected into a highly polar cyanosiloxane column (SP-2380, 30 mm × 0.25 mm × 0.20 µm film thickness) from Supelco (USA).

RESULTS AND DISCUSSION

pH

The pH for the formulated vaccines remained between 5.70-5.81 over period of 6 months (Fig. 2). The results indicate that there is no chemical change occurred during storage period and vaccine remains stable as pH is the prime indicator of any chemical change.
or physical change in the formulation. Low pH (values lower than 5) should be avoided as the electrostatic repulsion between emulsified oil globules is decreased, resulting in increased globule size and coalescence.

**Viscosity**

Viscosity was observed for the period of 6 months in order to assess any intermolecular changes and instability. The viscosity of the vaccine remained between 52.0 to 52.4 cps (Fig. 3) throughout the stability study period and indicated no significant change in the viscosity. The ratio between the continuous phase and the dispersed phase has a strong influence on the viscosity. An increase of the dispersed phase leads to an increase of the viscosity of the final emulsion, due to droplets close pack network. The continuous phase must increase in order to decrease viscosity. Hence, when water in oil emulsion are achieved with adjuvants adapted for a ratio of 70% of oil and 30% of aqueous phase, the viscosity can decrease to reach 50 mPa s, whereas a similar optimised formula for a ratio 50 : 50 will have a four times higher viscosity.

**Particle size**

Particle size measurement in nanovaccines is an important indicator of measuring emulsion instabilities. Particle size was found to be stable and ranged between 210 to 225.9 nm throughout the

Figure 7. ATR-FTIR spectrophotometer spectrum of vaccine

Figure 8. Total fatty acid content (%) of vaccine at 4°C, 25°C and 40°C
period of 6 months (Fig. 4). Stability of particle size at different temperatures indicated the efficiency of emulsification process and surfactant system. Emulsion with small particle size and a homogeneous distribution is generally considered more stable (23).

**Zeta potential**

The zeta potential is a function of the surface charge which develops when any material is placed in a liquid. It is defined as the difference in potential between the surface of the tightly bound layer of ions on the particle surface and the electro-neutral region of the solution. It is a very good index of the magnitude of the electrostatic repulsive interaction between the particles which directly affect the stability of the formulation. The zeta potential is used to predict and control dispersion stability. The measured zeta potential was $-30.7 \text{ mV}$ on the day of formulation and average zeta potential for 24 weeks were $-29.1 \text{ mV}$, $-30.9 \text{ mV}$ and $-29.06 \text{ mV}$ for $4^\circ\text{C}$, $25^\circ\text{C}$ and $40^\circ\text{C}$, respectively (Fig. 5). The zeta potential value is relatively high and the repulsive forces may exceed the attractive London forces and in result particles stay dispersed and the system are deflocculated.

**Transmission electron microscopy**

Transitional electron microscopy results suggest that the antigen is entrapped in the oil phase and distributed in the water phase (Fig. 6). Antigen entrapped in the oil phase because of the lipophilic nature of the bacterial cell wall. This will help the antigen to retain more time in the body as the oil will tend to stay in the tissue for longer time and develop a long term immunity and production of antibodies as longer time antigen stay in the body more antibodies will be produced and also the formulation will be stable for longer time. Because the antigen has not affinity for water phase so sedimentation of antigen will not takes palace.

**Fourier transform infrared spectroscopy**

The vaccine base (adjuvant) is composed of coconut and palm oil; therefore most of the peaks in the spectrum are attributed to the functional groups of fats and oils. This could also be seen in vaccine formulation as well (Fig. 7). Coconut and palm oil in terms of fatty acid compositions looks very similar, asymmetrical and symmetrical stretching vibration of methylene (-CH$_2$) group could be seen at 2924 and 2852/cm$^{-1}$, 1743/cm$^{-1}$ Ester carbonyl functional group of the triglycerides, 1402/cm$^{-1}$ = C-H bending vibration, 1117 and 1098/cm$^{-1}$, stretching vibration of the C-O ester group, and overlapping of the methylene (-CH$_2$) rocking vibration and to the out of plane vibration of olefins could be found at 721 cm$^{-1}$. -OH stretch at 3335 cm$^{-1}$ and 1638 cm$^{-1}$-NH$_2$ group of from *Pasteurella multocida* (antigen) was also spotted in FTIR spectrum. In the vaccine spectrum, 4000 cm$^{-1}$ to 500 cm$^{-1}$, all the peaks from palm oil, coconut oil and *Pasteurella multocida* was spotted as well, indicating that antigen and fatty acids from the palm and coconut oil are in a stable state.

**Gas chromatography**

Total fatty acid content was determined in order to determine oxidation or rancidity of oil phase inside the formulation. There was no significant difference in the amount of total fatty acid content in mixture at different temperature (Fig. 8). All of the fatty acid content was found between the range of 80-120% which means that there is an absence of any indication of oxidation of fatty acids or any other chemical or physical interaction which could effect on the stability of vaccine formulation. Fatty acid stability analysis revealed that the formulations are safe to be utilized as drug delivery vehicle as well.

**CONCLUSION**

Duck pasteurellosis nanovaccine using MCT-LCT lipid emulsion as adjuvant was prepared and analyzed for its physical and chemical stability at different temperatures ($4^\circ\text{C}$, $25^\circ\text{C}$ and $40^\circ\text{C}$) for the period of 6 months. During the stability study, it was revealed that the formulations showed no physical or chemical interaction and were stable at all temperatures. The palm oil based nanovaccine was also remained nano-sized ($210-225.9 \text{ nm}$) for the period of 6 months on all the temperature conditions, with the viscosity ranging 50-52 cps and did not showed any signs of oxidation, hydrolysis or rancidity as the pH and total fatty acid content for the vaccines were stable throughout the stability studies. Thus it could be concluded that the palm oil based nanovaccines were stable and can be utilized for delivering antigens or utilized as drug delivery vehicle.

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**Conflict of interest**

Authors have no conflict of interest.
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FORMULATION AND \textit{IN VITRO} ANALYSIS OF GASTRO RETENTIVE DRUG DELIVERY SYSTEM CONTAINING TIMOLOL MALEATE

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\textbf{Abstract:} The objective of current research work was to formulate sustained release matrix tablet of timolol maleate using natural and semi-synthetic drug release retardant polymers. Guar gum, xanthan gum and hydroxypropyl methyl cellulose (HPMC) were used in different concentration in formulation of timolol maleate. Nine formulations were formulated by using direct compression method. Gastroretentive floating tablet of timolol maleate employs polymers (guar gum, xanthan gum, HPMC) and gas generating agents (sodium bicarbonate, citric acid). FTIR showed no drug polymer interaction. All formulations were evaluated for pre-compression (bulk density, tapped density, Hausner’s ratio, compressibility index and angle of repose) and post-compression parameters (hardness, thickness, diameter, swelling index, weight variation, friability, disintegration and dissolution). All results were significant and within range. Among all formulations, F2 containing 37.5\% guar gum, float in 20 s showed 71.33\% drug release up to 8 h. Most of formulations (F1, F2, F4, F8 and F9) showed zero order drug release. Formulations F3, F5 and F6 showed first order drug release.

\textbf{Keywords:} timolol maleate, guar gum, xanthan gum, HPMC, floating tablets

Oral drug administration is prime method of drug delivery in comparison to all other methods. The reason of its extensive use is due to the beneficial quantity of drug going in the blood circulation. This drug quantity sustains anticipated plasma drug concentration in body. One of the most appropriate and economical means of attaining sustain release drug delivery phenomenon is matrix tablet. The most basic working principle of sustained release of drug relies on dissolution, diffusion and degradation (1). The characteristic physical appearance of timolol maleate includes white colored odorless crystalline powder with melting point at 202°C. Timolol maleate shows solubility in water, methanol and alcohol. Its half-life is 2.5 to 5 h with oral bioavailability up to 60\%. Timolol maleate has capability of blocking β-1 and β-2 adrenergic receptors with reduction in intraocular stress by lowering the process of producing aqueous humor. The therapeutic effect of this drug includes assistance in lowering of blood pressure by blocking adrenergic receptors and reducing sympathetic outflow. There is a negative chronotropic and inotropic activity by unidentified methodology (2). Natural polymers like guar gum and xanthan gum have hydrophilic, risk-free and nontoxic nature. They have capability of chemical modification along with potential characteristic of gel development. The main property of natural polymers is stomach specific drug delivery with sustained drug release feature also. The methodology of gel formation includes the steps of polymer interaction with gastric mucosa, followed by hydration and finally the stage of production. The phenomenon of diffusion is involved in drug release from gel layer of polymer (3). Synthetic polymer hydroxyl propylmethyl cellulose (HPMC) exhibits swelling in water for production of gel layer. This layer assists in impediment and regularizing the process of drug release. The proportional relation of drug to polymer and nature of polymer material are key factors for the forte of diffusion and/or erosion (4).

In current research work, effort had been made to formulate sustained release floating tablet of timolol maleate by engaging polymers (guar gum, xanthan gum, HPMC) and gas generating agents (sodium bicarbonate, citric acid) which offer the desired sustained release effect up to 12 h.

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Figure 1. IR spectrum of timolol maleate

Figure 2. IR spectrum of timolol + HPMC K4M
MATERIALS AND METHODS

Materials
Timolol maleate, guar gum, HPMC, xanthan gum (gifted by Schazoozaka (Pvt) Ltd., Lahore) citric acid, lactose, magnesium stearate, sodium bicarbonate, talc (purchased from Merck, Germany) PVP (gifted by Obson Pharmaceutical (Pvt) Ltd., Lahore). All polymers, excipients and reagent were of analytical grade.

Preparation
Natural polymers (guar gum, xanthan gum) semisynthetic polymer HPMC K4M were used as release retarding polymers. Sodium bicarbonate and citric acid were used as gas forming agents. Polyvinyl pyrrolidone (PVP) as binder, magnesium stearate, talc were lubricant and glidant, respectively. Lactose was used as diluent. All the ingredients were accurately weighed, sieved through sieve no. 40 and mixed for 15 min in mortar and pestle. At the end lubricant and glidant were added in powder blend and further mixed for 5 min. Tablets were compressed using single punch machine.

Pre-compression studies
Solubility profile
Different solvents were tried for the solubility of timolol maleate. The amount of 20 mg was used in various solvents for this purpose.

Melting point determination
Melting point of timolol maleate was determined by using melting point apparatus. Drug sample was filled in capillary and capillary inserted in chamber. The chamber was heated gradually and temperature at which drug sample melted was noted.

Angle of repose
The funnel method was used to measure the angle of repose of powder mixture. The calculation of angle of repose is done by measuring the height (h) of the pile and the radius (r) of the base.

\[ \theta = \tan^{-1}\left(\frac{h}{r}\right) \]

Where \( \theta = \) angle of repose; \( h = \) height; \( r = \) radius.

Bulk density
The weighed amount of powder was transferred to the graduated cylinder with a funnel to calculate bulk density. The bulk density was measured by proportion of the sample weight to the volume occupied and calculated by following formula:

\[ \text{Bulk density} = \frac{\text{Weight of powder}}{\text{Volume occupied}} \]

Tapped density
A measured quantity of powder was taken in a graduated cylinder. The cylinder was tapped. The
tapped density was concluded as the ratio of sample weight to tapped volume measured from cylinder and tap density values was calculated by following equation (6):

\[
\text{Tapped density} = \frac{\text{Weight of powder}}{\text{Tapped volume}}
\]

**Hausner’s ratio**

The mathematical proportional relation of bulk volume to tapped volume or tapped density to bulk density is called Hausner’s ratio. The equation of Hausner’s ratio is given below (9):

\[
\text{Hausner’s ratio} = \frac{\text{Tapped density}}{\text{Bulk density}}
\]

**Compressibility index**

The compressibility index determines free flow of powder blend (Carr index) and was calculated by measuring the tap density and bulk density values of powder (10):

\[
\text{Compressibility index} = \left(\frac{\text{Tapped density} - \text{Bulk density}}{\text{Tapped density}}\right) \times 100
\]

**Drug polymer compatibility studies**

The FTIR spectrophotometer (IRAffinity-1, Shimadzu) was employed to study the IR characteristics of pure and physical combined blend of drug with polymers. The wave number range from 4000 – 400/cm⁻¹ in FTIR was set for obtaining the spectra of the mixture. The spectra were elucidated for identification of drug mixtures.

**Post-compression studies**

**Thickness and diameter**

Tablet thickness and diameter were calculated by Vernier caliper (11).

**Friability**

The friabilator was employed for measuring the friability of the tablet in percentage (%). 20 tablets after weighing (W initial) were taken into the friabilator at 25 rpm/min for 4 min. The unhindered drops in apparatus gave rolling and frequent shudders to tablets. The weighing (W final) was done in the end (12).

The mathematical relation which was used to calculate the percentage friability is given as:

\[
F = \left(\frac{W_{\text{initial}} - W_{\text{final}}}{W_{\text{initial}}}\right) \times 100
\]

**Hardness**

Monsanto hardness tester was used for measuring hardness in kilogram per centimeter² (13).

**Weight variation test**

The electronic balance was employed to find out average weight values of arbitrarily selected 20 tablets. Later, the tablets were weighed one by one for comparison with the average weights earlier taken. If the tablets exceeding the limits in percentage are up to 2 with no tablet defying the percentage limit more than 2 times then the requirement of weight variation test is fulfilled (14).

![Figure 4. IR spectrum of timolol + guar gum](image-url)
In vitro buoyancy studies

*In vitro* buoyancy studies were done for all the formulations. The floating lag time (FLT) is the time engaged in the process of arising of tablet on medium surface. The total floating time (TFT) is the interval of time the dosage form takes to persistently stay on medium surface. 0.1 M HCl was prepared and in a 250-mL beaker and tablets were taken in it. The calculation of time interval needed for a tablet to rise and float on the surface was done (15).

Swelling index

The swelling index of floating tablet of timolol maleate was measured by studying the ability of tablet to absorb water. The water gain determined by placing tablet in Petri dish adding 50 mL of 0.1 M HCl. Swelling of tablets of each formulation studied for 1 to 6 h. After specified time intervals tablet was withdrawn and excess water remove with tissue paper and reweight. The swelling index is determined by using formula (16):

$$\text{Swelling index} = \frac{[\text{Final weight} - \text{Initial weight}]}{\text{Initial weight}} \times 100$$

Content uniformity

For the content uniformity test, twenty (20) tablets were taken. Ten tablets were randomly selected and analyzed separately. Nine tablets must contain not less than 85% or more than 115% of the labelled drug amount. The tenth tablet may contain less than 75% or more than 125% of the labelled drug content. If this condition is not fulfilled, thirty
In vitro dissolution studies

United States Pharmacopeia (USP) Dissolution Testing Apparatus II (paddle method) was used to find out the release rate of floating tablets of timolol maleate. 900 mL of 0.1 M HCl is used for dissolution tests at conditions of 37 ± 0.5°C temperature and frequency at 50 revolutions per minute (rpm). 5 mL sample solutions were interchanged with fresh medium after every 60 min from the dissolution apparatus. A 0.45 µm membrane filter was employed for the purpose of filtration of samples. The samples were diluted to appropriate concentration with 0.1 M HCl. Absorbance of these solutions was measured at 295 nm using a UV/Visible spectrophotometer at 295 nm. The curve of percentage drug release against time was drawn and studied to understand the release profile (18).

Analysis of in vitro drug release

The release data of floating tablet fitted into various kinetic model for release pattern study includes:

Zero order kinetics

\[ Q_t = Q_0 + K_o t \]

Q_t (amount of drug dissolved in time t), Q_0 (initial amount of drug in the solution) and K_o (zero order release constant expressed in units of concentration/time) represent the definition of zero order kinetics (19).

First order model

The first order kinetics of drug release is explained by the mathematical relation given below:

\[ \log C = \log C_0 + \frac{K_t}{2.303} \]

The \( C_0 \) represents the initial concentration of drug, \( K \) the first order rate constant and \( t \) is time log cumulative percentage of drug remaining against time with expected yield of a straight line with a slope of \(-K / 2.303\) (20).

Higuchi model

\[ Q = k t^{1/2} \]

In the above equation \( k \) represents the release rate constant and \( t \) is the release time. The rate of drug release is said to be proportional to the reciprocal of the square root of time (21).

Hixson-Crowell cube-root model

\[ Q_o^{1/3} - Q_t^{1/3} = KHC t \]

where \( Q_t \) = amount of drug released in time \( t \), \( Q_o \) = initial amount of the drug in tablet, \( KHC \) = rate constant for Hixson-Crowell rate equation (22).

RESULTS AND DISCUSSIONS

Evaluation of drug

Solubility profile

The solubility profile was established by the solubility tests of timolol maleate with various solvents. It showed solubility in water, ethanol, methanol and 0.1 M hydrochloric acid (HCl).

Melting point

The melting point of timolol maleate was found at 202°C, which verified the pure form of timolol maleate.
Drug compatibility studies

The compatibility studies of pure drug and polymers are implemented by FTIR spectrophotometer employing KBr method. The peaks obtained in polymers spectra correlate with peaks of drug spectra. The infrared spectroscopy of timolol maleate was performed and the IR values of timolol maleate were studied. The characteristic peak at 3303.24/cm⁻¹ showed the stretching vibrations of hydroxyl group (O–H) and the vibrations of bending type had peak at 1228.71/cm⁻¹. The peak at 956.73/cm⁻¹ showed the stretching vibrations of hydroxyl CóO. The stretching vibration bands of =C–O–C and C–O–C showed values at 1263.43 and 1119.73/cm⁻¹. The aliphatic CóH bonds had values at the 2965.68, 2890.45 and 2854.77/cm⁻¹ due to their stretching vibrations. The maleate group showed peaks of carbonyl C=O and NóH vibration of bending nature at 1707.8 and 1485.25/cm⁻¹. The bond of C=N had the peak at 1693.57/cm⁻¹ due to its stretching vibrating movement. The combinations of timolol maleate drug and different polymers like guar gum, xanthan gum and HPMC were also analyzed using IR spectroscopy. The IR values of all combinations confirmed the presence of timolol maleate with its all characteristics peaks representing its functional groups. The study revealed that there is no interaction between timolol and polymers used.

DISCUSSION

The vigilant analysis of the pre-compression studies depicted that all the formulations have appropriate worthy physical features. The angle of repose of powder blends (F1 – F9) was analyzed by fixed funnel method and the angle of repose was seen in array of 27.47° to 29.46°. The calculation and results demonstrated tremendous flow characteristics of powder blends. The powder blends (F1 – F9) displayed decent worthy compressibility and Carr compressibility index was employed to calculate the percentage compressibility index which was found to be in range of 16.21 – 19.35% (Table 2). The calculation of Hausner’s ratio was done by comparison of tap density with bulk density and it arrays from 1.19 to 1.24 for formulations (F1 – F9) (Table 2). The average diame-
ter of formulated tablets of timolol maleate diameter was found to be in range of 5.45 mm – 5.75 mm (Table 3) and the average thickness was from 4.0 mm to 4.35 mm (Table 3). The hardness tester (Curio, Pakistan) exhibited good mechanical strength of all formulation and the hardness values were found to from 7.3 – 8.6 kg/cm². The employment of friabilator (Curio, Pakistan) for the friability of all formulations displayed the crushing strength capability of timolol maleate floating tablets to endure stress and pressure in handling, packaging and transportation and the values of friability were found to be in range of 0.19% – 0.76%. For the weight variation test, twenty tablets from each formulation were randomly selected and assessed and subsequently all the formulations passed with result in pharmacopoeia limit 7.5%. The content uniformity test of timolol maleate floating tablets as per USP for all (F1 ñ F9) formulations exhibited that drug contents for all the formulations obey the official limit (Table 3). Floating lag time and total floating time was calculated by submersing the tablet in 0.1 M HCl in 100 mL beaker. The results of floating lag time and total floating time of all formulations (F1 ñ F9) was presented in Table 4. The carbon dioxide was generated and trapped within gel barrier formed. The hydration of polymers reduced density of tablets and formed tablets buoyant. The formulations F1, F2 and F3 formulated with guar gum gave values of FLT 16, 20 and 51 s, respectively, and the TFT as > 9 h, > 12 h and more than 16 h. The formulations F4, F5, F6 made with xanthan gum exhibited FLT 170, 143 and 128 s and TFT as > 9 h, > 12 h and more than 16 h, respectively. Then the formulations F7, F8, F9 with HPMC K4M gave FLT 22, 24 and 26 s, respectively, and the TFT 9 h, > 9 h and > 12 h, respectively. These values advocated that the concentration of polymers were related directly with the total floating period of formulated tablets. The escalation in con-

**Table 3. Post-compression parameters of timolol maleate.**

<table>
<thead>
<tr>
<th>Formulations</th>
<th>Diameter (mm)</th>
<th>Thickness (mm)</th>
<th>Hardness (kg/cm²)</th>
<th>Friability</th>
<th>Weight variation (mg)</th>
<th>Drug content uniformity</th>
<th>Floating lag time (s)</th>
<th>Total floating time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>5.70 ± 0.11</td>
<td>4.0 ± 0.22</td>
<td>7.3 ± 1.15</td>
<td>0.19 ± 0.005</td>
<td>247.5 ± 0.20</td>
<td>100.2 ± 0.15</td>
<td>16</td>
<td>&gt;9</td>
</tr>
<tr>
<td>F2</td>
<td>5.60 ± 0.11</td>
<td>4.2 ± 0.11</td>
<td>8.3 ± 0.57</td>
<td>0.19 ± 0.005</td>
<td>247.4 ± 0.11</td>
<td>99.6 ± 0.25</td>
<td>20</td>
<td>&gt;12</td>
</tr>
<tr>
<td>F3</td>
<td>5.55 ± 0.22</td>
<td>4.2 ± 0.13</td>
<td>8.6 ± 1.15</td>
<td>0.21 ± 0.01</td>
<td>251.8 ± 0.77</td>
<td>101 ± 0.10</td>
<td>51</td>
<td>&gt;16</td>
</tr>
<tr>
<td>F4</td>
<td>5.50 ± 0.22</td>
<td>4.1 ± 0.17</td>
<td>7.6 ± 2.08</td>
<td>0.41 ± 0.01</td>
<td>237.0 ± 2.75</td>
<td>98.2 ± 0.15</td>
<td>170</td>
<td>&gt;9</td>
</tr>
<tr>
<td>F5</td>
<td>5.45 ± 0.44</td>
<td>4.2 ± 0.11</td>
<td>8.6 ± 1.15</td>
<td>0.2 ± 0.005</td>
<td>250.8 ± 0.75</td>
<td>100.3 ± 0.40</td>
<td>143</td>
<td>&gt;12</td>
</tr>
<tr>
<td>F6</td>
<td>5.60 ± 0.11</td>
<td>4.2 ± 0.28</td>
<td>7.6 ± 1.52</td>
<td>0.76 ± 0.005</td>
<td>253.3 ± 0.47</td>
<td>102.2 ± 0.10</td>
<td>128</td>
<td>&gt;16</td>
</tr>
<tr>
<td>F7</td>
<td>5.75 ± 0.22</td>
<td>4.3 ± 0.44</td>
<td>7.3 ± 0.53</td>
<td>0.2 ± 0.005</td>
<td>248.1 ± 0.15</td>
<td>100.8 ± 0.20</td>
<td>22</td>
<td>&lt;9</td>
</tr>
<tr>
<td>F8</td>
<td>5.65 ± 0.35</td>
<td>4.0 ± 0.13</td>
<td>7.3 ± 0.53</td>
<td>0.2 ± 0.005</td>
<td>247.2 ± 0.26</td>
<td>99.4 ± 0.35</td>
<td>24</td>
<td>&gt;9</td>
</tr>
<tr>
<td>F9</td>
<td>5.45 ± 0.44</td>
<td>4.1 ± 0.61</td>
<td>7.6 ± 0.57</td>
<td>0.4 ± 0.01</td>
<td>252.0 ± 0.20</td>
<td>101.4 ± 0.20</td>
<td>26</td>
<td>&gt;12</td>
</tr>
</tbody>
</table>

**Table 4. Swelling index of formulations.**

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Formulation code</th>
<th>1 h</th>
<th>2 h</th>
<th>3 h</th>
<th>4 h</th>
<th>5 h</th>
<th>6 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>TG1</td>
<td>20</td>
<td>36</td>
<td>48</td>
<td>60</td>
<td>64</td>
<td>72</td>
</tr>
<tr>
<td>F2</td>
<td>TG2</td>
<td>52</td>
<td>72</td>
<td>84</td>
<td>104</td>
<td>108</td>
<td>112</td>
</tr>
<tr>
<td>F3</td>
<td>TG3</td>
<td>56</td>
<td>76</td>
<td>104</td>
<td>132</td>
<td>156</td>
<td>172</td>
</tr>
<tr>
<td>F4</td>
<td>TX1</td>
<td>16.6</td>
<td>29.1</td>
<td>45.8</td>
<td>58.3</td>
<td>66</td>
<td>75</td>
</tr>
<tr>
<td>F5</td>
<td>TX2</td>
<td>42.3</td>
<td>73</td>
<td>88</td>
<td>107</td>
<td>119.2</td>
<td>134</td>
</tr>
<tr>
<td>F6</td>
<td>TX3</td>
<td>44</td>
<td>80</td>
<td>96</td>
<td>116</td>
<td>140</td>
<td>168</td>
</tr>
<tr>
<td>F7</td>
<td>TK4M1</td>
<td>36</td>
<td>64</td>
<td>72</td>
<td>76</td>
<td>84</td>
<td>88</td>
</tr>
<tr>
<td>F8</td>
<td>TK4M2</td>
<td>40</td>
<td>56</td>
<td>76</td>
<td>80</td>
<td>88</td>
<td>100</td>
</tr>
<tr>
<td>F9</td>
<td>TK4M3</td>
<td>52</td>
<td>76</td>
<td>96</td>
<td>108</td>
<td>124</td>
<td>136</td>
</tr>
</tbody>
</table>
Formulation and in vitro analysis of gastro retentive drug delivery...

The concentration of polymers correspondingly amplified the value of total floating time. The swelling index study showed the direct relation of swelling with the time. The swelling amplified with passage of time and the hydrophilicity was found be the reason behind it because polymers steadily keep absorbing the water. The gel barrier was formed around the tablet as the polymer swelled. The regulation of the drug release from floating matrix tablet is done by the gel barrier formed. The results indicated the percent gain in weight of floating tablets upsurges as polymer concentration amplified (Table 4).

The calculation of percentage (%) drug release at different time intervals was performed and results presented in Table 5 and the cumulative percentage (%) drug release versus time for F1, F2, F3 (guar 10, 15, 20%), F4, F5, F6 (xanthan gum 10, 20, 30%) F7, F8, F9 (HPMC-K4M 15, 20, 30%) was plotted. The consequences of different polymers and variation in their concentrations were studied.

The drug released from formulations F1, F2, F3 after 8 h was displayed at 82.67%, 71.33%, and 43.60%, respectively. The formulations F4, F5, F6 released drug at values of 75.30, 66.09, 57.03, 48.29, 39.03, 30.13, and 21.23, respectively.

Table 5: In-vitro release profile of formulations (F1-F9).

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>F1</th>
<th>F2</th>
<th>F3</th>
<th>F4</th>
<th>F5</th>
<th>F6</th>
<th>F7</th>
<th>F8</th>
<th>F9</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>1</td>
<td>15.43 ±0.35</td>
<td>13.08 ±0.38</td>
<td>11.51 ±0.52</td>
<td>17.97 ±0.34</td>
<td>18.20 ±0.20</td>
<td>12.46 ±0.56</td>
<td>18.13 ±0.40</td>
<td>12.49 ±0.26</td>
<td>12.13 ±0.38</td>
</tr>
<tr>
<td>2</td>
<td>25.00 ±0.36</td>
<td>21.71 ±0.39</td>
<td>16.53 ±0.62</td>
<td>25.38 ±0.53</td>
<td>24.59 ±0.34</td>
<td>20.28 ±0.49</td>
<td>34.46 ±0.55</td>
<td>26.59 ±0.49</td>
<td>20.44 ±0.44</td>
</tr>
<tr>
<td>3</td>
<td>35.04 ±0.34</td>
<td>28.44 ±0.43</td>
<td>20.54 ±0.31</td>
<td>34.35 ±0.46</td>
<td>32.46 ±0.31</td>
<td>24.55 ±0.28</td>
<td>54.10 ±0.50</td>
<td>35.76 ±0.11</td>
<td>27.39 ±0.14</td>
</tr>
<tr>
<td>4</td>
<td>43.41 ±0.58</td>
<td>35.70 ±0.56</td>
<td>24.25 ±0.19</td>
<td>41.82 ±0.31</td>
<td>38.20 ±0.11</td>
<td>31.72 ±0.42</td>
<td>73.39 ±0.84</td>
<td>44.15 ±0.65</td>
<td>36.34 ±0.23</td>
</tr>
<tr>
<td>5</td>
<td>52.39 ±0.43</td>
<td>43.90 ±0.52</td>
<td>28.51 ±0.23</td>
<td>48.51 ±0.07</td>
<td>42.77 ±0.37</td>
<td>35.27 ±0.27</td>
<td>81.34 ±0.25</td>
<td>52.47 ±0.36</td>
<td>42.43 ±0.48</td>
</tr>
<tr>
<td>6</td>
<td>61.83 ±0.37</td>
<td>51.76 ±0.70</td>
<td>34.23 ±0.41</td>
<td>56.49 ±0.28</td>
<td>49.71 ±0.42</td>
<td>38.01 ±0.43</td>
<td>84.47 ±0.19</td>
<td>60.49 ±0.56</td>
<td>50.73 ±0.57</td>
</tr>
<tr>
<td>7</td>
<td>71.00 ±0.99</td>
<td>59.60 ±0.50</td>
<td>37.76 ±0.64</td>
<td>67.28 ±0.22</td>
<td>57.03 ±0.26</td>
<td>41.29 ±0.58</td>
<td>87.74 ±0.46</td>
<td>68.80 ±0.70</td>
<td>60.05 ±0.65</td>
</tr>
<tr>
<td>8</td>
<td>82.67 ±0.48</td>
<td>71.33 ±0.63</td>
<td>43.60 ±0.57</td>
<td>75.30 ±0.10</td>
<td>66.09 ±0.33</td>
<td>48.29 ±0.85</td>
<td>92.89 ±0.90</td>
<td>77.90 ±0.46</td>
<td>69.78 ±0.26</td>
</tr>
</tbody>
</table>

Table 6. Release kinetics of timolol maleate matrix tablet.

<table>
<thead>
<tr>
<th>Formulations</th>
<th>R² values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Zero order</td>
</tr>
<tr>
<td>F1</td>
<td>0.995</td>
</tr>
<tr>
<td>F2</td>
<td>0.993</td>
</tr>
<tr>
<td>F3</td>
<td>0.976</td>
</tr>
<tr>
<td>F4</td>
<td>0.980</td>
</tr>
<tr>
<td>F5</td>
<td>0.9713</td>
</tr>
<tr>
<td>F6</td>
<td>0.9489</td>
</tr>
<tr>
<td>F7</td>
<td>0.9104</td>
</tr>
<tr>
<td>F8</td>
<td>0.990</td>
</tr>
<tr>
<td>F9</td>
<td>0.9953</td>
</tr>
</tbody>
</table>
75.30%, 66.09% and 48.29% and the formulations F7, F8, F9 with HPMC K4M at 92.89, 77.90 and 69.78%, respectively. The formulation F2 was selected as best formulation based on floating lag time and total floating time. The study of release profile and values from in vitro dissolution studies with numerous kinetic equations like zero order, first order, Higuchi model and Hixson Crowell models showed the in-depth view and interpretations. The values in Table 6 revealed that F1, F2, F4, F8, F9 follows zero order and F3, F5, F6 follows first order equation. Coefficients (R²) values of Higuchi model were found to be in range of 0.932 – 0.987. It was concluded that drug release from formulated formulations (F1 – F9) goes by diffusion.

CONCLUSION

The productive preparation of gastro retentive floating tablets of timolol maleate was performed. Later the evaluation of tablets by natural polymers (guar gum, xanthan gum) and semisynthetic polymer like hydroxypropyl methylcellulose (HPMC K4M) with the technique of direct compression was performed. The drug compatibility with polymers was investigated and studied with help of FTIR technique. The studies revealed that the ratio of polymer has impact on drug release as polymer ratio escalates the release rate of timolol maleate reduces. The floating tablet of timolol maleate (F2) displayed adequate results using short floating lag time of 20 s and released drug up to more than 12 h. This research work has potential scope to be further elaborated and studies for in vivo analysis in rabbits and humans.

REFERENCES


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Transdermal drug delivery system is designed in a way that it must deliver drug at predetermined and controlled rate for systemic effect. This should also maintain the plasma drug concentration between minimum effective and minimum toxic level over a desired period of time (1). The oral route is an attractive site for the delivery of the drugs but faces several barriers like intestinal and or hepatic first pass elimination, high variance in bioavailability due to variable conditions of gastrointestinal tract, difficulty in long term and rate regulated absorption, and impossibility of arbitrary drug input and its interruption. Such problems of oral routes of delivery can be solved by using the transdermal administration of drugs due to low metabolic activity of the skin when compared with that of the gastrointestinal tract and liver.

The physicochemical properties of the drug are very important in the penetration of drugs through skin. Stratum corneum being a barrier allows only small amount of drug which could reach the systemic circulation making this route favorable only for lipophilic drugs (2). For a drug to permeate through skin, it must have high but balanced value of partition coefficient, because the stratum corneum offers much more resistance to hydrophilic compounds for diffusion comparatively to the cellular layers beneath it (3). Dermis layer is a significant barrier to the lipophilic compounds. Materials having partition coefficient greater than 400 are restricted by dermis but can be absorbed through the stratum corneum. Similarly, the molecular weight of drug and daily dose should be low to achieve therapeutic drug concentration because the drug has to pass through tight junctions of skin (4).

Drug permeation through skin is very slow and to reach steady state requires lag time of several hour (5). It is impossible to achieve therapeutic effective drug concentrations in blood without enhancing skin permeation (2). In order to facilitate drug diffusion across skin, penetration enhancers are commonly used. After partitioning into stratum corneum, penetration enhancers modify the skin properties, the mechanism of which is attributed to the disordering the stratum corneum lipid structure (6). The use of penetration enhancers is a long-standing and widely used approach to increase transdermal and topical drug delivery. Various natural oils are used as penetration enhancers. Lack of information on the composition and utilization of new natural compounds has created a considerable concern in exploring innovative resources to get safe and efficient active and inactive entities of pharmaceutical interest (7).

The Silybum marianum (Milk Thistle) belongs to family Asteraceae (compositae) (annul or biennial herb) (8), abundantly available as weed in Khyber Pakhtoon Khwah and in some areas of Punjab, Pakistan on unutilized barren ground. It is the safest oil and has extensively been used in European Traditional Medicines since long, particularly in the treatment of various liver diseases. The seeds yield 26-39% fixed oil that is rich in polyunsaturated fatty
acids. The other constituents of oil are free fatty acids, compesterol, stigmasterol, mono, di and triglycerides. The defatted seed contains an excellent quality and quantity of proteins, ranging from 20-24% containing amino acids such as aspartic acid, glycine, cystine and glutamic acid. The seed of *Silybum marianum* is a potential source of dietary oil and protein, an excellent source of unsaturated fatty acid i.e., linoleic acid (64.59%) and oleic acid (23.59%) which is of great medicinal significance (9, 10).

The study aims to design a typical zero order release transdermal patch using indigenous crude milk thistle oil extracted from the seeds of *Silybum marianum* collected locally from Mansehra and Abbottabad districts, KPK, Pakistan as natural penetration enhancers. The said formulation would help the patient to overcome the burden of repeated dosing frequencies and would ultimately help to get the better compliance. It would also help utilize the local plants to get various valuable natural compounds of pharmaceutical interest.

### MATERIALS AND METHODS

Materials used in the present study include: flurbiprofen received as a gift from Global Pharmaceuticals, Pakistan, *Silybum marianum* seeds collected locally from the surroundings of Mansehra and Abbottabad districts in the province of KPK, Pakistan. Potassium dihydrogen phosphate (KH₂PO₄), sodium hydroxide (NaOH), hydrochloric acid (HCl), ethanol and chloroform were purchased from Merck Germany, Olive oil (OLO) from Marhaba Pvt Ltd., Pakistan, polyvinyl alcohol (PVA) from Sigma Aldrich (Germany), ethyl cellulose ether derivative polymer Ethocel® Premium 10 FP (EC) and Di-N-butyl phthalate were purchased from Dow Chemical Co., Midland, USA. All chemicals and reagents used in the experiments were of molecular biology grade.

#### Extraction of milk thistle oil (MTO)

Fully ripened seeds of *Silybum marianum* were collected from Mansehra and Abbottabad districts, KPK, Pakistan. The seeds were dried in sunlight during the month of August. After drying, the seeds were cleaned and grinded using a common electric grinder model (Panasonic, BH 925P, Japan). The ground samples were stored in air tight containers in a cold dark room at temperature 25°C.

The oil was extracted using a Soxhlet extraction apparatus (Pyrex®, Sigma-Aldrich, USA) using n-hexane as a solvent. The solvent was removed with a rotary evaporator (Tokyo Rikakai Co, Ltd, N, No. 4030414) at 40°C and later in an oven at 50°C. The oil samples were stored in cool dry dark place at 25°C for further experimental use (11).

**Preparation of transdermal patch**

Backinc membrane of polyvinyl alcohol (PVA) 4% (w/v solution of PVA) (Sigma Aldrich, Germany) was prepared in distilled water. The mixture was heated under constant stirring at 80°C until a clear solution was obtained. The solution was then cooled to room temperature and was placed on the Sonicator (Elma D 78224, Germany) for 2 min to remove any entrapped air bubbles. About 12 mL of this solution was finally poured into Petri dishes (of about 20 cm² area) and was dried at room temperature in open air. Drug polymer dispersions was prepared by dissolving the drug (2 mg) and polymers (160 mg) (EC : PVP, 3 : 1) in solvent system (ethanol QS 10 mL) with continuous stirring at 300 rpm on the magnetic stirrer. Later, Di-N-butyl phthalate (15% w/w of polymer) was added to the solution as a plasticizer. Finally, the prepared solution (containing drug-polymer-plasticizers) was poured into a Petri dish containing the backing membrane. The solvent was allowed to evaporate under ambient conditions for 24 h. The Petri dish was covered with a funnel to control the rate of evaporation. Penetration enhancers (each 5%) milk thistle oil and

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Permeation Enhancers (w/w) MTO/OLO</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT1</td>
<td>1%</td>
</tr>
<tr>
<td>MT2</td>
<td>2%</td>
</tr>
<tr>
<td>MT3</td>
<td>3%</td>
</tr>
<tr>
<td>MT4</td>
<td>4%</td>
</tr>
<tr>
<td>MT5</td>
<td>5%</td>
</tr>
<tr>
<td>MT10</td>
<td>10%</td>
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<tr>
<td>OL1</td>
<td>1%</td>
</tr>
<tr>
<td>OL2</td>
<td>2%</td>
</tr>
<tr>
<td>OL3</td>
<td>3%</td>
</tr>
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<td>4%</td>
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<td>OL5</td>
<td>5%</td>
</tr>
<tr>
<td>OL10</td>
<td>10%</td>
</tr>
<tr>
<td>Control</td>
<td>0%</td>
</tr>
</tbody>
</table>

Flurbiprofen 1% w/w, Di-n-butyl phthalate 15% w/w of polymer, Ethanol QS 10 ml, Polymer (mg) EC:PVA 3:1
olive oil (w/w of the polymer) were finally added (see Table 1). After complete drying, the film was removed from the Petri dish and cut into square patches (3.14 cm²) (12).

**Physicochemical evaluation of patches**

Physicochemical evaluations of all the patches were performed in following order:

**Physical appearance, thickness and weight uniformity**

The patches were examined visually for appearance, color, homogeneity, smoothness and transparency. Thickness was determined at three different points in triplicates. The weight was checked by randomly selecting 10 patches and weighing in triplicates; the average was calculated and tabulated.

**Moisture content, % moisture uptake and moisture loss**

The test patch formulations were marked, weighed accurately and kept in a desiccator using silica maintained at room temperature for about 24 h. Percent moisture content was calculated using equation 1:

\[
\text{% moisture uptake} = \frac{W_f - W_i}{W_i} \times 100
\]  

Percent moisture uptake was calculated by selecting three patches randomly and weighing accurately. The selected patches were then placed in a desiccator at room temperature. To maintain the humidity, saturated solution of aluminium chloride was placed in the desiccator. After three days the patches were taken out from the desiccator and were weighed again. Percent moisture uptake was calculated using equation 1.

To determine the % moisture loss, three sample patches were randomly selected, weighed and placed in desiccator at 37°C. To maintain dry condition anhydrous calcium chloride was also placed. After three days the patches were taken out from the desiccator and were weighed again. The average % moisture loss was calculated using equation 2 (13):

\[
\text{% moisture loss} = \frac{W_i - W_f}{W_i} \times 100
\]  

**Folding endurance, tensile strength, and percent elongation**

The folding endurance for test patches was determined by swiftly folding patch at the same point till it broke. The number of times a patch can be folded at the same point without breaking defines the value of the folding endurance.

The mechanical properties were assessed by tensile strength and percent elongation of the patch. The fixed pulley arrangement was used to determine the tensile strength and % elongation. The initial length of the patch strip was determined with a common scale. One end of the patch was fixed and the other end of the patch was tied to a thread passing over a pulley. The hanging end of the thread was attached to a pan. Weight was gradually added to the pan until the patch was broken and the weight was noted. A pointer on the thread was used to measure the elongation of the patch. Finally, the tensile strength (in kg/cm²) of the patch was measured using equation 3:

\[
\text{Tensile strength} = \frac{F}{a \times b \times (1 + L/I)} \times 100
\]  

where, \(F\) = force required breaking the patch; \(a\) = width (cm) of the patch, \(b\) = thickness (cm) of the patch, \(L\) = length of the film (cm) and \(I\) = elongation (cm) of the film just before the patch is broken. Percent elongation of the patches was determined using equation 4:

\[
\text{% elongation} = \frac{L_f - L_i}{L_i} \times 100
\]  

where, \(L_f\) = final length just before the patch is broken. \(L_i\) = initial length.

**Drug contents**

The drug contents were determined by placing the patches in 100 mL phosphate buffer pH 7.4 in a volumetric flask and sonicated for about 8 h. After sonication, the solution was filtered and the drug contents were determined spectrophotometrically at 247 nm.

**Differential scanning calorimetry (DSC)**

The physicochemical compatibility of flurbiprofen drug and excipients was investigated by differential scanning calorimetric (DSC) analysis, using a DSC instrument (Mettler Toledo DSC 822e, Greifensee, Switzerland) with a thermal analysis data station system, computer and a plotter interface. The instrument was calibrated with indium as standard over a temperature range 100-350°C, at heating rate 10°C/min using nitrogen as purge gas at flow rate 50 mL/min. The samples were heated (50-300°C) at a constant scanning speed of 10°C/min in sealed aluminium pan size (1 cm diameter).

**Fourier transform infrared (FTIR)**

FTIR was used to study possible interaction of flurbiprofen and excipients. FTIR (FT-IR Spectrum spectrophotometer, Perkin Elmer, UK) was used in this study. Approximately 10 mg sample was placed on a previously cleaned diamond surface plate,
enough pressure was applied and absorption pattern was recorded over a wavelength range 500 to 4000 cm\(^{-1}\).

**X-ray diffraction (XRD)**

The samples were analyzed, using X-ray diffractometer, Phillips PW1830 powder diffractometer (Phillips, Eindhoven, Netherland), using nickel-filtered CuK\(\alpha\), 30 kV and 20 mA voltage and current, respectively. The spectra were taken in the range of 0-20\(^\circ\) and the time for each run was kept at 1\(^\circ\)/min.

**Scanning electron microscopy (SEM)**

Surface morphology of the drug were analyzed by photomicrographs of flurbiprofen using SEM (SEM; Joel JSM-5910, Japan). For such purpose physical mixtures and solid dispersions in ethanol were used to scrutinize the surface morphology of the drug, physical mixtures and solid dispersions. A suitable amount of sample was mounted on a metal stub using double sided adhesive tape and was coated with cold for conductivity. The micrographs were obtained at 10-50 \(\mu\)m to view the clear morphology.

**Skin irritation assay**

Skin irritation studies were performed according to Draize patch test for optimized patch (14). Fifteen healthy male rabbits were divided into five groups each comprising of three rabbits. Group-I was tagged as normal and was kept without any treatment. Group-II received marketed adhesive tape (Nichipore Surgical tape, Japan) and was tagged as control group. Test transdermal flurbiprofen patches were applied to Group-III, while the blank patches (without drug) to Group-IV. Standard irritant formalin (0.8% v/v aqueous solution) was applied to Group-V. The experiment was carried out for 7 days and application sites were graded according to visual scoring scale, by the same person. The erythema and edema were scored as follows: 0 for none, 1 for slight, 2 for well defined, 3 for moderate and 4 for scar formation and severe erythema and edema.

