Liver is the principal internal organ of body having hepatocytes which are its functional entities. Liver separately performs up to 500 functions, usually by interacting with other systems and organs. The chief task of liver is to detoxify toxic substances from body and to metabolize ingested substances (food, nutritional supplements, alcohol or medicines). Along with it, liver regulates glycogen storage and breakdown of red blood cells; produce plasma proteins; and synthesize hormones (1).

The injury produced in liver is hepatotoxicity and it is derived by chemicals (2). Hepatotoxicity is also defined as malfunction or destruction to liver due to drugs or xenobiotics known as hepatotoxins when they are given in overdose (3). Hepatic injury can also be induced as a result of oxidative stress, which is induced by activation of enzymes in CYP 450 system such as CYP2E (4). Hepatotoxicity damages liver in both mild and severe forms. The capability of any formulation, compound or product to prevent damage to the liver is known as hepatoprotection or anti-hepatotoxicity. Many synthetic drugs being used in the treatment of liver diseases have serious side effects, owing to which there is an increasing focus to follow systemic research methodology and to evaluate scientific basis for traditional herbal medicines that are claimed to possess hepatoprotective activity (5). There are a number of medicinal preparations in Ayurveda for treatment of liver disorders in the absence of a reliable liver protective drug in modern medicine (6).

Numerous studies have justified that phytotherapeutic agents which possess antioxidant activity are effective in producing hepatoprotection against paracetamol (PCM) and carbon tetrachloride (CCl4) induced hepatotoxicity (7). Melilotus officinalis, L. (MO), belongs to genus Melilotus Mill is a sweet smelling biennial herb, mainly distributed in Pakistan, Kashmir, India, Tibet, Russia, China, Turkey, Middle and Southern Europe (8). In folk medicine, it is used for treating jaundice and liver diseases (9, 10). Thus, on the basis of traditional use

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**EVALUATION OF HEPATOPROTECTIVE ACTIVITY OF MELILOTUS OFFICINALIS L. AGAINST PARACETAMOL AND CARBON TETRACHLORIDE INDUCED HEPATIC INJURY IN MICE**

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**Abstract:** Hepatic diseases are becoming common day by day and pose serious health threats to the life of humans. In order to treat these diseases, the attention of man is diverting towards herbal drugs, which are much safer and cost effective than synthetic drugs. The aim of present study was to investigate hepatoprotective activity of methanolic extract of *Melilotus officinalis* against paracetamol and carbon tetrachloride induced hepatic damage. *Melilotus officinalis* at selected oral doses of 50 mg/kg and 100 mg/kg showed significant hepatoprotective effects by decreasing the levels of serum marker enzymes such as total bilirubin, SGOT, SGPT, ALP, albumin and total protein, when compared with standard drug (silymarin) and negative control. Similarly, histopathological studies also supported biochemical estimations. It was concluded that extract of *Melilotus officinalis* has strong hepatoprotective activity against paracetamol and carbon tetrachloride induced hepatotoxicity, which might be due to free radical scavenging mechanisms exhibited by flavonoids and phenolics, thus affirming its traditional therapeutic role in liver injury.

**Keywords:** *Melilotus officinalis*, hepatoprotective, paracetamol, carbon tetrachloride, histopathology

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of *Melilotus officinalis* (MO) for hepatic ailments, it was thought worthwhile to scientifically investigate hepatoprotective effect of *Melilotus officinalis* against paracetamol (PCM) and carbon tetrachloride (CCl₄) induced hepatotoxicity in mice, by screening the levels of serum marker enzymes and by performing histopathological studies.

**MATERIALS AND METHODS**

**Chemicals**

The chemicals used were methanol (Sigma Aldrich), paracetamol (Sigma Aldrich), carbon tetrachloride (Sigma Aldrich), silymarin (Sigma Aldrich). All the other chemicals used were of analytical grade.

**Plant material**

The aerial parts of *Melilotus officinalis* (2 kg) were collected from Dhillam Ballaggan, Sialkot, Punjab, Pakistan during the months of April to May 2015. Plant was identified and authenticated by Professor Dr. Ashiq, Department of Botany, University of Agriculture, Faisalabad. Plant material was first washed, shade dried and ground into powder with a Chinese herbal grinder. The powdered material was stored in well closed cellophane bags at 4°C in refrigerator.