**Skin preparation, invitro permeation and release kinetics**

The skin from rabbit abdominal region was carefully removed by surgery after anesthesia. The adhering fats were removed by dip-
ping the skin in hot water for a while and then teasing the epidermis from the dermis. The skin was then washed with distilled water and was stored at -20°C till further use. Franz diffusion cell (FDC) (surface area 176 ± 0.34 mm²) apparatus was used to evaluate the permeation of the drugs from the patches across the rabbit skin. The prepared rabbit skin was allowed to hydrate for 1 h before the experiment. The skin was placed in between the donor and receptor compartment of (FDC) with the stratum corneum of the skin facing the donor compartment. Then, the patch having an area of 3.14 cm² was placed on the skin with the drug releasing surface of the patch facing the stratum corneum of the skin. The receptor compartment was filled with the phosphate buffer pH 7.4. The receptor fluid was maintained at 32 ± 0.5°C by means of water circulating in the water jacket around the receptor compartment and the medium was stirred with a magnetic bead. Two ml samples were taken from the receptor fluid at the time intervals of 0.5, 1, 1.5, 2, 4, 8, 12, 16, 20 and 24 h and an equal volume of fresh receptor fluid was added to receptor compartment to maintain the fluid volume. The samples were then analyzed spectrophotometrically at 247 nm. The drug permeated per cm² of patch was calculated and plotted against time and the flux was calculated as drug permeated per cm² per hour (14).

Invivo studies and pharmacokinetic analysis

For pharmacokinetic studies (15), the rabbits were divided into three groups, each group containing four rabbits. The rabbits were kept in cages with husk bedding. On the next day, early in the morning, flurbiprofen reference tablet (50 mg) was given orally through a feeding tube by making a solution in distilled water to group-I. The blood samples (3 mL) were collected at 0, 0.5, 1, 2, 3, 4 and 8 h after drug administration. The sparse hairs on the back of the animal were shaved carefully without damaging the stratum corneum with an electrical clipper one day before the scheduled experiment. The application

Figure 1. DSC thermograms of pure flurbiprofen drug, patch formulation (drug-polymer dispersions in ethanol) and drug-polymer physical mixture

![DSC thermograms](image-url)
area was swept with dry cotton. The flurbiprofen patch was applied on back of rabbit (group-II) patch and the attachment was sustained for 24 h. The blood samples were taken at 0, 0.5, 1, 2, 3, 4, 6, 8, 12, 16, and 24 h. The placebo transdermal formulation was given to group-II rabbits which acted as control group. The blood samples were taken regularly up to 24 h.

The centrifuged tubes containing sodium heparin (as anticoagulant) (Vacutainer, BD) were used for collecting blood samples of 3 mL at specified time intervals via an in-dwelling cannula placed in the marginal ear vein of rabbits following administration of test and reference formulations. The blood samples collected were centrifuged for 15 min at 3500 rpm and the plasma was transferred to new glass tubes and kept frozen at -20°C until analysis.

Acetonitrile (2 mL) was added to plasma sample (1 mL) in order to precipitate the proteins. This suspension was vortexed for 60 s, centrifuged for 5 min at 3500 rpm and supernatant was transferred to another Eppendorf (propylene) tube. The clear supernatant was evaporated to dryness under nitrogen flux, dissolved in 80 µL of mobile phase and 20 µL of it was injected into the injection port of reversed-phase HPLC (Perkin Elmer series 200, USA). Briefly, the HPLC system comprised of an
HPLC (Perkin Elmer series 200, USA) with binary pump solvent delivery system, reverse phase C-18 (ODS Hypersil, 4.6 × 250 mm, 5 µm) stainless steel analytical column (Thermo Electron Corporation, UK) fitted with a refillable guard column, UV/Visible wave length detector, integrator NCI 900, degasser and ChemStation software.

Following the injection 20 µL of plasma solution to a 20 µL sample loop in injection port using 50 µL syringe chromatographic peaks were recorded at λ 247 nm. The solvent used were degassed with the help of sonicator (Elma D 78224, Germany) before operation of HPLC and the pH of the mobile was adjusted by pH meter (Inolab Series, Germany). Mobile phase consisted of a mixture of acetonitrile and ammonium acetate buffer 0.01 M with pH 3.4 adjusted by glacial acetic acid (60 : 40, v/v). Optimum flow rate was 1.5 mL/min. The mobile phase prepared was filtered through the 0.45 µL membrane filter (Sartorius, Germany) and was then degassed by ultra-sonication.

In case of in vivo studies different pharmacokinetic parameters such as Cmax (peak plasma concentration), Tmax (time to reach maximum plasma concentration and AUC (area under the plasma concentration time curve) were determined. Plasma concentration data were used to calculate the peak plasma concentration Cmax and time of its occurrence Tmax.

Statistical analysis
A p value < 0.05 was considered to be statistically significant using t-test. The data were collected in triplicates and expressed as the mean ± standard deviation.

RESULTS
Physicochemical evaluation of patches
The patches were subjected for evaluation of different physicochemical properties (see Table 2). Morphologically all the test patches were found to be consistent, clear, non-sticky and smooth in appearance. The thickness and weight ranged between 0.37 ± 0.003 to 0.42 ± 0.004 mm and 668.62 ± 0.041 to 82.20 ± 0.032 mg, respectively.
Percent moisture absorbance and percent moisture loss were found to be 8.35 ± 1.23 to 11.13 ± 1.13 and 6.4 ± 0.32 to 7.8 ± 1.3, respectively. Folding endurance and drug content ranged from 200 to 243 and 97.21 ± 0.11 to 102.4 ± 0.13, respectively, while the tensile strength was found to be ranging from 9.03 to 12.22 kg/cm².

**Differential scanning calorimetry (DSC)**

DSC thermograms of pure flurbiprofen, drug-polymer physical mixture and drug-polymer dispersions are presented in Figure 1. All of the thermograms displayed a characteristic endothermic peak at ~117°C which corresponds to the melting of flurbiprofen. The melting peak demonstrated a negligible shift to low temperature following inclusion of excipients; this might be attributed to the presence of moisture or the solvent in the patch matrix. The fact that melting peak of flurbiprofen was persistently observed at 117°C demonstrates that the drug remains intact in the patches (16).

**Fourier transform infrared (FTIR) absorption spectroscopy**

FT-IR spectra of pure flurbiprofen, its physical mixture and drug polymer dispersions were taken to study any possible chemical interaction between drug and polymer (see Fig. 2). Pure flurbiprofen showed a sharp characteristic peak at 1695.82/cm¹ which corresponds to the carboxyl acid (COOH) present in drug molecule. Other smaller peaks found in the region 1000-1700/cm¹ are the indication of benzene ring in drug molecule. As the sharp characteristic peaks of flurbiprofen did not showed any stretching or bending in physical mixture or patch formulation suggesting no interaction between the drug and excipients.

**X-ray diffraction (XRD)**

X-ray diffraction results of flurbiprofen are shown in Figure 3 where drug polymer physical mixture and drug polymer solid dispersions were measured in θ angle from 0 to 50°. Due to crystalline nature of flurbiprofen drug, x-ray diffraction pattern showed sharp characteristic peaks for flurbiprofen powder at 20 degree which are slightly shifted to lower diffraction in patch formulation containing polymeric excipient and ethanol. The minor changes in the peaks might be attributed to the reduction in particle size of flurbiprofen drug in the physical mixture or drug-polymer dispersions. The XRD data suggest that the crystallinity of flurbiprofen is least influenced by the formulation components.

**Skin irritation studies**

No noticeable sign of erythema or edema was found on the rabbit skin for any group throughout study period. The skin irritation score (erythema and edema) with test formulations was found to be up to 1.50 ± 0.00 (see Table 3). Standard irritant formalin produced severe erythema and edema when used on the rabbit’s skin in Group-V i.e., 3.06 ± 0.19, 3.22 ± 0.48, respectively. According to the Draize test, score of less than 2.0 ± 0.00 is considered negative.

**In vitro permeation studies**

Figs. 5-8 show the permeation profiles of flurbiprofen through synthetic membrane and rabbit skin with and without different concentrations of MTO and OLO used as penetration enhancers in transdermal patches. The flux, permeability coefficient (Kp), lag time (Tlag) and enhancement ratio

<table>
<thead>
<tr>
<th>Formulation Coding</th>
<th>Visual Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Erythema</td>
</tr>
<tr>
<td>Group 1</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Group 2</td>
<td>0.96 ± 0.61*</td>
</tr>
<tr>
<td>Group 3</td>
<td>1.26 ± 0.77*</td>
</tr>
<tr>
<td>Group 4</td>
<td>1.01 ± 0.28*</td>
</tr>
<tr>
<td>Group 5</td>
<td>3.06 ± 0.19</td>
</tr>
</tbody>
</table>

Visual observation are expressed as the mean ± SD, n = 3; *Significant compared to formaline (p < 0.05).
Figure 5. Cumulative amount of flurbiprofen patch permeation containing different concentration of milk thistle oil as enhancer across synthetic membrane, the mean ± SD (n = 3).

Figure 6. Cumulative amount of flurbiprofen patch permeation containing different concentration of milk thistle oil as enhancer across rabbit skin, the mean ± SD (n = 3).

Figure 7. Cumulative amount of flurbiprofen patch permeation containing different concentration of olive oil as enhancer across synthetic membrane, the mean ± SD (n = 3).
(ER) are shown in Table 4. The maximum flux was achieved for flurbiprofen transdermal patch with 10% MTO (w/w) used as penetration enhancer giving the best permeability coefficient and ER. The permeation rate, permeability coefficient, and ER of the control formulation was found to be 12.25 ± 0.20, 0.199 ± 0.003 and 1.0 through synthetic membrane and 15.33 ± 0.21, 0.250 ± 0.002 and 1.0 through rabbit skin. The formulation containing 10% MTO as enhancer exhibited the parameters as 53.75 ± 1.71, 0.839 ± 0.002 and 4.09 through synthetic membrane and 77.15 ± 2.14, 1.771 ± 0.001 and 7.27 through rabbit skin, respectively. Similarly, the skin permeation parameters for olive oil used as permeation enhancer were found to be 50.53 ± 2.74, 0.784 ± 0.002 and 4.34 for synthetic membrane, while 66.03 ± 3.14, 2.08 ± 0.001 and 2.86 for rabbit skin as flux, permeability coefficient (Kp) and ER, respectively.

**Ex vivo permeation through different natural skins**

Since human skin may not be available for ex-vivo permeation studies. It might be important to select suitable animal skin model to conduct ex-vivo transdermal evaluation. The current study allows

![Figure 8. Cumulative amount of flurbiprofen patch permeation containing different concentration of olive oil as enhancer across rabbit skin, the mean ± SD (n = 3).](image1)

![Figure 9. Cumulative amount of flurbiprofen patch permeation containing different concentration of olive oil as enhancer across rabbit skin, the mean ± SD (n = 3).](image2)
comparing rabbit, mice, pig, sheep and dog’s skins using flurbiprofen transdermal patch formulated with MTO as natural permeation enhancer. It was observed that flurbiprofen permeation using different skins flux was increased in the order dog < sheep < pig < rabbit when 10% MTO was used and was found to be $50.32 \pm 2.32 < 57.34 \pm 1.87 < 60.62 \pm 0.98 < 67.21 \pm 1.54 < 70.55 \pm 2.11 < 77.15 \pm 2.14$, respectively (see Figure 9).

**Pharmacokinetic analysis**

Figure 10 demonstrates flurbiprofen plasma profile after transdermal application of patch and oral administration of conventional tablets to the albino rabbits. The $C_{\text{max}}$ of the different patch formulations MT10 and OL10 were found to be 39.92 ± 1.2 and 22.12 ± 1.5 µg/mL, respectively. The $C_{\text{max}}$ of formulation MT10 and OL10 were found to be 4.27 and 2.34 times higher than control patch formulation. Area under the curve for test patch formulations with MTO MT10 and with OLO OL10 were found to be 553.84 ± 5.5 and 343.80 ± 3.98 significantly higher than that of the control patch formulation 223.43 ± 5.14 (see Table 5).

**DISCUSSION**

The transdermal patch is considered as a user-friendly dosage form. It is more convenient and painless dosage form that generally leads to improved patient compliance. Inciting research technologies have shown that skin port is a potential route especially for delivery of lipophilic drugs to the systemic circulation. The patches are usually designed to control the delivery of drugs at desired controlled rates by employing appropriate hydrophilic and/or lipophilic polymers (17).

Fatty acids have been investigated by number of time and exhibited to enhance the skin permeation of various drugs when applied as penetration enhancers and the effect might be attributed to the increased fluidity of intercellular lipids (18). Numbers of pharmaceutical scientists have demonstrated the permeation enhancing effects of fatty acids in drug delivery using buccal mucosa. However, the mechanism of action has not been investigated yet due to the use of inappropriate models in the previous studies. Pluronic F-127 gels were designed, formulated and evaluated for insulin permeability using rat buccal mucosa (19). The presence of oleic acid in the gel also exhibited the hypoglycemic effect in addition to insulin (20). So the researchers should address these types of attentions while using such sort of combinations in their laboratories especially while dealing with human skins, as the rat buccal mucosa is already keratinized in comparison to the human buccal mucosa. Ergotamine tartrate permeability through keratinized epithelial-free hamster cheek pouch was investigated using natural cod liver oil as permeation enhancer (21). The above mentioned studies did not give any information about the mechanism of permeation

![Figure 10](image-url). Pharmacokinetic profile flurbiprofen transdermal patch (MT10 and OL10) to each other and also in comparison to oral and control formulation in albino rabbit.
Table 4. Skin permeation parameters for flurbiprofen transdermal patch formulated with milk thistle oil and olive oil as permeation enhancer using synthetic membrane and rabbit skin. The values are taken in triplicate, the mean and standard deviations were calculated.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Synthetic membrane</th>
<th>Rabbit skin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Flux (µg/cm²·h) ± SD</td>
<td>Kp (cm/h) ± SD</td>
</tr>
<tr>
<td>Control</td>
<td>12.25 ± 0.20</td>
<td>0.199 ± 0.003</td>
</tr>
<tr>
<td>MT-Oil1</td>
<td>13.23 ± 0.31</td>
<td>0.203 ± 0.002</td>
</tr>
<tr>
<td>MT-Oil2</td>
<td>16.72 ± 1.08</td>
<td>0.277 ± 0.001</td>
</tr>
<tr>
<td>MT-Oil3</td>
<td>20.41 ± 0.43</td>
<td>0.391 ± 0.001</td>
</tr>
<tr>
<td>MT-Oil4</td>
<td>23.31 ± 1.15</td>
<td>0.471 ± 0.002</td>
</tr>
<tr>
<td>MT-Oil5</td>
<td>25.61 ± 1.17</td>
<td>0.598 ± 0.001</td>
</tr>
<tr>
<td>MT-Oil10</td>
<td>53.75 ± 1.71</td>
<td>0.839 ± 0.002</td>
</tr>
<tr>
<td>Olive oil</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O-Oil1</td>
<td>18.673 ± 0.01</td>
<td>0.196 ± 0.001</td>
</tr>
<tr>
<td>O-Oil2</td>
<td>77.47 ± 2.13</td>
<td>0.254 ± 0.001</td>
</tr>
<tr>
<td>O-Oil3</td>
<td>44.08 ± 3.95</td>
<td>1.321 ± 0.005</td>
</tr>
<tr>
<td>O-Oil4</td>
<td>44.53 ± 2.74</td>
<td>0.411 ± 0.002</td>
</tr>
<tr>
<td>O-Oil5</td>
<td>37.741 ± 1.91</td>
<td>0.488 ± 0.001</td>
</tr>
<tr>
<td>O-Oil10</td>
<td>50.53 ± 2.74</td>
<td>0.784 ± 0.002</td>
</tr>
</tbody>
</table>
through skin. In another report published on in vitro permeability of propanolol using oleic acid as permeability enhancer using porcine buccal epithelial membrane, it was just assumed that oleic acid might have permeation enhancing effect attributed to the lipid-fluidizing effect of fatty acid. However, the mechanism by which drug permeation is enhanced through skin port has never been instigated and discussed so far.

Most researchers used co-solvents and solubilizing agents along with penetration enhancers in their studies while determining the penetration effect (22-24). Our study aimed at finding out the potential of these fatty acids as pure compounds without using co-solvents. Although this technique has been found to be used in volatile transdermal sprays (25) and topical gels as well (26). The results of this study imply three conclusions, first as penetration enhancers fatty acids in liquid state (OLA, LA) showed high efficiency in comparison to those fatty acids which are in solid state (SDA, TDA). Thus high melting point fatty acids show low permeation enhancement effect and are less effective than liquid fatty acids in formulations for topical applications (27). The second implication of the study points towards the existence of a linear relationship between the solubility and permeation enhancement (28). The solubility of the enhancer in lipids of SC is an important factor for penetration of fatty acid enhancers, effectively. Third, as the decreasing lipophilicity of penetration enhancers could indicate the decreased duration of the enhancement. The consequent enhancement effect cannot be maintained as with decreasing lipophilicity of enhancer (18).

The techniques used in the current study might allow the overall drug release through patch matrix via appropriate choice of polymers and their combinations utilizing different diffusion pathways to get overall desired steady state and release pattern both in vitro and in vivo. The cumulative amount of flurbiprofen drug released per cm² from different drug delivery systems of varied ratios of EC and PVP exhibited a variable release patterns (see Figs. 5-8). The drug release pattern in most of the controlled/sustained release devices is mostly governed by the diffusion and erosion mechanism. Since, the patch matrix formulated with compatible polymers comes into contact with the solvent system, the fluid is absorbed into the patch matrix initiating drug as well as polymer chain dissolution process in the matrix (15). The satisfactory folding endurance of patch revealed that the formulated patch might be able to maintain their reliability with general skin folding during handling and would be able to endure the cracks and ruptures (12). The flatness study might indicate that the patch formulation has the same strip length before and after the cuts. Moisture content in the formulation was related to ratios of EC and PVP. Low moisture uptake by the polymer matrix gives 100% flatness to the patch and might maintain smooth and uniform surface when applied onto skin.

Formulations containing high concentration of polymer show a greater tensile strength than the counterparts fabricated with low concentrations. Similar behavior was observed with the plasticizer, however the impact of plasticizer concentration is to a lower extent than the one recorded with polymer. The presence of the plasticizer also contributed in the production of smooth, uniform and flexible patches throughout. Its existence also prevents the cracking of the film by imparting it the flexibility and desirable mechanical properties. Similarly, the concentration of the PE has a relatively small positive correlation with the tensile strength (29). All the patches were found to have agreeable flexibility evidenced by getting high values of tensile strengths and % elongation. Uniform and homogenous distribution of drug in the patches is necessary to ensure uniform sustained delivery of the drug from the patches. Furthermore, no significant changes in the physicochemical properties and drug contents of the formulated patches were observed during the stabil-

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Cmax (µg/mL)</th>
<th>Tmax (h)</th>
<th>AUC0-24 (µg.h/mL)</th>
<th>Relative bioavailability</th>
<th>Half-life t1/2 (h)</th>
<th>MRT 0-24 (h)</th>
<th>Clearance (mL/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral</td>
<td>26.44 ± 2.11</td>
<td>1.00</td>
<td>77.78 ± 3.72</td>
<td>-</td>
<td>5.33 ± 0.13</td>
<td>7.21 ± 0.88</td>
<td>0.34 ± 0.32</td>
</tr>
<tr>
<td>Plain (control)</td>
<td>09.43 ± 0.62</td>
<td>6.0</td>
<td>223.43 ± 5.14</td>
<td>2.87</td>
<td>9.33 ± 0.07</td>
<td>14.29 ± 1.87</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>MT10</td>
<td>39.92 ± 1.2</td>
<td>8.0</td>
<td>553.84 ± 8.3</td>
<td>7.12</td>
<td>10.88 ± 0.01</td>
<td>15.89 ± 2.1</td>
<td>0.09 ± 0.02</td>
</tr>
<tr>
<td>OL10</td>
<td>22.12 ± 1.5</td>
<td>6.0</td>
<td>343.80 ± 3.98</td>
<td>4.42</td>
<td>11.22 ± 0.05</td>
<td>16.11 ± 1.34</td>
<td>0.04 ± 0.03</td>
</tr>
</tbody>
</table>
ity studies of the patches for a period of three months, thus ensuring the stability of the formulated patches.

The profiles of permeation enhancers showing dissimilar slopes are evident where the MTO and OLO concentration increases gradually from 1-10 percent. After twelve hours, formulations containing 5.0% MTO and OLO have shown significantly higher permeated (mg/cm²) cumulative amount of flurbiprofen through porcine skins with full thickness (p < 0.01 or 10% (p < 0.001)) in contrast to control formulations. Still, no significant difference was found between the thickness of two formulations containing 5.0% and 10.0% OLO, respectively (p > 0.05). The calculated flux (J) values of flurbiprofen from downwards slopes of steady state (linear) portion of in vitro permeation curves are shown in Table 4. As it is evident, the flux (J) is increased with the increase in concentrations of MTO and OLO. The only formulation containing 10.0% OLO (w/w) was found to be statistically different (p < 0.001) from control. A 2.4 times higher value for flurbiprofen flux (J) was found through the skin for this percentage. Thus the formulations having OLO concentrations of 2-5% showed statistically significant and varied values of fluxes (J) in comparison to formulation of 10% OLO. A diffusion model can also be interpreted form the values of permeated flurbiprofen values (mg/cm²) in Table 4, presenting a linear correlation (r). Thus rendering the proposed mechanism of MTO as penetration enhancer compatible with this observation i.e., the diffusion of the compound is facilitated by the increased SC permeability. Another suggested mechanism for MTO penetration is the phase separation between lamellar solids and fluids (1). The efficiency of MTO and OLO has been proven from highly hydrophilic than lipophilic compounds (ethanol/water < 0), which supports our rationale for using these compounds to enhance delivery of highly hydrophilic flurbiprofen through skin (23).

The potential formulations could be identified from the suggested in vitro models. It can be inferred statistically from this model that 5.0-10.0% facilitates the penetration of flurbiprofen in the skin, thus increasing its availability quantitatively in the tissue and to the deeper layers. This happens due to the enhancement effect of the oil, which interacts with the lipids or cellular membranes of SC.

Olive oil has been widely used to enhance the transdermal permeation of drug molecules. This compound acts by disordering the highly packed SC intercellular domains lipid. Another possible mechanism for the action of olive oil is lamellar solids fluid phase separation. When applied together with ethanol, olive oil is also believed to cause SC lipids extraction. In our study, we demonstrated that olive oil not only affects the SC lipids, but also influences the viable cells in the epidermis responsible for the skin immune function (29).

**CONCLUSION**

Flurbiprofen transdermal patch was successfully prepared and in vitro ex vivo and in vivo studies were performed using milk thistle oil as unique permeation enhancer. This compound was tested against artificial membrane, rabbit, rat, pig dog and human skin. The physicochemical parameters and in vitro drug permeation data showed that the formulation containing 5-10% milk thistle oil exhibited enhanced drug release; hence the one with 10% permeation enhancer was further selected for in vivo studies using rabbit as optimized animal model for further pharmacokinetics evaluation. It was concluded that the patch formulated with flurbiprofen in the current study using indigenous extracted milk thistle oil could serve as a potential formulation for transdermal delivery.

**Acknowledgment**

Thanks to global pharmaceuticals for providing gift samples of drug.

**REFERENCES**


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Cancer and its treatment cause pain in most patients. This pain may derive from the presence of tumors, medical tests, and surgical treatment. These pains may progress as acute, chronic or sudden pain. Keeping the pain under control is a key step in the treatment of cancer (1).

In the first stage, active ingredients of the non-steroidal anti-inflammatory drug (NSAID) group are preferred in order to be able to control these types of pains. Frequently used active ingredients are ibuprofen, naproxen, and acetaminophen. Alternatively, the reason for using dexketoprofen is for its use for relieving pain post-surgery to be effective, and the risk of experiencing side effects to be similar to other NSAIDs (2-6).

Another disorder which needs to be taken under control to increase the patient’s quality of life during cancer treatment is nausea and vomiting. Nausea and vomiting are generally seen during cytotoxic chemotherapy and radiotherapy, and after surgery.

Chemotherapy agents and radiotherapy causing the release of 5-HT in the small intestine triggers the vomiting reflex by stimulating vagal afferents through 5-HT3 receptors. However, the mechanism of action which takes part in the control of nausea and vomiting is not clearly known. Nonetheless, 5-HT3 receptor antagonists prevent the stimulation of his reflex (7-9).

NSAID and the 5-HT3 receptor antagonist active ingredients will be formulated in the form of orally disintegrating tablets to be able to increase patient compliance and decrease symptoms in cancer patients and patients having had surgical operation. Features which distinguish orally disintegrating tablet formulations are that neither water nor chewing is required during use, and that they also orally disintegrate in a short amount of time.

Oral rapidly disintegrating/dissolving tablet formulations are solid dosage forms which disintegrate/dissolve in a very short amount of time (under 30 s or under 1 min) without requiring water or chewing when taken orally (10).

DEVELOPMENT OF ORALLY DISINTEGRATING FIXED DOSE COMBINATION TABLETS CONTAINING ONDANSETRON HYDROCHLORIDE AND DEXKETOPROFEN TROMETAMOL

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Abstract: Orally disintegrating tablet formulations that involve active pharmaceutical ingredients in combination with a non-steroidal anti-inflammatory (dexketoprofen trometamol) and 5-HT 3 receptor antagonist (ondansetron hydrochloride) to prevent pain and nausea/vomiting during cancer therapy have been developed; in vitro dissolution profiles of these formulations have been examined and evaluated. 9 different formulations were generated to observe the effect of disintegrants that have various mechanisms of action, and the effect of lubricants on dissolution profiles in several media. Direct compression and wet granulation manufacturing methods were used throughout the trials of 9 formulations. Dissolution tests were conducted for both active pharmaceutical ingredients in pH 1.2 HCl acid, pH 4.5 acetate and pH 6.8 phosphate buffers for 9 formulations, then an optimized formulation was defined. Dissolution profiles of the optimized formulation were compared with two different reference products. Selected formulations, reference products and the optimized formulation were stored in accelerated stability conditions. Then, the alteration of tablets was evaluated. To our knowledge in this study, it was the first time that dexketoprofen trometamol and ondansetron hydrochloride were combined as an orally disintegrating fixed dose combination.

Keywords: dexketoprofen, ondansetron, ODT, pain, nausea
In this study, the objective was to develop *in vitro* examinations of and to evaluate oral rapidly disintegrating tablet formulations combining a non-steroidal anti-inflammatory drug (NSAID) dexketoprofen trometamol and 5-HT3 receptor antagonist ondansetron hydrochloride as active ingredients. Tablet formulations which increase patient compliance were developed by containing combined active ingredients, in which superdisintegrants are used to provide rapid oral disintegration and the bitter taste is masked through leaving a pleasant after-taste and sensation. To our knowledge in this study, it was the first time that dexketoprofen trometamol and ondansetron hydrochloride were combined as an orally disintegrating fixed dose combination.

**MATERIALS AND METHODS**

**Materials**

Ondansetron hydrochloride (Dr. Reddys Lab.), dexketoprofen trometamol (Enaltec Lab.), Ludiflash® (BASF), microcrystallinecellulose (Harke), croscarmellose sodium (FMC Biopolymer – Ac-di-sol®), cross linked polyvinyl pyrrolidone CL (BASF- Kollidon CL®), sodium starch glycolate (JRS Pharma – Explotab®), colloidal silicon dioxide (Evonik – Aerosil®), strawberry flavoring (Dallant), poloxamer 188 (BASF), sodium stearyl fumarate (FMC Biopolymer – Alubra®), magnesium stearate (Peter Greven), hydrochloride acid (Merck), ammonium acetate (JT Baker), glacial acetic acid (JT Baker), disodium hydrogen phosphate (JT Baker), citric acid (JT Baker), potassium chloride (JT Baker), acetonitrile (Merck), purified water (Onko), ethanol (Merck).

**Methods**

9 separate formulations containing the active ingredients dexketoprofen trometamol and ondansetron hydrochloride were developed. The unit formulas can be found in Table 1. While the F1, F2, F3, F7, F8, F9 formulas deriving from these formulas were manufactured with the direct compression method, the F4, F5, F6 formulas were manufactured with the wet granulation method.

The formulas prepared with the direct compression method (F1, F2, F3, F7, F8, F9) were consecutively manufactured. Dexketoprofen trometamol and ondansetron hydrochloride, Ludiflash®, sucralose, strawberry flavoring were separately passed through a 0.5 mm sieve. Poloxamer 188 used in the F9 formula was added after it had been sifted. The sifted raw materials were stirred for 10 min. The excipient functioning as a disintegrant (Kollidon CL®, Ac-di-sol®, Explotab®) was passed through a 0.5 mm sieve, added to the mixture and stirred for 5 min. Aerosil® was passed through a 0.5 mm sieve, added to the mixture and stirred for 1 min. Alubra®

<table>
<thead>
<tr>
<th>Table 1. Tablet formulations.</th>
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</thead>
<tbody>
<tr>
<td><strong>Raw material</strong></td>
</tr>
<tr>
<td>Dexketoprofen trometamol</td>
</tr>
<tr>
<td>Kollidon CL®</td>
</tr>
<tr>
<td>Ac-di-sol®</td>
</tr>
<tr>
<td>Explotab®</td>
</tr>
<tr>
<td>Aerosil®</td>
</tr>
<tr>
<td>Poloxamer 188®</td>
</tr>
<tr>
<td>Sucralose</td>
</tr>
<tr>
<td>Strawberry flavor</td>
</tr>
<tr>
<td>Alubra®</td>
</tr>
<tr>
<td>Magnesium stearate</td>
</tr>
<tr>
<td>Purified water</td>
</tr>
<tr>
<td>Ethanol</td>
</tr>
<tr>
<td>Tablet weight</td>
</tr>
</tbody>
</table>
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(F1, F2, F3, F8, F9) or magnesium stearate (F7) were passed through a 0.5 mm sieve, added to the mixture and stirred for 1 min.

The formulas prepared with the wet granulation method (F4, F5, F6) were consecutively manufactured. Dexketoprofen trometamol and Ondansetron hydrochloride, Ludiflash®, sucralose, strawberry flavoring were separately passed through a 0.5 mm sieve. The sifted raw materials were stirred for 10 min. These were then granulated with purified water (F4, F5) or a purified water/ethanol mixture (F6). This was dried in an incubator until the inner phase reached suitable moisture content. The dried granules were passed through a 0.5 mm sieve. The percent yield was then calculated. Kollidon CL®, Aerosil® and Alubra® were weighed according to this calculation. The excipient functioning as a disintegrant (Kollidon CL®) was passed through a 0.5 mm sieve, added to the mixture and stirred for 5 min. Aerosil® (F5, F6) was passed through a 0.5 mm sieve, added to the mixture and stirred for 1 min. Alubra® was passed through a 0.5 mm sieve, added to the mixture and stirred for 1 min.

Tablets of the formulations (F1-F9) were compressed in an eccentric tablet press, weighing approximately 200 mg and of a 20-40 N hardness value. The disintegration times (Table 2 and Figure 1) and in vitro dissolution rate profiles of these tablets were evaluated in pH 1.2 HCl, pH 4.5 acetate, pH 6.8 phosphate media. The tablet characteristics and disintegration times of the tablets were testes with a pharmacopeial method (11), and also the disintegration of the F10 formulation was observed with the naked eye in a pH 6.8 phosphate buffer in a Petri dish (Fig. 2). The dissolution rate profiles can be found in Figures 3-8, respectively.

<table>
<thead>
<tr>
<th>Formulation no.</th>
<th>Disintegration time, seconds (n : 6)</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>18</td>
<td>4.58</td>
</tr>
<tr>
<td>F2</td>
<td>23</td>
<td>6.32</td>
</tr>
<tr>
<td>F3</td>
<td>20</td>
<td>5.03</td>
</tr>
<tr>
<td>F4</td>
<td>25</td>
<td>2.27</td>
</tr>
<tr>
<td>F5</td>
<td>26</td>
<td>1.65</td>
</tr>
<tr>
<td>F6</td>
<td>&gt;180</td>
<td>2.02</td>
</tr>
<tr>
<td>F7</td>
<td>30</td>
<td>2.78</td>
</tr>
<tr>
<td>F8</td>
<td>21</td>
<td>2.15</td>
</tr>
<tr>
<td>F9</td>
<td>22</td>
<td>2.58</td>
</tr>
</tbody>
</table>

(F1, F2, F3, F8, F9) or magnesium stearate (F7) were passed through a 0.5 mm sieve, added to the mixture and stirred for 1 min.
The dissolution rate test was carried out in the following conditions: 500 mL, 50 rpm, paddle, 37 ± 0.5°C.

While the dissolution rate method of dexketoprofen trometamol and ondansetron hydrochloride was being developed, attempts were primarily made to develop a UV-spectrophotometric method. However, an HPLC method was developed as the active ingredients gave absorbance of the same wavelength and interfered with each other’s absorbance values.

The chromatographic analyses of dissolution rate test samples were conducted at a wavelength of 233 nm at 1.0 mL/min. flow rate conditions in an injection volume of 20 µL with a UV-VIS detector using an ACE 5 C18, (250 mm × 4.6 mm), 5 µm 100 Å column with an HPLC device (Agilent, USA). The column temperature was 10°C and a pH 3.5 acetate buffer : acetonitrile : purified water (20 : 430 : 550) was used as the mobile phase. The method was determined to be selective through analytical method validation. Ondansetron hydrochloride gives absorbance at approximately 3.5 min and dexketoprofen trometamol gives absorbance at approximately 10.5 min.

After the dissolution rate profiles were examined in three different media, the stability of the F1, F5, F7 and F9 formulas in accelerated conditions (40 ± 2°C, 75% RH ± 5% RH) was tested with a DSC analysis and assay. The DSC analysis (DSCQ2000, TA Instruments, Likrom, USA) was carried out through placing the sample in an aluminium cell, increasing the temperature 10°C per min and increasing the temperature from 0°C to 350°C. Whereas, the assay was conducted at a wavelength of 233 nm at 1.0 mL/min. flow rate conditions in an injection volume of 20 µL with a UV-VIS detector using an ACE 5 C18, (250 mm × 4.6 mm), 5 µm 100 Å column with an HPLC device (Agilent, USA). The column temperature was 10°C and a pH 3.5 acetate buffer : acetonitrile : purified water (20 : 430 : 550) was used as the mobile phase. Ondansetron hydrochloride gives absorbance at approximately 3.5 min and dexketoprofen trometamol gives absorbance at approximately 10.9 min in sample solutions, in which the mobile phase is used as the solvent.

**DISCUSSION AND RESULTS**

While Kollidon CL® was used in the F1 formula, the superdisintegrants Ac-di-sol® and Explotab® were respectively used in the F2 and F3 formulas. As used in commercially available products, frequently used superdisintegrants were preferred (12). Kollidon CL® is superior as it does not enable disintegration with a single mechanism, but enables disintegration by combining different mechanisms (13, 14). The wet granulation method was tested instead of the direct compression method with F4 and F5 formulas to further improve compression and corrosion characteristics. Aerosil® was removed from the F4 formula as wet granulation was to be carried out, and the amount of lubricant was reduced so as not to prolong the dissolution time. Also, a formula using Aerosil® was carried out in parallel with this. The flow properties were improved with the F4 and F5 formulas manufac-

![Figure 2. Disintegration simulation of F9 in a Petri dish A) at the beginning in pH 6.8 phosphate buffer; B) after 30 s. in pH 6.8 phosphate buffer](image)
Figure 3. Comparative ondansetron dissolution profiles of reference tablets and (F1-F9) tablets in a pH 1.2 (0.1 M HCl) acid buffer

Figure 4. Comparative dexketoprofen dissolution profiles of reference tablets and (F1-F9) tablets in a pH 1.2 (0.1 M HCl) acid buffer

Figure 5. Comparative ondansetron dissolution profiles of reference tablets and (F1-F9) tablets in a pH 4.5 acetate buffer
Figure 6. Comparative dexketoprofen dissolution profiles of reference tablets and (F1-F9) tablets in a pH 4.5 acetate buffer.

Figure 7. Comparative ondansetron dissolution profiles of reference tablets and (F1-F9) tablets in a pH 6.8 phosphate buffer.

Figure 8. Comparative dexketoprofen dissolution profiles of reference tablets and (F1-F9) tablets in a pH 6.8 phosphate buffer.
tured with wet granulation, however the flow properties containing Aerosil® were determined to be more suitable. However, the tablet surfaces were observed to be rough as the amount of lubricant had been reduced in these tablets. Ethanol was added to the binding solution in addition to purified water in the F6 formula to improve granulation properties and to render the tablet surface smoother. Sodium stearyl fumarate which was used in the F1 formula and was thought to display optimum properties and magnesium stearate which was used in the F7 formula were both tested to decide upon the lubricant through evaluating the formulas up to F6. However, the ratio of magnesium stearate was reduced so as not to delay the dissolution profile.

A decision was made to increase the concentration of Kollidon CL® to reduce the disintegration time, and to increase solubility in the first few minutes. An incremental increase was reported in the surface area as the concentration of polyvinylpyrrolidone CL (Kollidon CL®) reverse cross linked with croscarmellose sodium (Ac-di-sol®) and sodium starch glycolate (Explotab®) increased (15). Kruse (16) conducted studies using sodium stearyl fumarate and magnesium stearate in orally disintegrating studies with Ludiflash®. According to the results of this study, it was determined that the formula containing sodium stearyl fumarate became wet in a shorter period of time and thus, disintegration time was reduced (16). The F8 formula was tested by evaluating this information and data from previous batches.

Through evaluating the physicochemical test results of the formulas (F1-F8) and the in vitro dissolution rate results of the formulas (F1-F8) and reference products, and designing the F9 formula, the F9 formula was determined as the optimized formula. Although the amount of ondansetron and dexketoprofen dissolved at the initial sampling point had increased in comparison to other batches in the tablets of the F8 formula, a decision was made to use a surface active agent as the amount of ondansetron dissolved at 2 min was less than the amount of ondansetron dissolved at 2 min of Onzyd 8 mg Orally Disintegrating Tablets of the company PlatinKimya. A decision was made to use a low concentration of poloxamer 188 to increase the solubility at 2 min of our active ingredients with a BCS class I. A short period of wetness, a HLB value of about 29, not affecting the pH of the medium due to being non-ionic, and being less toxic were indicative in this selection (17, 18). Studies were observed to be conducted with the direct compression method in studies carried out in ADTs prepared with a solid dispersion (19, 20).

The length and thickness values of the formulas (F1-F9) complied with the weight and hardness values. That the weight and thickness values were homogeneous shows that the tablets will be less affected by the preparation method and small differences during compression.

The formulas (F1-F9) were pressed with an oblong punch in a single punch tablet press machine between the hardness values of 20–33 N. The effect of the wet granulation method and the used lubricant may be observed in the tablet hardness values. The corrosion values of batches manufactured with wet granulation and batches using magnesium stearate as a lubricant were lower than other batches.

Although they disintegrated in a similar period of time when the disintegration times were compared, the formula F1 has the longest disintegration

<table>
<thead>
<tr>
<th>Sample</th>
<th>Assay (%) (as is) Initial</th>
<th>Assay (%) (as is) 3 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dexketoprofen trometamol (as an API)</td>
<td>99.85</td>
<td>97.56</td>
</tr>
<tr>
<td>Ondansetron hydrochloride (as an API)</td>
<td>99.56</td>
<td>100.71</td>
</tr>
<tr>
<td>F1 tablets (Dexketoprofen trometamol )</td>
<td>99.20</td>
<td>99.56</td>
</tr>
<tr>
<td>F1 tablets (Ondansetron hydrochloride)</td>
<td>100.21</td>
<td>101.75</td>
</tr>
<tr>
<td>F5 tablets (Dexketoprofen trometamol )</td>
<td>100.44</td>
<td>99.61</td>
</tr>
<tr>
<td>F5 tablets (Ondansetron hydrochloride)</td>
<td>99.48</td>
<td>98.69</td>
</tr>
<tr>
<td>F7 tablets (Dexketoprofen trometamol )</td>
<td>100.70</td>
<td>96.75</td>
</tr>
<tr>
<td>F7 tablets (Ondansetron hydrochloride)</td>
<td>98.89</td>
<td>98.20</td>
</tr>
<tr>
<td>F9 tablets (Dexketoprofen trometamol )</td>
<td>100.30</td>
<td>98.19</td>
</tr>
<tr>
<td>F9 tablets (Ondansetron hydrochloride)</td>
<td>101.13</td>
<td>99.98</td>
</tr>
</tbody>
</table>
time (approximately 18 seconds). The F6 formula was granulated with a water/alcohol (1:1) mixture, but the tablets of the F6 formula did not disintegrate within 3 min as relatively hard granules were obtained. The disintegration times of the tablets of the F2, F3, F4, F5, F7, F8, F9 formulas ranged between 20 s and 30 s (Fig. 1).

The DSC thermograms of the active ingredients and the tablets of the F1 formula manufactured with the direct compression method with a rapid disintegration time, tablets of the F5 formula manufactured with the wet granulation method, and tablets of the F7 formula using magnesium stearate as a lubricant are shown in Fig. 9, respectively.

As seen in Fig. 9, the active ingredient begins to melt at 104.3°C and it corresponds to the literature data (104.8-105.1°C) even though there is a slight deviation, in the DSC thermogram of dexketoprofen trometamol. While the active ingredient began to melt at 104.4°C in the F1 formula, it began melting at 102.8°C and 104.6°C in the formulas F5 and F7, respectively. The active ingredient begins to melt at 183.0°C and it corresponds to the literature data (178.5-179.5°C) even though there is a slight deviation, in the DSC thermogram of ondansetron hydrochloride. However, melting which began at 131.5°C suggests that the active ingredient has a different polymorph. When the thermograms of the tablets of the formulas were examined, the tablets of the formulas F1, F6, and F7 began to melt at 163.55°C, 162.84°C and 163.76°C, respectively. Theoretically, Ludiflash® should melt at this temperature. Excipients are thought to have an effect as deviations were observed in the melting point in formulas of both direct compression and wet granulation.

The stability of these formulas and active ingredients selected as an example was followed in accelerated conditions in a tightly sealed glass vial (40 ± 2°C, 75% RH ± 5% RH). The stability conditions were followed by taking DSC thermograms and conducting assays. The DSC thermograms of ondansetron hydrochloride, dexketoprofen trometamol, F1, F5, and F7 tablets suspended in accelerated conditions for 3 months can be found in Figure 10, respectively. As seen in Figure 10, three endothermic peaks were observed at 136.7°C, 176.2°C, and 238.8°C in the DSC thermogram of

![Figure 9. DSC thermograms (initial) of A, C, and E) corresponded to ondansetron hydrochloride, dexketoprofen trometamol and F1 tablets, respectively. DSC thermograms of B, D and F) corresponded to ondansetron hydrochloride, dexketoprofen trometamol and F1 tablets respectively after 3 months in accelerated stability conditions.](image-url)
ondansetron hydrochloride, whereas in DSC thermogram of dexketoprofen trometamol, an endothermic peak was observed at 107.65°C. Endothermic peaks were observed at 94.16°C, 159.57°C, 320.13°C in the DSC thermogram of the F1 tablet; at 94.51°C, 161.15°C, 316.41°C in the DSC thermogram of the F5 tablet; and at 91.57°C, 159.67°C, 317.71°C in the DSC thermogram of the F7 tablet. A significant difference was not observed between the stability samples with the melting point temperatures in the DSC thermograms taken at baseline conditions.

In vitro dissolution rate studies were conducted for reference products and formulas (F1-F8) in pH 1.2 HCl acid, pH 4.5 acetate, pH 6.8 phosphate buffers to evaluate the dissolution rates of the tablets. As the dissolved amounts of substance in the formulations containing two active ingredients interacted with each other, they were not spectrophotometrically evaluated and the HPLC method was used.

The comparative dissolution rate profiles of the tablets of the formulas (F1-F9), Onzyd 8 mg Orally Disintegrating Tablets containing ondansetron of the company PlatinKimya, and conventional Arveles 25 mg Film Coated Tablets containing dexketoprofen trometamol of the company Menarini Group in pH 1.2 HCl acid, pH 4.5 acetate, pH 6.8 phosphate buffers may be found in Figures 3-8.

Reaching a high dissolution rate is aimed by enabling disintegration to begin from the oral cavity to increase bioavailability in orally disintegrating tablet formulations (21). Therefore, 10 min was determined as the limit. According to the ‘Ondansetron Orally Disintegrating Tablets’ monograph in the European Pharmacopoeia (USP38), at least 85% ondansetron should dissolve in 10 min (11). Onzyd 8 mg Orally Disintegrating Tablets displayed a high ratio of dissolution from the first few minutes in the pH 1.2 (0.1 M HCl) acid buffer. The cumulative amount of dissolved ondansetron at 10 min in the pH 1.2 (0.1 M HCl) acid buffer was 99.42%. The cumulative amount of dissolved ondansetron in the pH 4.5 acetate buffer was 95.74% and the cumulative amount of dissolved ondansetron in the pH 6.8 phosphate buffer was 64.86% at 10 min.

While low-alkali active ingredients are substantially ionized in media with a lower pH value

Figure 10. DSC thermograms (initial) of A, C, and E) corresponded to F5 tablets, F7 tablets and F9 tablets, respectively. DSC thermograms of B, D and F) corresponded to F5 tablets, F7 tablets and F9 tablets, respectively, after 3 months in accelerated stability conditions
such as the stomach, these active ingredients are less likely to be ionized in media varying between pH 6.0-8.0 such as the small intestine. While active ingredients displaying weak acid properties are more non-ionized in an acid media, they are more ionized between pH 6.0-8.0 (22).

According to the Biopharmaceutical Classification System, ondansetron is within Class I, i.e. the ‘High solubility, High permeability’ group (23). The pKa value of ondansetron is 7.4 (24), therefore while the highest solubility ratio is expected to be in the pH 1.2 (0.1M HCl) acid medium, a low solubility profile is expected in the pH 6.8 phosphate buffer. The obtained results confirm this pre-evaluation.

Dexketoprofen trometamol and tablets containing dexketoprofen trometamol are not registered in any pharmacopoeia, and dexketoprofen trometamol belongs to Class I – ‘High solubility, High permeability’ group according to the Biopharmaceutical Classification System (25). The pKa value of dexketoprofen is 5.02 (26), therefore its solubility is expected to be affected by the pH variations. When the obtained results were evaluated, the solubility of dexketoprofen trometamol at 3 different pH values was observed to be high.

Arveles 25 mg Film Coated Tablets have an immediate release profile; and display a slower release profile in the first few minutes as they are not in an orally disintegrating tablet form, yet displayed an increasing profile over time. Therefore, the results at 10 min are relatively low. The cumulative amount of dissolved dexketoprofen at 10 min in the pH 1.2 (0.1 M HCl) acid buffer was 36.81%. The cumulative amount of dissolved dexketoprofen in the pH 4.5 acetate buffer was 78.63% and the cumulative amount of dissolved dexketoprofen in the pH 6.8 phosphate buffer was 39.12% at 10 min.

However, when infinity conditions were evaluated by increasing the speed (150 rpm), the cumulative amount of dissolved dexketoprofen in the pH 1.2 (0.1 M HCl) buffer was 98.62% at 30 min. The cumulative amount of dissolved dexketoprofen in the pH 4.5 acetate buffer was 103.08% at 10 min and the cumulative amount of dissolved dexketoprofen in the pH 6.8 phosphate buffer was 100.88% at 30 min. Thus, dexketoprofen was also observed to have high solubility. Due to the abovementioned reasons, the aim was to design formulas that have high solubility as of the first few minutes and can reach the maximum solubility value at 10 min for both active ingredients.

When the tablets of the formulas (F1-F8) were evaluated in terms of 3 mediums with regard to ondansetron (Figs. 3-8), F1 was determined as the batch to reach the highest cumulative amount of dissolved ondansetron at 10 min in the pH 1.2 0.1 M HCl acid medium. As the % amount of dissolved ondansetron at 15 min was more than 85%, the f2 similarity factor was not calculated. The tablets of the F6 formula were observed to reach the lowest cumulative amount of dissolved ondansetron at 10 minutes in the pH 1.2 (0.1 M HCl) acid medium. Although the tablets of the F1 formula had dissolved at a high ratio, it failed to reach the % amount of dissolved ondansetron of Onzyd 8 mg Orally Disintegrating Tablets at 2 minutes. The reason for this thought to have derived from the difference in manufacturing technology, the source of the used active ingredient, or the particle size of the active ingredient. F8 was determined as the batch to have reached the highest cumulative amount of dissolved ondansetron and F6 was determined as the batch to have reached the lowest cumulative amount of dissolved ondansetron at 10 min in the pH 4.5 acetate buffer. The tablets of the F1, F2, F3 and F8 formu-
las were determined to have very close ratios of % cumulative amount of dissolved ondansetron. F8 was determined as the batch to have reached the highest cumulative amount of dissolved ondansetron and F4 was determined as the batch to have reached the lowest cumulative amount of dissolved ondansetron at 10 min in the pH 6.8 phosphate buffer. As cumulative dissolution at 15 min in the pH 6.8 phosphate buffer had not reached 85%, the f2 similarity factor was calculated. The similarity factor between the tablets of the F9 (optimized formula) formula and Onzyd 8 mg ADT was found to be 63.

When the tablets of the formulas (F1-F8) were evaluated in terms of 3 mediums with regard to dexketoprofen (Figs. 3-8), F4 was determined as the batch to reach the highest cumulative amount of dissolved dexketoprofen and F1 was determined as the batch to have the next highest solubility at 10 min in the pH 1.2 (0.1 M HCl) acid medium. As the % amount of dissolved dexketoprofen at 15 min was more than 85%, the f2 similarity factor was not calculated. The tablets of the F6 formula were observed to reach the lowest cumulative amount of dissolved dexketoprofen at 10 min in the pH 1.2 (0.1 M HCl) acid medium. F8 was determined as the batch to have reached the highest cumulative amount of dissolved dexketoprofen and F6 was determined as the batch to have reached the lowest cumulative amount of dissolved dexketoprofen at 10 min in the pH 4.5 acetate buffer. F1 was determined as the batch to have reached the highest cumulative amount of dissolved dexketoprofen and F6 was determined as the batch to have reached the lowest cumulative amount of dissolved dexketoprofen at 10 min in the pH 6.8 phosphate buffer.

In general, when we evaluated the physico-chemical properties and in vitro dissolution rate profiles of tablets containing formulas (F1-F8), F1 was determined as the batch to have optimum characteristics. The excipients used in its formula are excipients which are supported by literature and frequently preferred. However, when the in vitro dissolution rate profiles of this batch were examined, Onzyd 8 mg Orally Disintegrating Tablets was determined to be faster at 2 minutes in three media. Therefore, a decision was made to use the F1 formula as the basis, to use a surface active agent to increase solubility at 2 min, and to use sodium stearyl fumarate as a lubricant as it improves compression specifications, reduces wettability time compared to magnesium stearate and does not cause any incompatibilities in stability while the optimized formula was being designed. Poloxamer 188, which is a non-ionic surface active agent specified to generally have low toxic effects, was preferred while choosing the surface active agent. A decision was made to employ the direct compression method so as not to increase the process period and cost as the in vitro dissolution rate profiles of the tablets manufactured with the direct compression method were faster compared to tablets manufactured with the wet granulation method while choosing the manufacturing method of the optimized formula (F9). Tablets pressed with the powder belonging to the optimized formula (F9) displaying good flowability were pressed with a weight of about 200.04 mg and 25.2 N hardness. The average disintegration time of the tablets is 22 s. A DSC analysis was carried out before placing the F9 tablets into stability conditions and after being suspended in accelerated stability conditions for 3 months. The tablets of the formula F9 were observed to begin melting at 91.52°C and 157.83°C (Fig. 9). Endothermic peaks were observed at 94.48°C, 160.37°C and 320.35°C in the DSC thermogram after the stability studies (Fig. 10).

The results of the assay conducted within the scope of the stability studies are shown in Table 3. While a significant difference was not observed in the active ingredients, the F1, F5 and F9 formulas, and the samples suspended for 3 months in accelerated conditions, a loss of approximately 4% in the amount of dexketoprofen was observed in the tablet of the F7 formula. The tablet in the F7 formula became off-yellow and slight speckling was observed. Figure 11 shows the appearances of the tablets manufactured with the F7 formula at the baseline and end of the stability period. Also, there were no meaningful changes in dissolution, hardness and disintegration times of formulations (F1, F5, and F9) that were stored in the accelerated stability conditions for three months.

CONCLUSION

In this study, an oral rapidly disintegrating tablet formulation which combined the active ingredients dexketoprofen trometamol and ondansetron hydrochloride was designed to increase patient compliance, and to rapidly eliminate complaints of nausea, vomiting and pain following a surgical operation. With the conducted in vitro solubility studies, the rapid dissolution of both substances was observed in different media, and their solubilities were not affected by each other. The oral bioavailability of the developed product is expected to be high.
Acknowledgment

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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INFLUENCE OF ANTIOXIDANTS ON THE STABILITY OF BIORASOL®

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Abstract: Biorasol® was developed for flushing and storing body organs. The fluid is classified as a class 2a medical product. The influence of antioxidants on the fluid’s stability was tested. Stability of Biorasol® was tested by accelerated aging. Ascorbic acid, cysteine and fumaric acid were added at the concentrations of 0.1 mM/L, 0.3 mM/L, and 0.5 mM/L. The study was based on the kinetics of the change rate of the glucose content at elevated temperatures differing by 10°C. The stability of Biorasol® at 5°C lasts for 886 days. An addition of ascorbic acid at concentrations of 0.3 mM/L and 0.5 mM/L resulted in extending the stability of Biorasol® by 16% and 25%, respectively. Other modifications did not significantly affect the stability of the tested fluid. Enrichment of the composition of Biorasol® with an addition of ascorbic acid increases the fluid’s stability.

Keywords: Biorasol®, antioxidants, stability, accelerated aging test

The group of world-known perfusion fluids now includes a newly developed Polish fluid Biorasol® designed for perfusion, preservation and reperfusion of body organs and tissues (1). The new preparation was classified as a class 2a medical product. The influence of antioxidants on the fluid’s stability was tested. Stability of Biorasol® was tested by accelerated aging. Ascorbic acid, cysteine and fumaric acid were added at the concentrations of 0.1 mM/L, 0.3 mM/L, and 0.5 mM/L. The study was based on the kinetics of the change rate of the glucose content at elevated temperatures differing by 10°C. The stability of Biorasol® at 5°C lasts for 886 days. An addition of ascorbic acid at concentrations of 0.3 mM/L and 0.5 mM/L resulted in extending the stability of Biorasol® by 16% and 25%, respectively. Other modifications did not significantly affect the stability of the tested fluid. Enrichment of the composition of Biorasol® with an addition of ascorbic acid increases the fluid’s stability.

In order to optimize the stability of Biorasol®, its composition was modified by adding antioxidants, i.e., ascorbic acid, cysteine and fumaric acid. We chose the compounds with potent antioxidant properties, helping eliminate free oxygen radicals, which significantly increases the survival of transplanted organs and tissues. Cysteine is a substrate in the reaction of glutathione synthesis and it has antioxidant properties. It prevents peroxidation of cell membrane lipids and protects lymphocytes against chromosomal aberration (5). Ascorbic and fumaric acids exhibit potent antioxidant and protective activity for the transplanted organ. Vitamin C is
a powerful antioxidant of interventional and hydrophilic properties; it prevents oxidative damage to DNA (6-11). It improves the functions of endothelial cells in blood vessels as a result of tetrahydrobiopterin stabilization (12). It neutralizes the reactive forms of oxygen and nitrogen which initiate oxidative stress and are dangerous to humans. It forms the oxidation-reduction system with glutathione, cytochrome C and pyridine nucleotides (13). It exerts a nephroprotective effect and significantly affects the proper functioning of the kidneys (14, 15). The fumaric acid used, as an indirect metabolite of the Krebs cycle, shows cardioprotective effect, and it protects tissue from reperfusion damage (16).

Stability of the modified fluids was assessed using the accelerated aging test: a quick way to evaluate the stability of the fluid depending on the stabilizers used at various concentrations. This method allows us to assess the mechanism and speed of reaction. It is based on the quantitative relation between the reaction rate and the temperature which is described by the laws of kinetics of chemical reaction (6-9, 17).

**EXPERIMENTAL**

**Materials**

Biolasol® was modified with a suitable antioxidant, i.e. ascorbic acid (by Zakłady Farmaceutyczne “PLIVA” S.A. Kraków; analytical grade), cysteine (by Sigma-Aldrich, USA; analytical grade) and fumaric acid (by “CHEMZAK” Sp. z o.o. Kędzierzyn-Koźle; analytical grade). The stability test cycle was performed under laboratory conditions using a climate chamber (by POL-EKO-aparatura Sp.j.). Glucose in body fluids was determined using a kit for the quantitative determination of glucose (by Pointe Scientific Inc. Glukoza Oxy – Category No. G7521).

**Kinetic studies**

Stability analysis involved the application of an accelerated aging test (18) which is recommended for stability studies during the developmental drug phase. That is an isothermal method to help determine the degree of drug degradation in several constant temperatures. The test was performed according to ICH guidelines (19) in four temperatures differing by 10°C, i.e.: 50°C ± 0.05 (323 K), 60°C ± 0.05 (333 K) 70°C ± 0.05 (343 K) and 80°C ± 0.05 (353 K). Relative humidity was 75% RH; test time: 40 days. Under these conditions, glucose degraded with a measurable rate.

Five unit packages containing specific fluids were placed in a climate chamber. Sampling started when the temperature of the test fluid was the same as the temperature in the chamber. The experiment was continued until over 50% decomposition of glucose was achieved. Based on the results of analytical determinations of glucose in the collected fluids, it was found that decomposition of glucose occurs according to the equation for first-order reaction. Degradation rate constants (k) were calculated for first-order reaction at four temperatures based on the following formula:

\[ k = 2.303 \times \frac{\lg [C_0/(C_0-C_x)]}{t} \quad [h^{-1}] \]  

where: \( C_0 \) – baseline content of a decomposed substance determined at \( t = 0 \) (100%), \( C_x \) – the substance content at a given time.

Then, graphs were made showing the relation between lnk and the inverse of the absolute temperature. It was found that the relation of lnk = f(1/T) is

<table>
<thead>
<tr>
<th>Solution</th>
<th>k 10^{-5}/h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50°C/75%RH</td>
</tr>
<tr>
<td>Biolasol®</td>
<td>11.162 ± 0.130</td>
</tr>
<tr>
<td>+0.1 mM/L vit. C</td>
<td>9.158 ± 0.080</td>
</tr>
<tr>
<td>+0.3 mM/L vit. C</td>
<td>8.294 ± 0.071</td>
</tr>
<tr>
<td>+0.5 mM/L vit. C</td>
<td>6.102 ± 0.083</td>
</tr>
<tr>
<td>+0.1 mM/L cysteine</td>
<td>9.905 ± 0.080</td>
</tr>
<tr>
<td>+0.3 mM/L cysteine</td>
<td>9.407 ± 0.087</td>
</tr>
<tr>
<td>+0.5 mM/L cysteine</td>
<td>10.532 ± 0.120</td>
</tr>
<tr>
<td>+0.1 mM/L fumaric acid</td>
<td>10.155 ± 0.110</td>
</tr>
<tr>
<td>+0.3 mM/L fumaric acid</td>
<td>9.905 ± 0.080</td>
</tr>
<tr>
<td>+0.5 mM/L fumaric acid</td>
<td>10.909 ± 0.120</td>
</tr>
</tbody>
</table>
Influence of antioxidants on the stability of Biolasol®

A linear function, which allowed us to assume that Arrhenius equation in a logarithmic form was satisfied in the studied cases (8, 9). Within the range of temperatures, activation energy ($E_a$) and frequency factor ($A$) are therefore constant values. For the linear relationship of $\ln k = f(1/T)$, we used linear regression to determine slope ($a$) and intercept ($b$) and then, on that basis, we determined activation energy ($E_a$) and frequency factor ($A$).

$$E_a = a \ln k + b$$

where:

$$\ln k = a \cdot \frac{1}{T} + b = \frac{E_a}{R} \cdot \frac{1}{T} + \ln A$$

Reaction rate constant at a temperature of 5°C was calculated using the relationship:

$$\ln k_{5°C} = \ln A - E_a/(RT)$$

Stability of the test fluid at a temperature of 5°C was calculated by determining parameter $t_{90}$ (time after which the content of the active ingredient is reduced into 90% of the initial value):

$$t_{90} = \frac{0.1053}{k_{278}}$$

The results are presented as the mean values of at least five independent repetitions of the experiment. Periods of stability of Biolasol® and the modified fluids were subjected to ANOVA variance analysis and verification by Newman–Keuls method. Data analysis was performed using the Statistica 10 software and the Zestaw Ocena Stabilności package (StatSoft Polska).

**RESULTS**

Based on the results obtained, it was found that glucose transformations at all the test temperatures

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**Table 2. Activation energy ($E_a$) and frequency factor ($A$) determined for solutions.**

<table>
<thead>
<tr>
<th>Solution</th>
<th>$E_a$ [kJ/M] ± SD</th>
<th>lnA ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biolasol®</td>
<td>51.69 ± 0.13</td>
<td>15.06 ± 0.04</td>
</tr>
<tr>
<td>+0.1 mM/L vit. C</td>
<td>49.47 ± 0.07</td>
<td>8.89 ± 0.03</td>
</tr>
<tr>
<td>+0.3 mM/L vit. C</td>
<td>49.58 ± 0.10</td>
<td>12.11 ± 0.04</td>
</tr>
<tr>
<td>+0.5 mM/L vit. C</td>
<td>46.45 ± 0.08</td>
<td>10.00 ± 0.03</td>
</tr>
<tr>
<td>+0.1 mM/L cysteine</td>
<td>50.70 ± 0.03</td>
<td>3.51 ± 0.01</td>
</tr>
<tr>
<td>+0.3 mM/L cysteine</td>
<td>50.63 ± 0.06</td>
<td>7.36 ± 0.02</td>
</tr>
<tr>
<td>+0.5 mM/L cysteine</td>
<td>50.74 ± 0.07</td>
<td>8.88 ± 0.03</td>
</tr>
<tr>
<td>+0.1 mM/L fumaric acid</td>
<td>49.77 ± 0.06</td>
<td>7.95 ± 0.02</td>
</tr>
<tr>
<td>+0.3 mM/L fumaric acid</td>
<td>50.80 ± 0.20</td>
<td>24.01 ± 0.07</td>
</tr>
<tr>
<td>+0.5 mM/L fumaric acid</td>
<td>51.92 ± 0.11</td>
<td>13.14 ± 0.04</td>
</tr>
</tbody>
</table>

**Table 3. The stability of Biolasol® solution and modified solution at 20°C and 5°C.**

<table>
<thead>
<tr>
<th>Solution</th>
<th>20°C k10⁻⁵/h ± SD</th>
<th>20°C $t_{90}$ [days] ± SD</th>
<th>5°C k10⁻⁵/h ± SD</th>
<th>5°C $t_{90}$ [days] ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biolasol®</td>
<td>1.56 ± 0.15</td>
<td>282 ± 27</td>
<td>0.50 ± 0.05</td>
<td>886 ± 88</td>
</tr>
<tr>
<td>+0.1 mM/L vit. C</td>
<td>1.39 ± 0.08</td>
<td>316 ± 18</td>
<td>0.46 ± 0.03</td>
<td>947 ± 55</td>
</tr>
<tr>
<td>+0.3 mM/L vit. C</td>
<td>1.25 ± 0.10</td>
<td>350 ± 27*</td>
<td>0.42 ± 0.03</td>
<td>1050 ± 83*</td>
</tr>
<tr>
<td>+0.5 mM/L vit. C</td>
<td>1.04 ± 0.06</td>
<td>422 ± 27*</td>
<td>0.37 ± 0.02</td>
<td>1181 ± 77*</td>
</tr>
<tr>
<td>+0.1 mM/L cysteine</td>
<td>1.43 ± 0.03</td>
<td>306 ± 7</td>
<td>0.47 ± 0.01</td>
<td>941 ± 22</td>
</tr>
<tr>
<td>+0.3 mM/L cysteine</td>
<td>1.37 ± 0.06</td>
<td>322 ± 15</td>
<td>0.44 ± 0.02</td>
<td>987 ± 48</td>
</tr>
<tr>
<td>+0.5 mM/L cysteine</td>
<td>1.52 ± 0.08</td>
<td>289 ± 16</td>
<td>0.49 ± 0.03</td>
<td>888 ± 52</td>
</tr>
<tr>
<td>+0.1 mM/L fumaric acid</td>
<td>1.52 ± 0.08</td>
<td>288 ± 15</td>
<td>0.51 ± 0.03</td>
<td>869 ± 45</td>
</tr>
<tr>
<td>+0.3 mM/L fumaric acid</td>
<td>1.43 ± 0.22</td>
<td>308 ± 47</td>
<td>0.46 ± 0.07</td>
<td>948 ± 149</td>
</tr>
<tr>
<td>+0.5 mM/L fumaric acid</td>
<td>1.51 ± 0.13</td>
<td>291 ± 24</td>
<td>0.48 ± 0.04</td>
<td>919 ± 79</td>
</tr>
</tbody>
</table>

*Statistically significantly different from Biolasol®, p < 0.05.

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occurred according to the equation for first-order reaction. Table 1 presents the calculated degradation rate constants (k) for first-order reaction at four temperatures. The results obtained for each temperature are presented as the mean of 5 results (k). The determined reaction rate constants allowed us to establish their relation to temperature. A graphical representation of this relation of \( \ln k = f(1/T) \) is shown in Figures 1-3.

The observed linear relation between the reaction rate constant and the temperature is determined by Arrhenius equation (8, 9). A straight line of slope is equal to \(-E_a/RT\) which crosses the axis of ordinates at point \( \ln A \) for value \( 1/T = 0 \). For the linear relation of \( \ln k = f(1/T) \), we used linear regression to determine slope (a) and intercept (b) and then, on that basis, we determined activation energy (\( E_a \)) and frequency factor (\( A \)). The determined values of \( E_a \) and \( \ln A \) are presented in Table 2.

The obtained values for activation energy (Table 2) fit in the range of 49-52 kJ/M, which corresponds to the mean for solvolytic drug decomposition.

If Arrhenius equation is satisfied, we assume that there are constant values of \( E_a \) and \( A \) within the tested temperature range. That allows us to deter-

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**Figure 1.** Arrhenius plot for the first-order rate constant of reducing compounds degradation in Biolasol® solution and in Biolasol® solution modified by adding vitamin C over the temperature range of 50-80°C

**Figure 2.** Arrhenius plot for the first-order rate constant of reducing compounds degradation in Biolasol® solution and in Biolasol® solution modified by adding cysteine over the temperature range of 50-80°C
Influence of antioxidants on the stability of Biolasol® solution and in Biolasol® solution modified by adding fumaric acid over the temperature range of 50-80°C

Figure 3. Arrhenius plot for the first-order rate constant of reducing compounds degradation in Biolasol® solution and in Biolasol® solution modified by adding fumaric acid.
tions by organs after transplantation, the results of this study are important of the perspective of possibility increase the success of transplantation surgery.

The study was conducted in the implementation of project co-financed by the European Regional Development Fund under the Operational Programme Innovative Economy No. UDA-POIG.01.04.00-24-002/11-00 – “Research on development of production technology of effective kidney transplantation solution” in years 2011-2014.

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Multicompartment dosage forms, compared to the traditional one-unit formulations, provide high surface area of drug release and short diffusion way, which in the consequence enables improvement of therapeutic efficacy and reduction of drug toxicity (1, 2). Microspheres are an example of multicompartment carriers with diameter 1-500 µm, where the active substance is incorporated in natural or synthetic polymer matrix. The properties of microspheres depend on the type of polymer and the method of their preparation (3-5). One of the advanced methods used in microparticles production is the spray drying — process, in which a solution, emulsion or suspension is sprayed in a stream of drying gas — compressed air or nitrogen (6, 7).

Capsules are solid dosage form, where drug and excipients are enclosed in hard or soft gelatin shell. Hard gelatin capsules are divided into two pieces — cap and body, which are most often obtained from gelatin, sugar and water. Gelatin possesses ability to form non-toxic gel, which is readily soluble in biological fluids and is characterized by strong flexibility (8, 9). Capsules filling might consist of pure active substance, active substance with excipients or multiple units dosage forms such as pellets, microspheres, microcapsules or microgranes. In comparison to tablets production, capsules filling avoids process of compression, which could destroy structure of multi units forms. Moreover, the number of excipients involved in capsules production is significantly lower (9-11).

Sodium alginate (ALG) is a natural, biocompatible and non-toxic heteropolysaccharide polymer with mucoadhesive and swelling properties. Mucoadhesive dosage forms with prolonged gastric residence time are particularly suitable for drugs, which are absorbed in the stomach, are unstable in the intestines or are poorly soluble in the high pH (12, 13). Our earlier studies have shown that ALG microspheres containing commonly used active substances — metronidazole (MT), ranitidine hydrochloride (RNT) and metformin hydrochloride (MF) were successfully prepared by the spray drying method and possessed mucoadhesive properties (14-16).

MT is slightly water soluble nitroimidazole chemotherapeutic drug, which activity includes strictly anaerobic bacteria and protozoa. ALG microspheres with MT after oral administration may
extend the residence time of the drug in the stomach and improve the effectiveness of *Helicobacter pylori* eradication. MT is also used in the treatment of common vaginal infections – bacterial vaginosis and trichomoniasis (17, 18). In the case of vaginal infections, ALG might prolong residence time of the microspheres in the application site and, in the consequence, increase therapeutic efficacy of MT. RNT is histamine receptor antagonist, mainly used in the treatment of gastro-esophageal reflux disease, gastric and duodenal ulceration and Zollinger-Ellison syndrome. However, in the lower segment of the gastrointestinal tract, RNT is poorly absorbed and may be degraded (19, 20). MF is an orally administered antidiabetic agent from biguanide group, which is the first line therapy in type 2 diabetes. MF is freely soluble in water and its bioavailability after oral administration in conventional dosage forms is below 50% (21-23). It is assumed that ALG microspheres with RNT and MF can increase drug bioavailability by prolonged contact of the dosage form with the stomach mucus membrane. It is necessary to notice that sustained release forms with MT and RNT are not available. In case of MF, film coated, polymer matrix or osmotic technology tablets providing extended release are commercially available. However, no multicompartment dosage forms with MF are registered.

The aim of this research was to prepare multicompartment hard gelatin capsules with ALG microspheres containing MT, RNT and MF in order to improve drug residence. In case of ALG microspheres with MF, to prolong drug release, crosslinking with chitosan was additionally performed. 0.1% chitosan solution in acetic acid pH 4.5 was added to 2% water solution of ALG and then obtained mixture was spray dried. Parameters of the process were set as follows: inlet temperature 150°C, aspirator flow 37 m³/h, feed flow 5 mL/min, spray flow 600 L/h.

### EXPERIMENTAL

#### Materials

Metronidazole (MT) was purchased from Amara (Kraków, Poland), ranitidine hydrochloride (RNT) was obtained from Zakłady Farmaceutyczne Polpharma S.A. (Starogard Gdański, Poland), metformin hydrochloride (MF) was obtained from Debao Fine Chemical CO (Henan, China). Sodium alginate (ALG) low viscosity (2%, 100-300 cP) and chitosan (medium molecular weight, viscosity of 1% solution in 1% acetic acid: 200 cP) were purchased from Sigma Aldrich (Steinheim, Germany). Potassium dihydrogen phosphate, sodium hydrogen carbonate, methanol, acetonitrile, sodium chloride, potassium hydroxide, calcium hydroxide, lactic acid, acetic acid, glycerol, urea and glucose were obtained from Chempur (Piekary Śląskie, Poland). Water was distilled and passed through a reverse osmosis system Milli-Q Reagent Water System (Billerica, MA, USA). Porcine stomach and vaginal mucosa from Large White Pigs weighing 200 kg were obtained from the veterinary service (Turośnio Kościelna, Poland). Samples were stored at -20°C and before the experiment were defrosted and cut into 5 mm in diameter and 2 mm thick pieces. Empty hard gelatin capsules were obtained from Eprus (Bielsko-Biała, Poland).

Microspheres with MT, RNT and MF were prepared using Mini Spray Dryer B-290 (Büchi, Flawil, Schweiz). Parameters of the spray drying process and characterization of obtained microspheres were described earlier (14-16). Microspheres formulations with the highest drug loading were selected for capsule preparation. In order to prolong MF release, microspheres crosslinking with chitosan was additionally performed. 0.1% chitosan solution in acetic acid pH 4.5 was added to 2% water solution of ALG and then obtained mixture was spray dried. Parameters of the process were set as follows: inlet temperature 150°C, aspirator flow 37 m³/h, feed flow 5 mL/min, spray flow 600 L/h.

#### Microspheres flow properties

**Angle of repose**

The angle of repose was examined with using manual powder flow tester (Electrolab EFT-01, Mumbai, India) according to European Pharmacopoeia (24) using 5 g of microspheres. The angle of repose (α) was calculated as follows:

\[ \tan \alpha = \frac{h}{r} \]

where, \( h \) – height of the cone formed, \( r \) – radius of receipt disc (24).

**Bulk and tapped density**

Bulk and tapped densities of 50 g microspheres were determined using Electrolab ETD-1020 Tap Density Tester (Electrolab, Mumbai, India) accord-
Evaluation of hard gelatin capsules with alginate microspheres...

Compressibility (Carr’s index and Hausner ratio)

Carr’s compressibility index (CI) and the Hausner ratio (HR) were determined according to the European Pharmacopoeia (24):

\[
CI = \left( \frac{\rho_{\text{tap}} - \rho_{\text{bulk}}}{\rho_{\text{tap}}} \right) \times 100
\]

\[
HR = \frac{\rho_{\text{tap}}}{\rho_{\text{bulk}}}
\]

where \( \rho_{\text{tap}} \) is the tap density and \( \rho_{\text{bulk}} \) the bulk density.

Moisture content

In order to determine the moisture content, 20 mg of microspheres were placed in the aluminium pan of moisture analyzer Radwag WPS 50SX (Warsaw, Poland), heated from 30°C to 120°C.

Capsules preparation

In each series, fifty hard gelatin capsules were filled by using manual capsule machine Capsunorm® (Eprus, Bielsko-Biała, Poland). Composition of prepared capsules is presented in Table 1.

Capsules evaluation

Weight variation test and drug content uniformity

Weight uniformity was evaluated by weighing accurately twenty randomly selected capsules and the mean mass of capsules were calculated (24).

To assess drug content uniformity, ten randomly chosen capsules were weighted accurately and microspheres were placed in 100 mL of water in flat-bottomed flasks. The flasks were shaken for 24 h in a shaking water bath at 25 ± 1°C with a rotation speed of 250 rpm to extract the total entrapped drug in microspheres. The solution was then filtered through 0.45 µm syringe nylon filters (Witko, Łódź, Poland). Samples (1 mL) were suitably diluted and analyzed by HPLC method.

HPLC analysis

The amount of MT, RNT and MF was determined by the HPLC system Agilent Technologies 1200 equipped with a G1312A binary pump, a G1316A thermostat, a G1379B degasser and a G1315B diode array detector (Agilent, Waldbronn, Germany). Isocratic separation of MT and RNT was achieved on a Zorbax Eclipse XDB-C18, 4.6 x 150 mm, 5 µm column (Agilent, Waldbronn, Germany). MF analysis was performed using Waters Spherisorb® 5.0 µM ODS 4.6 x 250 mm, 5 µm column (Waters Corporation, MA, USA). Mobile phase for MT was acetonitrile : 0.01 M phosphate buffer pH 4.7 (15 : 85, v/v), methanol : phosphate buffer pH 7.0 (1 : 3, v/v) for RNT and for MF – acetonitrile : methanol : phosphate buffer pH 3.0 (20 : 20 : 60, v/v). UV detection was performed at a wavelength of 319 nm (MT), 224 nm (RNT) and 240 nm (MF) (14-16). The flow rate was 1.0 mL/min and the column temperature was maintained at 25°C. For injection into the HPLC system, 20 µL of sample was used. All reagents used for analysis were HPLC grade. The retention time of MT and RNT was 3.0 min, and MF – 2.8 min. The standard calibration curves in all substances were linear over the range of 1 – 100 µg/mL with the coefficient of determination (\( R^2 \)) of 0.999.

In vitro drug release profile

The in vitro MT, RNT and MF release tests were conducted using apparatus type I (Erweka Dissolution tester type DT 600HI, Heusenstamm, Germany). Capsules were placed in the basket, immersed in 900 mL of 0.1 M HCl (pH 1.2) and stirred at 75 rpm (24). In case of capsules with MT, modified simulated vaginal fluid (SVF, pH 4.2) as dissolution medium was additionally used. Non modified commercial available tablets with studied substances were used as controls. In case of MF, extended release polymer matrix tablet as control was additionally used. Samples were withdrawn and filtered through 0.45 µm cellulose acetate Millipore filters (Billerica, MA, USA) at predetermined time intervals and replaced with fresh dissolution medium. During the dissolution process the temperature was maintained at 37 ± 1°C. The amount of released drugs was analyzed by HPLC method as described above.

Mathematical modeling of drug release profile

MT, RNT and MF release data were analyzed according to zero order kinetic, first order kinetic,

<table>
<thead>
<tr>
<th>Formulation</th>
<th>MT 1</th>
<th>MT 2</th>
<th>RNT 1</th>
<th>RNT 2</th>
<th>MF 1</th>
<th>MF CL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amount of drug (mg)</td>
<td>250</td>
<td>500</td>
<td>75</td>
<td>150</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>Weight of microspheres (mg)</td>
<td>402.1</td>
<td>804.3</td>
<td>105.8</td>
<td>211.6</td>
<td>661.4</td>
<td>653.6</td>
</tr>
<tr>
<td>Size of capsules</td>
<td>0</td>
<td>00</td>
<td>2</td>
<td>1</td>
<td>00</td>
<td>00</td>
</tr>
</tbody>
</table>
Higuchi model, Korsmeyer–Peppas equation and Hixson-Crowell cube root law. The constants of release kinetics and the regression coefficients ($R^2$) were calculated from the slope of plots by linear regression analysis. The constants of release kinetics and the regression coefficients ($R^2$) were calculated from the slope of plots by linear regression analysis.

**Zero order kinetics:**

$$F = k \times t,$$

**First order kinetics:**

$$\ln F = k \times t,$$

**Higuchi model:**

$$F = k \times \sqrt{t},$$

**Korsmeyer-Peppas model:**

$$F = k \times t^n,$$

**Hixson-Crowell model:**

$$1 - (1 - F)^{1/3} = k \times t,$$

where $F$ is the fraction of drug release, $k$ – the release constant and $t$ – the time. For the Korsmeyer–Peppas model, the fraction of drug remaining at time $t$ was determined for every time interval log ($M_t/M_0$) and plotted against the log of time $t$. The slope of the line was taken as the value of $n$ – diffusional release exponent used to interpret the mechanism of release (25, 26).

**Disintegration time test**

The disintegration test was carried out for six capsules from each formulation by using disintegration tester (Electrolab ED-2L, Mumbai, India). The study was conducted in 500 mL of water, 0.1 M HCl (pH 1.2) or SVF (pH 4.2) at 37 ± 0.5°C (24).

**Residence time test**

Residence time was evaluated by the *in vitro* adhesion test, known as “wash off” method. It was performed using self-constructed apparatus (by modifying USP disintegration tester), according to Nakamura et al. (27), where plexiglass cylinder (6 cm × 14 cm × 10 cm) was placed inside the graduated cylinder (6 cm × 14 cm × 10 cm) containing 10 mL of thermostatic water bath at 37°C. The porcine stomach or vaginal mucosa membrane was placed on the bottom of the plexiglass cylinder (6 cm × 10 cm). The disintegration time test was carried out for six capsules from each formulation by using disintegration tester (Electrolab ED-2L, Mumbai, India). The study was conducted in 500 mL of water, 0.1 M HCl (pH 1.2) or SVF (pH 4.2) at 37 ± 0.5°C (24).
cm diameter, weight 280 g) moving up and down was vertically fixed (Fig. 1). In case of microspheres and capsules intended for oral administration, segments of porcine stomach mucosa (2 cm long) were glued to the internal side of a beaker above the level of 500 mL 0.1 M HCl (pH 1.2) at 37 ± 0.5°C. To study residence time of microspheres and capsules with MT, segments of porcine vaginal mucosa (2 cm long) and 500 mL of SVF (pH 4.2) at 37 ± 0.5°C were additionally applied. After moisturizing the microspheres and capsules with 100 µL of HCl or SVF, a hydrated surface was put in contact with the mucosal membrane and immersed completely in the medium. Microspheres were used in the amount corresponding to their content in the capsules. Time required for entire detachment of the microspheres and capsules from the mucosa was noted (28).

Stability test

Organoleptic properties, weight, drug content uniformity and drug release from capsules were assessed directly after preparation and after 14, 30 and 180 days of storage at 25 ± 2°C, RH 60 ± 5% and 40 ± 2°C, RH 75 ± 5%.

Statistical analysis

Quantitative variables were expressed as the mean and standard deviation. A statistical analysis was performed using nonparametric techniques: the Kruskal–Wallis and Mann-Whitney U-test with the Statistica 10.0 software. Differences between groups were considered to be significant at p < 0.05.

RESULTS AND DISCUSSION

Determination of flow powder properties is crucial for tablet designing and capsule filling process. The powder flowability is directly related to the physical features of material and depends on particle size, shape, surface area and density. Cohesive forces (e.g., van der Waals and various electrostatic forces) possess the higher values when the degree of organization of molecules is greater (29, 30).

The angle of repose is a traditional method for powder flow properties evaluation and is related to internal friction and resistance movement among particles. When angle of repose is higher – the powder is more cohesive (24, 31, 32). The angle of repose of microspheres (α) is shown in Table 2. The flow of analyzed microspheres was rated as “passable which may hang up” for MT (42.25 ± 2.6°) and RNT microspheres (44.75 ± 1.7°). In case of MF microspheres, angle of repose was 38.56 ± 1.3°, which indicates “fair” flow and in chitosan crosslinked ALG microspheres with metformin hydrochloride (MF CL) it was “good flow” (33.62 ± 2.4°).

The bulk density is the ratio of untapped powder mass to its volume and depends on density of particles and their spatial arrangement. Tapped density is obtained after mechanically tapping of powder sample (24). Microspheres flow properties are presented in Table 2. Carr’s index (CI) and Hausner ratio (HR) values are directly related with bulk and tapped density. Hence, in case of good powder flow properties, particles interactions are lower, the bulk density and tapped density possess lower values, and CI and HR are also characterized by lower results (24, 31, 32). Obtained data suggest that MT and RNT microspheres were characterized by “very poor” flow properties, MF – by “poor” flow, and MF CL – by “passable” flow.

As hard gelatin capsules can absorb humidity and lose their shape, therefore moisture content is a key factor in capsules production process. Moisture content in analyzed microspheres was below 10% (Table 2), which should not affect capsules stability (33).

Hard gelatine capsules with ALG microspheres containing MT, RNT and MF were prepared by using manual capsule machine Capsunorm®. All capsules were characterized by low values of mean

<table>
<thead>
<tr>
<th>Drug loading (%)</th>
<th>MT</th>
<th>RNT</th>
<th>MF</th>
<th>MF CL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture content (%)</td>
<td>62.17 ± 1.89</td>
<td>70.9 ± 1.52</td>
<td>75.6 ± 1.54</td>
<td>76.51 ± 3.41</td>
</tr>
<tr>
<td>Angle of repose (°)</td>
<td>42.25 ± 2.61</td>
<td>44.75 ± 1.71</td>
<td>38.56 ± 1.32</td>
<td>33.62 ± 2.44</td>
</tr>
<tr>
<td>Bulk density (g/mL)</td>
<td>0.21 ± 0.02</td>
<td>0.22 ± 0.01</td>
<td>0.25 ± 0.02</td>
<td>0.24 ± 0.03</td>
</tr>
<tr>
<td>Tapped density (g/mL)</td>
<td>0.36 ± 0.05</td>
<td>0.38 ± 0.06</td>
<td>0.34 ± 0.07</td>
<td>0.32 ± 0.04</td>
</tr>
<tr>
<td>Carr's index (%)</td>
<td>41.62 ± 2.15</td>
<td>42.13 ± 1.65</td>
<td>26.54 ± 1.61</td>
<td>25.47 ± 2.41</td>
</tr>
<tr>
<td>Hausner's ratio</td>
<td>1.72 ± 0.42</td>
<td>1.73 ± 0.57</td>
<td>1.36 ± 0.32</td>
<td>1.33 ± 0.91</td>
</tr>
</tbody>
</table>
mass deviation and high uniformity of drug content (Table 3). However, in order to improve the flowability of microspheres, for preparation capsules by automatic capsule filling machine, using of lubricants seems to be necessary (34).

**In vitro drug release profile**

The *in vitro* release profiles of MT, RNT and MF from formulated capsules and commercially available tablets (used as controls) are shown in Figure 2. ALG ability to gel formation in acidic pH leads to closing of pores in the microspheres matrix and hinders the entrance of water. As a result, swelling and gelling of microspheres creates reservoir which prolongs drug dissolution. Differences in drug release profiles were associated with different drug solubility, and drug and ALG content in the microspheres. Therefore, the higher amount of microspheres was used, the drug release was more sustained. Entrapment of microspheres in capsule shell decreased surface access to medium, decreased the dissolution area and prolonged drug release in comparison to pure microspheres. In case of
MT, which is characterized by slightly solubility (24, 35), drug release was the most extended. After 8 h, about 70% and 90% of MT was released from MT 2 and MT 1 capsules, respectively (Fig. 2A, 2B). RNT and MF are characterized by freely solubility (24, 35) and after just 4 h about 80% of RNT and 90% of MF was released (Fig. 2C, 2D). Crosslinking of microspheres containing MF and electrostatic attraction between anionic carboxyl groups of alginate and cationic amino groups of chitosan provided sustained dissolution of drug. Release profile from capsules formulation MF CL was similar to the modified commercially available tablet – after 6 h of the study, about 80% of MF was released.

In order to investigate mechanism responsible for drug release from prepared capsules, obtained dissolution data were fitted to zero order, first order equations, Higuchi, Korsmeyer-Peppas and Hixson-Crowell models (Table 4). It was found that in all capsules formulations (except MT 2) the plots showed the highest regression correlation coefficient in the Higuchi model. The best fit curve in this model with \( R^2 (0.994) \) was observed for capsules formulation MF CL. Drug release from capsules MT 2 was according to the zero order kinetics, where the release rate is independent of drug concentration. The obtained data from Korsmeyer-Peppas equation (\( n \) value was ranged from 0.14 to 0.33) indicate that drug release from capsules (except MT 2) was controlled by Fickian diffusion mechanism. The high values of \( R^2 \) obtained in the Hixson-Crowell model indicate that this equation can also describe the release of drugs and suggest that drug release rate is limited by the capsules erosion and diffusion process (36-38).

Disintegration time test revealed that disintegration of capsules was related with their weight, solubility of drugs and type of medium used. Disintegration time evaluated in water was ranged from 38.6 ± 3.5 min (RNT 1) to 57.3 ± 4.2 min (MT 2) (Table 3). Because of gel formation, all prepared capsules did not disintegrate in acidic pH (0.1 M HCl or SVF pH 4.2) for 48 h of the study.

### Table 3. Characteristics of capsules with ALG microspheres containing metronidazole (MT 1, MT 2), ranitidine hydrochloride (RNT 1, RNT 2), metformin hydrochloride (MF 1) and chitosan crosslinked ALG microspheres with metformin hydrochloride (MF CL).

<table>
<thead>
<tr>
<th>Capsules formulation</th>
<th>Weight (mg)</th>
<th>Drug content (mg)</th>
<th>Disintegration time (min)</th>
<th>Residence time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>H₂O</td>
<td>0.1 M HCl</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>**</td>
</tr>
<tr>
<td>MT 1</td>
<td>405.1 ± 6.3</td>
<td>247.7 ± 16.7</td>
<td>44.5 ± 2.6</td>
<td>-</td>
</tr>
<tr>
<td>MT 2</td>
<td>809.2 ± 5.8</td>
<td>493.5 ± 18.0</td>
<td>57.3 ± 4.2</td>
<td>-</td>
</tr>
<tr>
<td>RNT 1</td>
<td>115.7 ± 8.4</td>
<td>75.7 ± 5.4</td>
<td>38.6 ± 3.5</td>
<td>-</td>
</tr>
<tr>
<td>RNT 2</td>
<td>221.5 ± 7.3</td>
<td>153.1 ± 8.7</td>
<td>39.3 ± 2.4</td>
<td>-</td>
</tr>
<tr>
<td>MF 1</td>
<td>668.2 ± 5.6</td>
<td>513.2 ± 16.4</td>
<td>41.4 ± 3.8</td>
<td>-</td>
</tr>
<tr>
<td>MF CL</td>
<td>641.7 ± 4.3</td>
<td>493.5 ± 7.3</td>
<td>52.2 ± 4.1</td>
<td>-</td>
</tr>
</tbody>
</table>

*capsules did not disintegrate over 48 h of experiment; ** test performed in SVF (pH 4.2).

### Table 4. Mathematical modeling of drug release from capsules with ALG microspheres containing metronidazole (MT 1, MT 2), ranitidine hydrochloride (RNT 1, RNT 2), metformin hydrochloride (MF 1) and chitosan crosslinked ALG microspheres with metformin hydrochloride (MF CL).

<table>
<thead>
<tr>
<th>Capsules formulation</th>
<th>Zero order kinetics</th>
<th>First order kinetics</th>
<th>Higuchi model</th>
<th>Korsmeyer-Peppas model</th>
<th>Hixson-Crowell model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( R^1 )</td>
<td>( K_0 )</td>
<td>( R^2 )</td>
<td>( K_1 )</td>
<td>( R^3 )</td>
</tr>
<tr>
<td>MT 1</td>
<td>0.864</td>
<td>9.12</td>
<td>0.706</td>
<td>0.17</td>
<td>0.986</td>
</tr>
<tr>
<td>MT 2</td>
<td>0.996</td>
<td>9.32</td>
<td>0.818</td>
<td>0.25</td>
<td>0.962</td>
</tr>
<tr>
<td>RNT 1</td>
<td>0.831</td>
<td>5.9</td>
<td>0.743</td>
<td>0.76</td>
<td>0.935</td>
</tr>
<tr>
<td>RNT 2</td>
<td>0.927</td>
<td>8.77</td>
<td>0.865</td>
<td>0.15</td>
<td>0.958</td>
</tr>
<tr>
<td>MF 1</td>
<td>0.767</td>
<td>6.72</td>
<td>0.652</td>
<td>0.11</td>
<td>0.891</td>
</tr>
<tr>
<td>MF CL</td>
<td>0.950</td>
<td>9.55</td>
<td>0.855</td>
<td>0.07</td>
<td>0.994</td>
</tr>
</tbody>
</table>
In order to assess capsules residence time, “wash off” test was performed using a self-constructed apparatus, according to Nakamura et al. (27). ALG is characterized by high bioadhesive properties, which mechanism is defined by interaction between carboxyl groups of polymer and mucin through electrostatic adsorption, van der Waals and hydrogen bonds. Carboxyl groups of ALG penetrate into the mucus network and finally the formation of bonds between the mucus and the polymer occurs. Porcine stomach and porcine vaginal mucosa were used as adhesive models due to their similar anatomy and physiology to the human tissue (39). All examined microspheres and capsules adhered to the mucosal surface. However, the residence time of free microspheres was significantly higher (>24 h) than when they were encapsulated (Table 3). This might be due to the higher contact area of microspheres with the mucosa membrane.