**Preparation of plant extract**

The aqueous methanolic (30 : 70) extract of plant was prepared by cold maceration process. The plant was soaked in 3 L of solvent and it was kept at room temperature for 3 days (72 h) while, it was occasionally shaken daily. After 3 days, filtration was carried out using a porous cloth. The filtrate was collected and again plant material was soaked for 3 days, in 3 L solvent twice. At the end of procedure, all of the three filtrates were collected and again filtered through muslin cloth and Whatman filter paper I. The filtrate was evaporated under reduced pressure in rotary evaporator at 50°C. At the end, a solid mass was obtained after air drying extract. The color of crude extract was dark brown (11). For administration, the extract was dissolved in distilled water and administered as solution.

**Animals used**

Young and healthy, both male and female albino mice (20-40 g), obtained from NIH, Islamabad were used. Mice were kept in a controlled room with 12 h light and dark cycles, at room temperature of 22 ± 0°C at animal house of University of Sargodha, Sargodha. Animals were fed standard diet and tap water. All the experiments performed complied with the rules of National Research Council (12).

**Paracetamol induced hepatotoxicity**

For this experiment, mice were divided into 5 groups of 4 mice each. Group I was maintained as control and received normal saline (1 mL/kg, p.o.) once daily for 7 days. Group II received normal saline and PCM (250 mg/kg, p.o.) was given in single dose on 7th day. Group III mice served as standard group and were treated with silymarin (100 mg/kg/10 mL, p.o.), once daily for 7 days, followed by single administration of PCM (250 mg/kg, p.o.) on 7th day. Group IV mice were treated with MO (50 mg/kg, p.o.) once daily for 7 days, followed by single administration of PCM (250 mg/kg, p.o.) on 7th day, 1 h after MO treatment. Group V received MO (100 mg/kg, p.o) once daily for 7 days, followed by single administration of PCM (250 mg/kg, p.o.) on 7th day, 1 h after MO extract treatment.

The hepatoprotective activity of plant extract was biochemically and histopathologically assessed. After 24 h of drug treatment, the animals were anesthetized using ether. The blood was withdrawn from carotid artery of neck of each mouse, preserved in centrifugation tubes and allowed to clot for 30 min at room temperature. Serum was separated by centrifugation at 3000 rpm for 15 min. This serum was utilized for evaluating biochemical parameters like ALT, SGOT, SGPT, albumin, bilirubin and total protein. Livers of all the animals were dissected out and were placed in 10% formalin solution for histopathological study (13).

**Carbon tetrachloride induced hepatotoxicity**

The mice were divided into 5 groups of 4 mice each. Group I mice were maintained on distilled water (1 mL/kg, p.o.) once daily for 7 days, followed by 1 mL/kg of olive oil subcutaneously on the last day, 1 h after distilled water feeding. Group II mice were administered distilled water for 7 days, followed by 20% v/v CCl₄ in olive oil (1 mL/kg, s.c.) on 7th day, 1 h after distilled water administration. Group III served as standard and mice received silymarin (100 mg/kg/10mL, p.o.), once daily for 7 days, followed by single administration of 20% v/v CCl₄ in olive oil (1 mL/kg, s.c.) on last day, 1 h after silymarin treatment. Group IV mice were treated orally with MO (50 mg/kg, p.o) once daily for 7 days, followed by single administration of 20% v/v CCl₄ in olive oil s.c. at 1 mL/kg on last day, 1 h after MO treatment. Group V mice were given MO (100 mg/kg, p.o.) once daily for 7 days, followed by single administration of 20% v/v CCl₄ in olive oil s.c. at 1 mL/kg on last day, 1 h after MO treatment.
The hepatoprotective activity of plant extract was biochemically and histopathologically assessed. After 24 h of drug treatment, the animals were anesthetized using ether. The blood was withdrawn from carotid artery of neck of each mouse, preserved in centrifugation tubes and allowed to clot for 30 min at room temperature. Serum was separated by centrifugation at 3000 rpm for 15 min. This serum was utilized for evaluating biochemical parameters like ALT, SGOT, SGPT, albumin bilirubin and total protein. Livers of all the animals were dissected out and were placed in 10% formalin solution for histopathological study (14).