Stability evaluation plays significant role during development of pharmaceutical delivery systems. Designed capsules were examined directly after preparation and after 14, 30 and 180 days of storage at 25 ± 2°C, RH 60 ± 5% and 40 ± 2°C, RH 75 ± 5%. No significant changes in physical appearance, drug and mass content uniformity, and drug release profiles from capsules with microspheres containing MT and MF were observed. However, after one month storage of RNT 1 and RNT 2 capsules in 40 ± 2°C, RH 75 ± 5%, brown decomposition product of RNT (40, 41), which absorbed moisture and dissolved gelatin capsule shell was observed.

CONCLUSIONS

The results of this study suggest that designed capsules with ALG microspheres could be considered as multicompartment carriers for slightly soluble MT. Capsules with MT were characterized by sustained drug release (according to zero order kinetics in MT 2 formulation). Capsules containing microspheres with freely soluble RNT and MF did not sufficiently prolong the release time of drugs. Moreover, capsules with RNT did not successfully pass the accelerated stability test. Chitosan crosslinking of ALG microspheres containing MF provided sustained dissolution of drug and release profile from capsules formulation MF CL was similar to the modified commercially available tablet – after 6 h of the study, about 80% of MF was released.

Acknowledgments

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APPLICATION OF SUPERCRITICAL CARBON DIOXIDE TO ENHANCE DISSOLUTION RATE OF BICALUTAMIDE

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Abstract: Bicalutamide solid dispersions were prepared by supercritical fluid method using carbon dioxide as a solvent. Approximately 8-fold dissolution improvement was noticed for system prepared with supercritical carbon dioxide containing Poloxamer 407 as a carrier, when compared to physical mixture. The system was characterized using scanning electron microscopy, X-ray powder diffraction and differential scanning calorimetry. We found that applying the supercritical fluid treatment led to significant decrease in bicalutamide crystals size and their partial amorphization. The studies confirmed that use of supercritical fluid technology might be efficient for dissolution rate enhancement of poorly water soluble drugs.

Keywords: bicalutamide, supercritical carbon dioxide, solid dispersion, dissolution, Poloxamer 407

Poorly water soluble active pharmaceutical ingredients (APIs) may undergo incomplete dissolution in gastrointestinal fluids and thus be only partially absorbed into systemic circulation. As it is reported, ca. 40% of commercialized APIs and almost 70% of potential new drugs are poorly water soluble (1). Formation of solid dispersions that leads to dispersion of insoluble APIs among well soluble polymeric matrix is potential approach to enhance their bioavailability. The processing methods like fast cooling from melting, spray drying or micronization, may result in crystalline drugs amor-phization. However, solids are often characterized by combined properties, i.e. partially crystalline, containing stable and metastable forms and partially amorphous. The result of applied process can be very complexed and tough to control (2).

Bicalutamide (BCL) is a non-steroidal antiandrogen used in treatment of prostate cancer (3). It is administered orally as 50 mg tablets. Since the drug is practically insoluble in water (4 µg/mL at 37°C) (4) and has high membrane permeability (LogP 2.92) (5), it is assigned to class II according to Biopharmaceutical Classification System (6). Its pKa = 12 is higher than the upper limit of intestinal fluids pH, therefore BCL solubility does not seem to be facilitated in any region of gastrointestinal tract (5). The BCL crystals exhibit polymorphism - forms I and II have already been identified (7-9). While form I referred as monoclinic is more stable than triclinic form II, the form II is 2.4-times more soluble than form I. Nevertheless, both polymorphs are very poorly soluble in aqueous media (10).

BCL dissolution rate can be improved by development of API-cyclodextrins complexes or formulation of solid dispersions. Complexation of BCL with β-cyclodextrin in 1 : 1, 1 : 2 and 1 : 5 w/w ratios was carried out by three techniques, i.e., solvent evaporation, spray-drying and kneading. The most significant improvement of drug dissolution rate was observed for 1 : 5 inclusion complex prepared by kneading (11).

BCL solid dispersions were formulated by hot melt extrusion, solvent evaporation or melting methods. Gavin et al. described the manufacture of BCL and polyvinylpyrrolidone K25 (PVP) systems in 1 :...
10, 2:10 and 3:10 w/w ratios prepared by hot melt extrusion. The drug dissolution enhancement was attributed to BCL amorphization and the extrudates’ wettability improvement caused by PVP hydrophilic matrix (12).

The solvent evaporation method was applied for solid dispersions preparation containing BCL and PVP or Poloxamer® 407 and F68 (PLX) (13-15). The drug-carrier ratio was the most important factor affecting the drug dissolution rate. In particular, the highest dissolution rate was observed for solid dispersion prepared in 1:5 w/w ratio, for both carriers i.e., PVP K30 and PLX 407. This phenomenon may be caused by transformation of crystalline BCL into amorphous form. The other study demonstrated that improvement of BCL dissolution rate was the most effective for the 1:1 BCL–Poloxamer® F68 solid dispersion prepared by melting method (15). The effect was attributed to partial amorphization of the API. Surprisingly, increase in Poloxamer® F68 quantity resulted in prolongation of BCL dissolution that was caused by the gelling of PLX.

Improvement in BCL dissolution rate was also achieved by solid dispersion prepared with Aeroperl® 300, a highly porous grade of spherically shaped fumed silica and hydrophilic Macrogol.
The API and Macrogol 400 were dissolved in an organic solvent - acetone, and then mixed with silica. After the solvent was evaporated, particles of amorphous BCL surrounded by Macrogol 400 were uniformly adsorbed within the silica pores. As a result, the dissolution rate of BCL was improved 15-fold in comparison to unprocessed active substance. The effect was attributed to amorphization of the API and the phase stabilization by the drug incorporated into AeroperlÆ 300 pores, as well as by improvement of the particles wetting by the presence of Macrogol 400 (16).

In recent years, the supercritical fluid (SCF) technique was applied to solid dispersion formation. This method has become popular in the pharmaceutical sciences and recognizable by industry. Numerous applications, e.g., extraction of thermally sensitive substances, drug particles’ size reduction, mixing and complexation (17-19) may be much easier with SCF technology. Carbon dioxide (CO2), is the most popular medium, since it is non-toxic, non-flammable, inexpensive, easy to remove and possesses low critical temperature and pressure (31.1°C and 73.8 bar). The molecule has linear shape (O-C-O), so its dipolar momentum is zero and the quadrupolar momentum is very small. Hence, the nonpolar (lipophilic) substances can be easily extracted from polar (hydrophilic) matrices simply by dissolution in supercritical CO2 at relatively low temperature. Such properties enable supercritical carbon dioxide (scCO2) to replace organic solvents as well as prevent drug degradation (20, 21). Up to now the supercritical fluid technique was successfully applied for dissolution enhancement of carbamazepine (22), nifedipine (23) and furosemide (24).

The present study was aimed to improve solubility and dissolution rate of bicalutamide by preparation of solid dispersion using SCF and melting method. PoloxamerÆ 407, a low-meltable polymeric surfactant was chosen as a carrier. The effect of preparation method on drug dissolution was investigated. The solid dispersions and physical mixture containing BCL and PLX in 1 : 1 w/w ratio were characterized by scanning electron microscopy.
EXPERIMENTAL

Materials
Bicalutamide (BCL, Pharmaceutical Research Institute, Poland), Poloxamer® 407 (PLX, BASF, Germany), carbon dioxide in form of dry ice (Linde, Poland). All materials were of analytical grade.

Procedures
Two methods, i.e., supercritical carbon dioxide method or melting method were used for preparation of bicalutamide and Poloxamer® 407 solid dispersions while the physical mixture was prepared by mixing with carrier in a mortar. Samples were prepared in 1:1 (w/w) drug to carrier ratio.

Supercritical carbon dioxide method
The materials, BCL and PLX were loaded into high pressure reactor BR-300 (Berghof Products + Instruments GmbH, Germany) equipped with magnetic stirrer MR Hei-Standard (Heidolph Instruments, Germany) and thermometer CHY 700T (CHY Firemate Co., Taiwan). The calculated quantity of CO₂ in form of dry ice was placed in the reactor to achieve supercritical pressure of 130 bar at 60°C. Temperature, pressure, and mixing speed were monitored during the process.

Melting method
Polymer was melted in a water bath and BCL was poured while stirring. Dispersion of BCL in melted polymer was cooled down to the ambient temperature. After 24 h of storage in desiccator the solidified mass was crushed, pulverized and sieved through the 500 µm sieve.

Characterization of solid dispersion

Morphological analysis by scanning electron microscopy
Particles were examined using scanning electron microscope S-4700 (Hitachi Inc., Japan). The powder was adhered to a sample holder by a double-sided carbon tape and coated with gold using the 208 HR sputter coater (Cressington Scientific, USA). The images were taken at the magnification of 50× and 400×.

X-ray powder diffraction analysis
The diffraction pattern was recorded in terms of 2θ at a range of 2θ - 60° using PW 1830 diffractometer (Philips, The Netherlands) equipped with nickel filtered Cu-Kα radiation (λ = 1.5406 Å). Diffraction patterns used later for Rietveld analysis were recorded with the rate of 0.02°/10 s. Analyses were performed in MAUD (25) environment, FITYK (26) was used as auxiliary software for data preparation.

Differential scanning calorimetry analysis
The measurements were performed using DSC7020 calorimeter (Hitachi Inc., Japan) equipped with dedicated electric cooling unit. The apparatus was calibrated using indium and tin standards. The analyses were performed in nitrogen atmosphere with a flow rate of 50 mL/min. The 6.8 - 8.1 mg of samples were placed in aluminum pans.

Figure 2. Experimental XRPD pattern of bicalutamide sample, simulated patterns of polymorph I and polymorph II. Structural data for polymorphs I and II were taken from 2014 CCDC database.
and sealed. The following thermal protocol was used: equilibrating at 25°C for 15 min, then heating to 220°C, except PLX, which was heated to 200°C, at heating rate of 10°C/min. With the exception of PLX all determined $\Delta H$ values of samples within the study were normalized to the weight of BCL.

**Solubility studies**

An excess of drug or binary system was dispersed in 25 mL of distilled water in a conical flask. The suspension was shaken at room temperature using the KS 130 Basic shaker (IKA, Germany) for at least 24 h to achieve the equilibrium solubility. Samples were centrifuged at 3600 rpm for 30 min in the MPW 221 apparatus (MPW, Poland) and filtered through a 0.2 µm membrane filter. The diluted samples were assayed at 270 nm with UV-VIS spectrophotometer V-500 (Jasco Analytical Instruments, USA). The reported data represents the averages from three series of measurements.

**Dissolution rate studies**

Dissolution studies were carried out using the method recommended by the FDA for BCL tablets, i.e. USP type II apparatus (paddle) at 50 rpm, 1000 mL of water containing 1% SLS at 37 ± 0.5°C. The analysis was performed in the SR8 Plus type II Dissolution Test Station (Hanson Research, USA). A certain amount of powder samples, i.e., solid dispersions, physical mixtures or pure drug, equivalent to 50 mg of active substance, were poured into the beakers. 5 mL of solution were withdrawn from each dissolution vessel at predefined intervals, filtered, diluted and assayed spectrophotometrically at $\lambda = 272$ nm. After collection of the each sample the dissolution medium was replaced with the same volume of dissolution medium. The tests were carried out in triplicate. The dissolution data were analyzed by Open Source software KinetDS 3.0 with standard settings including non-linear regression methods. Different models i.e., zero order, first order, second
order, third order, Korsmeyer-Peppas model, $y = a \cdot \ln(x) + b$, model, Weibull models, Hixson-Crowell, Higuchi, Baker-Lonsdale, Michaelis-Menten, Hill, were evaluated to found the best fit to obtained data [27].

RESULTS AND DISCUSSION

Effects of preparation methods on samples physical state

The both methods i.e., supercritical carbon dioxide (SCF) and melting (Melt) were suitable for preparation of BCL solid dispersions, however they impacted the samples properties. Referring to table 1, solid dispersion prepared by SCF was in form of freely flowing powder, while prepared by melting method was a little waxy but easy to pulverize.

The SEM micrographs of raw materials and solid dispersions are compared in Figure 1. Bicalutamide (Fig. 1a) appears as aggregates composed of plate-like crystallites of different size, from 15 µm up to 200 µm in length. The individual plates exhibit shape of elongated hexagons. Poloxamer® 407 (Fig. 1b) is in form of smooth particles of droplet-like elliptical shape. The solid dispersion obtained by melting (Fig. 1c) demonstrates aggregates with sharp edges covered by carrier, however a fraction of uncovered crystals is also noticeable. Reduction of particle size was observed. The solid dispersion prepared by SCF method (Fig. 1d) shows random aggregates of small block-like objects with edges rounded as if they were covered with polymer layer. It is worth to emphasize that significant fraction of the grains has smaller size than the drug substance presented in Figure 1a. In our opinion during SCF process PLX covered the drug crystals entirely and after cooling and depressurization a partial recrystallization of BCL occured.

In the crystallographic databases two polymorphs of BCL are listed. The first reference structure corresponds to plate-like crystals [7] while the second to the prism-like ones [3]. The micrograph in Figure 1a presents plate-like crystals of the non-treated BCL, which suggests that structure of polymorph I is dominant. The finding is fully consistent with the set of diffraction patterns presented in Figure 2.

The XRPD patterns of pure BCL, PLX, solid dispersions prepared by both methods and corresponding physical mixture are shown in Figure 3. Broad peaks in PLX diffractogram are typical for polymeric substances, in which structural elements show only short-range ordering. These peaks were mathematically removed from diffraction patterns of binary systems with right proportion to their weight fraction before further interpretation of powder diffractograms. The curve obtained for physical mixture indicate unchanged diffraction pattern of BCL. This is confirmed by analysis of diffractogram appearing after removal of PLX results from the curve of physical mixture. The diffraction pattern of

![Figure 5. Dissolution rate profiles tested for non-processed bicalutamide (BCL), solid dispersions (BCL/PLX) prepared by melting (Melt), the supercritical carbon dioxide method (SCF) or by physical mixing (PhM). Method: Ph. Eur. ap. 2, 50 rpm, 1000 mL of 1% SLS solution in water](image-url)
the SCF treated sample becomes more complex. It reveals that BCL peaks are broader than observed in the unprocessed substance. The Scherrer formula was used to determine mean diameter of monocrystal zones:

\[
D = \frac{0.89 \lambda}{B \cos \theta}
\]

where: \(D\) stands for the mean diameter of monocrystal, \(\lambda\) for wavelength of X-ray used, \(B\) is half-width of a peak and \(\theta\) stands for the diffraction angle.

SCF process leads to decrease in monocrystalline BCL domains size from 230 ± 7 nm before, to 170 ± 5 nm after treatment. The comparison of the XRPD data with SEM micrographs points to the fact that almost all individual grains of BCL and BCL/PLX SCF are much larger than monocrystals, and thus they are considered as polycrystalline aggregates. At least two mechanisms may be responsible for lowering of BCL crystal size. The first is dissolution and subsequent quick recrystallization of the drug during treatment by SCF. It is supported by Figure 1d which shows the aggregates composed of tiny crystals, which are all covered with PLX and stack together. However, the smallest visible crystals are about 20-times larger than the mean monocrystallite size. The recrystallization was so quick that it stopped shortly after nucleation of BCL crystals. It is worth noting that PLX prevented formation of larger BCL crystals. Strong interactions between BCL and PLX are also suggested by the fact that BCL recrystallizes in the shape of block-like grains (cf. Fig 1d).

The DCS curves of the pure drug, the physical mixture and the solid dispersions are shown in Figure 4. The DSC curve of crystalline BCL exhibited a single endothermic response corresponding to melting of the drug. BCL reveals significant changes of the melting temperature in the presence of PLX. Drug and carrier interact strongly even when physically mixed. In this case (cf. Table 2) the onset temperature goes down by 12°C (from 194.2 to 181.9°C). Sample treated by SCF is characterized by onset temperature as low as 159.9°C, which is ca. 34°C lower than the onset temperature of pure bicalutamide. The DSC curve of BCL/PLX Melt is similar to SCF curve.

The melting energies \(\Delta H\) recalculated for the pure drug and obtained for the above mentioned physical mixture and solid dispersion are 150.0 mJ/mg and 61.0 mJ/mg, respectively. Such a considerable difference (89.0 mJ/mg) and a wide temperature shift indicate destabilization of the crystal structure of BCL and its partial amorphization. It means that morphology of both PLX and BCL has been changed, which has previously been illustrated in SEM images. The above results indicate that the treated sample contains BCL as amorphous, small, recrystallized grains and some unchanged, crystalline large grains.

### Improvement of bicalutamide dissolution rate

The determined solubility of bicalutamide in water at 25°C was low, i.e., 3.7 µg/mL. Poloxamer® 407 as nonionic surfactant with HLB value 18-23 being usually used as a solubilizer for many active ingredients should improve the solubility of BCL. However, in case of physical mixture, solubilizing effect of PLX on BCL was only slightly pronounced. The solubility of BCL from solid dispersion prepared by SCF method was greater than that of pure drug by a factor of 2.8.

The results of dissolution studies were in agreement with solubility data (Fig. 5). Importantly, in case of solid dispersions the use of PLX led to improvement of dissolution rate; the highest amount of BCL was dissolved from solid dispersion prepared by SCF method. After 1 h of testing, the amount of dissolved BCL was 1.7-times greater than from samples prepared by melting method and 8-times greater than from pure drug. At the same time only 30% of drug dissolved from physical mixture. The solid dispersion prepared by SCF method exhibited also higher burst release - 70.9% of drug after 15 min, twice greater than in the case of solid dispersion prepared by the melting method. Among all evaluated kinetic models the best fitting were found for Korsmeyer-Peppas model. Using it, the dissolution rate constants were determined. In Table 3 kinetic data obtained for solid dispersions systems, physical mixture as well as pure drug are presented. The s data confirm that the SCF method allowed to enhance the dissolution of BCL in more effective way that melting method.

The differences among dissolution profiles of BCL solid dispersion were mostly resulted from wetting effect of hydrophobic drug crystals surrounded by carrier’s hydrophilic particles. The other reasons of dissolution profiles enhancement was reduction of particles size by SCF method and partial amorphization of BCL crystals.

### CONCLUSIONS

Two methods: supercritical fluid method and melting method were used for preparation of solid dispersions containing bicalutamide and Poloxamer® 407. The formulations were investigated using scanning electron microscopy, differential
scanning calorimetry as well as X-ray powder diffractron analyses. The SCF as solvent-free method proved to remarkable increase in the bicalutamide solubility and dissolution characteristics. The effects are attributed to the reduction of the size of drug crystals and partial amorphization what was confirmed by SEM, DSC and X-ray studies.

Application of melting method for preparation of solid dispersion was not satisfactory for BCL solubility and dissolution rate enhancement.

Acknowledgments

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REFERENCES


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Pulsatile Drug Delivery System (PDDS) is an upcoming technique to combat patient’s non-compliance, achieve optimum drug target actions and it leads to availability of the right amount of drug on right site at right time using right dosage (1). These systems release their active moiety within a short period of time to produce its therapeutic action immediately after predetermined off release period (2). The pulsatile effect in this system is to release the active drug in a pulsation form after lag time in such a manner that rapid drug release pattern should follow lag time (3, 4). Disparate studies enlighten the use of PDDS in xenobiotic having chronopharmacological behavior (circadian rhythm), drug undergoing hepatic first pass metabolism, several ailments like asthma, allergic rhinitis, cardiovascular diseases, attention deficits in children, diabetes, gastric ulcers, cancer, neurodegenerative disease, infectious disease and hypercholestremia (5-7). An underlying pathophysiology behind above disorders and their treatment strategies necessitates the development of Pulsatile Delivery System (8-10).

Circadian rhythms are produced by natural factors within the body, but they are also affected by signals from the environment (11). Biological cycle is the main cue influencing circadian rhythms, turning on or turning off genes that control an organism’s internal clocks (12). Many branded and generic products of amlodipine formulations are available in the market. Less side effects of amlodipine may include headache, swelling (edema) of the lower extremities, dizziness, drowsiness, tired feeling, stomach pain, or flushing. The usual initial antihypertensive oral dose of amlodipine is 5 mg once daily, and the maximum dose is 10 mg (13-15).

The main aim of this research work to develop and evaluate the microsphere of amlodipine to provide a drug release at a specific time and provide the medicament at particular time when the patient required the medicine which leads to the maximum efficacy and improved efficiency of the drug with minimum side effect.

EXPERIMENTAL

Amlodipine was obtained as gift sample from Shasun Chemicals & Drugs, India, Eudragit S100, polyvinyl alcohol, PVA, (MW ~ 70 KDa, 88%
hydrolyzed, Sigma, Germany), ethanol and dichloromethane (Merck, Germany) were used. The other additives and solvents were of analytical grade.

Preparation of the amlodipine loaded microspheres

Different amount of Eudragit S100 were added into a chloroform solution containing 10 mg of the drug. The aqueous phase was prepared by dispersing 0.2% PVA (polyvinyl alcohol) in water. The drug polymer solution was added to the aqueous phase with constant mixing. Microsphere formulations F1-F3 were prepared with RPM 500 with various concentration of Eudragit S100 (Table 1). Formulation F4-F7 were prepared at different RPM such as 750, 1000, 1250 and 1500 using 500 mg of Eudragit S100 in each formulation (Table 1). The mixture was stirred with a propeller at different rpm for 3 h at 25°C till the complete removal of solvent. The mixture was filtered and the microspheres collected, which were then washed with deionized water. These microspheres were dried at room temperature for 24 h.

Evaluation parameters
Differential scanning calorimetry

The thermal characteristics of pure drug were determined using a differential scanning calorimeter (DSC60, Shimadzu, Japan). After calibration with indium and lead standards, samples of the crystals (3–5 mg) were heated (range 25–300°C) at 10°C/min in hermetically sealed aluminium pans. DSC is used to measure the heat flow into and out of a sample.

Scanning electron microscopy

The shape and surface morphology of optimized amlodipine microsphere formulation F5 were investigated using scanning electron microscopy (SEM). The sample of SEM study was prepared by lightly sprinkling the formulation on a double-adhesive tape stuck to an aluminium stub. The stubs were then coated with gold to a thickness of ~300 Å under an argon atmosphere using a gold sputter module in a high-vacuum evaporator. The coated samples were then randomly scanned and photomicrographs were taken with a scanning electron microscope (Jeol, JSM-6510, Tokyo, Japan).

Table 1. Composition of different formulations.

<table>
<thead>
<tr>
<th>Serial no.</th>
<th>Formulation code</th>
<th>Drug (mg)</th>
<th>Eudragit S100 (mg)</th>
<th>0.2 % PVA (mL)</th>
<th>Chloroform (mL)</th>
<th>RPM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F1</td>
<td>10</td>
<td>250</td>
<td>100</td>
<td>10</td>
<td>500</td>
</tr>
<tr>
<td>2</td>
<td>F2</td>
<td>10</td>
<td>500</td>
<td>100</td>
<td>10</td>
<td>500</td>
</tr>
<tr>
<td>3</td>
<td>F3</td>
<td>10</td>
<td>750</td>
<td>100</td>
<td>10</td>
<td>500</td>
</tr>
<tr>
<td>4</td>
<td>F4</td>
<td>10</td>
<td>500</td>
<td>100</td>
<td>10</td>
<td>750</td>
</tr>
<tr>
<td>5</td>
<td>F5</td>
<td>10</td>
<td>500</td>
<td>100</td>
<td>10</td>
<td>1000</td>
</tr>
<tr>
<td>6</td>
<td>F6</td>
<td>10</td>
<td>500</td>
<td>100</td>
<td>10</td>
<td>1250</td>
</tr>
<tr>
<td>7</td>
<td>F7</td>
<td>10</td>
<td>500</td>
<td>100</td>
<td>10</td>
<td>1500</td>
</tr>
</tbody>
</table>

Table 2. Characteristics of different amlodipine microsphere formulations.

<table>
<thead>
<tr>
<th>Serial no.</th>
<th>Formulation code</th>
<th>Particle size (µm)</th>
<th>Percentage yield</th>
<th>Entrapment efficiency (%)</th>
<th>Drug loading capacity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F1</td>
<td>289.94 ± 0.893</td>
<td>61.531 ± 0.052</td>
<td>84.020 ± 0.125</td>
<td>3.230 ± 0.023</td>
</tr>
<tr>
<td>2</td>
<td>F2</td>
<td>262.90 ± 1.693</td>
<td>88.235 ± 0.042</td>
<td>94.860 ± 0.103</td>
<td>1.692 ± 0.012</td>
</tr>
<tr>
<td>3</td>
<td>F3</td>
<td>345.89 ± 1.409</td>
<td>81.578 ± 0.045</td>
<td>85.755 ± 0.122</td>
<td>1.128 ± 0.013</td>
</tr>
<tr>
<td>4</td>
<td>F4</td>
<td>368.90 ± 1.892</td>
<td>35.714 ± 0.033</td>
<td>94.860 ± 0.154</td>
<td>1.692 ± 0.012</td>
</tr>
<tr>
<td>5</td>
<td>F5</td>
<td>289.90 ± 0.029</td>
<td>74.946 ± 0.029</td>
<td>97.734 ± 0.142</td>
<td>1.743 ± 0.015</td>
</tr>
<tr>
<td>6</td>
<td>F6</td>
<td>389.45 ± 1.346</td>
<td>63.267 ± 0.048</td>
<td>94.860 ± 0.132</td>
<td>1.692 ± 0.011</td>
</tr>
<tr>
<td>7</td>
<td>F7</td>
<td>409.60 ± 0.235</td>
<td>87.589 ± 0.053</td>
<td>86.236 ± 0.221</td>
<td>1.538 ± 0.014</td>
</tr>
</tbody>
</table>
Measurement of micromeritics properties

The microspheres were indicated for their micrometric characteristics such as particle size (by optical microscopy), bulk density, tapped density, Carr’s index, Hauser ratio and angle of repose as noted in various previous studies (16-18).

Determination of production yield

Microspheres dried at 50°C were then weighed and the percentage yield of amlodipine loaded microsphere preparation was calculated by dividing the weight of the prepared microspheres by total amount of drug and excipients used (Table 2).

\[
\text{Percentage yield} = \frac{\text{Practical amount (microspheres)}}{\text{Theoretical amount (polymer + drug)}} \times 100 \tag{1}
\]

Percentage yield was estimated to confirm that there is expected amount of drug present in the product. The formulation with the maximum percentage yield was selected.

Percentage encapsulation efficiency

Different formulations of amlodipine loaded microsphere (25 mg) were dissolved in 10 mL methanol and the resulting solution was filtered through 0.45 µm membrane filter paper (MDI, Ambala). The filtrate was diluted and analyzed for drug content using ultraviolet spectrophotometer (Shimadzu 1800, Kyoto, Japan) at 239 nm. The entrapment efficiency was calculated using the following equation:

\[
\text{Entrapment efficiency} = \frac{\text{Actual loading}}{\text{Theoretical loading}} \times 100 \tag{2}
\]

Drug loading

This was determined following the method of Semalty. Microspheres (25 mg) were dissolved in 10 mL methanol and the resulting solution was filtered through 0.45 µm membrane filter paper. The filtrate was diluted and analyzed for drug content at 239 nm. Drug loading capacity was calculated using equations 3:

\[
\text{Loading capacity} = \frac{\text{Drug loaded in microsphere}}{\text{Total weight of microsphere}} \times 100 \tag{3}
\]

FTIR studies

The interaction study between the drug and polymer was observed using FT-IR spectrophotometer in the Perkin-Elmer FTIR. Different samples of pure drug (amlodipine), pure polymers (Eudragit S100), mixture of amlodipine + Eudragit S100 and selected final formulations microsphere F5 were scanned.

X-ray diffraction:

Crystalline nature of the amlodipine, Eudragit S-100, mixture of amlodipine and Eudragit S100 and prepared microspheres formulation F5 were evaluated by powder XRD technique using Philips model “X” Pert, diffractometer attached to the digital graphical assembly and a computer with Cu target X-ray tube-4 as the Cu α-radiation source in the range of -3° to 136° of 2θ.

In vitro release studies

In vitro dissolution profiles of all formulations were performed employing USP 36-NF31 dissolution apparatus. Microspheres equivalent to 2.5 mg amlodipine of all formulations F1 to F7 were placed into the dissolution apparatus containing 900 mL of acid buffer of pH 1.2 for first 2 h and 900 mL of phosphate buffer of pH 6.8 was used for the next 10 h and speed at 100 rpm and temperature 37 ± 0.5°C were maintained. Five millilitres of the sample was withdrawn from the dissolution media at particular time intervals and the same amount was replaced with fresh buffer. Samples were filtered through membrane filter 0.45 µm (Millipore). The absorbance of the filtrate was determined spectrophotometrically at 239 nm. Cumulative percent of drug release of the formulation were reported and all data were compared with the marketed formulation (Amlopress 2.5, Cipla).

Kinetic analysis of drug release

Model dependent methods are based on different mathematical functions, which describe the release profile. Once a suitable function has been selected, the release profiles are evaluated depending on the derived model parameters. The results obtained from in vitro release studies were plotted in different model of data treatment as follows:

Zero order kinetics

The zero order rates describe the systems where the drug release rate is independent of its concentration. A zero-order release would be predicted by the following equation;

\[
A_t = A_0 - K_0 t
\]

Where \(A_t\) is the amount of drug released in time \(t\), \(A_0\) is the initial concentration of drug (most times, \(A_0 = 0\)) and \(K_0\) is the zero order release constant expressed in units of concentration/time. To study the release kinetics, data obtained from in vitro drug release studies were plotted as amount of drug released versus time, if the plot is linear then the data obeys zero-order release kinetics, with a slope equal to \(K_0\).

First order kinetics

The first order describes the release from system where release rate is concentration dependent.
A first-order release would be predicted by the following equation:

\[
\log C = \log C_0 - \frac{Kt}{3.303}
\]

where \( C \) is the amount of drug released in time \( t \), \( C_0 \) is the initial concentration of drug and \( K \) is the first order rate constant.

**Higuchi’s Model**

The first example of a mathematical model aimed to describe drug release from a matrix system was proposed by Higuchi in 1961. Initially conceived for planar systems, it was then extended to different geometrics and porous systems. This model is based on the hypotheses that (i) initial drug concentration in the matrix is much higher than drug solubility; (ii) drug diffusion takes place only in one dimension (edge effect must be negligible), (iii) drug particles are much smaller than system thickness, (iv) matrix swelling and dissolution are negligible, (v) drug diffusivity is constant, and (vi) perfect sink conditions are always attained in the release environment. Higuchi was the first to derive an equation to describe the release of a drug from an insoluble matrix as the square root of a time-dependent process based on Fickian diffusion. Simplified Higuchi equation is:

\[
Q_t = K_H (t)^{0.5}
\]

where \( Q_t \) is the amount of drug released in time \( t \) and \( K_H \) is the release rate constant for the Higuchi model.

**Korsmeyer and Peppas model**

The release rates from controlled release polymeric matrices can be described by the equation proposed by Korsmeyer:

\[
Q = K t^n
\]

where, \( Q \) is the percentage of drug released at time ‘\( t \)’, \( K \) is a kinetic constant incorporating structural and geometric characteristics of the tablets and ‘\( n \)’ is the diffusional exponent indicative of the release mechanism.

**Statistical analysis**

The results were expressed as the mean ± standard deviations (SD). Statistical analysis was carried out using analysis of variance (ANOVA) on Graphpad Prism 4.0 (Graphpad Software Inc. San Diego, CA, USA), \( p < 0.05 \) was considered significant.

**RESULTS**

**Optimization of the pulsatile microspheres**

In this research work, the pulsatile (time-specific) microspheres of amlodipine besylate were prepared using solvent evaporation technique and studied effect of different concentration of Eudragit S100 and variation of RPM.

**Effect of different concentration of polymer**

Formulation F1 to F3 containing polymer concentration 250 to 750 mg, respectively, were studied and found F2 showed maximum percentage yield 88.235 ± 0.042 % and entrapment efficiency 94.860 ± 0.103 % as compared to formulation F1 and F3.

**Effect of RPM in microspheres formulations**

Formulation F4 to F7 containing 500 mg of polymer and 10 mg of drug were microspheres prepared at 750 to 1500 RPM, respectively. It was found that F5 formulation prepared at 1000 rpm showed optimum entrapment efficiency 97.73 ± 0.142 % and percentage yield 74.946 ± 0.029.

**Table 3. Flow and bulk properties of amlodipine microspheres.**

<table>
<thead>
<tr>
<th>Sr. no.</th>
<th>Formulation code</th>
<th>Angle of repose</th>
<th>Bulk density</th>
<th>Tapped density</th>
<th>Carr’s Index</th>
<th>Hausner’s ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>F1</td>
<td>27.16 ± 0.153</td>
<td>0.792 ± 0.013</td>
<td>0.871 ± 0.021</td>
<td>9.070 ± 0.012</td>
<td>1.99 ± 0.011</td>
</tr>
<tr>
<td>2.</td>
<td>F2</td>
<td>28.16 ± 0.171</td>
<td>0.873 ± 0.023</td>
<td>0.941 ± 0.047</td>
<td>7.226 ± 0.023</td>
<td>1.77 ± 0.012</td>
</tr>
<tr>
<td>3.</td>
<td>F3</td>
<td>24.74 ± 0.120</td>
<td>0.768 ± 0.018</td>
<td>0.912 ± 0.035</td>
<td>15.789 ± 0.019</td>
<td>1.187 ± 0.014</td>
</tr>
<tr>
<td>4.</td>
<td>F4</td>
<td>23.74 ± 0.221</td>
<td>0.899 ± 0.024</td>
<td>1.032 ± 0.037</td>
<td>12.887 ± 0.024</td>
<td>1.147 ± 0.012</td>
</tr>
<tr>
<td>5.</td>
<td>F5</td>
<td>29.13 ± 0.127</td>
<td>0.872 ± 0.038</td>
<td>1.014 ± 0.028</td>
<td>14.003 ± 0.021</td>
<td>1.162 ± 0.013</td>
</tr>
<tr>
<td>6.</td>
<td>F6</td>
<td>27.81 ± 0.153</td>
<td>0.558 ± 0.026</td>
<td>0.606 ± 0.031</td>
<td>7.920 ± 0.028</td>
<td>1.86 ± 0.011</td>
</tr>
<tr>
<td>7.</td>
<td>F7</td>
<td>25.23 ± 0.128</td>
<td>0.632 ± 0.034</td>
<td>0.781 ± 0.028</td>
<td>19.078 ± 0.032</td>
<td>1.235 ± 0.028</td>
</tr>
</tbody>
</table>
Comparatively high encapsulation values were obtained with the microspheres with high drug loading. Also, increase in the entrapment efficiency was observed with the increase in the polymer concentration and RPM (Table 2).

**Micromeritic properties**

All the formulations were free flowing as indicated by the angle of repose value between 23 to 30°. The values of bulk and tapped densities have shown good packing ability. The values of Carr indices (Ci) were 7.2-19.07% with the lowest Ci value, indicating its excellent compressibility. The Hausner ratios for the formulations were in the range 1.12-1.86, showed good flow properties (Table 3). Density was in the range of 0.729 to 0.820 g/mL. Particle size was in the range of 262.09 to 409.60 µm. With the increasing concentration of Eudragit S100, particle size of the microspheres formulations were increased (F1 to F3). It was observed that formulation F5 containing 500 mg of polymer at 1000 RPM showed optimum particle size 289.90 ± 0.029 µm while other formulation F4, F6 and F7 showed higher particle size due to less percentage yield, entrapment efficiency and drug loading capacity at RPM 750, 1250 and 1500 respectively.

![Figure 1. Scanning electron microscopy of formulation F5 of amlodipine loaded using Eudragit S100 microspheres](image1)

![Figure 2. FTIR of drug, Eudragit S100, drug + Eudragit S100 and formulation F5](image2)
Surface morphology

Scanning electron microscopy of selected formulation F5 showed that the microspheres prepared using polyvinyl alcohol and chloroform Eudragit S100 had smooth surface and were spherical in shape (Fig. 1).

FTIR studies

There was no significant difference in the FTIR spectra of amlodipine drug and amlodipine loaded pulsatile microspheres of optimum formulation F5 which indicated the stable nature of amlodipine of the prepared formulations (Fig. 2). There were no change of shifting of the characteristics peaks in the drug and excipients mixture suggested that there was no significant drug polymer interaction which indicates the stable nature of the drug in all the formulations. From Figure 2, it was observed that similar peaks were also reported in optimized formulations.

X-ray diffraction

The crystalline natures of pure amlodipine, Eudragit S-100, mixture of amlodipine + Eudragit S100 and prepared final formulation F5 have been evaluated by XRD. XRD diffraction of a) amlodipine, b) mixture of amlodipine + Eudragit S-100, c) Eudragit S-100, d) formulation F5 (amlodipine loaded microsphere) (X1 to X4) is shown in Figure 3. The XRD diffractograms of different formulations do not showed the same peaks as amlodipine, indicating that amlodipine underwent a transition from a crystalline to an amorphous state. This study indicates the dispersion of drugs at a molecular level in the formulation and also intensity of XRD was independent on particle size and its distribution. The XRD intensities depend on crystal size but in this study for amlodipine-loaded microsphere formulation F5, the characteristics intensities of amlodipine have been overlapped with the noise of coated Eudragit S-100 indicating molecular level disper-

Figure 3. XRD diffractograms of X1 pure amlodipine, X2 mixture of amlodipine and Eudragit S-100, X3 Eudragit S-100 and X4 amlodipine loaded microsphere formulation F5
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sion of amlodipine in formulation. Hence, no crystals were found in amlodipine-loaded microspheres.

**In vitro drug release**

*In vitro* drug release results are shown in Figure 4. Formulations F1 to F7 showed different drug release pattern in first 6 h while formulation F1 showed immediate drug release pattern similar to the marketed product due to poor entrapment. F2 and F3 showed slow release drug profile for initial first 6 h and after 6 h (lag time) drug release pattern showed fast release of drug because of good entrapment of drug in the formulation but decreased in 12 h. Formulation F4 and F5 showed slow release in first 6 h and after a particular lag time at 8 h drug released was 90.219% in the formulation F5. F6 and F7 showed increased drug release in first 6 h as compared to F5 and decreased in drug release after 8 h i.e., pulsatile release effect were not maintaining for long time while in the case of marketed product cumulative percentage drug release after 8 h was only 5.8%. It showed that formulation F5 followed pulsatile drug released pattern after a particular lag time (Fig. 4).

**Kinetics of drug release**

The data obtained from *in vitro* release studies of all formulation F1 to F7 were fitted to various kinetics equations such as zero-order, first-order, Higuchi model and Korsmeyer-Peppas model to find out the mechanism of drug release from microspheres are presented in Table 4. $R^2$ (regression coefficient) values of different release kinetic models are shown in Table 4. Release data of F1, F2 and F7 obeys first order kinetics whereas F3 and F4 formulations followed zero order drug release kinetics. Formulation F5 follows Huguchi model because $R^2$ was found to be more for Huguchi model as compared to other models. Higuchi model revealed that the drug release occurs through diffusion process.
that is based on Fick’s law of diffusion and is inversely related to square root of time.

DISCUSSION

Pulsatile microspheres have been investigated for improving amlodipine release after a particular lag time for maintaining normal blood pressure. The average sizes of the microsphere were found in between 262.09 to 409.60 µm that showed optimum size. The average particle size of the microsphere was varied with the change in concentration of polymer and RPM. The formulation F5 showed optimum particle size containing 500 mg of Eudragit S100 and prepared at 1000 RPM due to good entrapment of drug polymer and maximum percentage yield while other formulations prepared at RPM 750 to 1500 were larger in particle size due to poor entrapment. FT-IR spectra study showed no change in fingerprint of drug spectra. Scanning electron microscopy of formulation F5 proved the natures of the microspheres were found discrete, spherical and uniform.

Drug release of all formulations were compared with the marketed product. It was observed that the formulation F5 prepared by solvent evaporation method has potential to deliver amlodipine in a pulsatile manner i.e., after a particular lag time drug release was maximal. Drug release studies were incorporated in kinetics models and comparing the correlation coefficient (R²), it was found that formulation F5 followed Higuchi model. The pulsatile release effect was increasing with the time and later showed Higuchi model kinetics and in comparison with marketed product concentrations were decreases with time.

CONCLUSION

The present research work concluded that microsphere with Eudragit S100 showed release pulsatile result pattern at particular lag time as compared to conventional marketed product. It was also found that prepared microspheres showed time controlled release properties. Thus, the result from this study of microspheres provided a potential pulsatile drug delivery effect for the delivery of amlodipine in the treatment of hypertension.

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Conflict of interest

No conflict of interest associated with this work.

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Benign prostatic hyperplasia (BPH/LUTS) is one of the most common urinary disorders in elderly men. The symptoms of the disease include prostate gland enlargement, bladder outlet obstruction, and lower urinary tract symptoms (LUTSs) (1). BPH predisposes patients to bladder infections and bladder stone formation and increases their risk of urinary retention, which in turn causes renal failure (2). The disease is becoming an important diagnostic-therapeutic and socioeconomic problem, considering the increase in the life expectancy of men. BPH often requires surgical treatment (3); however, in recent years, the number of surgical interventions performed has significantly decreased because of the high efficacy of pharmacotherapy, including combination treatment mostly with 2 drug classes, namely, 5α-reductase inhibitors and α-1-adrenolytics with a different pharmacological activity (4). α-1-Adrenolytics contribute to decreasing urinary symptoms, by improving objective parameters, increasing maximum urine flow rate, and decreasing urine retention after miction (1). Noradrenaline acts at α-1-adrenergic receptors (α-1-ARs) in the neck and sphincter of the urinary bladder. It promotes contraction and urinary retention, and controls the smooth muscles in the prostate capsule and prostate urethra. The selective α-1-AR blockers relieve obstruction by relaxing the smooth muscle in the prostate and bladder neck (1). The most common drugs available from this class are doxazosin, tamsulosin, alfuzosin, and terazosin (Fig. 1).

Alfuzosin known chemically as N-[3-((4-amino-6,7-dimethoxyquinazolin-2-yl)-methylamino)propyl]tetrahydrofuran-2-carboxamide, is a quinazoline derivative α-adrenergic blocking agent active after oral administration. It is a selective antagonist of postsynaptic α-1-ARs located in the prostate gland, at the base of the urinary bladder and
in the prostatic urethra. Inhibition of these adrenoceptors leads to the relaxation of smooth muscle in the bladder neck and prostate, resulting in the improvement of urine flow and a reduction in symptoms in BPH. Alfuzosin also inhibits the vasoconstrictor effect of circulating and locally released catecholamines (epinephrine and norepinephrine), resulting in peripheral vasodilation (5). Alfuzosin is rapidly absorbed from the alimentary tract, reaching peak plasma concentrations on average 1.5 h after intake. Food does not impact alfuzosin bioavailability. In patients over 75 years of age, this drug has a faster absorption rate and reaches higher blood plasma concentrations. Alfuzosin metabolites are not pharmacologically active. The half-life is about 4.8 h, and it is not significantly prolonged in patients with renal failure. Alfuzosin undergoes extensive metabolism by the liver using CYP3A4 as the principal hepatic enzyme isoform, and is metabolized by 3 metabolic pathways: oxidation, O-demethylation,
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and N-dealkylation. Only 11% of the administered dose is excreted unchanged in the urine (5). Doxazosin known chemically as 1-(4-amino-6,7-dimethoxy-2-chinazolinyl)-4-(2,3-dihydro-1,4-benzodioxin-2-ylcarbonyl)piperazin is a selective \(\alpha\)-1-AR antagonist. Doxazosin acts by inhibiting postsynaptic \(\alpha\)-1-ARs in vascular smooth muscles. Doxazosin competitively antagonizes the pressor effects of phenylephrine (an \(\alpha\)-1-AR agonist) and the systolic pressor effect of noradrenaline. The antihypertensive effect of doxazosin results from a decrease in systemic vascular resistance, and the parent compound doxazosin is primarily responsible for the antihypertensive activity. It is easily absorbed from the alimentary tract. Peak blood plasma concentration is reached on average after 2 h of drug intake. Protein binding is 98%, and biological half-life is 22 h (5).

Terazosin chemically known as 1-(4-amino-6,7-dimethoxy-2-quinazolinyl)-4-((tetrahydro-2-furanyl)carbonyl)piperazine is a selective \(\alpha\)-1-AR antagonist. Inhibition of \(\alpha\)-1-ARs in the vasculature and prostate results in muscle relaxation, decreased blood pressure, and improved urinary outflow in symptomatic BPH. As there are relatively few \(\alpha\)-1-ARs in the body of the bladder, terazosin decreases obstruction syndromes without any impact on contraction of the whole urinary bladder. Obstruction of \(\alpha\)-1-ARs leads to decreased blood pressure following a decrease in peripheral vascular resistance. This drug causes a decreased systolic and diastolic pressure in both standing and sitting positions, but a stronger drug activity on diastolic pressure has been reported. It is easily absorbed from the alimentary tract. Bioavailability is 90% (5).

Tamsulosin known chemically as 5-[2-[2-(ethoxyphenoxy)ethylamino]propyl]-2-methoxybenzenesulfonamide is a selective \(\alpha\)-1-AR antagonist. Tamsulosin acts by inhibiting postsynaptic \(\alpha\)-1-ARs in vascular smooth muscles. This drug decreases symptoms in irritation syndromes and in obstruction, in which contraction of smooth muscles in the lower urinary tract plays a significant role. Tamsulosin increases maximum flow rate following smooth muscle relaxation in the prostate gland and urethra. Tamsulosin is rapidly absorbed from the intestine and is almost completely bioavailable (6).

The aim of this study was to evaluate \(\alpha\)-1-adrenolytics in combination therapy with finasteride in patients diagnosed with BPH.

METHODS

The clinical trial was conducted in 2 stages: Stage I from October 2008 to November 2009, and Stage II from November 2009 to November 2010. A total of 10,066 patients, \(n_1 = 4,315\) and \(n_2 = 5,751\), respectively, for each stage were enrolled in 50 urological centers in Poland participating in the clinical trial. This clinical trial was organized by Moneo Pharma Group. The researchers who conducted the trial were urologists from different regions of Poland. The clinical trial involved 6 follow-up visits – the time interval between every visit was 2 months.

The most frequently reported discomforts were as follows: frequent urination in 77.5% (\(n = 7,804\)) of the patients, nocturia in 78.13% (\(n = 7,865\)), respectively, for each stage were enrolled in 50 urological centers in Poland participating in the clinical trial. This clinical trial was organized by Moneo Pharma Group. The researchers who conducted the trial were urologists from different regions of Poland. The clinical trial involved 6 follow-up visits – the time interval between every visit was 2 months.

Most frequently reported discomforts were as follows: frequent urination in 77.5% (\(n = 7,804\)) of the patients, nocturia in 78.13% (\(n = 7,865\)), decreased size and strength of the urinary stream in 67.33% (\(n = 6,778\)), and compelling urge to urinate (urgency) in 62.43% (\(n = 6,285\)). The detailed data are presented in Fig. 2.

The following data on co-morbidities were collected: hypertension, diabetes, elevated cholesterol...
and lipid levels, erectile dysfunctions, and neurological diseases (Fig. 3).

The data, including those self-reported by the patients, were stored in a database available to urologists to facilitate the selection of a suitable pharmacotherapy.

The pharmacotherapy selected was applied to all patients. The patients were prescribed finasteride at a dose of 5 mg/day because of BPH and urinary dysfunctions. In addition, the combination therapy of finasteride and α1-adrenolytics consisting of the following: alfuzosin (10 mg/day), doxazosin (4 or 6 mg/day), tamsulosin (0.4 mg/day), and terazosin (10 mg/day).

Patients were included in the study if they met the following inclusion criteria: LUTSs, age > 40 years, BPH, and finasteride use for at least 2 weeks. The mean age of the patients was 67 years (range, 45–93 years; median, 67.00; SD, 8.304). The clinical trial involved 6 follow-up visits and lasted 25 months. On the first visit, a self-reported evaluation and physical examination, including a digital rectal examination (DRE), were performed. The data pertaining to LUTSs were evaluated using IPSS (International Prostate Symptoms Score) with regard to the intensification or attenuation of the urinary system symptoms. Moreover, the patients underwent additional examinations, including serum concentrations of prostate-specific antigen (PSA), urinary tract ultrasonography (USG) with evaluation of total prostate volume (TPV), and uroflowmetry with evaluation of residual urine volume. These all examinations were repeated on the sixth visit, after 12 months of treatment.

On the succeeding visits, the data pertaining to LUTSs were evaluated using IPSS with regard to the intensification or attenuation of the urinary system symptoms. Furthermore, whether the treatment was continued or modified after the first visit was recorded; in addition, the possible adverse effects of the pharmacotherapy were also documented. On the second and on the third visit, uroflowmetry with evaluation of residual urine volume was again performed.

Based on the results of the physical examination, BPH occurred to be concomitant with other diseases such as hypertension in 51.50% of patients (n = 5184), diabetes in 20.01% (n = 2216), hypercholesterolemia and hyperlipidemia in 16.03% (n = 1614), erectile dysfunctions in 16.77% (n = 1689), neurological diseases (Parkinson’s disease and multiple sclerosis) in 3.92% (n = 395), and other comorbidities in 4.10% (n = 413; Fig. 2).

On the second visit, in addition to the administration of finasteride at a dose of 5 mg/day in 10 066 patients, the combination of drug therapy consisting of finasteride + an α1-adrenolytic was administered to 63.65% of the patients (n = 6408/10 066), including 52.30% (n = 3352), 34.14% (n = 2189), 7.59% (n = 487), and 5.93% (n = 380) of the patients receiving tamsulosin, doxazosin, alfuzosin, and terazosin, respectively (Fig. 4).

This combination of drug therapy was caused by a low efficiency of monotherapy applied earlier.

RESULTS

The analyzed group, in which the combination of drug therapy was prescribed on the second visit, included 6408/10 066 (63.05%) patients. On the third visit following the administration of an α1-adrenolytic, attenuation of BPH symptoms was observed in 3882/6 408 (60.58%) patients. The improvement was most common within the spectrum of the following symptoms: residual urine after miction (62.3%), frequent urination (61.7%), decreased size and strength of the urinary stream (61.5%), and nocturia (60.2%).

Adverse effects were observed in 2306 patients (36%) after combination of drug therapy. The most common side effects were reported in patients using doxazosin (n = 897; 14%) and included palpitations.
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In the group of patients using alfuzosin, the following adverse effects were observed (n = 450; 7%): orthostatic hypotension (n = 347; 5.41%), stomatitis (n = 311; 4.85%), chest pain (n = 201; 3.13%), palpitations (n = 86; 1.34%), rash (n = 68; 1.06%), and pruritus (n = 58; 0.90%). In the group of patients using terazosin, the following adverse effects were observed (n = 449; 7%): orthostatic hypotension (n = 257; 4.01%), palpitations (n = 172; 2.68%), chest pain (n = 36; 0.56%), irritability (n = 28; 0.43%), anxiety (n = 31; 0.48%), weakness (n = 18; 0.28%), and drowsiness (n = 23; 0.35%). The least number of side effects were observed in patients using tamsulosin (n = 255; 4%) and included retrograde ejaculation (n = 83; 1.29%), decreased ejaculation (n = 124; 1.93%), orthostatic hypotension (n = 46; 0.71%), intraoperative floppy iris syndrome (n = 1; 0.01%) (Fig. 5).

The results of the following were analyzed: PSA levels, urinary tract USG with evaluation of TPV, and uroflowmetry with the evaluation of residual urine volume and maximal flow rate.

α-1-Adrenolytics showed high efficiency in decreasing dysuric discomforts, improving objective parameters, increasing maximum flow rate, and decreasing residual urine after miction. The most efficient α-1-adrenolytics were doxazosin and tamsulosin, which contributed to significant improve-
ment of maximum flow rate ($Q_{\text{max}}$) and time of miction (T flow), and decreased residual urine after miction $R_v$ (Table 1).

From the abovementioned drugs, it is important to emphasize the uroselective activity of tamsulosin as it had the lowest influence on arterial blood pressure. Furthermore, finasteride confirmed its overall efficiency by the significant decrease in TPV by 35% and in PSA level by 30% observed even after 12 months of treatment (on the sixth visit).

In this clinical trial, on the second to the sixth visits, progressive improvement in the spectrum of LUTSs was observed as self-reported by patients (Table 2).

The McNemar’s test showed a significant difference in improvement of the spectrum of LUTSs between the second and sixth visits ($p < 0.001$). The prevalence of adverse effects after pharmacotherapy was also evaluated. ANOVA test was applied to elaborate the significance of difference between various parameters. The level of significance was set at 0.05. ANOVA test showed a significant difference in the total amount of adverse effects reported by patients between the second and sixth visits ($p < 0.03$).

The clinical trial was completed after the sixth visit, and the data on urological symptoms were analyzed at the last visit. Figure 5 shows the noticeable decrease of LUTSs owing to the efficiency of the combination therapy. In 376/6408 (5.86%) patients, the efficiency of combination of drug therapy with finasteride and an $\alpha$-1-adrenolytic was low and patients qualified for surgical treatment.

**DISCUSSION**

The aim of this study was to evaluate the efficacy of $\alpha$-1-adrenolytics in combination therapy with finasteride in patients with diagnosed BPH based on the clinical trial PLESS (Proscar Long-term, Efficacy and Safety Study) (6). The different pharmacological activities of the 2 drug groups, $\alpha$-1-adrenolytics and 5-$\alpha$-reductase blockers, probably influenced the long-term effects of BPH treatment in combination drug therapy. The combination therapy was more effective than placebo, $\alpha$-1-adrenolytic monotherapy, or finasteride monotherapy in the suppression of BPH progression (6).

Until date of this study, few randomized studies have been conducted evaluating the long-term efficiency of BPH treatment with $\alpha$-1-adrenolytics. The results of previous studies on combination therapy consisting of terazosin, alfuzosin, tamsulosin, and doxazosin after approximately 4 years demonstrated a long-lasting improvement in the degree of symptoms and uroflow rate (7). The $\alpha$-1-adrenolytic doxazosin was demonstrated to suppress the duration of BPH. However, it did not contribute to the slowing down progression of the disease. $\alpha$-1-Adrenolytics were also prescribed to patients with hypertension and BPH. In these patients, combination therapy normalized arterial blood pressure and eliminated LUTSs. In the present study, doxazosin showed the highest number of side effects, such as palpitation, arrhythmia, tachycardia, and orthostatic hypotonia. However, according to Kirby (7), patients receiving doxazosin or terazosin with accurate arterial blood pressure data available did not report any discomforts, which is of clinical importance.

Furthermore, in both studies doxazosin showed a positive effect on lipid profiles by reducing LDL-cholesterol and triglyceride levels, and increasing HDL-cholesterol levels. Hence, doxazosin provides a peculiar health protection from cardiac infarction.
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or stroke. It is important to emphasize the uroselective activity of tamsulosin, since it had the lowest influence on arterial blood pressure compared to the other agents (8).

However, it should be underlined that BPH can occur as full-blown LUTSs without prostate gland enlargement or as independent symptoms of benign prostatic obstruction. Therefore, tamsulosin is recommended for full-blown LUTSs, in which LUTSs are dominant. When symptoms of benign prostatic obstruction intensify, doxazosin is recommended (8). However, the Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial does not recommend pharmacotherapy with α-1-adrenolytic drugs to patients with hypertension and congestive heart failure (7). In the present study, tamsulosin was the safest and the most efficient α-1-adrenolytic but it should not be prescribed in sexually active patients because it may cause ejaculation disorders, such as decreased ejaculate or retrograde ejaculation. α-1-Adrenolytics are very efficient because they decrease dysuric discomforts, improve objective parameters, increase maximum flow rate, and decrease residual urine (9).

The Medical Treatment of Prostatic Symptoms (MTOPS) study reported a decrease in TPV by 25% in comparison with the placebo group, in patients administered only finasteride or finasteride + doxazosin. The decrease in TPV in the MTOPS study was comparable with that in our study, wherein patients underwent pharmacotherapy with finasteride at a baseline TPV of 40 mL or higher. Furthermore, compared to that in the placebo group, the estimated decrease in TPV by 25% in patients treated with finasteride and with a baseline prostate volume of 25 mL < TPV > 40 mL contributed to the beneficial value of the combination therapy with doxazosin in the clinical improvement of BPH. A decrease in TPV by 20% was observed in patients with a baseline prostate volume of 25 mL < TPV > 40 mL, who only took doxazosin as monotherapy and placebo (10).

The Veteran’s Administration Cooperative Study conducted a contrastive analysis between the activity of terazosin and that of finasteride and the combination of both with placebo (8). The study showed that α-1-adrenolytic drugs were more effective than finasteride after 1 year of pharmacotherapy. However, they were associated with more adverse drug reactions such as dizziness (9).

This medical trial conducted in Poland (n = 10 066) in 2 stages – Stage I from October 2008 to November 2009 and Stage II from November 2009 to November 2010 – demonstrated that in the 4806 patients (75%) who were receiving both α-1-adrenolytic drugs and finasteride, disease progression and LUTSs were suppressed, and micturition improved significantly within Qmax, T flow, and Rv and TPV decreased by 35%.

The data from the present medical trial confirmed the high efficiency of α-1-adrenolytics in combination therapy with finasteride administered to patients with extensive LUTSs related to BPH. The α-1-Adrenolytics evaluated in the study demonstrated high efficiency in improving dysuric discomforts, Qmax, T flow, and Rv. Furthermore, finasteride confirmed its overall efficiency by the significant decrease in TPV (35%) observed even after 12 months of treatment. It was also found to contribute to the attenuation of LUTSs, improvement in maximum flow rate, decrease in nocturia, and improvement in quality of life.

There is no conflict of interest. There is absence of any interest to disclose.

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ANTIBACTERIAL ACTIVITY OF SELECTED MEDICINAL PRODUCTS FOR MOUTH DISINFECTION IN SOLID FORM, ASSESSED IN ACCORDANCE WITH PN-EN 1040

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Abstract: There are many products on the Polish market used for mouth disinfection, with antibacterial activity declared by the manufacturers. These products appear in the form of tablets, powders for the preparation of suspensions and liquids. They contain different active substances of natural or synthetic origin. The purpose of this study was to examine the bactericidal activity of selected medicinal products in solid form, mainly tablets, used for mouth disinfection which are available on the Polish market. In a previous study, the antibacterial activity of liquid antiseptics was analyzed. Products selected for the tests were mainly synthetic, in tablet (16 types) or powder (one type) form. The study was performed according to the standard PN-EN 1040:2006 with the use of reference strains and clinical oral streptococcal isolates. Suspension method based on dilution–neutralization was used. At the end of contact time, the bactericidal activity was immediately stopped by a validated neutralizer. At the same time the test, validation and control were performed. Only some of the tested medicinal products for mouth disinfection showed antimicrobial activity, as required by the standard PN-EN 1040:2006. Clinical isolates of oral streptococci were found to be more susceptible to these products than the reference strains. A favorable feature of the tested products was their more effective antibacterial activity at 36°C in comparison to 30°C. Antimicrobial activity declared by the manufacturers on the package or in the leaflet is sometimes not accurate. It is necessary to introduce a more efficient control system for the antimicrobial activity of these antiseptic products before their registration.

Keywords: mouth disinfection, antiseptics, antibacterial activity, EN 1040:2006

There are many products on the Polish market used for mouth disinfection, possessing antibacterial activity as declared by the manufacturers. These products come in the form of tablets, liquids for the throat and mouthwashing and powders for oral suspension. They differ in their qualitative and quantitative composition and in the concentration of active substances and excipients which may be of the natural or synthetic origin.

Research into the antibacterial activity of selected commercial products for mouth washing and disinfection in liquid form has been conducted and published (1). The purpose of this study was to examine the bactericidal activity of selected medicinal products for mouth disinfection available on the Polish market in solid form, mostly as tablets. These studies were conducted in accordance with PN-EN 1040:2006 (Polish-European Standard) (2). In order to obtain more information, some clinical oral streptococcal strains were included in the study.

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The Department of Antibiotics and Microbiology at the National Medicines Institute, Warsaw possesses attestation from the European Directorate for the Quality of Medicines (EDQM/ MJA-078) for microbiological tests carried out in accordance with ISO/EN 17025 and accreditation from the Polish Centre for Accreditation No. AB 774 for microbiological testing of disinfectants and antiseptics, according to several European Standards (ENs) including PN-EN 1040.

EXPERIMENTAL

Material and methods

Products: Products selected for the tests were in tablet (16 types) or powder (one type) form. Table 1 presents the characteristics of the products applied in this study.

Bacterial strains: The study was conducted using reference strains recommended by PN-EN
1040 : 2006 – phase 1 (2) and EN 13727 : 2012 – phase 2, step 1 (3): Staphylococcus aureus ATCC 6538 and Pseudomonas aeruginosa ATCC 15442, Escherichia coli NCTC 10538 and Enterococcus hirae ATCC 10541. Additionally, four oral streptococcal isolates recovered from tongue swabs, i.e., Streptococcus sanguinis and Streptococcus mitis and tooth swabs, i.e., Streptococcus oralis and Streptococcus salivarius were used.

Standard: The study was performed according to PN-EN 1040 : 2006 – “Chemical disinfectants and antiseptics – Quantitative suspension test for the basic bactericidal activity of chemical disinfectants and antiseptics – Test method and requirements (phase 1)” (2). It is assumed that the product possesses antibacterial properties if it causes at least a 5 log reduction in the number of viable bacteria after 60 min or less of contact time. In this study, the dilution-neutralization method was applied.

The tablet of each product was dissolved in 5 mL of sterile water. This amount was set by the previous empirical measurement of the amount of saliva secreted in the mouth during the dissolution of an individual tablet. Additionally, 5 g of the tested powder was dissolved in 250 mL of sterile water. Prior to performing the test, the density of bacterial suspension was adjusted to a value of 1.5-5 × 10^8 cfu/mL.

The test procedure was as follows: 1 mL of water and 1 mL of bacterial test suspension were added to 8 mL of the examined product test solution. Analysis of the reference strains was conducted at 30°C and 36°C for two contact times, 15 min and 60 min. For the clinical streptococcal isolates, the following conditions were applied: temperature of the test 36°C, the contact time 5 min and 15 min, and incubation in an atmosphere enriched with 5% CO₂. At the end of the contact time, 1 mL sample of the test mixture was transferred into a tube with 8 mL of neutralizer mixed with 1 mL of water. The neutralizer contained the following composition: lecithin (AppliChem) 3 g/L, polysorbate 80 (POCH Gliwice) 30 g/L, sodium thiosulfit (Sigma) 5 g/L, L-histidine (Merck) 1 g/L, saponin (Sigma) 30 g/L, tryptone (Difco) 1 g/L, NaCl (POCH Gliwice) 8.5 g/L and water up to 1 L.

After 5 min ± 10 s of neutralization time, 1 mL sample of the neutralized test mixture was taken in duplicate and inoculated using the pour plate technique. The plates were incubated at 37 ± 1°C. After 24–48 h, the number of colony forming units (cfu) was determined. Verification of the absence of toxicity of the neutralizer and validation of the dilution-neutralization method test was performed at the same time and under the same conditions.

RESULTS

In the first stage of the study, the bactericidal activity of the products with declared antibacterial activity was investigated against reference strains. For the products: Chlorchinaldin, Faringosept, Halset, Menthocept, Sebidin Plus, Strepsils Intensive and Gargarin, residual bactericidal activity was observed, as required by standard PN-EN 1040 : 2006, at 30°C and for both contact times. The logarithm of the reduction of viable bacteria was very low and ranged from 0.01 to 1.98.

The products Neo-Angin and Orofar possessed bactericidal activity required by the standard against all tested strains at 30°C after 15 min of contact time. The products Propolki and Cholisept caused, as required by the standard, a reduction in the number of all tested bacteria after 60 min of contact time. After 15 min of contact time, a reduction in the number of bacteria less than the required 5 log decrease was observed for the product Cholisept in the case of E. hirae ATCC 10541 and for the product Propolki against E. coli NCTC 10538.

In the case of the product Cholinex, a log reduction of bacteria above 5.0 was observed against S. aureus ATCC 6538, P. aeruginosa ATCC 15442 and E. coli NCTC 10538 after 60 min of contact time. The bactericidal activity of the Sebidin product complied with the standard after 15 min of contact time, but only in the case of P. aeruginosa ATCC 15442, and after 60 min of contact time only toward S. aureus ATCC 6538. The product Septolete D possessed bactericidal activity required by the standard after 15 min of contact time only against E. hirae ATCC 10541, and after 60 min of contact time against S. aureus ATCC 6538. The product Septolete Plus showed appropriate activity after 60 min of contact time only against E. hirae ATCC 10541. Two other products, Strepsils and Tantum Verde, caused the required reduction in the number of bacteria in three out of four reference strains after just 15 min of contact time. In the case of the product Strepsils, S. aureus ATCC 6538 was the only strain for which the product was not sufficiently active (after 60 min contact time the reduction was 4.7). Moreover, in the case of the product Tantum Verde, the most tolerant strain was E. hirae ATCC 10541 (the reduction in cell number after 60 min of contact time was 4.7).

A comparison of the antimicrobial activity of the tested products for mouth disinfection in solid form applied for 15 min or 60 min at 30°C is presented in Figure 1. Only two products, Neo-Angin and Orofar, which represent 12% of the tested prod-
ucts, showed bactericidal activity against all tested reference strains after 15 min of contact time. Nine of the 17 tested products (53% of the products) did not show efficacy against any strain as required by the standard PN-EN 1040:2006. The remaining 35% (n = 6) of the products showed partial effectiveness, but the required degree of reduction was found only against some of the tested strains (Fig. 1).

Differences were observed when the products were tested after 60 min of contact time. In this case, four products (24%), i.e., Neo-Angin, Orofar, Cholisept, and Propolki, showed total effectiveness against all tested strains, but eight products (47%) were ineffective. The remaining five products (29%) were partly active against the reference strains (Fig. 1).

In the next step of the study, five tested medicinal products showing the greatest difference in the log reduction number of bacteria tested at 30°C were retested at 36°C. Increasing the testing temperature from 30°C to 36°C allowed to observe increased antimicrobial activity of products.

A comparison of the bactericidal activity of the product Cholinex tested at two temperatures is shown in Figure 2. There was an increase in the bactericidal properties to 5.0 log against *P. aeruginosa* ATCC 15442 and *E. coli* NCTC 10538 after 15 min of contact time. In the case of *S. aureus* ATCC 6538, the reduction in bacterial number increased by about 1 log, while against *E. hirae* ATCC 10541 only a small change was observed. Extension of the contact
time from 15 to 60 min did not result in significant changes in the log of reduction against tested strains apart from \emph{S. aureus} ATCC 6538.

In the case of the Sebidin Plus product (Fig. 3) after 15 min of contact time, the greatest reduction in the number of viable bacteria was observed against \emph{P. aeruginosa} ATCC 15442. For other strains, the logarithms of bacterial reduction increased by about 2 (for \emph{S. aureus} ATCC 6538 and \emph{E. coli} NCTC 10538 strains) and 0.5 (for \emph{E. hirae} ATCC 10541). In the case of 60 min of contact time, bactericidal activity was in accordance with the requirements of the standard against \emph{S. aureus} ATCC 6538, \emph{P. aeruginosa} ATCC 15442 and \emph{E. coli} NCTC 10538. The Sebidin Plus product did not possess sufficient bactericidal activity against \emph{E. hirae} ATCC 10541, regardless of the test conditions of temperature and contact time.

In the case of Septolete D (Fig. 4), changes to the test conditions did not cause a significant increase in bactericidal activity against \emph{S. aureus} ATCC 6538, \emph{E. hirae} ATCC 10541 and \emph{E. coli} NCTC 10538. Only in the case of \emph{P. aeruginosa} ATCC 15442 was increase in activity by 1 log at 36°C shown, but the product still did not meet the requirements of the standard.

In the case of the Septolete Plus (Fig. 5) at a temperature 36°C after 15 min of contact time, the reduction in the number of \emph{S. aureus} ATCC 6538, \emph{P. aeruginosa} ATCC 15442 and \emph{E. coli} NCTC 10538 as greater. The product complied with the standard requirements only against \emph{E. coli} NCTC 10538 and \emph{E. hirae} ATCC 10541 after 60 min of contact time. The changes to the test conditions slightly increased the bactericidal activity of the product against other strains.

The Strepsils Intensive (Fig. 6) showed bactericidal activity according to the standard only against \emph{S. aureus} ATCC 6538 after 60 min of contact time. The active substance, Flurbiprofen, has
analgesic and anti-inflammatory effects, so we did not expect any bactericidal activity and the manufacturer did not declare bactericidal activity for this product.

In the next stage of this study, the following clinical oral streptococci isolates were used: *Streptococcus sanguinis*, *Streptococcus mitis*, *Streptococcus oralis* and *Streptococcus salivarius*. The study was carried out at 36°C for two contact times, 5 min and 15 min.

Investigation of the bactericidal activity of the products Chlorochinaldin, Faringosept, Menthosept and Gargarin did not show efficacy against the reference strains and clinical isolates.

The products Cholisept, Neo-Angin, Orofar, Propolki, Strepsils and Tantum Verde met the requirements of the standard against clinical isolates as the log reduction in bacterial count was above 5 after 5 min of contact time. The products Sebidin and Strepsils Intensive did not meet the requirements of the standard only in the case of *S. sanguinis* after a contact time of 5 min. The Septolete D product showed bactericidal activity according to the standard against *S. sanguinis* and *S. oralis* after both contact times; however, against the *S. salivarius* isolate, this was seen only after 15 min. In the case of activity against *S. mitis*, the log of reduction in the number of bacterial cells was slightly below 5 (4.6 log). The evaluation of the efficacy of Cholinex as an antiseptic showed bactericidal activity in accordance with the standards for all clinical isolates, but only after 15 min of contact time. For the Septolete Plus product, a reduction in the number of viable bacteria above 5 log was observed after 15
min for all isolates except *S. salivarius*. In the case of the Halset product, a reduction above 5 log units was observed only for the *S. oralis* isolate after 15 min of contact time. Similar results (log reduction in cell numbers greater than 5 after 15 min of contact time) were observed for the Sebidin Plus product against *S. oralis* and *S. mitis*.

A comparison of the bactericidal activity of the tested products at 36°C after 5 min and 15 min of contact time are shown in Figure 7. After 5 min of contact time, 47% (8 out of 17) of the products did not show bactericidal activity against the tested clinical isolates; 18% of the products (3 out of 17) showed a reduction required by the standard, only against some of the strains. Almost 35% of products (6 out of 17) showed satisfactory antibacterial activity (Fig. 7). In the case of 15 min of contact time, most of the tested products (53%) were effective against clinical microorganisms, but 23.5% of products (4/17) showed bactericidal activity required by the standard only against some strains. Four (23.5%) products showed no bactericidal activity against the studied bacterial strains (Fig. 7).

**DISCUSSION**

Every fourth person who complains of a sore throat uses antiseptics (4). Regardless of the cause of the infection, treatment is used to reduce the pain of the affected organs. On the Polish market, the medicinal products which are available in the form of compressed lozenges, gargle liquids, mouthwashes or powders for oral suspension are marketed as antimicrobial, anti-inflammatory or analgesic agents. However, not all products demonstrate the desired effect, and their actual antimicrobial activity is not in agreement with the information provided by the manufacturers in the leaflet (1). The products tested in this study, which are used as a support in the treatment of oral cavity infections, are antiseptics. As defined by the Regulations of the Polish Ministry of Health (5), the group of antiseptics includes medicines which are microbicidal or inhibit microbial growth and are applied to mucous membranes, intact skin and damaged tissue in order to reduce and prevent infections. Now, the question arises as to whether products used for the mouth disinfection available on the Polish market show the expected antibacterial activity. Products in liquid form for mouth washing and disinfection with declared antimicrobial activity as medicinal products, examined in a previous study, in most cases did not exhibit the antibacterial activity required by the PN-EN 1040 : 2006 standard (2) at the recommended concentration (1). There are actually no standardized methods for testing the effectiveness of biocidal products for mouth disinfection, so research into the activity of antiseptics should be carried out in accordance with the methods described in international standards dedicated to antiseptics and disinfectants. In the absence of such standards, it is recommended to test products according to national methods developed by authorized institutions. This study describes an attempt to verify the basic bactericidal activity of medicinal products used for the mouth disinfection, available on the Polish market. For this purpose, the methodology used in phase 1 of the study is described in EN: 1040 : 2006, which possesses the status of Polish standard (PN) (2). It is assumed that the product possesses antibacterial properties if it causes at least a 5 log reduction in the number of viable bacteria after 15 min of contact.
It is assumed that 15 min can be considered the longest dissolution time for a tablet in the mouth. The obligatory contact time (bacterial suspension with the product) recommended by the standard is 5 min. However, as was empirically demonstrated, some of the tested tablets dissolve in the mouth within 15 min. In the case of the reference strains, due to a small reduction after 5 and 15 min, the contact time was extended to 60 min, the longest time recommended by PN-EN 1040 : 2006 (2). Because the investigated products are dedicated to mouth disinfection, representatives of four oral streptococcal species were included in the study. Streptococci, as strains that are more sensitive to antiseptics when compared to reference strains, were tested after 5 and 15 min.

One of the active ingredients in the products used for the treatment of sore throat is chlorhexidine dihydrochloride, which shows differential antimicrobial activity. The strongest activity of chlorhexidine dihydrochloride was observed against Gram-positive bacteria, which are a large group of oral pathogen flora. However, chlorhexidine dihydrochloride turns out to be ineffective against spores and *Mycobacteria tuberculosis* (6). Addition of a salicylic acid derivative to tablets, i.e., choline salicylate or sodium salicylate, increased the biocidal properties of the product against pathogenic microorganisms. The use of medicinal ingredients such as essential oils, 2,4-dichlorobenzyl alcohol or cetyl pyridinium chloride, present in a chewable tablet, also increases their biocidal properties.

### Table I. Information of analyzed products in form of tablets (16) and powder (1) in this study.

<table>
<thead>
<tr>
<th>No.</th>
<th>Name of the product</th>
<th>Manufacturer</th>
<th>Composition - active substances</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tablets</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>Chlorchinaldin ICN Polfa Rzeszów S.A.</td>
<td></td>
<td>chlorquinaldol 2 mg</td>
</tr>
<tr>
<td>2.</td>
<td>Cholinex GlaxoSmithKline Pharmaceuticals S.A.</td>
<td></td>
<td>choline salicylate 150 mg</td>
</tr>
<tr>
<td>3.</td>
<td>Cholisept GlaxoSmithKline Pharmaceuticals S.A.</td>
<td></td>
<td>hexylresorcin 12.5 mg, benzalkonium chloride 1.2 mg</td>
</tr>
<tr>
<td>4.</td>
<td>Faringosept S.C. Terapia S.A.</td>
<td></td>
<td>ambazole 10 mg</td>
</tr>
<tr>
<td>5.</td>
<td>Halset Biofarm Sp. z o.o.</td>
<td></td>
<td>cetylpyridine chloride 1.5 mg</td>
</tr>
<tr>
<td>6.</td>
<td>Menthosept Polfa - Łódź S.A.</td>
<td></td>
<td>cetylpyridine chloride 1.5 mg</td>
</tr>
<tr>
<td>7.</td>
<td>Neo-Angin DIVAPHARMA GmbH</td>
<td></td>
<td>2,4-dichlorobenzyl alcohol 1.2 mg, amyl metacresol 0.6 mg, menthol 5.72 mg</td>
</tr>
<tr>
<td>8.</td>
<td>Orofar Novartis</td>
<td></td>
<td>benzoxonium chloride 1 mg, lidocaine chloride 1 mg</td>
</tr>
<tr>
<td>9.</td>
<td>Propolki Nepentes S.A.</td>
<td></td>
<td>extract of propolis, vitamin C</td>
</tr>
<tr>
<td>10.</td>
<td>Sebidin GlaxoSmithKline Pharmaceuticals S.A.</td>
<td></td>
<td>chlorhexidine hydrochloride 5 mg, ascorbic acid 50 mg</td>
</tr>
<tr>
<td>11.</td>
<td>Sebidin Plus GlaxoSmithKline Pharmaceuticals S.A.</td>
<td></td>
<td>chlorhexidine hydrochloride 5 mg, ascorbic acid 75 mg</td>
</tr>
<tr>
<td>12.</td>
<td>Septolete D KRKA</td>
<td></td>
<td>benzalkonium chloride 1 mg, menthol 1.2 mg, peppermint oil 1 mg, eucalyptus oil 0.6 mg, thymol 0.6 mg</td>
</tr>
<tr>
<td>13.</td>
<td>Septolete Plus KRKA</td>
<td></td>
<td>benzocaine 5 mg, cetylpyridine chloride 1 mg</td>
</tr>
<tr>
<td>14.</td>
<td>Strepsils Intensive Reckitt Benckiser Healthcare International Ltd.</td>
<td></td>
<td>amyl metacresol 0.6 mg, 2,4-dichlorobenzyl alcohol 1.2 mg</td>
</tr>
<tr>
<td>16.</td>
<td>Gargarin Farmina Sp. z o.o.</td>
<td></td>
<td>5 g of powder contains: sodium tetraborate 1.74 g, sodium bicarbonate 1.74 g, sodium benzoate 0.75 g, menthol 0.02 g</td>
</tr>
</tbody>
</table>
Research by Karolewska et al. (7) revealed the activity of laboratory-produced antiseptic tablets against *S. aureus* ATCC 6538 and *P. aeruginosa* ATCC 27853. The most effective biocidal activity was observed in the case of tablets with chlorhexidine, choline salicylate and essential oils (research was carried out by a suspension method at 20°C with a contact time of 16 min). In this case, the requirements of the European Committee for Standardization (Comité Européen De Normalisation – CEN) were met for all strains. Satisfactory results for both bacterial strains were also obtained in the case of tablets containing chlorhexidine, choline salicylate and 2,4-dichlorobenzyl alcohol. Tablets containing chlorhexidine, choline salicylate and cetylpyridinium chloride were effective only against *S. aureus* ATCC 6538 (7). Currently, there are no chewable tablets available on the Polish market containing the same mixture of components as those used in the test by Karolewska (7). However, products having in their composition one or two previously tested compounds can be found on the market. These products are Cholinex (choline salicylate, peppermint oil), Halsel (cetylpyridinium chloride, peppermint oil), Menthocept (cetylpyridinium chloride), Neo-Angin throat lozenges (2,4-dichlorobenzyl alcohol, oil of anise, peppermint oil), Sebidin (chlorhexidine dihydrochloride), Sebidin Plus (chlorhexidine dihydrochloride), Septolete plus (cetylpyridinium chloride, peppermint oil) and Strepsils (2,4-dichlorobenzyl alcohol). In this study, the only products containing chlorhexidine dihydrochloride were Sebidin and Sebidin Plus. Sebidin met the requirements of the standard against *P. aeruginosa* after both contact times but against *S. aureus* only after 60 min (tested at 30°C). No satisfactory bactericidal activity was observed in the case of the product Sebidin Plus. Comparing the microbial effects of these products against *S. aureus* ATCC 6538 and *E. coli* NCTC 10538, much better activity against *E. coli* was noticed. Chlorhexidine dihydrochloride has a destructive influence on the bacterial cell membrane, causing leakage of potassium ions from the cytoplasm. Castillo et al. (8) observed weaker activity of chlorhexidine dihydrochloride against *E. coli* ATCC 10536 and stronger activity against *S. aureus* ATCC 9144. Tablets containing 5 mg of chlorhexidine dihydrochloride, used to treat infections of the upper respiratory tract, may also contribute to the reduction of dental plaque. Kaufman et al. (9) demonstrated in an *in vivo* study that products containing chlorhexidine dihydrochloride reduce dental plaque by nearly 63% after the first week of tablet administration three times a day. Kałowski et al. (10) have shown in clinical trials of a product containing chlorhexidine dihydrochloride (Sebidin) that even three days of application reduces or even abolishes gingivitis and periodontitis. This product showed the greatest efficacy in the case of surface periodontitis, due to the scattering effect of chlorhexidine on biofilm-forming bacteria on the teeth. Magas et al. (11) checked the suitability of the Sebidin product for the treatment of chronic aphthas, a chronic inflammation of the mouth. This product caused not only pain relief (although it did not contain any anesthetic in its composition) and removed aphtha eruptions after using just three tablets, but also led to the healing of aphtha erosions after seven days of treatment. It was also found that chlorhexidine in the form of chewable tablets was better tolerated by patients than in an aqueous solutions such as a mouthwash. The essential oil most commonly added to lozenges available on Polish market is peppermint oil. The use of natural compounds increased the microbicidal activity of the products. The Kalemba (12) study showed that peppermint and sage oil or tea tree oil are highly effective biocidal agents against 15 bacterial strains isolated from the oral cavity. Features such as fast action and a long-lasting bactericidal effect make essential oils especially valuable in practical applications.

Excipients used in medicinal products may also affect the efficiency of lozenges. Karolewska and Dobrucki (13) studied a pilot series of tablets containing chlorhexidine and choline salicylate. A differential effect of macromolecular excipients on antimicrobial activity was observed (studies were carried out using a quantitative suspension method according to EN 1040 with the tested strains *S. aureus* ATCC 6538 and *P. aeruginosa* ATCC 27853). The use of a 2% solution of methylcellulose as a binder substantially reduced the biocidal activity of the tested tablets. It was found that among all the analyzed substances which increased viscosity, the best agent was polyvinylpyrrolidone, however it affected the antimicrobial activity of the active substances (13). Medicinal products containing several active substances may show greater biocidal activity. Zones of inhibition of microbial growth in the case of tablets with chlorhexidine and choline salicylate were higher than in the case of tablets containing only chlorhexidine (7, 13). Cholinex is most commonly used product in oral cavity infections. An evaluation of the bactericidal activity of this product showed that it did not meet the requirements of the standard in relation to two reference strains, *S. aureus* ATCC 6538 and *E. hirae* ATCC 10541, after 15 min of contact time. The required biocidal activity was
observed after 60 min of contact time for the reference strains (except *E. hirae*) and after 15 min for all clinical isolates. The Department of Pharmaceutical Bacteriology at the University of Medical Sciences in Poznań conducted a study to test the antimicrobial activity of the product Cholinex (14). The study was performed according to the recommendations of PN-EN 1040 and PN-EN 1275 by the suspension method, the same as that used in this study. The results of Muszynski and Mirska (14) showed that the product Cholinex was the most active, meeting the requirements of the standards against *Streptococcus pyogenes*. The weakest bactericidal activity of Cholinex was demonstrated against *S. aureus* and *P. aeruginosa* with reduction rates below the requirements of the standard. The results obtained by Muszynski and Mirska (14) were confirmed by our study for *S. aureus* and *P. aeruginosa* in the test performed at 30°C with 15 min of contact time. The research conducted in this study showed that the reduction in the number of bacteria in the tests carried out with the products Halset, Menthosept and Septolete D was too small for these products to be considered effective antiseptics. Interactions between excipients and cetylpyridinium chloride, which is the active substance of the above products, may have reduced the biocidal activity, as reported by Richards et al. (15). They found that the antimicrobial activity of cetylpyridinium chloride is stronger in tablets consumed like a candy, than other forms of tablets. Magnesium stearate, used as a lubricant in some types of tablets, absorbs cetylpyridinium chloride. A content of magnesium stearate less than 0.3% in tablets (by weight) does not influence the antimicrobial activity of the product. Our research concerning products containing cetylpyridinium chloride confirmed the results of Richards (15). There is no information in the leaflets of Halset, Menthosept and Septolete D about the weight content of excipients and therefore it is impossible to determine whether the minimal bactericidal effect of these products is caused by the interaction of magnesium stearate with cetylpyridinium chloride.

The product Propolki, containing the propolis extract (a natural bee product) was tested with a contact time 15 min at 30°C, and exhibited standard antibacterial properties against all clinical and reference strains, except *E. coli* NCTC 10538. The healing properties of propolis have been known for a long time. In the literature, information can be found that propolis at a concentration of 3 mg/mL completely inhibits the growth of *S. aureus* (including MRSA), *S. epidermidis*, *Enterococcus* spp, *Corynebacterium* spp, and *B. cereus*. However, the growth of *P. aeruginosa* and *E. coli* is only partly inhibited (16). In the 1970s, higher activity of propolis against Gram-positive than Gram-negative bacteria was confirmed (17).

Gargarin, an oral product available on the market in the form of a powder for preparing a suspension for mouthwashing, prepared according to the manufacturer’s instructions, did not possess the desired bactericidal activity.

The product Strepsils, despite the lack of bactericidal activity required by PN-EN 1040:2006 against *S. aureus* ATCC 6538 at 30°C, showed good activity against the tested clinical streptococcal isolates. A study by McNally et al. demonstrated the in vivo analgesic activity of Strepsils in cases of acute throat infection (18).

The results of this study, as well as already published data (1), show that a number of the products for mouth disinfection available on the Polish market are not fully effective when tested according to appropriate standards (PN-EN). More effective biocidal activity was observed against clinical isolates than against reference bacterial strains. Taking this into account, registration trials of medicinal products should investigate the actual biocidal activity, and test methods should be harmonized. There is also the need to verify the declaration of the manufacturers concerning the antimicrobial activity of their products, in order to remove from the market products which do not meet the requirements. It should be remembered that products used in oral cavity infections, apart from their microbicidal activity, may also have anti-inflammatory and analgesic activity. All information concerning the activities of these products should be specified in the leaflet.

Due to the problem of insufficient antimicrobial activity of antiseptic products on the market, experts of the European Pharmacopeia Committee decided to establish an appropriate monograph, titled “Determination of bactericidal, fungicidal or yeasticidal activity of antiseptic medicinal products”, which will hopefully force manufacturers to perform tests evaluating the antimicrobial activity of antiseptic products. This monograph number 5.1.11 will be published in one of the supplements of Ph. Eur. 9 edition.

**CONCLUSIONS**

Only some of the tested medicinal products for mouth disinfection showed antimicrobial activity, required by the standard PN-EN 1040:2006.

Clinical isolates of streptococci were found to be more susceptible to products for mouth disinfection than the reference strains.
A favorable feature of the tested products is their more effective antimicrobial activity at a temperature of 36°C.

Antimicrobial activity declared by the manufacturers on the package or on the leaflets does not include accurate information.

It is necessary to introduce a more efficient control system of determining the antimicrobial activity of the products before their registration.

REFERENCES

2. Standard PN-EN 1040:2006 Chemical disinfectants and antiseptics – Quantitative suspension test for the evaluation of basic bactericidal activity of chemical disinfectants and antiseptics – Test method and requirements (phase 1).
5. Regulation of the Minister of Health on 11 August 2005 on defining groups of medicinal products and the requirements for documentation of test results of these products (2005-08-24, No. 160, item. 1358).

Received: 1. 08. 2016
Cystic fibrosis (CF) is a rare disease (1) and its prevalence in Poland is around 1 : 5000, and adult patients comprise as little as 32% of all patients suffering from this disease. CF treatment is based on a properly selected and effective pharmacotherapy. The aim of this study was to understand the regimen of pharmacotherapy in adult CF population in Poland, and to calculate the costs of drugs used in this group of patients. The researchers conducted a retrospective study. Following an analysis of the patients’ medical documentation, 46 adult patients were included in the study group. Data were sourced from case history forms, doctors’ order sheets and medical treatment records made by physicians. Total pharmacotherapy cost in 2013 amounted to EUR 467876.66 giving EUR 10171.23 (±2818.39) per patient. The most popular group of drugs used by CF patients was mucolytic drugs, with the estimated cost of EUR 382054.21. Only 14 patients were treated with antibiotics. The total cost of using antibiotics in these 14 patients was estimated at EUR 15393.01. Analysis of the treatment regimen for adult CF patients has shown that treatment regimen of Polish patients differed from the regimen applied in another countries.

Keywords: cystic fibrosis; costs of pharmacotherapy; regimen of cystic fibrosis treatment; treatment of cystic fibrosis

Cystic fibrosis (CF) is a rare disease (1) and its prevalence in Europe is estimated at 1:8000-10000 cases (2). Prevalence of cystic fibrosis in Poland is approximately 1 : 5000 (3) while the register kept by the Polish Cystic Fibrosis Society (PTM) had 1518 CF patients recorded at the end of September 2010, of which 32.3% where adult patients (4). This low prevalence of cystic fibrosis among adults makes their treatment more difficult because, according to the data of the Ministry of Health, in Poland there are only two centers partially meeting the criteria for care of adult CF patients, and these centers have major limitations in ensuring effective care over such patients. CF treatment is based on a properly selected and effective pharmacotherapy (5). In order to support lung function, pharmaceuticals such as bronchodilators, mucolytic agents, or anti-inflammatory drugs need to be used. Oral, inhaled, or intravenous antibacterial therapy is of special importance for patients who have problems with chronic bacterial colonization of the lungs and airways. In case of pancreatic insufficiency, digestive enzymes have to be substituted with every meal. Furthermore, patients frequently require additional vitamin supplementation as well as high-calorie food (5, 6). Properly managed pharmacotherapy and diagnostics are the major basic factor affecting the patient’s survival, contributing to improvement of their comfort and quality of life (7, 8). Studies report that effective drugs have increased average life expectancy for patients with cystic fibrosis to a median survival of 37.4 years (9). Unfortunately, although effective pharmacotherapy affects survival chances of CF patients, it is suggested that Polish patients still have a difficult access to innovative and effective CF treatment. Currently, the National Health Fund offers CF patients only therapeutic health programs using tobramycin to treat chronic pulmonary infections (10) but only for patients with a documented intolerance of colistin – a cheaper and less effective antibiotic.

Given the large number of new treatments being developed for CF and related complications, it is critical to consider the economic costs associated
with CF care. Numerous cost of pharmacotherapy studies have been conducted over the past decades across a range of diseases, however few have addressed rare diseases, especially cystic fibrosis. Moreover, as cystic fibrosis is usually considered a pediatric complaint, in this context the aim of this study was to understand the regimen of pharmacotherapy in adult CF population in Poland, and to calculate the costs of drugs used in this group of patients.

**EXPERIMENTAL**

**Study population**

The researchers conducted a retrospective study. Having analyzed medical documentation of patients, they included in the study a group of 46 adults patients (28 female, 18 male) treated in 2013 by a team of pulmonologists from one of Poznań hospitals. The study population was 100% of the adult population treated in the adopted time period. Inclusion criteria for the study: a) age above 18, b) cystic fibrosis diagnosed in accordance with ICD-10, c) regular pulmonologist supervision between January and December 2013.

**Calculation of costs**

Calculation of pharmacotherapy costs included pharmacotherapy for both in- and out-patients. Data were sourced from case history forms, doctors’ order sheets and medical treatment records made by physicians, specifying the name of the medication, its dosage and pharmaceutical form. As the medications are mostly used on a long-term basis, use of the resources was evaluated using the largest packaging sizes available. Prices of originator medicines were used to calculate the costs of active substances taken by the patients. Prices were sourced from the 2013 Galenica Pnax medicines wholesale price list. All amounts are expressed in Euros according to the average exchange rate table of the National Bank of Poland of 02 JAN 2013 (exchange rate: EUR 1.00 = PLN 4.0671 ) (11). Study time horizon was 1 calendar year (01/01/2013 – 31/12/2013). The study received approval of the Bioethics Committee of the University of Medical Sciences in Poznań.

**RESULTS**

The mean age of the study group patients was 27. The youngest study subject was 19 and the eldest 42 years old. Most of the study subject were non-Poznań residents (n = 42) with only 4 patients being Poznań residents (Table 1).

A total of 322 pharmaceutical records from CF patients were collected. Total pharmacotherapy cost in 2013 amounted to EUR 467876.66 giving EUR 10171.23 (± 2818.39) per patient. Pharmacotherapy costs amounted to 52% of total costs (EUR 900729.78 ± 19581.08).

The most popular group of drugs used by CF patients was mucolytic agents (Table 2), of which 89% of the patients were treated with dornase alpha and 57% with ambroxol (Table 2). Total annual cost of dornase alpha usage was estimated at EUR 380,322.80 giving EUR 9,276.16 per patient (Table 3). Total cost of ambroxol treatment was much lower at EUR 1337.27 (EUR 51.43 per patient on average). Total cost of mucolytic agents was estimated at EUR 382054.21 (EUR 5233.62 per patient on average) (Table 3). Pancreatin, being a pancreatic enzyme, was also popular (85%) (Table 2). Average cost of pancreatin treatment was EUR 540.28 (Table 3). On the other hand, total annual cost of bronchodilatory treatment was estimated at EUR 10393.84 giving EUR 176.17 per patient (Table 3). 65% of the patients were treated with short-acting inhalation anti-

<table>
<thead>
<tr>
<th>Variables</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients (n)</td>
<td>46</td>
</tr>
<tr>
<td>Female/Male (n)</td>
<td>28/18</td>
</tr>
<tr>
<td>Mean age in years (± SD)</td>
<td>27.4 (8.01)</td>
</tr>
<tr>
<td>Youngest/oldest, in years (± SD)</td>
<td>19 (7.2)/42 (10.01)</td>
</tr>
<tr>
<td>Poznań residents / Non-Poznań residents (n)</td>
<td>4/42</td>
</tr>
<tr>
<td>Average number of days at the hospital ward (± SD)</td>
<td>27.8 (10.01)</td>
</tr>
<tr>
<td>Average number of outpatient visits (± SD)</td>
<td>2.74 (1.48)</td>
</tr>
</tbody>
</table>

SD - standard deviation
Table 2. Active substances most frequently used in CF treatment.

<table>
<thead>
<tr>
<th>Therapeutic group*</th>
<th>Active substance</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibiotics</td>
<td>Azithromycin</td>
<td>1 (2)</td>
</tr>
<tr>
<td></td>
<td>Colistin</td>
<td>13 (28)</td>
</tr>
<tr>
<td>Mucolytic agents</td>
<td>Dornase α</td>
<td>41 (89)</td>
</tr>
<tr>
<td></td>
<td>Ambroxol hydrochloride</td>
<td>26 (57)</td>
</tr>
<tr>
<td></td>
<td>Acetylcysteine</td>
<td>6 (13)</td>
</tr>
<tr>
<td>Bronchodilators</td>
<td>Fenoterol hydrobromide</td>
<td>24 (52)</td>
</tr>
<tr>
<td></td>
<td>Salmeterol</td>
<td>7 (15)</td>
</tr>
<tr>
<td></td>
<td>Ipratropium bromide</td>
<td>30 (65)</td>
</tr>
<tr>
<td></td>
<td>Formoterol fumarate</td>
<td>8 (17)</td>
</tr>
<tr>
<td></td>
<td>Salbutamol</td>
<td>4 (9)</td>
</tr>
<tr>
<td>Glicocorticosteroids</td>
<td>Ciclesonide</td>
<td>20 (44)</td>
</tr>
<tr>
<td></td>
<td>Budesonide</td>
<td>13 (28)</td>
</tr>
<tr>
<td>Pancreatic enzymes</td>
<td>Pancreatin</td>
<td>39 (85)</td>
</tr>
<tr>
<td>Non-steroid anti-inflammatory drugs</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Vitamins and supplements</td>
<td>Phytomenadione</td>
<td>10 (22)</td>
</tr>
<tr>
<td></td>
<td>L-ornithin L-aspartate</td>
<td>8 (17)</td>
</tr>
<tr>
<td></td>
<td>β-Carotene</td>
<td>6 (13)</td>
</tr>
<tr>
<td></td>
<td>Retinol</td>
<td>5 (11)</td>
</tr>
<tr>
<td></td>
<td>Tocopherol</td>
<td>4 (9)</td>
</tr>
<tr>
<td></td>
<td>Calcium carbonicum</td>
<td>2 (4)</td>
</tr>
<tr>
<td></td>
<td>Cholecalciferol</td>
<td>1 (2)</td>
</tr>
<tr>
<td>Concomitant diseases and GI complications</td>
<td>Ursodeoxycholic acid</td>
<td>22 (47)</td>
</tr>
<tr>
<td></td>
<td>Omeprazole</td>
<td>21 (46)</td>
</tr>
<tr>
<td></td>
<td>Phospholipids</td>
<td>10 (22)</td>
</tr>
<tr>
<td></td>
<td>Mebeverine hydrochloride</td>
<td>6 (13)</td>
</tr>
<tr>
<td></td>
<td>Alendronic acid</td>
<td>5 (11)</td>
</tr>
<tr>
<td></td>
<td>Rupatadine</td>
<td>4 (9)</td>
</tr>
<tr>
<td></td>
<td>Ivabradine</td>
<td>3 (7)</td>
</tr>
<tr>
<td></td>
<td>Spironolactone</td>
<td>2 (4)</td>
</tr>
<tr>
<td></td>
<td>Silybum marianum fruit extract</td>
<td>2 (4)</td>
</tr>
<tr>
<td></td>
<td>Theophylline</td>
<td>2 (4)</td>
</tr>
<tr>
<td></td>
<td>Trimebutine</td>
<td>2 (4)</td>
</tr>
<tr>
<td></td>
<td>Bisoprolol fumarate</td>
<td>2 (4)</td>
</tr>
<tr>
<td></td>
<td>Fluticasone furoate</td>
<td>2 (4)</td>
</tr>
<tr>
<td></td>
<td>Losartan potassium</td>
<td>1 (2)</td>
</tr>
<tr>
<td></td>
<td>Human insulin</td>
<td>1 (2)</td>
</tr>
</tbody>
</table>

*therapeutic groups isolated according to the guidelines of the Polish Cystic Fibrosis Society (18).

cholinergic agents with ipratropium bromide as the active substance, with the total annual cost estimated at EUR 4984.91. Fenoterol hydrobromide was equally frequently used by the patients (52%), with the estimated per patient cost of EUR 168.10 (Table 3).

Only 14 patients were treated with antibiotics. 13 patients (28%) were treated with an antimicrobial peptide – colistin and one with a macrolidic antibiotic for oral use – azithromycin (Table 2). Average cost of colistin was EUR 1134.74, and the cost of azithromycin EUR 641.33. The total cost of using
antibiotics in these 14 patients was estimated at EUR 15393.01 (Table 3).

Glycocorticosteroids were used by 71% of patients (Table 2). The total cost of using glycocorticosteroids was EUR 13108.52, (EUR 397.23 on average). The most commonly used glycocorticosteroid was ciclesonide (43%) and budesonide (40%) (Table 3). The annual cost of using glycocorticosteroids was EUR 13108.52 (Table 3). CF patients were also taking a number of drugs used to treat concomitant diseases as well as vitamins supporting CF treatment. The most commonly used vitamins and supplements were phytoenadione (22%), l-ornithine l-aspartate (17%), as well as β-carotene (13%) (Table 2). Vitamin E (tocopherol) was used by 9% of patients, calcium by 4%, while vitamin D by as few as 2% of patients (Table 2). Of drugs used to treat concomitant diseases and CF complications, the patients most frequently used omeprazole (46%) which inhibits secretion of hydrochloric acid in the gastric acid, phospholipids used in hepatic therapy, and a number of other substances strengthening the respiratory, gastrointestinal, skeletal, and cardiovascular systems (Table 2).

**DISCUSSION**

To our knowledge, this is the first study attempting to estimate costs of pharmacotherapy of adult patients with cystic fibrosis in Poland. No reference on the cost of CF treatment in Poland has been found. Perhaps this small interest of researchers in cystic fibrosis is due to the fact that it is rare, and thus it is difficult to find the patients and assess the costs of their disease. The conducted analysis assumed that the study group would comprise adult patients which posed an extra difficulty for the researchers because – as CF is associated with death at a very young age – the disease is usually considered a pediatric problem. Therefore, the age criterion adopted in the study (> 18) was a strong barrier in collecting a large study population.

### Table 3. Calculation of pharmacotherapy costs in CF patients.

<table>
<thead>
<tr>
<th>Therapeutic group</th>
<th>Active substance</th>
<th>Number of patients (%)</th>
<th>Total annual substance cost [EUR]</th>
<th>Average cost per patient [EUR]</th>
<th>Total cost of the substance [EUR]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mucolytic agents, expectorants</td>
<td>Domase α</td>
<td>41 (89)</td>
<td>380322.80</td>
<td>9276.16</td>
<td>382054.21 (5233.62)</td>
</tr>
<tr>
<td></td>
<td>Ambroxol hydrochloride</td>
<td>26 (56)</td>
<td>1337.27</td>
<td>51.43</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Acetylcysteine</td>
<td>6</td>
<td>394.14</td>
<td>98.54</td>
<td></td>
</tr>
<tr>
<td>Pancreatic enzymes</td>
<td>Ancreatin</td>
<td>39 (84)</td>
<td>21071.00</td>
<td>540.28</td>
<td>21071.00 (540.28)</td>
</tr>
<tr>
<td>Bronchodilators</td>
<td>Fenoterol hydrobromide</td>
<td>24 (52)</td>
<td>4034.33</td>
<td>168.10</td>
<td>10393.84 (176.17)</td>
</tr>
<tr>
<td></td>
<td>Ipratropium bromide</td>
<td>30 (65)</td>
<td>4984.91</td>
<td>166.16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Salmeterol</td>
<td>5</td>
<td>1374.60</td>
<td>274.92</td>
<td></td>
</tr>
<tr>
<td>Glycocorticosteroids</td>
<td>Ciclesonide</td>
<td>20 (43)</td>
<td>7619.65</td>
<td>380.98</td>
<td>13108.52 (397.23)</td>
</tr>
<tr>
<td></td>
<td>Budesonide</td>
<td>13 (28)</td>
<td>5488.87</td>
<td>422.22</td>
<td></td>
</tr>
<tr>
<td>Antibiotics</td>
<td>Colistimethate sodium</td>
<td>13 (28)</td>
<td>14751.68</td>
<td>1134.74</td>
<td>15393.01 (1099.50)</td>
</tr>
<tr>
<td></td>
<td>Azithromycin</td>
<td>1 (2)</td>
<td>641.33</td>
<td>641.33</td>
<td></td>
</tr>
<tr>
<td>Concomitant diseases and GI</td>
<td>Ursodeoxycholic acid</td>
<td>22 (47)</td>
<td>5470.65</td>
<td>248.66</td>
<td></td>
</tr>
<tr>
<td>complications</td>
<td>Omeprazole</td>
<td>17 (37)</td>
<td>1315.1</td>
<td>77.36</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phytomenadione</td>
<td>10 (22)</td>
<td>295.41</td>
<td>29.54</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ornithine (ornithine aspartate) + vitamins</td>
<td>8 (17)</td>
<td>631.00</td>
<td>78.88</td>
<td></td>
</tr>
<tr>
<td></td>
<td>β-Carotene + vitamins</td>
<td>6 (13)</td>
<td>181.16</td>
<td>30.19</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mebeverine hydrochloride</td>
<td>6 (13)</td>
<td>1194.59</td>
<td>199.10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phospholipids</td>
<td>6 (13)</td>
<td>752.30</td>
<td>125.38</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Retinol</td>
<td>5 (11)</td>
<td>42.80</td>
<td>8.56</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rupatadine</td>
<td>4 (9)</td>
<td>377.43</td>
<td>94.36</td>
<td></td>
</tr>
</tbody>
</table>
The study included 46 adult patients who were 100% of the adult population treated at the Poznań hospital in 2013.

The present study addresses several important issues usually overlooked by researchers. Most of foreign references concerning costs of treatment of CF patients contains only general pharmacotherapy costs. The studies specify either average or total pharmacotherapy cost, while the costs of individual drug types are usually either not specified or the authors focus only on those most recommended and most frequently used by CF patients. The present study, in addition to estimating the annual pharmacotherapy cost, analyses treatment regimen in detail, taking into account the most frequently used active substances.

It has been assessed that in spite of the low prevalence rate, cystic fibrosis is a major economic problem. It has been confirmed that pharmacotherapy cost was a particular burden from the economic perspective. In the Polish study, this cost was assessed in 2013 at EUR 467876.66 (mean: EUR 10171.23), making up 52% of the total costs. A similar structure of CF treatment costs observed in a study by Huot and co-authors (12) where pharmacotherapy costs made up 45% of the total costs. In the study by Chevreul et al. (13), average pharmacotherapy cost of adult patients was lower than in the Polish study and amounted to EUR 8277.00. Such a large difference in the value of estimated costs may result from the differences in study methodologies. Chevreul sourced the unit cost of drugs from Statutory Health Insurance tariffs, while the Polish study assessed pharmacotherapy cost based on the wholesale drug price list.

In the study by Horvais et al. (14), average pharmacotherapy cost of a CF patient was similar to the value estimated by Chevreul and co-authors and amounted to nearly EUR 8000.00. Study by Dewitt et al., in turn, had a much larger part attributed to drug costs (15). This is not surprising as drugs are notoriously more expensive in the US than in Europe. In the US study, costs were assigned to 39 different types of concomitant medications and averaged $33394.00 per participant. These medications accounted for more than 86% of total direct medical costs, with dornase α accounting for 41.5%, inhaled tobramycin accounting for 21.3%, and pancreatic enzymes accounting for 21.6% of the estimated cost for concomitant medications. In the study conducted in Poland, dornase α was used to treat 89% of the patients, while pancreatin for 85%. These drugs were one of the most frequently used in therapy of CF patients in the United Kingdom (16). Total cost of dornase α treatment in the Polish study amounted to 42% of total costs of CF treatment which corroborated with the results obtained by Johnson et al. (17) where dornase α treatment accounted for 44% of the total costs.

In the study by Huot et al. (12) conducted in France in 2000 and 2003, dornase α was used by as few as 32% of the patients in 2000 and 42% in 2003. The difference in the share of patients taking this drug may result from the fact that the details of pharmacotherapy was derived from various sources. Data on pharmacotherapy conducted in 2000 were derived from questionnaires filled in by the patients while cost calculation for pharmacotherapy conducted in 2003 was based on the NHI system. Results of another French study corroborated with reports of Huot et al. from 2000 and also show that only 31% of CF patients were treated with dornase α (14).

Although in accordance with scientific reports antibiotics are recommended in cystic fibrosis treatment (18, 19), only 30% in the Polish study used them, and similarly in the study (17) antibiotics were used by 44% of the patients. The use of antibiotics in CF treatment is much more popular in the United Kingdom (16) and in France where oral antibiotics were used by 84% of the patients in 2003 (12). This small use of antibiotics in CF treatment in Poland may be due to their high cost (20). Moreover, use of treatment regimen based on only two antibiotics proves limited access of Polish patients to state-of-the-art medicines recommended by international drug agencies, such as ivacafitor (Kalydeco) (5, 21) which is the first drug for cystic fibrosis with a marketing authorization which acts on the cause of the disease and increases activity of non-functional trans-membrane channels in patients with G551D mutation or 8 other gating mutation types [22].

The study has also shown that many patients had concomitant diseases and CF complications. Analysis of pharmacotherapy used has shown that the patients usually used drugs for gastrointestinal symptoms (including gastroesophageal reflux, drugs used to treat and prevent gastric and duodenal ulcers), and drugs used in hepatic disorders. According to the reports of the Polish Cystic Fibrosis Society concerning the rules of diagnosing and treating CF, these organs are most frequently affected by CF complications (18).

Moreover, consumption of vitamins K, D, and Ca may be a sign of problems with skeleto-articular system which are common in CF patients (23, 24). Poland is currently taking efforts to implement the European Cystic Fibrosis Standards of Care. The
key issue raised nowadays by CF patients, their families, and their organizations, is the limited access to state-of-the-art medicines due to lack of reimbursement by state authorities. Monthly cost of treatment including medicines, nutrients, and antibiotics in case of frequently recurring infections, amounts approximately to a few thousand PLN. The greatest challenge for Poland at present is to introduce full reimbursement of costs incurred for cystic fibrosis treatment.

Countries with GDP similar to Poland provide CF patients with a much better access to treatment. To give an example of this, in the present study only 13 patients were treated with antibiotics. In Poland, the first-line drug is colistin iv (the patient pays a lump sum) while azithromycin — reimbursed at 50% rate up to the financing limit — is the second-line drug. The only inhalation antibiotic available in the National Health Fund’s drug program is tobramycin (25). Unfortunately, this program is very limited and thus access to inhalation antibiotic therapy is very limited as well, as confirmed by the results of the present study showing that none of the patients included in the study participated in the said drug program or received tobramycin treatment.

CONCLUSION

Standards of treatment and care of adult CF patients have improved significantly in the recent years, however the burden associated with CF therapy remains high and, moreover, keeps increasing every year (26). Analysis of the treatment regimen for adult CF patients has shown that treatment regimen of Polish patients differed from the regimen applied in another countries. Antibiotics are used much more frequently in foreign patients. Although chronic inhalation antibiotic administration is indicated in persistent infection of respiratory tract by Pseudomonas aeruginosa, and their efficacy in bacterial eradication has been repeatedly documented in studies and clinical practice, as few as 30% of the Polish patients used antibiotics, none of which used tobramycin. This may be a sign of hindered access of these patients to treatment.

Cystic fibrosis remains an untreatable disease, although numerous studies are being conducted to find new methods of treating disease symptoms or even eliminate its causes. Owing to the development of molecular techniques and understanding of pathophysiological mechanisms responsible for development of cystic fibrosis, therapeutic strategies can be continuously extended and improved, and the greatest hopes are set on gene therapy (27, 28).

No conflicts of interest were reported.

REFERENCES


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In contemporary healthcare, there is a strong need to create a professional culture which would fuel expectations towards the development and practice of interprofessional collaboration, especially among pharmacists and physicians (1). Coluccio and Maguire defined professional collaboration as “joint communicating and decision making process with the goal of satisfying the patient’s wellness and illness needs while respecting the unique qualities and abilities of each professional” (2). According to FIP/WHO, the relationship between pharmacists and other healthcare professionals involved in patient care, especially physicians, “should be established as a therapeutic collaborative partnership that involves mutual trust and confidence in all matters relating to pharmacotherapy” (3). The concept of pharmaceutical care is intended to foster closer collaboration between healthcare professionals engaged in patient management (4). According to the Polish definition, pharmaceutical care is “a documented process where a pharmacist, in collaboration with patients, physicians and, where necessary, other healthcare professionals, monitors the proper course of pharmacotherapy to achieve specific outcomes that improve patient’s quality of life” (5). However, pharmaceutical care as such has been neither generally known (6), nor practiced in pharmacy settings in Poland. Also, it seems that pharmacists and physicians in Poland play separate roles and do not collaborate (7).

The study objective was to describe the pharmacists’ interactions with physicians and the interrelation between selected factors determining the pharmacist-physician relationship from the perspective of a community pharmacist. The self-administered questionnaire contained questions about interprofessional interactions, as well as types of, limiting factors and general assessment of the pharmacist-physician relationship. Four constructs were used as latent variables: co-responsibility (4 items), collaboration (3 items), pharmacist behavior (2 items), and GP behavior (1 item). A total of 1496 pharmacists took part in the study. Hypothetical relationships between observable variables and/or latent variables were analyzed with structural equation modeling. Pharmacists and physicians in Poland are isolated in their interprofessional relationship, with physicians in the dominant position, and pharmacists uninvolved in patient care. Communication with physicians is one-sided, mostly over the phone, and concerns only formal issues. The perceived co-responsibility for drug therapy outcomes prompts pharmacists to consult physicians on medication therapy related issues, and therefore needs to be enhanced.

**Keywords**: interprofessional interactions, pharmacists, physicians, Poland

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maceutical care delivered by the Centre of Postgraduate Training of the Medical University of Warsaw, Faculty of Pharmacy. The course was addressed to pharmacists working at pharmacies, dispensaries, and pharmaceutical wholesalers. The course was a one-day symposium. The course was delivered free of charge and was open to all eligible pharmacists, conditional on prior registration. The course was delivered according to the same curriculum and by the same lecturers in 16 different cities across Poland. Continuing education of pharmacists is compulsory in Poland and this particular symposium was heavily attended by community pharmacists. It did not cover the area of four Regional Pharmaceutical Chambers (Środkowopomorska, Kaliska, Beskidzka and Częstochowska Regional Pharmaceutical Chambers, 20% of total chambers). Inclusion criteria for the study participants were as follows:

- licensed pharmacists working at community pharmacies,
- licensed pharmacists working at dispensaries,
- non-pharmacists, including owners of pharmacies who did not hold the Masters degree in pharmacy, students of pharmacy and pharmacy technicians,
- incomplete data (missing response to more than 20% of scale items).

Survey

The questionnaire consisted of two parts and demographic characteristics. The first part contained questions on pharmacist’s interactions with physicians. The second related to pharmacist’s interactions with patients and will be referred elsewhere.

The survey instrument contained questions concerning the frequency of interprofessional relationships; channels of interprofessional communication; issues discussed with physicians; respondents’ personal opinion about the type of pharmacist-physician relationships, general evaluation of their relationships with physicians, and the factors which hamper their relationships with physicians.

In order for pharmaceutical care to succeed, pharmacists would have to accept co-responsibility for medication therapy outcomes and the perceived collaboration with physicians, both of them depending on physician behavior and influencing pharmacist behavior. Four constructs developed specifically for this study were used as latent variables. Construct co-responsibility (COR) was made up of 4 items, and collaboration (COL) covered by 3 items. These included statements referring to pharmacists’ views and opinions concerning their involvement in medication therapy and interprofessional relationships with physicians. The third construct (pharmacist behavior, BEH) was based on 2 items made up of statements which referred to pharmacists’ contribution to medication therapy. The fourth construct (general practitioner behavior, GP-BEH) relied on a single item which described GP behavior (initiating interaction with a pharmacist concerning medication therapy-related issues). A 5-point Likert scale was applied.

Co-responsibility

Pharmacist co-responsibility (COR) for patient medication therapy was defined as the personal beliefs on the perceived causative power and responsibility for medication therapy outcomes.

Collaboration

In this study, pharmacist-physician collaboration (COL) was defined as beliefs on co-dependence of medication therapy outcomes, safety and efficacy on the joint efforts of GP and pharmacist.

Pharmacist behavior

Two specific pharmacist behaviors (BEH) having significant effect on medication therapy represented this construct: reporting identified medication-related problems to physicians.

GP behavior

Initiating contact with pharmacists to discuss controversial medication therapy-related issues was the GP behavior (GP-BEH) selected for this analysis.

Data analysis

STATISTICA 10 software was applied for statistical analysis. Structural equation modelling (SEM) with LISREL 8.80 was performed by an external service provider.

RESULTS

Of 1722 completed questionnaires (response rate: 38.0%), 226 were rejected as incomplete or
non-compliant with the inclusion criteria. 1496 responses were qualified for further analysis. Sociodemographic characteristics of respondents is provided in Table 1. As compared to the Central Statistical Office in Poland data (8), the percentage share of female respondents was slightly higher than in the general population of pharmacists at large (88% vs. 85%), and the percentage share of pharmacy managers was lower (42% vs. 48%). The sample size was large enough to determine whether the results were influenced by sociodemographic factors; however, no statistically significant differentiation attributed to these factors was demonstrated (p < 0.05).

Pharmacist-physician interactions
Interactions between pharmacists and physicians are generally initiated by pharmacists, mostly over the phone, and typically concern formal issues, such as prescription illegibility or prescribing errors (n = 1337, 89.4%). Pharmacists claim that physicians contact them mainly to consult formal issues, such as the prices or availability of medicinal products (n = 333, 22.3%). Significantly fewer respondents indicated medication therapy-related issues as the reason for initiating pharmacist-physician interactions. If this is the case, pharmacists contact physicians to discuss medication therapy problems they have identified (n = 275, 18.4%). In those very rare cases where physicians initiate interaction with pharmacists for medication therapy-related reasons (n = 67, 4.5%), they do it to consult pharmacists on drug indications and drug action. In medication therapy, physicians play the dominant role (54.2%); pharmacists and physicians in Poland do not collaborate (66.3%). 3.50 ± 0.82 was the mean score in the

<table>
<thead>
<tr>
<th>Independent variables</th>
<th>n = 1496 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>40 ± 11 years</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>1312 (87.7)</td>
</tr>
<tr>
<td>Male</td>
<td>184 (12.3)</td>
</tr>
<tr>
<td>Years in practice</td>
<td></td>
</tr>
<tr>
<td>≤ 5</td>
<td>445 (30.1)</td>
</tr>
<tr>
<td>6-10</td>
<td>201 (13.6)</td>
</tr>
<tr>
<td>11-15</td>
<td>202 (13.6)</td>
</tr>
<tr>
<td>16-20</td>
<td>219 (14.8)</td>
</tr>
<tr>
<td>&gt; 20</td>
<td>413 (27.9)</td>
</tr>
<tr>
<td>Job position *</td>
<td></td>
</tr>
<tr>
<td>Pharmacist (with a Masters degree in pharmacy)</td>
<td>1496 (100.0)</td>
</tr>
<tr>
<td>Pharmacy manager</td>
<td>630 (42.1)</td>
</tr>
<tr>
<td>Pharmacy owner</td>
<td>156 (10.4)</td>
</tr>
<tr>
<td>Other job position</td>
<td>2 (&lt; 0.1)</td>
</tr>
<tr>
<td>Pharmacy setting</td>
<td></td>
</tr>
<tr>
<td>Rural area</td>
<td>115 (7.7)</td>
</tr>
<tr>
<td>Urban area of up to 20 000 inhabitants</td>
<td>279 (18.6)</td>
</tr>
<tr>
<td>Urban area of 20 000 to 100 000 inhabitants</td>
<td>365 (24.4)</td>
</tr>
<tr>
<td>Urban area of 100 000 to 500 000 inhabitants</td>
<td>291 (19.5)</td>
</tr>
<tr>
<td>Urban area of over 500 000 inhabitants</td>
<td>431 (28.8)</td>
</tr>
<tr>
<td>No response</td>
<td>15 (1.0)</td>
</tr>
<tr>
<td>Pharmacy</td>
<td></td>
</tr>
<tr>
<td>Independent pharmacy</td>
<td>913 (61.0)</td>
</tr>
<tr>
<td>Chain pharmacy</td>
<td>500 (33.4)</td>
</tr>
<tr>
<td>Other type of pharmacy</td>
<td>83 (5.5)</td>
</tr>
</tbody>
</table>

* Answers do not sum up to 100% as multiple answers were possible.
evaluation of pharmacists’ past experience of relationships with physicians. Interactions between pharmacists and physicians are presented in Table 2.

Participants were asked to choose three factors which they found to affect their professional relationships with physicians most. According to respondents, the circumstances in which the interaction is established (e.g., illegibility or prescription errors) carry the highest risk of conflict or misunderstanding (51.0%). Table 3 lists these factors in the descending order by score.

### Study constructs

Hypothetical relationships between observable variables and/or latent variables were analyzed with the structural equation modelling (Fig. 1). All relationships between observable and latent variables were confirmed to be statistically significant. The model indicates that there exists a strong relationship between all of the analyzed latent variables. The percentage distribution of responses is provided in Table 4.

| Table 2. Interactions between pharmacists and physicians (n = 1496). |
|-------------------------------------------------------------|-----------------|-----------------|
| **Statement**                                               | **Number of responses** | **Percent share** |
| Frequency of pharmacist-physician interactions initiated by pharmacists |                         |                 |
| At least once a week                                       | 593              | 39.6%           |
| At least once a month                                      | 449              | 30.0%           |
| Few times a year or less often                             | 415              | 27.7%           |
| Never                                                      | 39               | 2.6%            |
| Frequency of pharmacist-physician interactions initiated by physicians |                       |                 |
| At least once a week                                       | 322              | 21.5%           |
| At least once a month                                      | 357              | 23.9%           |
| Few times a year or less often                             | 581              | 38.8%           |
| Never                                                      | 236              | 15.8%           |
| Communication channels                                      |                   |                 |
| Phone                                                      | 1076             | 71.9%           |
| Direct contact (face-to-face)                              | 572              | 38.2%           |
| Patient-mediated                                           | 335              | 22.4%           |
| Nurse-mediated in an outpatient setting                    | 137              | 9.2%            |
| Types of pharmacist-physician relationships                |                   |                 |
| Physician plays the dominant role, pharmacist plays an important supportive role | 311              | 20.8%           |
| Physician plays the dominant role by prescribing drugs to patients, pharmacist only passively dispenses the prescribed drugs | 499              | 33.4%           |
| Physician and pharmacist are equal partners, their work is based on collaboration | 194              | 13.0%           |
| Physician and pharmacist are equal in their professional status, but they do not collaborate | 492              | 32.9%           |
| Evaluation of relationships with physicians                 |                   |                 |
| Very good                                                  | 79               | 5.3%            |
| Good                                                       | 781              | 52.2%           |
| I cannot tell for sure                                     | 473              | 31.6%           |
| Bad                                                        | 132              | 8.8%            |
| Very bad                                                   | 28               | 1.9%            |
| No response                                                | 3                | 0.2%            |

* Answers do not sum up to 100% as multiple answers were possible.
Weak to medium correlations were found to exist between individual items belonging to different constructs (from 0.1 to 0.5), but they were statistically very significant (p < 0.0001).

The pharmacist behavior construct has a strong direct positive impact on the GP behavior construct (β = 0.69, p < 0.05), GP behavior directly influences the co-responsibility construct (β = 0.10, p < 0.05), whereas co-responsibility exerts direct influence on pharmacist behavior (β = 0.39, p < 0.05), which means GP behavior has a positive indirect impact on pharmacist behavior. The influence of the latent variable of collaboration is not statistically significant.

The collaboration construct has a strong direct influence on co-responsibility (β = 0.27, p < 0.05). The same impact working in the reverse direction was found to be statistically insignificant. The co-responsibility construct indirectly influences collaboration through the pharmacist behavior variable (COR on BEH, β = 0.39, p < 0.05 and BEH on COL, β = 0.11, p < 0.05). The influence of collaboration construct on the pharmacist behavior construct is statistically insignificant.

The model can be approached from different angles by being analyzed from the perspective of four variables:

1. **GP behavior.** Pharmacist behavior is the only variable which directly influences the GP behavior construct. The model did not reveal any indirect influence on the GP behavior variable.

2. **Pharmacist behavior.** The co-responsibility variable has the biggest direct influence on the pharmacist behavior variable. Also, the latent variable of pharmacist behavior is indirectly influenced by the GP behavior variable through co-responsibility construct. The collaboration construct has no significant influence on the pharmacist behavior construct.

3. **Collaboration.** The pharmacist behavior construct has the biggest direct impact on the latent variable of collaboration. Also, the co-responsibility variable indirectly influences the collaboration variable through the pharmacist behavior construct. GP behavior did not have any statistically significant indirect or direct influence on the latent variable of collaboration.

4. **Co-responsibility.** This variable has the highest number of correlations, both indirect and direct. It is statistically significantly influenced by all model variables. The collaboration variable has the biggest influence, followed by pharmacist behavior, and GP behavior. The pharmacist behavior variable influences the co-responsibility construct both indirectly and directly, i.e. through the collaboration construct.

The correlation matrix analysis revealed that only two pairs of variables intercorrelate at > 0.90, which means collinearity had no significant effect on the analyzed model. A close fit hypothesis was confirmed and accepted for the designed model (9):

- RMSEA = 0.031 is within the range of (0.0; 0.05) for good fit acceptance,
- 90% confidence interval for RMSEA population value (0.020; 0.041) is within the range of (0.0; 0.05) for good fit acceptance,
- p-value for close fit hypothesis (RMSEA < 0.05) equals 1.0.

### Table 3. Factors that hamper relationships between pharmacists and physicians.

<table>
<thead>
<tr>
<th>No.</th>
<th>Factor</th>
<th>n = 1496</th>
<th>100% *</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Unfavorable circumstances in which the interaction is established</td>
<td>763</td>
<td>51.0</td>
</tr>
<tr>
<td>2.</td>
<td>Lack of preparation for collaboration</td>
<td>694</td>
<td>46.4</td>
</tr>
<tr>
<td>3.</td>
<td>Lack of a computer system which would allow exchange of information</td>
<td>676</td>
<td>45.2</td>
</tr>
<tr>
<td>4.</td>
<td>Lack of physicians' trust in pharmacists</td>
<td>617</td>
<td>41.2</td>
</tr>
<tr>
<td>5.</td>
<td>Lack of regulations regarding collaboration</td>
<td>505</td>
<td>33.8</td>
</tr>
<tr>
<td>6.</td>
<td>Difficulty in establishing direct contacts</td>
<td>419</td>
<td>28.0</td>
</tr>
<tr>
<td>7.</td>
<td>Unclear regulations with regard to filling prescriptions</td>
<td>297</td>
<td>19.9</td>
</tr>
<tr>
<td>8.</td>
<td>Lack of collaboration between professional organizations</td>
<td>268</td>
<td>17.9</td>
</tr>
<tr>
<td>9.</td>
<td>Physicians' competencies being questioned by pharmacists</td>
<td>125</td>
<td>8.4</td>
</tr>
<tr>
<td>10.</td>
<td>Lack of time</td>
<td>83</td>
<td>5.5</td>
</tr>
<tr>
<td>11.</td>
<td>Other</td>
<td>10</td>
<td>&lt; 1%</td>
</tr>
</tbody>
</table>

*Answers do not sum up to 100% as multiple answers were possible.
Table 4. Number of responses (n = 1496).

<table>
<thead>
<tr>
<th>Construct / item code</th>
<th>Item</th>
<th>n</th>
<th>Median [IQR]</th>
<th>Strongly agree</th>
<th>Agree</th>
<th>Neither agree nor disagree</th>
<th>Disagree</th>
<th>Strongly disagree</th>
<th>No response</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Co-responsibility</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COR Q1</td>
<td>Pharmacist is co-responsible for drug therapy outcomes</td>
<td>1492</td>
<td>4 (3-5)</td>
<td>447 (30%)</td>
<td>580 (39%)</td>
<td>254 (17%)</td>
<td>155 (10%)</td>
<td>56 (4%)</td>
<td>4 (&lt;1%)</td>
</tr>
<tr>
<td>COR Q2</td>
<td>Pharmacist is co-responsible for drug therapy errors</td>
<td>1477</td>
<td>3 (2-4)</td>
<td>232 (16%)</td>
<td>483 (32%)</td>
<td>333 (22%)</td>
<td>305 (20%)</td>
<td>124 (8%)</td>
<td>19 (1%)</td>
</tr>
<tr>
<td>COR Q3</td>
<td>Pharmacist has significant influence on the safety of drug therapy</td>
<td>1486</td>
<td>4 (3-4)</td>
<td>371 (25%)</td>
<td>654 (44%)</td>
<td>253 (17%)</td>
<td>159 (11%)</td>
<td>49 (3%)</td>
<td>10 (&lt;1%)</td>
</tr>
<tr>
<td>COR Q4</td>
<td>Pharmacist has significant influence on the efficacy of drug therapy</td>
<td>1481</td>
<td>4 (3-4)</td>
<td>278 (19%)</td>
<td>512 (34%)</td>
<td>352 (24%)</td>
<td>257 (17%)</td>
<td>82 (5%)</td>
<td>15 (1%)</td>
</tr>
<tr>
<td><strong>Collaboration</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COL Q1</td>
<td>Physician and pharmacist collaborate to provide for the safety of drug therapy</td>
<td>1491</td>
<td>3 (2-5)</td>
<td>394 (26%)</td>
<td>287 (19%)</td>
<td>217 (15%)</td>
<td>365 (24%)</td>
<td>228 (15%)</td>
<td>5 (&lt;1%)</td>
</tr>
<tr>
<td>COL Q2</td>
<td>Physician and pharmacist collaborate to provide for the efficacy of drug therapy</td>
<td>1487</td>
<td>3 (2-4)</td>
<td>349 (23%)</td>
<td>301 (20%)</td>
<td>243 (16%)</td>
<td>350 (23%)</td>
<td>244 (16%)</td>
<td>9 (&lt;1%)</td>
</tr>
<tr>
<td>COL Q3</td>
<td>Physician and pharmacist collaborate to provide for optimum drug therapy</td>
<td>1481</td>
<td>3 (2-4)</td>
<td>337 (23%)</td>
<td>273 (18%)</td>
<td>234 (16%)</td>
<td>363 (24%)</td>
<td>274 (18%)</td>
<td>15 (1%)</td>
</tr>
<tr>
<td><strong>Pharmacist behavior</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BEH Q1</td>
<td>I contact a physician if I discover any medication-related problems</td>
<td>1484</td>
<td>4 (3-5)</td>
<td>642 (43%)</td>
<td>410 (27%)</td>
<td>161 (11%)</td>
<td>180 (12%)</td>
<td>91 (6%)</td>
<td>12 (&lt;1%)</td>
</tr>
<tr>
<td>BEH Q2</td>
<td>I contact a physician if I a patient decides not to buy a drug</td>
<td>1476</td>
<td>2 (1-3)</td>
<td>152 (10%)</td>
<td>145 (10%)</td>
<td>177 (12%)</td>
<td>363 (24%)</td>
<td>639 (43%)</td>
<td>20 (1%)</td>
</tr>
<tr>
<td><strong>GP behavior</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GP-BEH Q1</td>
<td>Physician seeks my advice if s/he has any difficulty choosing the drug therapy</td>
<td>1480</td>
<td>2 (1-4)</td>
<td>207 (14%)</td>
<td>181 (12%)</td>
<td>192 (13%)</td>
<td>323 (22%)</td>
<td>577 (39%)</td>
<td>16 (1%)</td>
</tr>
</tbody>
</table>

IQR, interquartile range
These results indicate that the proposed model represents a relatively good approximation of the analyzed process. Table 5 presents the results of a confirmative factor analysis of the model.

**DISCUSSION**

The study results indicate that pharmacists and physicians in Poland are not considered equal partners, they do not collaborate, play separate roles (10) and are apparently isolated in their interprofessional relationships (11-13). Pharmacies and outpatient settings are geographically separated; pharmacists do not exercise any pharmaceutical care over patients, neither do they provide any other cognitive services.

To define the role of partners is one of the most important factors which determine the pharmacist-physician relationship (14, 15). The roles of pharmacists and physicians in Poland are clearly defined; the role of pharmacists appears to be marginalized and limited to issues essentially unrelated to medication therapy. In order to change this, pharmacists would have to demonstrate their professional capability to significantly contribute to successful clinical outcomes (15, 16).

Communication between pharmacists and physicians in Poland was demonstrated to be one-sided and initiated mainly by pharmacists. The same conclusions were arrived at in other studies, suggesting physicians were very reluctant to reach out to pharmacists and expected pharmacists to initiate contact, especially if the patient condition so required or if they identified any problem (17). Although the majority of respondents contacted physicians at least once a month, these consultations mainly concerned formal irregularities instead of issues specifically related to medication therapy. This limited scope of pharmacist-physician communication could be perhaps explained by the lack of knowledge or skills among pharmacists who do not feel competent enough to intervene (18). Even if a pharmacist has the social and material skills to initiate consultations with a physician or a patient on medication therapy-related issues, this does not necessarily translates into real action if the pharmacist feels discouraged by the expected negative consequences of such move. It would be oversimplistic to assume that the lack of professional skills is the only explanation, perhaps another issue lies in the fact that pharmacists do not have access to comprehensive information on patients’ medical history and medication therapy. Respondents considered the lack of access to patients’ medical records as one of key factors hampering pharmacist-physician relationships as it simply prevents them from being able to correctly evaluate the medication therapy prescribed to a patient. Unfortunately, based on the past experience from other countries, one might expect physicians to be reluctant to provide pharmacists with more information except for a plain list of medications a patient has to take (19).

**Table 5. Confirmative factor analysis.**

<table>
<thead>
<tr>
<th>Variable</th>
<th>SRW</th>
<th>URW</th>
<th>SE URW</th>
<th>Cronbach’s α</th>
<th>AVE [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Co-responsibility</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COR1</td>
<td>0.64</td>
<td>0.69</td>
<td>0.0</td>
<td>0.80</td>
<td>44</td>
</tr>
<tr>
<td>COR2</td>
<td>0.60</td>
<td>0.71</td>
<td>0.036</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COR3</td>
<td>0.68</td>
<td>0.70</td>
<td>0.066</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COR4</td>
<td>0.72</td>
<td>0.81</td>
<td>0.074</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Collaboration</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COL1</td>
<td>0.90</td>
<td>1.34</td>
<td>0.0</td>
<td>0.96</td>
<td>87</td>
</tr>
<tr>
<td>COL2</td>
<td>0.95</td>
<td>1.39</td>
<td>0.018</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COL3</td>
<td>0.95</td>
<td>1.42</td>
<td>0.032</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pharmacist behavior</strong></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>BEH1</td>
<td>0.50</td>
<td>0.62</td>
<td>0.0</td>
<td>0.48</td>
<td>33</td>
</tr>
<tr>
<td>BEH2</td>
<td>0.64</td>
<td>0.87</td>
<td>0.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>GP behavior</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GP-BEH1</td>
<td>1.00</td>
<td>1.00</td>
<td>0.062</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

SRW = standardized regression weight; URW = unstandardized regression weight; SE = standard error; AVE = average variance extracted.
The majority of respondents contacted physicians over the phone. Using telephone inherently involves a number of potential limitations, i.e., physicians can have no immediate access to patient records or cannot recall patient history details, they can be preoccupied with other patients, there can be some technical difficulties. Also, pharmacists typically report prescribing irregularities, which can be a sensitive issue for physicians (16). Moreover, pharmacists may experience objective difficulties in initiating contact with physicians (16) as they usually have to call a registration desk instead of directly calling a physician.

A relatively large share of pharmacists (38%) declared they have been contacting physicians personally. Face-to-face meetings are most suitable at the early stage of pharmacist-physician relationship. Face-to-face interaction can evolve into a more personal relationship and can help pharmacists develop their credibility and trustworthiness (18). Personal interactions help pharmacists better identify individual needs of physicians, and make physicians aware of the support pharmacists can offer. Also, it is easier to discover and eliminate any concerns physicians may have (20), abolish stereotypes and prejudice, and change attitudes (21). It seems that interprofessional collaboration can be consolidated by face-to-face contact between physicians and pharmacists, opinion exchange and information sharing, as well as participation in joint scientific meetings. These conclusions fit in the implementation model of pharmaceutical care at local level proposed by Chen et al., who argued that the implementation of new cognitive collaboration-based pharmaceutical services must be accompanied by establishing and maintaining personal interprofessional relationships. Hence, to be successfully implemented, pharmaceutical care in Poland would have to go hand in hand with personal interprofessional relationships (22).

Unlike in the initial stages of pharmacist-physician relationships where face-to-face contact is a must, later on, as the relationship progresses, it is desirable to consult issues related to medication therapy in writing (18). By keeping pharmaceutical patient records and regular medication therapy evaluation, pharmacists could provide physicians with explicit and valuable information (23), increasing the chances for relationship consolidation.

The respondents claimed their pharmacist-physician contacts were sporadic. Limited interaction initiated by physicians can be the consequence of stereotypical perception of pharmacists, lack of recognition for their professional competence, or lack of trust (15, 18). Lack of trust in pharmacists’ professional competencies was demonstrated to be one of the key factors affecting mutual relationships between pharmacists and physicians. Trust in pharmacists is critical for maintaining, or even more so for initiating a successful interpersonal relationship (15). Pharmacist credibility builds on professional competence and experience; therefore, in order to win the trust of physicians, pharmacist should demonstrate professional expertise and skills at the very beginning of the relationship. Many physicians

![Figure 1. A structural equation model of the pharmacist-physician relationships. Chi-square = 55.48, df = 23, p-value = 0.0002, RMSEA = 0.031; COL – collaboration; COR – co-responsibility; BEH – behavior.](image-url)
in Poland consider a pharmacy to be a point of sale rather than a healthcare setting (24). Unfortunately, this approach is very likely to negatively affect the credibility of pharmacists.

Lack of preparation for collaboration was named among the most common factors which affect professional relationships between pharmacists and physicians. Pre- and post-graduate interprofessional education can be one of the means to improve pharmacist-physician collaboration (25), although some further research is needed to confirm the benefits of such an education model (26).

The perceived co-responsibility for medication therapy outcomes among respondents appeared to be limited. According to literature sources, the provision of pharmaceutical care is directly positively influenced by the perceived co-responsibility of pharmacists for the medication therapy outcomes (27). The results of this study are apparently consistent with preceding studies since the respondents’ perceived co-responsibility for medication therapy outcomes was proved to influence the initiation of pharmacist-physician interactions concerning issues specifically related to the medication therapy. In the present study, pharmacists showed higher perceived co-responsibility for medication therapy outcomes, and lower perceived co-responsibility for medication therapy errors. This result may come as a surprise since pharmacists in Poland generally do not have any influence over the medication therapy choices (apart from OTCs); however, they can identify interactions or medication errors while handling prescriptions. Pharmacists’ opinions can be attributed to the absence of procedures for identifying, solving and preventing medicine-related problems (28).

Reluctance to changes among Polish pharmacists is perhaps another reason why pharmaceutical care had not been widely adopted by Polish pharmacies (28). People generally grew accustomed to the tried and tested settings and are not enthusiastic about changes, even more so about changes which they did not initiate (29). Even those who initially welcome changes are likely to yield to social pressure; for this reason, as postulated by Chen et al. (1), there is a strong need to create a professional culture which would fuel expectations towards the development and practice of interprofessional collaboration. Pharmacists will be able to stand up to the new challenges and meet the new expectations only if the desired attitudes and behaviors are deeply rooted in their professional practice so that pharmacists use them intuitively.

**Limitations**

This study consisted of an auditorium survey with a self-administered questionnaire; though the respondents were able to see, or even communicate with each other.

Measurement error was another possible limitation. It could have been caused mainly by the data collection method used in the study, where respondents self-reported their attitudes and behavior, and the behavior of other individuals (physicians). The study results could have been exposed to an error attributed to social expectations. Respondents could have been compelled to respond in a socially desirable manner instead of truthfully. With a relatively low response rate, an error attributed to non-responses cannot be excluded. Moreover, it should be noted that the study covered the time period of nine months, during which some factors determining the studied relationships could have changed considerably.

The pharmacist-physician relationship model incorporated only one item to measure GP behavior and only two items to measure pharmacist behavior. Future research would need to use more items to study these latent variables.

Future research would also need to include other items intended to examine the pharmacist-physician relationship. The pharmacist perspective was adopted in this study, and future research should also compare how physicians respond to the same relevant questions.

Despite these limitations, the study findings provide a new insight into pharmacist-physician relationships in Poland and how they interrelate.

**CONCLUSION**

It is desirable to create conditions favorable for pharmacist-physician collaboration in Poland, for example by promoting electronic patient information exchange systems. Before pharmaceutical care becomes widespread in Poland, more personal pharmacist-physician relationships should be fostered at local level. Pharmacists should demonstrate their professional competencies and their involvement has to be clinically relevant. The perceived co-responsibility for medication therapy outcomes prompts pharmacists to consult physicians on medication-therapy related issues, and therefore needs to be enhanced. In order to implement pharmaceutical care, assistance should be provided to pharmacists as they prepare to embrace their new role, by helping them develop their attributes and competencies,
and by establishing a clearly defined scope of professional duties.

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**Declaration of interest**

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

**REFERENCES**


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Schizophrenia is a severe form of a serious mental disorder, characterized by a chronic and recurrent course (1-3). The disease has onset usually in early adulthood (4) and requires a long-lasting and comprehensive treatment (5). Schizophrenia generates huge economic burden for the patients, their families, and for the society as well (3, 4); in addition to this, it is associated with direct and indirect costs (3). Direct costs concern inpatient and outpatient care and also medications (3, 6). Indirect costs are mainly the effect of productivity loss of both the patients and their caregivers (3, 6).

Although indirect costs constitute approximately 2/3 of total costs of schizophrenia, direct costs are responsible for a significant burden likewise (3, 7, 8). Usually, the main component of direct medical costs is inpatient care (2, 3, 9, 10); thus, a comprehensive and efficient pharmacotherapy is crucial for cost-effective treatment of schizophrenia.

In spite of the fact that in high-income countries the proportion spent on pharmacotherapy of schizophrenia is predominantly between 1 and 9 percent of all direct costs (3), medications used play a key role in achieving the main goals of schizophrenia treatment. These goals include: decrease of the recurrence rate, improved social functioning of the patients, and reduction of treatment costs (5, 7, 11). These objectives are related to the level of compliance, because non-compliance is considered a risk factor for relapses and rehospitalizations and it increases economic burden of the disease (7, 11). Moreover, it has been estimated that the main reasons for non-compliance are side effects of pharmacotherapy (5, 6, 11).

In spite of the fact that in high-income countries the proportion spent on pharmacotherapy of schizophrenia is predominantly between 1 and 9 percent of all direct costs (3), medications used play a key role in achieving the main goals of schizophrenia treatment. These goals include: decrease of the recurrence rate, improved social functioning of the patients, and reduction of treatment costs (5, 7, 11). These objectives are related to the level of compliance, because non-compliance is considered a risk factor for relapses and rehospitalizations and it increases economic burden of the disease (7, 11). Moreover, it has been estimated that the main reasons for non-compliance are side effects of pharmacotherapy (5, 6, 11). Although there are studies indicating that typical and atypical neuroleptics have similar efficacy (12), several authors emphasize differences in side effects of antipsychotic medicines (13, 14). They take into consideration advantages of the use of newer neurolep-
tics which may initially push up the drug cost contribution to the total cost but may lead to their cost-effectiveness in the long run (3, 15, 16).

It seems that a comprehensive and individually tailored pharmacotherapy should be the right of every patient and should be considered a priority of mental health care. Due to differences between health care systems and in economic status, however, treatment schedules and related costs may significantly differ even between neighboring countries (10). The differences in pharmacotherapy of schizophrenia also may result from differences in drug prices between typical and atypical neuroleptics (15), hence affordability of newer antipsychotics may be significantly limited in some social groups.

Presented herein are the costs of detailed pharmacotherapy used during hospitalization of patients with schizophrenia admitted to hospitals in Poznań (Poland) and in Lviv (the Ukraine).

The objective of this study was to evaluate and compare expenditures on typical and atypical antipsychotics and adjunctive pharmacotherapy. This analysis also allows observation of the differences between treatment schedules related to dissimilarities in mental health care funding in Poland and the Ukraine. It could be interesting not only for researchers but for health care decision makers as well.

Methodology

The study presents the results of cost analysis of pharmacotherapy used in adult patients hospitalized at Karol Jonscher Hospital of Poznań University of Medical Sciences (Poland) and at the Psychiatric Hospital of Lviv National Medical University (the Ukraine). The evaluation included 50 patients from Poznań (25 women and 25 men) and 58 from Lviv (33 women and 25 men) hospitalized between January 2010 and January 2011. Inclusion criteria were: diagnosis of schizophrenia based on the International Classification of Diseases, Tenth Revision (ICD-10), adult age of the patients (> 18 years old). Patients were excluded from the study if they had different time of hospitalization, if their hospital stay was less than 10 days, and in the case of discharge against medical advice.

The necessary information concerning pharmacotherapy used was gathered from hospital records. In addition to this, data concerning economic aspects of the study and drug prices data were obtained from hospital accounting departments and from the wholesale price list, respectively. Every hospital record was analyzed meticulously and the amount of each drug was counted individually. Afterwards, quantities of drugs used were converted into monetary values. The medicines were assigned into 8 groups (typical neuroleptics, atypical neuroleptics, antidepressants, normothymic drugs, anxiolytics, antiparkinson agents, benzodiazepines, and medicines related to concomitant disorders). Despite the fact, that anxiolytics and benzodiazepines might be a one group, these drugs are presented separately. It is related to hospital records, especially from Lviv, where benzodiazepines were considered independently. The cost of pharmacotherapy was calculated individually for every patient. The outcome was the total cost of pharmacotherapy for the medicine group and for patients in the analyzed hospitals.

Money values obtained in PLN (Poland) and in UAH (the Ukraine) were converted to the European currency based on the average euro exchange rate published by the National Bank of Poland (NBP) on October 9, 2012 (EUR 1 = PLN 4.07; EUR 1 = UAH 10.55). Monetary values used in the study are a rounding of the calculated amounts because of conversion of the local currencies into the common European currency.

Relevant permissions to conduct the analysis were obtained from the hospital decision-makers in Poznań and in Lviv. The study was approved by the Bioethics Committee. Moreover, the study complies with the Act on Protection of Personal Data and every patient was treated anonymously. In addition to this, each hospital record was analyzed in cooperation with the physician involved in the study.

Statistics

The data are shown as the mean values ± SEM. The data distribution pattern was not normal (unlike Gaussian function). Statistical analyses were conducted using the non-parametric Kruskal-Wallis test for unpaired data.

RESULTS

Patients in Poznań were treated using polytherapy. In Lviv, however, 10 patients received monotherapy and 48 patients – polytherapy. There were patients treated with all pharmaceutical groups included in this study. In Poznań, atypical neuroleptics were the most popular, although typical antipsychotics were used as well. In adjunctive pharmacotherapy, the most commonly used drugs were benzodiazepines, followed by antidepressants and normothymic drugs. Several patients hospitalized in Poznań were treated for concomitant disorders. In Lviv, on the other hand, typical neuroleptics were the most common drugs, followed by atypical neuroleptics. Adjunctive pharmacotherapy was used in
Lviv, likewise, but was not as widely used as in Poznań. The most popular medicines used as adjunctive pharmacotherapy were normothymic drugs, followed by benzodiazepines and antidepressants. In Lviv, pharmacotherapy related to concomitant disorders was not registered in hospital records.

The cost of typical neuroleptics used by patients in Poznań was estimated at EUR 175.78 (EUR 126.24 and 49.54 for women and men, respectively). The cost of atypical neuroleptics was EUR 9109.49; EUR 3349.71 for women and EUR 5759.78 for men. The cost of antidepressants was EUR 166.25 and in the female group it was EUR 92.30 and men EUR 73.95. The adjunctive normothymics’ cost was EUR 298.91; EUR 65.05 for women and EUR 233.86 for men. Expenditure on anxiolytic drugs was EUR 20.92, EUR 5.08 for women and EUR 15.84 for the analyzed group of men. The cost of antiparkinson agents was EUR 33.63 (women – EUR 23.57 and men – EUR 10.06). Benzodiazepines and benzodiazepine receptor agonists cost EUR 84.95, of which EUR 41.66 for women and EUR 43.29 for men. EUR 703.27 was spent on pharmacotherapy of concomitant disorders, EUR 245.71 and EUR 457.56 for women and men, respectively.

In Lviv, the proportion of money spent on pharmacotherapy was different than in Poznań. Typical neuroleptics cost EUR 1026.88, for women

<table>
<thead>
<tr>
<th></th>
<th>Poznan</th>
<th>Lviv</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Women</td>
</tr>
<tr>
<td>Typical neuroleptics</td>
<td>17.578€</td>
<td>8.37</td>
</tr>
<tr>
<td>Atypical neuroleptics</td>
<td>9109.49€</td>
<td>3349.71</td>
</tr>
<tr>
<td>Antidepressants</td>
<td>166.25</td>
<td>€</td>
</tr>
<tr>
<td>Normothymic drugs</td>
<td>298.91</td>
<td>€</td>
</tr>
<tr>
<td>Anxiolytic drugs</td>
<td>20.92</td>
<td>€</td>
</tr>
<tr>
<td>Antiparkinsonian drugs</td>
<td>33.63</td>
<td>€</td>
</tr>
<tr>
<td>Benzodiazepines and benzodiazepine receptor agonists</td>
<td>84.95</td>
<td>€</td>
</tr>
<tr>
<td>Drugs related to the treatment of concomitant disorders</td>
<td>703.27</td>
<td>€</td>
</tr>
<tr>
<td>Average spending</td>
<td>211.86</td>
<td>€</td>
</tr>
</tbody>
</table>

*p statistically significant result Poznań vs. Lviv (p < 0.05); #statistically significant result women vs. men (p < 0.05)
it was EUR 779.55 and for men – EUR 247.33. The cost of atypical neuroleptics was EUR 786.31 – EUR 496.68 for women and EUR 287.63 for men. The cost of antidepressants was EUR 48.69 – EUR 12.55 and EUR 36.14 for women and men, respectively. EUR 31.91 was spent on normothymic drugs, EUR 1.59 for women and EUR 30.32 for men. The cost of antiparkinson drugs used per 1 patient was EUR 3.33. Pharmaceutical agents from the group of benzodiazepines entailed an economic burden of EUR 91.12, EUR 83.52 for women and EUR 7.60 for men. In Lviv, medications related to concomitant disorders were not registered in hospital records.

**DISCUSSION**

Atypical neuroleptics proved to be the most expensive group (EUR 9109.49) of medicines used in the treatment of schizophrenia in Poznań. It is not surprising, because this kind of drugs was widely used at the Polish center. Atypical neuroleptics are known to be popular medications requiring significant financial outlays, especially in their long-acting injectable (LAI) forms (2, 12, 14, 15, 17). In Lviv, however, expenses for atypical neuroleptics were almost twelve-fold lower than in Poznań and the most cost-intensive (EUR 1026.88) medications were typical neuroleptics, reaching the value nearly six times higher than in Poznań.

Despite the discrepancy between Poznań and Lviv concerning the scope of neuroleptics used, Lieberman et al. (12) indicated that an older and less expensive neuroleptic (perphenazine) was as effective as newer medications. This opinion was confirmed by Davies et al. (18) and by Kilian et al. (19) who concluded that there was no aversion to suggest that atypical neuroleptics were more cost-effective than typical ones. It might confirm the statement that older antipsychotics which were widely used in Lviv were as effective as newer medications frequently used in Poznań. There are also several studies, however, demonstrating advantages of the use of atypical neuroleptics instead of typical antipsychotics resulting in better compliance, less burdensome side effects like extrapyramidal symptoms, lower hospitalization rates, and – consequently – resulting in cost-effectiveness considered in the long run (3, 6, 14, 15, 20-22). Despite similar antipsychotic efficacy of older and newer neuroleptics, it seems that atypical neuroleptics (also in their LAI forms) should be considered overall more valuable in the treatment of schizophrenia. It does not exclude the importance of typical neuroleptics which are still popular and effective (8, 10, 12).

Furthermore, differences in expenditures related to adjunctive pharmacotherapy were observed between Poznań and Lviv likewise. With the exception of the group of benzodiazepines, the cost of adjunctive pharmacotherapy was significantly higher in Poznań than in Lviv where the use of adjunctive pharmacotherapy seemed to be limited.

The observed discrepancies in the costs of detailed pharmacotherapy and *ipso facto* in the treatment schedules used may be the result of different funding and organization of health care systems in Poland and the Ukraine. Although in the Ukraine mental health care is theoretically free of additional charge, the patients or their relatives actually have to pay for newer and more expensive therapeutic options (23). Therefore, older and cheaper medicines dominated in pharmacotherapy (23), the prices of atypical neuroleptics also in LAI forms, lack of reimbursement, and low salaries significantly restricted access to these drugs in the Ukraine (10).

In Poland, mental health care also has some limitations and should be financially supported (10); nevertheless, analysis of detailed costs of pharmacotherapy allows a statement that pharmacological treatment of patients hospitalized due to schizophrenia in Poznań was comprehensive, especially compared to Lviv. Furthermore, analysis of frequency of schizophrenia relapses among patients from Poznan and Lviv (10) indicated that patients from Lviv had significantly more relapses than patients from Poznan within the same time horizon. Therefore, it seems that investment in newer atypical neuroleptics and in more comprehensive treatment could nominally increase the total cost of pharmacological care of patients with schizophrenia but could lead to a decrease in total expenditure e.g., owing to length of hospital stay reduction (7, 10, 15, 22, 24-27).

Moreover, an increased access to pharmaceutical drugs and, thus, a more wide-spread use of atypical neuroleptics and adjunctive pharmacotherapy would substantially boost the cost of pharmacotherapy in Lviv. It would increase the share of pharmacotherapy cost in the total expenditures of inpatient care of schizophrenia in the Ukrainian center. It would not be surprising because in countries in which mental health care has some limitations, the cost of pharmacotherapy tends to be higher (3). In addition to this, Salize et al. (28) provided the evidence that there were remarkable discrepancies in schizophrenia treatment expenses also among developed countries which have been members of the European Union for many years.

Our study also has some limitations. The study sample could have been larger and, thus, the time...
The cost of pharmacotherapy of patients with schizophrenia... 1287

horizon could have likewise been longer. It could be interesting to use the Gross Domestic Product (GDP) per capita and/or Purchasing Power Parity (PPP) to assess affordability of medicines and to compare results from the analyzed centers. It could be also interesting to conduct an assessment of the quality of life among patients to compare subjective efficacy of pharmacotherapy.

Despite these limitations, it can be concluded that the observed discrepancies in the costs and scope of pharmacotherapy used between Poznań and Lviv are the effect of insufficient health care funding, especially in the Ukrainian center. Access to comprehensive treatment should be the right of every patient, regardless of their economic status. Although a wider range of pharmaceutical agents used in Lviv could lead to equalization of drug expenses, comprehensive treatment might contribute to a cost-effective treatment of schizophrenia in the long run.

Conflict of interest

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In any healthcare organization, nurses belong to the group of principal serving staff, who are in direct contact to the patients. They perform various duties of which the medication administration is the most important activity in which pharmacists and doctors are also involved, since the doctor is responsible for prescribing of drugs and the pharmacist is responsible for drug provision (1). Thus, it becomes necessary to develop a collaborative relationship among various healthcare professionals to improve the patients’ health condition (2).

Pharmacists can take the necessary information from the nurse for optimizing the patient therapy and developing a pharmaceutical care plane according to the patient needs (3). However, most of the hospital pharmacies are distributing the medicines to the nursing stations by using the floor stock system, so the inter-professional relationship among the nurses and pharmacists is of great value (4).

In relation to WHO international standards, the ratio of nurses to doctors should be 3 : 1, that is opposite in Pakistan where the ratio of nurses to doctors is 1 : 3, whereas no such data is available for the nurse to pharmacist ratio (5).

Pharmacy profession is shifting from drug oriented to the patient oriented approach that is known as pharmaceutical care approach. The pharmaceutical care practice involves the responsible provision of drug therapy for improvement of patients’ health conditions. The pharmaceutical care practitioner in collaboration with other healthcare providers is responsible for provision of pharmaceutical care services to the patients (6).

As compared to the developed countries, healthcare system in developing countries like Pakistan is not well established. Healthcare system faces various hurdles including economic limitations, lack of qualified and trained staff and unavailability of standard practice guidelines.

To promote the rational drug use, pharmaceutical care practice has been extended in private hospitals in Pakistan but improvements can only be possible when the pharmaceutical care practitioner will work as a part of healthcare team including nurses and doctors. Around the world, researchers are focusing on the evaluation of pharmacists and physicians but very limited attention has been given to the nurses and pharmacists relationship. In Pakistan, a limited study has been conducted on the nurse’s perception. These studies were emphasized on the pharmacist’s role in public sector hospitals in Punjab province, Pakistan (2) and private sector hospitals in Karachi city, Pakistan (7).
This study was intended to assess the nurse’s perception regarding the pharmaceutical care services in healthcare system of Khyber Pakhtunkhwa province of Pakistan.

METHODS

Study design

This quantitative study was carried out among the nurses working in public sector hospitals of Khyber Pakhtunkhwa province. The study was implemented after taking approval from Departmental Research Ethical Committee.

In quantitative part of the study, cross-sectional survey was conducted after developing a questionnaire on the basis of findings of qualitative part of study and after an extensive review of literature (2, 7, 8). The participants were selected for assessment of face validity of the questionnaire. They were asked for their opinions on the worth, significance and simplicity of each item of questionnaire. They were additionally asked for identification of questions “to be removed” for making the questionnaire brief. The questionnaire was tested for its reliability resulted in a Cronbach’s $\alpha$ value of 0.62. The questionnaire consists of four sections: (i) Demographic profile, (ii) Awareness regarding pharmaceutical care, (iii) Experience with other healthcare professionals, (iv) Perception regarding patient counseling. By using Raosoft sample size calculator, sample size was calculated with confidence interval of 95 ± 5% error margin. The calculated sample size was 278 and with addition of dropout rate of 20%, total sample size became 334. Self-administered survey was carried out among the nurses in government hospitals of seven major divisions of KPK Province including, Bannu, Dera Ismail Khan, Hazara, Kohat, Malakand, Mardan and Peshawar.

Statistical data analysis

Data analysis was done by using Statistical Package for Social Sciences (SPSS version 20). The results were described as percentages and frequencies. Chi-square test was applied to test the level of significance among the independent variables (age, gender, current position, year of practice) and dependent variables (awareness of pharmaceutical care, experience with other healthcare professionals and perception regarding patient counseling). Statistical significance was acceptable at p value of $\leq 0.05$.

RESULTS

Total of 281 questionnaires were returned from a sample of 334 from seven major divisions: Bannu (25), Dera Ismail Khan (48), Hazara (48), Kohat (23), Malakand (33), Mardan (46) and Peshawar (58) showing a response rate of 84.13%.

The responses of the respondents to questions on their interaction with pharmacists are given in Table 2. A majority of the participants, 56.2% (n = 158) told that they contacted the pharmacists regarding patient’s medications once a week while 31.0% (n = 87) of the nurses contacted once a day/more and only 12.8% (n = 36) never contacted. The main reasons for interaction are drug availability (71.2%), side effects (2.8%), drug alternative (36.7%), drug dosage (18.5%) and drug interactions (11.0%).
Table 3. Awareness regarding Pharmaceutical care.

<table>
<thead>
<tr>
<th>How often pharmacist</th>
<th>Responses</th>
<th>p value†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Always n (%)</td>
<td>Often n (%)</td>
</tr>
<tr>
<td>Provide pharmaceutical care services to your patient</td>
<td>28 (10.0)</td>
<td>76 (27.0)</td>
</tr>
<tr>
<td>Make an effort to improve your patient outcomes</td>
<td>27 (9.6)</td>
<td>56 (19.9)</td>
</tr>
<tr>
<td>Participate in higher education programs to maintain and improve their competence</td>
<td>15 (5.3)</td>
<td>37 (13.2)</td>
</tr>
<tr>
<td>Participate in organizing health awareness programs for patients</td>
<td>16 (5.7)</td>
<td>34 (12.1)</td>
</tr>
</tbody>
</table>

* Significant relationship with the respective variables, chi-square†.

reason for interaction was drug availability in the hospital pharmacy (71.2%, n = 200), side effects (8%, n = 2.8), drug alternatives (36.7%, n = 103), drug dosage (18.5%, n = 52) and drug interactions (11.0%, n = 31).

Nurse’s perception regarding their awareness of pharmaceutical care is listed in Table 3. A majority 35.2% (n = 99) of nurses responded that pharmacists sometimes provide pharmaceutical care to their patients which is statistically significant with respect to age (p = 0.023) and years of practice (p = 0.024). A major number 44.5% (n = 125) of respondents sometimes make an effort to improve their patients outcomes that is significant with gender (p = 0.019). A large number of the respondents told that pharmacists sometimes participate in higher education programs to maintain and improve their competence 49.8% (n = 140) and participate in organizing such awareness programs for patients 47.3% (n = 133).

Responses of the participants regarding their experience with other healthcare professionals are summarized in Table 4. A majority 56.2% (n = 158) of respondents strongly agreed that pharmacists should be a part of healthcare team. A large number 56.6% (n = 159) of nurses agreed that better patient outcomes can be achieved by team work of all. While a majority 51.2% (n = 144) of the nurses agreed that a collaborative relationship was found among the pharmacists, doctors and nurses in hospitals. Out of 281 nurses, 56.9% (n = 160) were agreed and accepted the pharmacists as to be a reliable source of general drug information for patients and other professionals 52.0% (n = 146). Most 56.9% (n = 160) of the respondents agreed that pharmacists suggestions regarding drug related problems were considered by the physicians that was statistically significant with respect to age (p = 0.002) and current position (p = 0.027).

Table 5 indicates the perception of nurses regarding pharmacist’s involvement in patient counselling. From 281 nurses, 72.6% (n = 204) told that pharmacists are involved in educating the patients regarding the drugs. A majority of the respondents 71.9% (n = 202) told that pharmacists were not informing the patients about drug and food interactions. While 73.3% (n = 206) have told that pharmacists instruct the patients how to use their medication. A large number 63.7% (n = 179) of the respondents have informed that pharmacists do not spend enough time on each patient that is statistically significant with respect to current position of the respondents (p = 0.016).

DISCUSSION

Nurses are important members of healthcare team because they hold a special place in direct
patient care. Due to their close interaction with patients, it is needed to assess the nurse’s perception. Keeping this objective in mind, the present study was designed to explore the nurses’ perception regarding the pharmaceutical care practice in healthcare system of Khyber Pakhtunkhwa, Pakistan.

The findings of the study explored that majority of the nurses interact on weekly bases with the pharmacists and the main purpose of interaction was regarding the availability of the drugs rather than other purposes (i.e., side effects, drug alternative, dosage and interactions). This finding is in line with the study conducted by Azhar et al. (2), where a majority of the respondents told that they were majorly contacted for queries related to the availability of drugs.

Majority of the nurses were unaware of the pharmaceutical care. The possible reason could be that pharmacists were not given the opportunity to share their drug expertise with other healthcare providers. This finding supports another Pakistani study (7), where a poor perception was seen regarding pharmacists and pharmacy practice.

There is a deficiency of pharmaceutical care programs for other healthcare professionals and patients. Pharmacists are sometimes but not always

<table>
<thead>
<tr>
<th>Responses</th>
<th>Strongly Disagree n (%)</th>
<th>Disagree n (%)</th>
<th>Agree n (%)</th>
<th>Strongly agree n (%)</th>
<th>p value?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Do you think that pharmacists should be a part of healthcare team?</td>
<td>3 (1.1)</td>
<td>15 (5.3)</td>
<td>105 (37.4)</td>
<td>158 (56.2)</td>
<td>0.104 0.363 0.829 0.385</td>
</tr>
<tr>
<td>A better patient care outcomes can be achieved by team work of all healthcare professionals</td>
<td>5 (1.8)</td>
<td>19 (6.8)</td>
<td>159 (56.6)</td>
<td>98 (34.9)</td>
<td>0.181 0.238 0.153 0.521</td>
</tr>
<tr>
<td>There is a collaborative relationship between pharmacists, doctors and nurses in this hospital</td>
<td>10 (3.6)</td>
<td>42 (14.9)</td>
<td>144 (51.2)</td>
<td>85 (30.2)</td>
<td>0.869 0.190 0.076 0.717</td>
</tr>
<tr>
<td>Pharmacists are seen as a reliable source of general drug information for patients</td>
<td>8 (2.8)</td>
<td>30 (10.7)</td>
<td>160 (56.9)</td>
<td>83 (29.5)</td>
<td>0.774 0.130 0.781 0.460</td>
</tr>
<tr>
<td>Pharmacists are seen as a reliable source of general information for patients drug information for other healthcare professionals</td>
<td>14 (5.0)</td>
<td>29 (10.3)</td>
<td>146 (52.0)</td>
<td>92 (32.7)</td>
<td>0.140 0.353 0.699 0.819</td>
</tr>
<tr>
<td>Pharmacists suggestions regarding drug related problems are considered by physicians</td>
<td>18 (6.4)</td>
<td>43 (15.3)</td>
<td>160 (56.9)</td>
<td>60 (21.4)</td>
<td>0.002* 0.524 0.027* 0.208</td>
</tr>
</tbody>
</table>

* Significant relationship with the respective variables, chi-square?.
Table 5: Perception regarding patient counselling.

<table>
<thead>
<tr>
<th>Response</th>
<th>Yes n (%)</th>
<th>No n (%)</th>
<th>Age</th>
<th>Gender</th>
<th>Current position</th>
<th>Years of practice</th>
</tr>
</thead>
<tbody>
<tr>
<td>In my experience pharmacists are involved in following counseling services</td>
<td>204 (72.6)</td>
<td>77 (27.4)</td>
<td>0.310</td>
<td>0.147</td>
<td>0.103</td>
<td>0.287</td>
</tr>
<tr>
<td>to patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Involved in educating patients regarding the drugs</td>
<td>79 (28.1)</td>
<td>202 (71.9)</td>
<td>0.320</td>
<td>0.374</td>
<td>0.434</td>
<td>0.800</td>
</tr>
<tr>
<td>Inform about drug and food interaction</td>
<td>206 (73.3)</td>
<td>75 (26.7)</td>
<td>0.249</td>
<td>0.159</td>
<td>0.284</td>
<td>0.636</td>
</tr>
<tr>
<td>Instruct how to use their medications</td>
<td>102 (36.3)</td>
<td>179 (63.7)</td>
<td>0.001*</td>
<td>0.613</td>
<td>0.051</td>
<td>0.036*</td>
</tr>
<tr>
<td>Inform the patients about side effects of drugs</td>
<td>192 (68.3)</td>
<td>89 (31.7)</td>
<td>0.222</td>
<td>0.520</td>
<td>0.087</td>
<td>0.776</td>
</tr>
<tr>
<td>Inform the patients regarding storage conditions of drugs</td>
<td>129 (45.9)</td>
<td>152 (54.1)</td>
<td>0.315</td>
<td>0.185</td>
<td>0.016*</td>
<td>0.609</td>
</tr>
<tr>
<td>Do they spend enough time on each patient</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Significant relationship with the respective variables, chi-square?.

participating in higher education programs for maintaining and improving their competence and organizing health awareness programs for patients that is persistent with study conducted in the Khyber Pakhtunkhwa province by Murtaza et al. (9), where a fewer of the respondents have participated in pharmaceutical care programs.

Most of the nurses want to collaborate and communicate with the pharmacists since they consider the pharmacist as a reliable source of drug information that is in line with the study by Gillespie et al. (10), where the respondents agreed on the collaboration as it was proved to be helpful in the improvement of patient care outcomes. Nurses are involved in direct patient care as they are responsible for patient drug administration. Administration errors can also be reduced by collaboration of nurses, pharmacists and other healthcare professionals (11). Another study explored that both nurses and physicians want to communicate with the pharmacists (2). This is in contrast to an earlier study (12), where a negative perception was found among the nurses regarding the pharmacists’ involvement in the patient care as it will decrease their worth and will cause an intrusion in their work. The finding of our study shows that nurses’ perception is becoming positive along with the passage of time.

Almost all of the nurses responded that the pharmacists are not much more involved in the patient counselling. They are informing the patients about the drug use and storage conditions but missing the knowledge regarding drug/food interactions and side effects that will results in poor patient adherence and compliance to the drug therapy. Additionally, the pharmacists do not spend enough time on each patient. According to them, the pharmacists do not have an interaction with the hospital in-patients. This finding of our study is supported by another study (2), in which majority of the respondents agreed that the pharmacists do not counsel their patients. The main reason of non-counselling is again lack of availability of the pharmacists in healthcare settings.

Limitation of the study

This study involved the nurses working in government hospitals of seven divisions of Khyber Pakhtunkhwa province of Pakistan so the findings of this study may not be applicable to the private sector.

CONCLUSION

Nurses in Pakistan are not much familiar with the pharmaceutical care practice, so there is a need to arrange awareness programs for nurses as well as other healthcare professionals and the patients. However, nurses showed a positive response and accept the pharmacists to be a part of healthcare team and want to collaborate with the pharmacists. They accept the pharmacists to be a reliable source of drug information who can improve their drug knowledge and can reduce their workload.
Conflict of interest

The authors declare no conflicts of interest. The authors alone are responsible for the content of article.

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The evidence base of health policy decisions has been increased in the last decades, as societies require more objective and verifiable criteria for the allocation of scarce public resources. This is especially true for the purchasing decisions of innovative pharmaceuticals (1). In addition to the main drug registration criteria, including efficacy, safety and quality, public payers in many countries mandate cost-effectiveness evidence and budget impact analysis prior to the pricing and reimbursement decisions of new pharmaceuticals (2-4).

As opposed to public investment decisions related to new drugs, the evidence base of pharmaceutical disinvestment decisions, such as increased utilization of generic medicines is more limited (5, 6). Policy makers often measure the success of generic drug policies by the market share of generics and their price erosion (7, 8). These two easy-to-measure indicators are important to estimate savings in the pharmaceutical budget; however, they cannot provide information on savings in total health expenditure or on maintaining equal health outcomes. In general, from public health perspective generic drug policies are successful, if savings in the health care budget are achieved without any deterioration in health outcomes of patients (9).

In chronic diseases, such as hypertension, the most crucial factor in the implementation of generic drug policies is the continuation of antihypertensive therapy after patent expiry of the original product. If generic reference pricing system is applied to facilitate the multiple switching of antihypertensives, the evidence shows that patients can experience increased cardiovascular events after treatment change (5, 6).

The objective of this study was to estimate the impact of multiple switching of generic antihypertensive therapy on health outcomes and total health expenditure in a retrospective analysis of payer’s database. We included patients with hypertension from the National Health Insurance Fund database into our analysis who were treated with losartan or losartan/hydrochlorothiazide (HCT) in 12-month prior to patent expiry of losartan or losartan/HCT. We compared 3-year MACEs (major cardiovascular events, including stroke, myocardial infarct or death) and treatment costs (antihypertensive drugs, other cardiovascular drugs, cardiovascular treatments, non-cardiovascular interventions) from payer’s perspective of those patients with multiple switching of generic drug therapy in 12-month after patent expiry (n = 3,280) with those patients who had been switched to a generic losartan or losartan/HCT brand after patent expiry (single switch) without any additional switching in 12-month (N = 3,101). 9.8% of patients with single switch had MACEs over 36-month compared to 10.7% of patients in the multiple switch group (p = 0.247). Compared to baseline year the difference in total annual treatment costs were higher by € 461 in the multiple switch group than in the single switch group (p < 0.001). Further studies are needed to strengthen the evidence on the suboptimal consequences of multiple switching of generic drugs on health outcomes and total treatment costs compared to single switch.

**Keywords:** generic drug policy, adherence, health outcomes, price erosion, switching, losartan
tate generic price erosion (10), the reference generic product may change frequently (11).

Generic medicine policy in Hungary: internal price referencing system

In Hungary, internal price referencing is applied for generic drugs. Until 2011, the generic drug with the lowest price became the reference product, and a new reference product was announced in every 3 months. Products with significant price differential over the reference drug were delisted from the reimbursement list. Products with minor price differential compared to the reference product had the same amount of reimbursement but increased copayment (i.e., resulting lower % of reimbursement). Before 2011 entrance of new generic drugs was the main driver of generic price erosion with a predefined mandatory reduction for new entrants of 30-10-10% for the first, second and third generic product. Manufacturers of already marketed generic products had less incentive to reduce drug prices.

From 2011, a new blind bidding procedure for the generic reference pricing scheme has been introduced to further facilitate price erosion for all manufacturers. Every 6 months generic manufacturers have to submit price reduction proposals via an online application system operated by the Hungarian National Health Insurance Fund (NHIF) for their drugs, but without knowing proposals by their competitors. After the closure of the bidding process, the lowest price drug becomes the reference product. Products with less than 15% price differential compared to the reference product have equal amount of reimbursement with the reference product, which translates to a relatively higher percentage of copayment due to higher total price. Products with more than 15% price differential to the reference product can have only 85% reimbursement of the reference product, so there is significant penalty for minor price reductions. The blind bidding procedure has been proved to be a very efficient tool to facilitate price erosion of generic medicines resulting regularly changing generic reference products.

Potential treatment scenarios after patent expiry

In any internal price referencing systems chronic patients on antihypertensive drug therapy may choose from several options, if the reference product changes over time. The most specific case is when the patent of a successful antihypertensive medicine expires, and the first generic products enter the market.

In such cases patients may stay on the original brand, if they are willing to pay higher copayment or even the full public price once the original product is delisted from reimbursement (Option 1). In real world some patients may also discontinue drug therapy after the copayment of their original medication increases rapidly, although this option does not make sense from the clinical point of view (Option 2). Alternatively they may switch to another patented original medicine which is not subject internal price referencing or generic substitution at pharmacy level (Option 3). Patients can also switch to a generic product with the same active ingredient of...
their original drug therapy (Option 4); or they can maximize savings from generic price erosion by always choosing the generic product with the lowest actual copayment (Option 5).

Should the patient choose either of these scenarios, it is not sufficient to evaluate the success of generic drug policy only in terms of savings in drug budget, changes in health outcomes and overall health care expenditures also have to be taken into account (12). If significant proportion of patients discontinue the antihypertensive therapy after patent expiry or maintain their treatment on the delisted original product, the generic drug policy cannot be considered successful even despite significant savings in drug budget (Option 1-2). Also, switching to another patented original medicine without price erosion cannot be considered as a successful generic drug policy (Option 3). However, it is difficult to choose between those two policy scenarios in which patients are switched to generic products only once or multiple times (Option 4-5).

Objective

Our objective was to estimate the impact of multiple switching of generic antihypertensive drug therapy on health outcomes and total health expenditure for those patients, who had been adherent and persistent with their chronic maintenance antihypertensive losartan therapy before its patent expiry by comparing to patients with only a single switch to a generic losartan brand after the patent expiry.

MATERIALS AND METHODS

Study design, inclusion and exclusion criteria

A retrospective analysis of NHIF database was applied with a full coverage of health care data of all insured Hungarian citizens. We selected those patients from the database into our analysis who had at least 304 days of losartan or losartan/hydrochlorothiazide (HCT) treatment (DOT) with International Statistical Classification of Diseases and Related Health Problems (ICD) I10-I15 codes on prescriptions in the 12 months prior to patent expiry of losartan in July 2007 or losartan/HCT January 2009, respectively (see inclusion period in Fig. 1). ICD-10 codes are mandated on prescriptions in Hungary to indicate primary disease according to the ICD, and these codes confirmed drugs were prescribed to treat hypertension of patients. More than 10 months of treatment was assumed to indicate that patients were persistent with their mono or combination losartan therapy before its patent expiry. We excluded those patients from the analysis who had major myocardial infarct or stroke within 12 months after patent expiry, as for these patients any changes in antihypertensive drug therapy could be attributable to deterioration in their medical status.

Patient allocation

We allocated patients to 5 groups based on switching history in 12 months after patent expiry (see allocation period in Fig. 1) similarly to the theoretical scenarios described above (Options 1-5). Patients in Group 1 stayed on the original brand in the next 12 months after patent expiry. Patients in Group 2 discontinued reimbursed medical treatment for hypertension. Group 3 patients were switched to other original antihypertensive products. Group 4 patients were switched to generic losartan or losartan/HCT, but did not change the generic brand in 12 months. Group 5 patients were switched at least twice among different generic products in 12 months after patent expiry.

As original losartan and losartan/HCT were delisted from reimbursement after 11 and 9 months to patent expiry, respectively, patients in Group 1 could not be tracked in the NHIF database after delisting of original losartan or losartan/HCT (no reimbursement consequences of delisted products). Therefore, we reallocated Group 1 patients between Groups 2-5 based on their prescription history in 12 months after their last original losartan or losartan/HCT prescription (see dotted lines in the allocation period in Fig. 1). Those patients who had no subsequent records of any reimbursed antihypertensive drugs were moved to Group 2. Those patients who were switched to another original antihypertensive drug, were reallocated to Group 3. Those patients who were switched to generic losartan or losartan/HCT, but did not change the generic brand in the next 12 months were moved to Group 4. Those patients who had multiple switch among different generic antihypertensive products at in the next 12 months were moved to Group 5. Group 2 and Group 3 patients provided evidence for the failure of generic drug policy, as they did not continue their antihypertensive therapy on a cheaper alternative medicine, therefore were also excluded from further analysis.

Cost and health outcomes

For patients in Group 4 and 5 we retrieved clinical records of major cardiac events (MACEs), including myocardial infarction, stroke or death for an additional 36 months period in the NHIF database, and calculated the odds ratio for MACEs in Group 4 compared to Group 5.
Total treatment costs after the allocation period over the additional 36 months were also estimated for Group 4 and 5. Treatment costs were censored after death for those patients who died during the 3-year study period. Average annual treatment costs were calculated in the following categories: antihypertensive drugs, other cardiovascular drugs (excluding antihypertensive drugs), cardiovascular treatments excluding non-hospital drugs (e.g., acute or chronic inpatient health services, hospital drugs related to cardiovascular indication defined as ICD I10-I15), non-cardiovascular drugs, non-cardiovascular services excluding non-hospital drugs (e.g., acute or chronic inpatient health services, hospital drugs related to non-cardiovascular indication). As NIHF database has no payment records on non-reimbursed medicines (e.g., over-the-counter drugs), only drugs with partial or full reimbursement could be included into the analysis. Drug costs were calculated at public price, which included NHIF reimbursement and copayment of patients. As services in the primary care are reimbursed by capitation, cost of general practitioner visits was excluded from the analysis. A third party payer’s perspective was applied for health services.

Average annual treatment costs in the study period were compared to the annual treatment costs in the inclusion period prior to patent expiry (see study period in Fig. 1). Difference-in-differences in annual treatment costs compared to baseline year were calculated between Group 4 and 5 (13).

We employed 174.5 HUF/EUR purchasing power parity exchange rate (2012) to report treatment costs in Euro (14). Fisher’s exact test and Welch two sample t-test were used to compare the two groups. The levels of significance were set to 0.05 unless stated otherwise. Statistical analyses were performed in R statistical software.

### RESULTS

Figure 1 summarizes the allocation of patients, who were persistent with losartan or losartan/HCT treatment prior to its patent expiry, to 5 different groups listed in the Materials and Methods section. Approximately one-third of patients had a single switch to generic losartan during the 12-month allocation period (Group 4), and another one-third of patients had multiple switches among different generic products (Group 5) resulting a total study population of 3101 + 3280, respectively.

9.8% of patients with single switch had MACEs over the 3 year study period, 10.7% of patients in the multiple switch group had MACEs, with no statistically significant difference between the two groups (p = 0.247) (see Table 1).

As patients became older and their disease progressed, the annual treatment costs were increased in both groups compared to baseline year (see Table 2). The difference in total treatment costs, however, were significantly higher by 461 € among those patients with multiple switches among generic products in 12-month after patent expiry (p < 0.001), and the same was true for all cost categories except cardiovascular treatments excluding non-hospital drugs. Interestingly, the annual savings in antihypertensive drug costs were less for patients in Group 5 than in Group 4.

### DISCUSSION AND CONCLUSION

The policy objective of switching stable chronic patients to generic drugs after patent expiry is to reduce health care costs without compromising health outcomes (i.e., same health gain at lower costs) (Group 4). However, current Hungarian generic medicines policy incentivizes multiple

#### Table 1. Impact of treatment scenarios on major cardiovascular events (MACE).

<table>
<thead>
<tr>
<th></th>
<th>Single switch to reference generic drug</th>
<th>Multiple switches to generic drugs</th>
<th>Difference between groups</th>
<th>Odds ratio</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>3,101</td>
<td>3,280</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acute myocardial infarct n(%)</td>
<td>60 (1.93%)</td>
<td>74 (2.26%)</td>
<td>0.32%</td>
<td>0.855</td>
<td>0.384</td>
</tr>
<tr>
<td>Stroke n(%)</td>
<td>115 (3.71%)</td>
<td>125 (3.81%)</td>
<td>0.10%</td>
<td>0.972</td>
<td>0.844</td>
</tr>
<tr>
<td>Death n(%)</td>
<td>187 (6.03%)</td>
<td>208 (6.34%)</td>
<td>0.31%</td>
<td>0.948</td>
<td>0.640</td>
</tr>
<tr>
<td>Total MACEs n(%)</td>
<td>303 (9.77%)</td>
<td>350 (10.67%)</td>
<td>0.90%</td>
<td>0.907</td>
<td>0.247</td>
</tr>
</tbody>
</table>

* Fisher's exact test
switching of drug therapy which may not result in reduction of total health expenditure (Group 5). Regular switch of chronic drug therapies may lead to poorer adherence and persistence (15, 16) and increase in hospitalization events (13, 17-19). Frequent substitution of generic drugs may result in increased side-effects and decreased tolerability (20) and patient confusion (21-23) especially among patients with several comorbidities.

Unfortunately, persistence of delisted products could not be directly assessed in the NHIF database, as these products have no reimbursement impact to be recorded. However, when we designed the study we assumed that poorer adherence potentially translates to negative health outcomes, and the impact of MACEs on total treatment cost may be greater than potential savings from multiple switching of generic drugs (24-27).

Direct causal relationship between multiple switching and increased total health care costs or negative health outcomes cannot be proven in real world database analysis. Patients with longer treatment period (i.e., better persistence) have higher chance for multiple switching, especially if several previous reference products are delisted. In order to prevent such bias, we stratified our study into separate time periods. Patient allocation into treatment groups based on their switching history over 12 months after patent expiry preceded the measurement of total treatment costs and health outcomes.

Overall, our observational study was not powered enough to detect statistically significant increase in 3-year MACEs in patients with multiple generic switch compared to those patients with single switch to generic losartan in the allocation period. However, increase in annual treatment costs were significantly greater in the multiple switch group compared to the single switch group.

Potential confounding factors may limit the generalizability of our conclusions. Higher treatment costs in Group 5 compared to Group 4 might be attributable to additional factors beyond frequency of switching drug therapy, including differences in baseline risks and switch of patients to antihypertensive medicines with different active ingredient(s). Unfortunately, no data on health state factors could be retrieved from the NHIF database. However, we still need to answer why patients who had strong persistence with a chronic antihypertensive drug therapy during the inclusion period were switched back and forth among different generic products after patent expiry.

The impact of potential confounding variables could have been minimized by multiple regression analysis based on individual patient records, or additional subgroup analyses. Unfortunately, the NHIF provides only aggregate results in order to protect privacy of personal data. Therefore, we were unable to conduct multiple regression analysis controlling for disease severity, individual compliance, demographic characteristics or comorbidities. NHIF even cannot disclose aggregate results for outcomes with less than 10 cases. Consequently, we could not make further subgroup analyses, including separation of losartan patients from losartan/HCT patients, or reporting separately those patients in the multiple switching group, who took several different brands of the same active ingredient.

### Table 2. Annual treatment costs and incremental annual costs compared to baseline period per patient.

<table>
<thead>
<tr>
<th>Treatment categories</th>
<th>Single switch to reference generic drug</th>
<th>Multiple switches to generic drugs</th>
<th>Difference-in-differences between groups</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Annual treatment costs</td>
<td>Increase to baseline year</td>
<td>Annual treatment costs</td>
<td>Increase to baseline year</td>
</tr>
<tr>
<td>Antihypertensive drugs</td>
<td>311 €</td>
<td>-201 €</td>
<td>385 €</td>
<td>-156 €</td>
</tr>
<tr>
<td>Other cardiovascular drugs</td>
<td>292 €</td>
<td>108 €</td>
<td>336 €</td>
<td>139 €</td>
</tr>
<tr>
<td>Cardiovascular treatments excluding non-hospital drugs</td>
<td>329 €</td>
<td>198 €</td>
<td>527 €</td>
<td>361 €</td>
</tr>
<tr>
<td>Non-cardiovascular drugs</td>
<td>908 €</td>
<td>478 €</td>
<td>1,096 €</td>
<td>610 €</td>
</tr>
<tr>
<td>Non-cardiovascular services excluding non-hospital drugs</td>
<td>492 €</td>
<td>240 €</td>
<td>631 €</td>
<td>330 €</td>
</tr>
<tr>
<td>Total annual treatment costs</td>
<td>2,332 €</td>
<td>823 €</td>
<td>2,976 €</td>
<td>1,284 €</td>
</tr>
</tbody>
</table>

* Welch two sample t test
In conclusion, the evidence base of pharmaceutical disinvestment decisions has to be strengthened. Further studies are needed to better understand the negative implications of multiple switching of generic drugs on health outcomes and total treatment costs in other chronic conditions. Real world data analysis can provide complementary evidence in addition to randomized controlled clinical trials, as prospective trials are not suitable to estimate costs (protocol driven costs) or persistence.

Conflict of interest

The study was financially supported by Abbott; however, authors summarized their independent professional opinion and take full responsibility for potential errors in the manuscript. Abbott had no influence on the review process, nor were they involved in writing the manuscript.

REFERENCES


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Pharmaceutical companies are eager to show themselves as actively pursuing the idea of corporate social responsibility – CSR (1, 2). It means they are engaging in activities that are not only good for business but also for the good of society. Such actions are beyond what is required by the law and do not necessarily take into account the financial interests of the company (3). Researchers indicate that CSR gives the company competitive advantages (4) and customer trust (5).

Furthermore, there is also possible link with better financial performance (6, 7). With regard to the pharmaceutical business, CSR activity is widely discussed in the literature (8-11). CSR by the pharmaceutical companies is directly related to ethical, legal as well as compliance-related issues in this heavily regulated industry (9).

The nature of the pharmaceutical industry enforces socially responsible behavior. Several aspects of CSR are reported in the literature to include: pricing (8, 11, 12); access to medicines (11, 12); quality of supply chain and distribution (8, 11) and research and development (R&D) practices (3, 8, 11) and specialized development of drugs for tropical diseases (10). Additionally, it includes appropriate management of intellectual property (9) and increased transparency (12). Transparency can be increased by disclosure of data, which can normally be hard to get, by publishing the full results of clinical trials and making it accessible to third parties (11) and by providing full access to industry payments in the form of consulting fees to clinical investigators and publication of information about sales incentives.

CSR also engages business to help governments build public capacity, public policy and new institutions as well as deliver public goods (13). CSR encourages the pharmaceutical industry to

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cooperate with governments (11), take part in discussion about reform of healthcare systems and cooperate to counter bioterrorism fights. CSR also suggests a responsibility towards development of generics, flu vaccines, genomics and biologics.

The media perceive issues related to pharmaceutical industry’s CSR as significant and are willing to publicize its favorable effects. However, the media is also quick to discern any irregularities, e.g., drug safety, and report them. The purpose of this article is to analyze which topics connected to ethics and CSR are most frequently reported in one media vehicle, newspapers. Newspapers not only have wide distribution but also reach people, who do not have access to electronic media. Importantly this analysis is unique because is made on the most widely circulated newspapers from two different countries, the U.S. and Poland, and it shows the different approach of this medium to theme ethically charged and CSR frequently associated with the pharmaceutical industry.

The idea of comparisons between/among countries in news coverage is present in the scientific literature. In 1961 Markham discussed the impact of news coverage in the U.S. on international relations with South America (14) and Potter (15) looked at the influence of U.S. news on third-world countries. More recently, in 1992, Lee and Craig (16) com-

<table>
<thead>
<tr>
<th>Table 1. Comparison of the U.S. and Polish Pharmaceutical Markets*.</th>
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<tbody>
<tr>
<td><strong>Poland</strong></td>
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<tr>
<td>Percent of drug expenses compared to total healthcare expenses</td>
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<td>Expansion of the pharmaceutical market due to:</td>
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<tr>
<td>Market in 2013</td>
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<td>Main drugs sold in the market</td>
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<td>Drug price</td>
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<td>Invesments in R&amp;D</td>
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<td>Biggest companies</td>
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<td>No. of REPS</td>
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*Based on: (51)
pared U.S. newspaper coverage of labor strikes in Poland and South Korea. In 2006, Wu contrasted news coverage between the U.S. and China about AIDS (17). A review of the literature that indicated there were no previous articles comparing newspaper coverage in the U.S and Poland, established the basis for this study. It also suggested that this cross-cultural analysis of newspaper coverage in the two countries could reveal interesting perspectives that would have implications for the stakeholders in healthcare delivery, especially pharmaceutical companies.

Comparing the pharmaceutical markets in Poland and the U.S.

A comparison of the U.S. and Poland compares two very different markets. Poland made a social, political and economic transformation from a communist government to a democratic government in 1989. In 1990, drug sales were 6.2 billion PLN. By 1999, sales had risen to 11.3 billion (18) and, in 2013, mushroomed to 27.7 billion PLN (19). Poland became the second largest market in Eastern Europe behind Russia (20) and is the seventh largest pharmaceutical market based on ex-factory prices (21). Several factors contributed to this meteoric growth, most prominent of which was Poland’s approval of pharmaceutical advertising in 1993 (22). Accompanying growth in the pharmaceutical industry is regulation. Currently, the Polish market is more controlled by their government’s regulatory agencies than the pharma industry is controlled in the U.S. Despite regulation, the pharmaceutical markets in both countries are significant. The pharmaceutical market in the U.S. is the largest in the world; its value in 2013 amounted to $ 329 billion (23). Comparatively, the Polish market has a value of PLN 27.6 billion or approximately $ 8.6 billion (19). A comprehensive summary comparing the U.S. and Polish markets is portrayed in Table 1.

Comparing the law and ethical regulations in Poland and the U.S.

Polish law and its regulations are stricter than Directives 2001/83/EC and 2004/24/EC of the European Commission. For example: gifts to physicians cannot be worth more than PLN 100 or $33 and must have a connection with their medical practice; Polish law forbids physicians to meet with reps in their offices during working hours since 2008 (it is not strictly enforced and not an effective deterrent). There is no direct-to-consumer advertising (DTCA) in contrast to U.S. Furthermore, there are the ethical guidelines from pharmaceutical companies and physicians’ organizations. However, these guidelines are not as strict as the law and state what constitutes good practice. While changes in Polish law (24) have limited marketing practices by pharmaceutical companies, they still attempt to influence doctors.

In the U.S., the pharmaceutical industry, through its trade association, the Pharmaceutical Research and Manufacturers’ Association (PhRMA), adopted a new code to address negative reactions to their marketing and promotional programs in July 2002 (25). Additionally, and importantly, the PhRMA guidelines moved companies’ activities away from aggressive, inappropriate promotional efforts toward greater educational and patient-benefit programs (26-28). The PhRMA Code was updated and implemented in January 2009 to reinforce the effectiveness of the 2002 Code because spending on advertising totaled nearly $20 billion in 2007 (29). There is still some “wiggle room” in the PhRMA guidelines that enables companies to promote actively, e.g., allowing educational gifts if their value does not exceed $100 (30). Another example is the PhRMA Code’s provision that prevents sales reps from meeting with physicians during their office hours, but only when it does not disrupt patients’ appointments (31, 32). All together, these efforts are not as strict as the Polish laws.

Finally, the 2010 U.S. healthcare law, the Physician Payments Sunshine Act (Sunshine Act), created the Open Payments Program. It requires manufacturers of drugs, medical devices, biological and medical supplies covered by the three federal health care programs, Medicare, Medicaid and State Children’s Health Insurance Program (SCHIP), to collect and track all financial relationships with physicians and teaching hospitals. Then, report these data to the Centers for Medicare and Medicaid Services (CMS) with the goal of increasing the transparency of financial relationships between healthcare providers and the manufacturers of medical products as well as to uncover potential conflicts of interest (33). Initial assessment of the Sunshine Act revealed connectedness and/or associative relationships between these newly disclosed data regarding drug firms’ payments to physicians and the overall financial health/strength of the paying companies (34). In Poland, the organization INFARMA published The Transparency Code, which governs disclosures regarding cooperation between innovative pharmaceutical companies and Polish healthcare professionals. Disclosures are voluntary and do not cover as much information as the Sunshine Act. This is being done to deter the EU
from following the U.S.’s lead and implementing a version of the Sunshine Act.

EXPERIMENTAL

Methodology

To compare newspaper coverage in the U.S. and Poland, we focused on the leading newspapers in each country based on circulation and notoriety. These newspapers in each country are the New York Times (NYT) in the U.S. and the Gazeta Wyborcza (GW) in Poland. We use the term ‘ethical issues’ to describe all issues connected with the CSR and ethically charged issues related to the pharmaceutical industry as explained in the introduction.

The two newspapers were selected carefully as those with a significant influence to society in their respective countries. Despite their prominent positions, the authors are aware that analyzing only one newspaper from each country is not enough to make the generalizations about the populations in each country. Although broad generalizations cannot be made, each newspaper has its own political position and style of reporting about issues affecting the pharmaceutical industry in their countries. Because health and healthcare is something that everyone needs or will need, the newspapers reporting has the ability to influence perspectives about one of the key stakeholders in healthcare delivery, the pharmaceutical industry, in both the U.S. and Poland.

The ethical issues covered by newspapers are issues are familiar to the majority of people. As background, there are differences between ethical and legal issues that are based on the underpinnings of ethics and law. Ethics are based on moral standards that govern what a person should or should not do while law is based on rules that control what a person can and cannot do. Generally, the law and ethics are in agreement but not always (35). This is especially true about newspaper coverage of ethical issues related to the pharmaceutical industry. For example, selling a drug that is not safe is both illegal and unethical. However, there are times that an issue is within the limits of the law, such as a pharmaceutical company’s setting the price of a new drug significantly, above what a healthcare plan will reimburse or what a patient can afford may be legal but is morally wrong.

The purpose of the audit was to shed light on the following questions:

1. Is there a difference between the newspaper coverage of ethical issues affecting the pharmaceutical industry in the U.S. and Poland?
2. Do the headlines and articles in the NYT and the GW support or oppose the positions taken by the industry?
3. How often do reporters include the industry’s perspective in the stories that cover the issues of the day in the NYT and the GW?
4. What pharmaceutical companies are identified and discussed in the U.S. and Polish articles?
5. What are the implications of these findings for the pharmaceutical industry?

To be included in this study, an article had to be published between the October 1, 2010 and September 30, 2013 in the NYT and GW. The article also had to (a) focus on an ethical or legal issue facing the pharma industry and (b) In the NYT, it had to appear either as a front-page story or on the editorial page – an indication of major news and public sentiment.

In the GW, it had to appear on the front page or one of the main sections, such as the second page, Poland World Economy, Opinions, Business/People/Money, Science/Science Extra and Informer that were selected due to absence of an editorial page in Polish newspapers.

Focus was placed on newspapers rather than the broadcast media or weekly magazines for a number of reasons. The newspapers cover a broader range of issues and in more depth than the sound bites reported on radio and TV. Business and news magazines are also constrained by their weekly or monthly formats while the newspapers have the advantage of editorial coverage that takes a specific and unambiguous position – pro or con – toward the controversies in question. For each article, four elements were examined:

Issues – We identified and categorized the hot-button issues that were discussed in each article. Many articles covered two or more issues that were included in relevant sections.

Headlines – We analyzed the headlines and categorized them as positive, negative, or neutral toward the industry. Positive, negative, neutral – are determined from the perspective of the pharmaceutical industry. For example, the October 27, 2010 NYT headline “$750 Million Dollar Fine for Drug Maker of Tainted Goods” and the August 23, 2012 GW headline, “Popular Medicines for Hypertension...
and Cholesterol Will Go Up in Price”, were assessed as negative.

If a headline or article is determined to be favorable to the industry, then it is “positive”. For example July 14, 2011 GW headline “Antiviral drugs are protecting before HIV infection.”. If an article or headline cannot be determined as positive or negative from the industry’s perspective, it is “neutral”.

**Tone** – We also analyzed each complete article to determine whether it took a positive, negative, or neutral position toward the pharmaceutical industry. For example, any article that called for restrictions or a prohibition on DTC advertising – a position that the industry opposes – was deemed negative. In contrast, an article that claimed that DTC advertising resulted in more informed patients was designated as positive from the industry’s point of view.

**Balance** – Regardless of the dominant position taken by the article, we also looked to see whether the stories included the opposing point of view. When an explicit statement about an opposing view was included in the article – even if the two sides did not receive equal coverage – we concluded that the article covered both sides. When no mention of the opposing view was presented, the article was labeled as one-sided.

As the human coders were involved, the quality of content analysis was assured by following a few principles followed. In both languages, there was a written coding scheme that consisted of the codebook and coding form. Coders also had detailed training (see: 36). The articles were identified by searching the online archive for the presence of key words. Then, coders received a list of articles to read from the identified articles. After reading their assigned articles, the coders met with researchers and presented a progress report. At the meeting assessments of the individual elements of a code key – such as tone, balance were discussed. In case of

<table>
<thead>
<tr>
<th>Table 2. Three-year analysis of issues covered by the NYT and GW.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>New York Times ranking</strong></td>
</tr>
<tr>
<td>Ethical issue</td>
</tr>
<tr>
<td>Drug safety</td>
</tr>
<tr>
<td>Healthcare reform</td>
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<tr>
<td>High drug prices in the US</td>
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<tr>
<td>Interaction with FDA</td>
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<tr>
<td>Research &amp; development for new drugs</td>
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<tr>
<td>Clinical study design and sponsorship</td>
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<tr>
<td>Data disclosure</td>
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<tr>
<td>Generics</td>
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<tr>
<td>Marketing and sales incentives</td>
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<tr>
<td>Developing countries</td>
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<tr>
<td>Medicare/medicaid coverage for drugs</td>
</tr>
<tr>
<td>Genomics and Biologics</td>
</tr>
<tr>
<td>Reimportation/importation</td>
</tr>
<tr>
<td>Differential pricing/distribution</td>
</tr>
<tr>
<td>Intellectual property</td>
</tr>
<tr>
<td>Flu vaccines</td>
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<tr>
<td>Bioterrorism</td>
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<tr>
<td>DTC advertising</td>
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<tr>
<td>Other</td>
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</table>
disagreement about the rating of an article, the article was read by another coder and researcher. All statistical analyses to compare the newspapers were done using \[ \tilde{\text{analysis}}. \]

**RESULTS**

We identified and analyzed a combined total of 323 articles from the two newspapers on a subject. The NYT accounted for 35 in 2011, 48 in 2012 and 81 in 2013 for a total of 164 articles that concerned ethical issues in pharmaceutical industry. From the GW, there were 61 articles in 2011, 37 in 2012 and 61 in 2013 for a total of 159 articles. The number of articles analyzed from the NYT has steadily increased over the past three years while the number of related articles in the GW has fluctuated. In 2013, the NYT had 33% more relevant articles than the Polish newspaper. During the three-year period, 46.6% of relevant NYT articles were on the front page while in the GW, it was only 6.9%. These findings suggest that the issues received more prominent coverage in the NYT than in the GW.

**Identifying the issues**

In the period 2011-2013, the most analyzed articles from the NYT were devoted to the issue of drug safety followed by healthcare reform. The third most frequently reported issue was interaction with the FDA, which ranges from review for product approval to citation for product recall. In the U.S., the fourth most reported issue was high drug prices followed by research and development for new drugs in fifth place.

In Poland, the most popular issue for the study time period was the need for healthcare reform. Data disclosure was second. The third most reported issue was high drug prices, followed by the Polish reimbursement system, and drug safety.

For both countries, three of the top five issues reported were the same – drug safety, healthcare reform and high drug prices. Interaction with the FDA was an important subject in the U.S. However, in Poland, the work of the Drug Administration was not a subject of interest. Data disclosure is one of the most important issues in the GW, perhaps due to lack of trust in post-communist countries (37), such as Poland. From 2011-2013, both countries had similar rankings for generic drugs (U.S. - 8, Poland - 9) and marketing incentives (U.S. - 9, Poland - 8). In Poland during the last three-year period, the issues that merited coverage by the GW remained the same, while their position in the ranking changed.

Table 2 has a summary of these results.

**Analyzing the headlines**

In both countries for every analyzed year, headlines were more negative than positive toward the industry. In the U.S., the number of positive headlines increased with the increasing number of articles. In Poland, it was the opposite, with the percent of positive headlines decreasing each year. Headlines in the U.S. were more neutral than negative or positive for all three years. The same is true for all years in Poland.

<table>
<thead>
<tr>
<th>Year</th>
<th>Positive</th>
<th>Negative</th>
<th>Neutral</th>
</tr>
</thead>
<tbody>
<tr>
<td>2011</td>
<td>6 (17.1%)</td>
<td>7 (20%)</td>
<td>22 (62.9%)</td>
</tr>
<tr>
<td>2012</td>
<td>9 (18.8%)</td>
<td>15 (31.2%)</td>
<td>24 (50.0%)</td>
</tr>
<tr>
<td>2013</td>
<td>19 (23.5%)</td>
<td>24 (29.6%)</td>
<td>38 (46.9%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>34 (20.7%)</strong></td>
<td><strong>46 (28.1%)</strong></td>
<td><strong>84 (51.2%)</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
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<th>Year</th>
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<th>Negative</th>
<th>Neutral</th>
</tr>
</thead>
<tbody>
<tr>
<td>2011</td>
<td>9 (14.8%)</td>
<td>21 (34.4%)</td>
<td>31 (50.8%)</td>
</tr>
<tr>
<td>2012</td>
<td>4 (10.8%)</td>
<td>12 (32.4%)</td>
<td>21 (56.8%)</td>
</tr>
<tr>
<td>2013</td>
<td>6 (9.8%)</td>
<td>29 (47.5%)</td>
<td>26 (42.6%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>19 (11.4%)</strong></td>
<td><strong>62 (39.0%)</strong></td>
<td><strong>71 (49.1%)</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
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<th>Negative</th>
<th>Neutral</th>
</tr>
</thead>
<tbody>
<tr>
<td>2011</td>
<td>5 (14.3%)</td>
<td>2 (5.7%)</td>
<td>28 (80.0%)</td>
</tr>
<tr>
<td>2012</td>
<td>10 (20.8%)</td>
<td>16 (33.4%)</td>
<td>22 (45.8%)</td>
</tr>
<tr>
<td>2013</td>
<td>29 (35.8%)</td>
<td>32 (39.5%)</td>
<td>20 (24.7%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>44 (26.8%)</strong></td>
<td><strong>50 (30.5%)</strong></td>
<td><strong>70 (42.7%)</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Year</th>
<th>Positive</th>
<th>Negative</th>
<th>Neutral</th>
</tr>
</thead>
<tbody>
<tr>
<td>2011</td>
<td>11 (18.0%)</td>
<td>38 (62.3%)</td>
<td>12 (19.7%)</td>
</tr>
<tr>
<td>2012</td>
<td>8 (21.6%)</td>
<td>22 (59.5%)</td>
<td>7 (18.9%)</td>
</tr>
<tr>
<td>2013</td>
<td>9 (14.8%)</td>
<td>43 (70.5%)</td>
<td>9 (14.8%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>28 (17.6%)</strong></td>
<td><strong>103 (64.8%)</strong></td>
<td><strong>28 (17.6%)</strong></td>
</tr>
</tbody>
</table>
in Poland for 2011 and 2012. In 2013, however, most of the Polish headlines were negative (47.5%) and focused on the need for healthcare reform and high drug prices. Total results from the three-year period showed a statistically significant difference in the headlines between both countries (p < 0.05). In the NYT, a smaller number of headlines were negative (28.1%) compared to the GW (39.0%). The NYT headlines were more often positive (20.7%) than the GW (11.4%).

Comparing the tone of the articles

In both countries, it was easier to assess tone of the articles than the headlines. Indicative of this was the smaller number of articles classified as neutral. In the GW, this tendency is more visible.

Over 49 percent (49.1%) of the headlines were neutral and only 17.6 percent of the articles were neutral. By comparison, 51.2% of the headlines in the NYT were neutral and 42.7% of articles were neutral.

In the NYT, the number of positive articles rose every year, but so did the number of negative articles. The tone of the articles in 2011 was mostly neutral (80.0%) while in 2013 only 24.7% of them were classified as neutral. Taking the three-year period into account, it became clear that most of the articles in the NYT had a neutral tone towards the pharmaceutical industry (42.7%), but there were more negative (30.5%) than positive (26.8%) articles. In the GW, every year more than a half of the articles had a negative tone toward the pharma industry. In 2013, there were 70.5%. For the three-year period, 64.8% of the articles were negative. Only 17.6% of the articles were positive; there was exactly the same percentage of neutral articles as positive ones.

Comparing the tone of the articles, there was a statistically significant difference between the two countries (p < 0.001) for the three-year period. Articles in the NYT more often had a positive or neutral tone than in the GW, where 64.8% of the analyzed articles had a negative tone toward the pharmaceutical industry.

The balance of coverage in the NYT and GW

In 2011, 40.0% of the articles in the NYT presented not only the dominant position taken by the article but also the opposing point of view and were judged to have fair balance. In 2012, it was more than half (62.5%) of the articles but in 2013, fair balance dropped to 28.4%. This was related to one-sided coverage about the tragic deaths associated with the New England Compound Center’s production and sale of contaminated medicines. Additionally, there was one-sided coverage about GlaxoSmithKline’s diabetes drug, Avandia®, and its link to cardiovascular problems. Coverage about one of Pfizer’s generic products, Levongestrel, was also one-sided due to potential side effects associated with this birth control medication.

In the GW, every year the percentage of fair balance articles increased from 31.1% in 2011 to 32.4% in 2012 and 39.3% in 2013. Conversely, in the GW, every year had more than 60 percent of one-sided articles. For the study period, there was fair balance in 40.8% of the U.S. articles compared to 34.6% in the Polish articles. The difference
between countries for fair balance is statistically significant (p < 0.05)

Analyses of the pharmaceutical companies’ names mentioned in articles showed that top five reported in both countries were mostly international corporations. Only the Polish Polfa broke this tendency. GSK and Pfizer were the most reported companies in both the NYT and GW. The companies reported are in Figure 1.

DISSCUSSION

The results yielded some commonality and divergence in the way ethical issues affecting the pharmaceutical industry are reported in the NYT and GW. Pharmaceutical companies in both the U.S. and Polish markets employ promotional strategies and are involved with ethically charged situations. Different history, culture and regulatory environments cause similar ethical situations to be perceived and described differently in newspapers. For example, coverage was more prominent in the NYT than the GW based on the larger number of front-page articles in the NYT. Only two sections of the NYT (front-page and editorials) had more articles than the entire GW without regional additions (159 vs. 164) over the three-year period of the study.

This different attention to ethical issues and the Pharm industry was also observed in other spheres of public life. In the U.S., there are many non-governmental organizations, such as No Free Lunch or Public Citizens, which pay attention to the type of marketing efforts the pharmaceutical industry is deploying. However, in Poland, the public believes that Polish laws (which is much stickier than law in U.S.) will protect them from questionable advertising and/or promotions by the pharmaceutical industry. Unlike the U.S., that same belief has also discouraged and continues to discourage creating codes of conduct to guide the pharmaceutical industry’s behavior with doctors, hospitals and medical schools. Conversely, in the U.S., codes of conduct of self-regulation have made somewhat of a difference (31).

Understanding why the issues were reported

Three of the top five issues affecting the pharmaceutical industry were common between the two countries, such as healthcare reform. During the investigated period, the U.S. was on the verge of approving the Affordable Care Act (ACA or Obamacare). Justification for the Obamacare reform was due to increasing costs of healthcare, growing profits of the healthcare institutions and rising personal debt and bankruptcy from medical bills. In Poland, discussion about the need for healthcare reform is open-ended. Costs of healthcare in Poland grew very quickly due to the aging of the society, incidence of chronic diseases, availability of medical technologies and greater expectations concerning health. Meanwhile, the percent of private payment in Poland is one of the OECD countries where out-of-pocket expenses as a percentage of total healthcare expenditures are the highest, including in the U.S. (38). The intersection of issues covered by the NYT and GEW are depicted in Figure 2.

![Figure 2. GW and NYT coverage of top five ethical issues over the last three years](image-url)
Drugs are more expensive in the U.S. than other countries, which attracts coverage in the NYT (39). Due to minimal control of drug prices, U.S. consumers pay more for their prescriptions but the industry contends that it enables the pharma industry to invest in R&D (40). However, according to 2012 IMS data, per capita expenditure on pharmaceuticals in US is $ 947, the highest in the world, nearly twice the OECD average (41). Eurostat research indicates that expenditures for medicines in Poland, at a per capita of $ 129, are one of the lowest in Europe (42). However, IMS statistics show that drugs in Poland are relatively expensive when the prices of medicines were compared to salary. In fact, Rx drugs are, on average, five percent (5%) more expensive in Poland than in other EU countries (43). That is the reason why the subject attracts so much attention in the GW.

Safety concerns and related lawsuits are always issues that garner coverage. Even though a medicine has been approved as “safe and effective,” it must be used according to specified instructions in the package insert. Yet, it is estimated that 10000 people die each year due to improper use (44). This is especially concerning and newsworthy when approved drugs reveal dangerous side effects not found in clinical studies. One such example for Americans and Poles was Vioxx®. The drug was in the market for five years. It has been prescribed for more than 80 million people globally, more than 20 million in the US (45), and about nine thousand in Poland (46).

In the U.S., R&D investments are the largest in the world (21) so it is not surprising that the issues of R&D for New Drugs and Clinical Study Design and Sponsorship are the subject of many NYT articles. In Poland, very little is invested in R&D for the development of new drugs (47). This is due to political, legal, fiscal and administrative barriers. Consequently, the GW was relatively quiet on these issues.

Articles related to data disclosure in Poland mainly focus on the transparency of what doctors get from the pharma companies, disclosure of problems with pharmacies and manufacturing problems or previously unreported interests connected with pharmaceuticals. This issue resonates with the lack of trust that people have in a post-communist society. Newspaper coverage about this also has a sensational nature as illustrated by the headline of one of the GW articles, “FBI on Polish Track”, which described how the FBI assisted the Polish prosecutor’s office with an investigation of whether the U.S.-company, Stryker, set up a fund to bribe Polish doctors and hospitals. Conversely, data disclosure in the U.S., while important, was ranked sixth among the issues identified.

Reimbursement for patients’ medicines or related issues always draws the media’s attention. This was the case in Poland when a new reimbursement was passed in January 2012 (48). It established regular retail prices for drugs along with uniform profit margins in pharmacies. It also introduced a limit of public funds for reimbursement for medicines at 17 percent of health spending. When this amount is exceeded, the pharmaceutical company will be forced to cover half the cost (49). Not surprisingly, the new law contributed to a slowdown in growth of the pharmaceutical market in Poland (50). For many months before and after the law, many articles in the GW discussed its potential impact. During that same time period, there were no articles in the NYT about Medicare/Medicaid Coverage for Drugs. With health costs continuing to rise in the U.S., any efforts negotiating lower prices for drugs will certainly receive newspaper coverage but the law that created pedicar part D forbids price negotiation (Negotiating prices for drugs reimbursed by medicare part D 2015).

Comparing Coverage in the NYT and the GW

Between the countries, there were statistically significant differences in support of or opposition to the positions taken by the industry by the headlines and articles. Over the past three years, the majority of U.S. headlines and articles were neutral (51.2% for headlines and 42.7% for articles), with the remaining being split fairly evenly between negative and positive. Whereas in Poland 49.1 percent of headlines were neutral with the remaining balance mostly negative (39.0%). The majority of articles (64.8%) were negative with the remaining being split evenly between positive and neutral. This phenomenon may be explained by the cultural differences and dissatisfaction by Poles.

When fair balance was compared between the NYT and the GW, the results were different. In the NYT, 40.0% of the articles had fair balance in 2011, and increased to 62.5% in 2012 but plunged to 28.4% in 2013. In the GW, the number of articles with fair balance remained stable with 31% in 2011, 32.4% in 2012 and 39.3% in 2013.

CONCLUSION/IMPLICATIONS

Comparing newspaper coverage in the U.S. and Poland identified similarities and differences in the types of issues covered as well as differences
in the reasons for the coverage. This was especially true for the top three issues, healthcare reform, drug safety and drug prices. The impetus behind the coverage usually had similar roots, such as healthcare reform. The U.S. recently passed Obamacare and Poland was anticipating reform. Differences that were observed were largely attributable to the cultural differences between the two countries, particularly the post-Communism effect in Poland. These differences manifested themselves in the tone of coverage in the NYT and the GW (p < 0.001). Articles in the NYT articles more often had a positive or neutral tone compared to the GW, where 64.8 percent were in negative tone toward the pharma industry. When fair balance was considered, the NYT had more articles during the first two years of the study period but the GW had more during the third year due to a decrease in the number of fair-balance articles in the NYT (p < 0.05).

Implications of the findings for the pharma industry

Key implications for the pharmaceutical industry from this study include the need for pharma companies to know the differences in attitudes towards pharmaceutical marketing to communicate effectively with customers worldwide. This cross-cultural analysis reveals some pertinent results. In the U.S., the issues connected to the pharmaceutical industry are more frequently reported on the front page, which is indicative of greater public interest about the issues than in Poland. Frequent coverage of those issues on the front pages in the NYT but quite rarely in GW may validate the belief that newspapers add “validity” of the issues to the public. They are trying to increase newspaper sales by choosing hot-button issues for the front page. It can be concluded that the Polish society is still not seen by them as “very sensitive” to issues related to the pharmaceutical industry and/or CSR, in contrast to the American public.

Importantly, the negative coverage about the industry in both the GW and NYT is also a reflection of the actions taken by the pharmaceutical industry countries. The media’s role is to report what it observes. Optimistically, newspaper coverage can serve as short-term motivation for change as well as long-term motivation for preservation of its reputation. Furthermore, experience suggests that a perceived lack of the ineffectiveness of industry self-regulation leads to increased government intervention and regulation.

To help ensure more positive newspaper coverage, the pharmaceutical industry should implement the principles of CSR in their everyday practices. They should not use the idea just as the marketing tool. This research showed that there are similar issues connected with ethics and CSR in the U.S. and Poland, which are important for the journalists and society. They are: drug safety, good interaction with drug administration, investments in R&D, responsible participation in healthcare reform, appropriate drug prices and data disclosure. These are the issues that need to be addressed in pharmaceutical companies’ strategies if they want to get more positive newspaper coverage.

No matter where you are located in the world, including the U.S. and Poland, drug safety and drug prices will be watched carefully and covered frequently. The interest in these two issues is heightened when healthcare reform is being discussed as is the case in the U.S. and Poland. Given the anticipation of healthcare reform in Poland and the implementation of Obamacare in the U.S., a future study comparing these differences can generate results to assess how the industry has responded to these findings.

For future research, these studies have the opportunity to be developed further. A longer time period can be considered to assess whether the reputation of the pharmaceutical industry has changed. It can also be expanded to the other daily newspapers in Poland and compared to the four other newspapers (Wall Street Journal, Los Angeles Times, USA Today and Washington Post) being studied in the U.S. since 2004 with analyses stored in the EthicsTrak database. Another research opportunity is to evaluate how other media sources, e.g., social media, radio and television, compare to newspaper coverage in the U.S. and Poland.

Acknowledgments

We want to recognize the analyses on the NYT by the EthicsTrak Administratrix, Lauren Lang, and GW by the Coders, Marcin Półtorak, Malwina Bugajak and Piotr Gołębiowski.

REFERENCES


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The science of chromatography constitutes the separation of different components based on their affinity to the non-miscible phases (1). One of two phases which is usually referred to as the stationary phase that can be either a liquid or a solid is usually fixed and the second is a liquid or gas flows through the stationary phase. Hence they both in combination constitute the chromatography system. There are two types of chromatography i.e., one is liquid chromatography and the other is gas chromatography (2). In chromatography, the compounds move along the natural velocity depending upon their affinities. Every molecule which is called analyte has different affinity and distribution depending upon which net migration velocity is calculated. It explains the principle of separation in chromatography (3).

Thin layer chromatography (TLC) is a separation and analytical technique which is widely used for the separating action of different analytes such as non-volatile, polar, non-polar substances, molecules or compounds natural or artificial present in the mixture to be analyzed by chromatography (4). Thin layer chromatography contains a supporting material made up of plastic or glass or aluminum foil which is then coated with a specific coating substance usually made of silica, cellulose or alumina of allowed thickness which acts as base or adsorbent or stationary phase (5). The analyte or sample is usually applied to the stationary phase and is than placed with an appropriate solvent(s) making the mobile phase or solvent system present in a TLC chamber which is pre-saturated to attain an equilibrium phase between the solvent system and its surrounding.
vapor phase. This pre-saturation results in a uniform and homogenized travelling of sample or analytes due to capillary action along the TLC plate inside the TLC chamber (6). The speed at which the sample or analyte moves along depends on the respective affinity of that particular substance to the stationary phase and mobile phase. Different molecules have different rates thus different ascending rates, and hence different time for every separation (7,8).

*Glycyrrhiza glabra* constitutes a drug called Liquorice which has somewhat sweetish flavor. The liquorices belong to the pea family widely known as legume family. Glycyrrhiza is indigenous to southern Europe and different areas of Asia (9). Glycyrrhiza is an herb having perennial cycle. The plant contains pinnate arrangement of leaves which are 7-15 cm in length usually with a varying number of leaflets around 9-17. Flowers of glycyrrhiza are 0.8–1.2 cm in length, purple, whitish blue, producing a sweet aroma. The plant as a whole or its part including the root, stem, leaves, fruits and seeds differ in their components when extracted under different conditions, also under different experimental extraction procedures, two different extraction methods and solvents systems have been used in this study.

**EXPERIMENTAL**

**Chemicals**

The chemicals used in this study were sulfuric acid (H$_2$SO$_4$), chloroform, anhydrous sodium sulfate, glacial acetic acid, methanol, water, ethyl acetate, vanillin, ammonia and ethanol. All these chemicals were obtained from Merck, Germany.

**Plant used**

Glycyrrhiza has many identified species but the most common species *Glycyrrhiza glabra* has been used for this study. *Glycyrrhiza glabra* was purchased from local market and authenticated by a Botanist. Its sample was kept in the institutional herbarium with a specific code number.

**Extraction Method A**

Powdered drug 2 or 20 g of glycyrrhiza was heated under reflux for 1 hour with 30 mL of 0.5 M H$_2$SO$_4$. The filtrate was shaken twice with 20 mL of chloroform. The combined extracts of both chloroform portions were then passed over anhydrous sodium sulfate which acted as a drying agent and absorbed the extra water molecules (13). The resulting filtrate was then filtered with the help of filter paper and was heated on an optimum temperature to facilitate drying process. The residue was dissolved in 10 mL of chloroform-methanol solvent system in 1 : 1 ratio and 10 µL of this solution was used for the detection of glycyrrhizic acid on TLC (14).

**Extraction Method B (without hydrolysis)**

Powdered drug 2 or 20 g of glycyrrhiza was heated under reflux for 1 hour with 30 mL of 0.5 M H$_2$SO$_4$. The filtrate was shaken twice with 20 mL of chloroform. The combined extracts of both chloroform portions were then passed over anhydrous sodium sulfate which acted as a drying agent and absorbed the extra water molecules (13). The resulting filtrate was then filtered with the help of filter paper and was heated on an optimum temperature to facilitate drying process. The residue was dissolved in 10 mL of chloroform-methanol solvent system in 1 : 1 ratio and 20 µL is usually used for TLC but for the species *Liquiritae radix* and ethanolic extract was evaporated and the dried residue is dissolved in 2 mL of chloroform and ethanol.

**Table 1. Compounds isolated under the studied experimental conditions.**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Liquiroside</th>
<th>Glycyrrhizin</th>
<th>Glycyrrhizic acid</th>
<th>Chalcones</th>
<th>Flavanon glycosides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method A + solvent system A</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>Method B + solvent System B</td>
<td></td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Method A + solvent system B</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Method B + solvent system A</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>Spraying with VS reagent 42</td>
<td>✓</td>
<td></td>
<td></td>
<td>✓</td>
<td></td>
</tr>
</tbody>
</table>

Spraying with VS reagent 42 in all methods and solvent systems.
methanol (1 : 1) mixture, out of which, 20 µL was
used for detection of glycyrrhizin on TLC (15).

Thin layer chromatography (TLC) conditions
Silica gel GF 254 (pharmaceutical grade) pre-
coated TLC plates (Science-Center, Merck) were
used in this study. Using TLC saturation chamber
(Peshawar, Pakistan), two solvent systems were
employed in this study, i.e., solvent system A which
consisted of chloroform, glacial acetic acid,
methanol and water in a ratio of 64:25:9:1 while sol-
vent system B contained ethyl acetate, ethanol, water

Methods of detection
Without chemical treatment
The detection method are designed to detect
glycyrrhizin and glycyrrhizic acid but cannot detect
saponins with low concentrations. All the com-
ponents are checked under UV 254 nm and UV 365
nm. In solvent system A, glycyrrhizin appears as
violet zone having Rf 0.35-0.4, violet brown zones
0.1-0.65 (0.3-0.4) shows the presence of gly-
cyrrhizin. In solvent system B, glycyrrhizic acid was
found at Rf 0.45 (21).

With chemical treatment
The 10 mL of solution A from VS 42 was
sprayed on TLC plate immediately followed by 10
mL of solution B from VS 42 (VS 42 : Vanillin-sul-
furic acid reagent preparation, chemicals needed for
VS 42 are 1% ethanolic vanillin (solution 1) and 10%
ethanolic sulfuric acid (solution 2). TLC plate is then
heated at 110°C almost for 5-10 minutes, evaluation
of plate is done in visible region and is implicated for
the presence of components of essential oils such as
terpenoids and phenylpropanoids), saponins are
detected in blue or blue violet and rarely as red or
brownish yellow bands or zones (22).

RESULTS AND DISCUSSION
This study isolates different organic fractions
of the glycyrrhiza root extract using TLC employing
different experimental methods and solvent systems.
As we know that any compound extracted from
whole plant, root or any part of the plant is depend-
ent on the isolation procedure However, the results
differ if the environmental conditions are not simi-
lar. Hydrolysis also has a great impact on the con-
stituents derived (23). TLC development, solvent
system, saturation chamber, and pre-coated TLC
plates all play a varying and distinctive role in the
isolation and detection of compounds (24).

This particular study was to elaborate the fine disti-
ctions in the noble field of chromatography using
specifically thin layer chromatography elaborating two
different extraction procedures resulting in two differ-
ent groups of compounds being isolated. Moreover, the
detected compounds were different at different wave-
lengths, i.e., UV 254 nm and UV 365 nm.

The detection methods were specifically
designed as a sequel to the fact that some com-
ponds are visible without UV light, whereas some
require UV fluorescence to be detected. Also some
compounds are not immediately visible under UV
lamp and thus a number of chemical treatments are
implicated which result in further detection of com-
ounds either permanently or for some transient
temporary time frame (25).

Experiment performed with solvent system A and
extraction method A
Glycyrrhizic acid was detected at Rf 0.40 and
liquiroside was detected without chemical treatment
with VS reagent at Rf 0.45. A broad range at solvent
front was also detected characterizing flavones.

Experiment performed with solvent system B and
extraction method B
This method being specific to detect gly-
cyrrhizin and so using solvent system A it was
detected. Glycyrrhizin was detected at UV 365 nm
at Rf 0.32. After chemical treatment with VS 42 still
there was no change in the detection of glycyrrhizin.
At Rf 0.45-0.62 chalcones were detected, with no
individual detection at UV 254 nm. No compounds
were detected at UV 254 nm, only undistinguished
full bands were traced along the solvent front.

Experiment performed with Solvent system B and
extraction method A
The primary compound in liquorice, gly-
cyrrhizin, was detected at UV 365 nm at Rf 0.30. After chemical treatment with VS 42, orange yellow
brown zone is visible showing the presence of
liquiroside and corresponding chalcones as a band
Rf at the visible and UV 365 nm.

Experiment performed with solvent system A and
extraction method B
Aglycone glycyrrhizic acid was detected at Rf
0.44. All the chalcones were detected in the Rf range
of 0.45-0.75 with individually unidentified chal-
cones at 0.47, 0.52 and 0.55 and flavanone glyco-
sides at Rf 0.70 under UV 365nm.

Two different extraction procedures and the
development of TLC plates with two different sol-
vent systems were designed according to the compound’s affinity for polar and non-polar solvents (26-30). In TLC systems, solvent systems play key role in carrying the compounds. The compounds which are to be detected depend on the extraction procedure, chemical purity, experimental errors, following the protocol, environmental factors and sensitivity of the equipments. If all the criteria are met as the protocol states, even then some extraction procedures have merits over others. In this experiment, there is an evident merit of method B over method A, method without hydrolysis. It is specifically designed to reduce time of experiment using vacuum for efficient extraction of compounds under reduced pressure. Both methods have their own merits but method B is proven better than method A for the detection of glycyrrhizin (31). The VS 42 reagent system which is designed to detect compounds of terpenoids and phenylpropanoids in nature does not have a valid significance as most of the compounds were detected without the chemical treatment.

CONCLUSION

The experimental results prove the significance of method B over method A for the detection and isolation of glycyrrhizin in short span of time. Hydrolysis converts glycyrrhizin to glycyrrhetic acid which then loses its pharmacological activity so method B shows major significance as compared to method A. Solvent system B being more basic (polar) having ammonia is an excellent choice for detection of glycyrrhizin – a triterpenoid saponin glycoside. Also ammonia forms salts with glycyrrhizin which is more water soluble and hence more effective if formulated as a drug.

REFERENCES


Received: 9.08.2016
In the paper entitled: “Synthesis and biological evaluation of sulfonamide derivatives as antimicrobial agents” by Hebat-Allah S. Abbas, Somaia S. Abd El-Karim, Nayera A.M. Abdel Wahed, published in vol. 74 (2017), issue no. 3, pp. 849-860, scheme no. 2 (p. 857) should read:

Scheme 2. Synthesis of the target compounds 11-19. Reagents and conditions: i) dimethyl malonate/AcOH, reflux; ii) acetyl acetone/AcOH, reflux; iii) ethyl cyanoacetate/AcOH, reflux; iv) ethylacetoacetate/AcOH, reflux; v) sodium nitrite/HCl/malononitrile/EtOH, stirring 0°C; vi) 2-(bis(methylthio)methylene)-malononitrile/DMF/TEA, reflux; vii) ethoxymethylene malononitrile/EtOH/TEA, reflux; viii) cyano guanidine/EtOH, reflux; ix) 2-(naphthalen-2-yilmethylene) malononitrile/EtOH, reflux

In the paper entitled: “Synthesis, characterization and biological screening of diandrine A” by Rajiv Dahija and Sunil Singh, vol. 74 (2017), issue 3, pp. 873-880, the following corrections should be introduced:

page 876, 2nd paragraph, last line, 10 mg/mL should be corrected as 10 mcg/mL,
page 878, 1st paragraph, 18th line, 10 mg/mL should be corrected as 10 mcg/mL,
page 878, Table 1, MIC value unit should be mcg/mL instead of g/mL,
page 879, below Table 2 bc and c should be as superscripts.

In the paper entitled “Pharmaceutical care in opinion of Polish medical and pharmaceutical students: an exploratory study” vol. 74 (2017), issue 3, pp. 1021-1030, the correct affiliation of authors should be:

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In the same paper, on page 1030 the following information should be added:

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