Statistical analysis
The results were expressed as the mean ± standard error of mean (S.E.M). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Dunnett’s post-hoc test, using Graph Pad Prism 5. The value of significant difference was considered at p < 0.05.

RESULTS
The present investigation suggested that paracetamol administration alone caused a significant increase in levels of serum marker enzymes, which can be evident as hepatotoxicity as compared to normal control group (Table 1). However, when plant extract was administered, the elevated marker enzymes were reduced as compared to negative control. Plant extract at 50 mg/kg produced significant reduction (p < 0.05) in the levels of total bilirubin. The reduction in ALP, SGOT and SGPT was highly significant (p < 0.01), (p < 0.001). However, a non-significant (p > 0.05) reduction in the levels of albumin and total bilirubin was observed. The reduction in levels of liver marker enzymes was found to be

### Table 1. Effect of methanolic extract of *Melilotus officinalis* on biochemical parameters in paracetamol induced hepatotoxicity.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total bilirubin (mg/dL)</th>
<th>SGOT (U/L)</th>
<th>SGPT (U/L)</th>
<th>ALP (U/L)</th>
<th>Albumin (g/dL)</th>
<th>Total protein (g/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control (N.S 1 mL/kg)</td>
<td>0.533 ± 0.067*</td>
<td>72.333 ± 2.333*</td>
<td>79.333 ± 4.055*</td>
<td>135.667 ± 5.364**</td>
<td>3.000 ± 0.058*</td>
<td>5.867 ± 0.467**</td>
</tr>
<tr>
<td>Paracetamol (250 mg/kg)</td>
<td>0.833 ± 0.120</td>
<td>85.333 ± 18.985</td>
<td>82.667 ± 15.452</td>
<td>209.333 ± 5.206</td>
<td>4.300 ± 0.889</td>
<td>7.533 ± 0.623</td>
</tr>
<tr>
<td>Silymarin (100 mg/kg/10 mL)</td>
<td>0.533 ± 0.033*</td>
<td>14.667 ± 1.856**</td>
<td>19.000 ± 1.528***</td>
<td>193.333 ± 4.410**</td>
<td>3.900 ± 0.577**</td>
<td>6.533 ± 0.318**</td>
</tr>
<tr>
<td>MO (50 mg/kg)</td>
<td>0.500 ± 0.000*</td>
<td>19.667 ± 8.172**</td>
<td>23.667 ± 8.212***</td>
<td>203.000 ± 14.224**</td>
<td>3.200 ± 0.300*</td>
<td>6.700 ± 0.379*</td>
</tr>
<tr>
<td>MO (100 mg/kg)</td>
<td>0.567 ± 0.033*</td>
<td>23.333 ± 7.333**</td>
<td>24.000 ± 6.000***</td>
<td>201.333 ± 14.438**</td>
<td>3.267 ± 0.267**</td>
<td>7.500 ± 0.551**</td>
</tr>
</tbody>
</table>

Results are expressed as the means ± SEM (n = 4), where, * = (p < 0.05), ** = (p < 0.01), *** = (p < 0.001) vs PCM (250 mg/kg).

### Table 2. Effect of methanolic extract of *Melilotus officinalis* on biochemical parameters in carbon tetrachloride induced hepatotoxicity.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total bilirubin (mg/dL)</th>
<th>SGOT (U/L)</th>
<th>SGPT (U/L)</th>
<th>ALP (U/L)</th>
<th>Albumin (g/dL)</th>
<th>Total protein (g/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control (N.S 1 mL/kg)</td>
<td>0.533 ± 0.067***</td>
<td>72.333 ± 2.333*</td>
<td>79.333 ± 4.055***</td>
<td>135.667 ± 5.364***</td>
<td>3.000 ± 0.058**</td>
<td>5.867 ± 0.467***</td>
</tr>
<tr>
<td>Carbon tetrachloride (1 mL/kg)</td>
<td>1.600 ± 0.153</td>
<td>72.000 ± 16.166</td>
<td>175.000 ± 15.373</td>
<td>441.000 ± 28.931</td>
<td>6.700 ± 1.353</td>
<td>9.333 ± 0.176</td>
</tr>
<tr>
<td>Silymarin (100 mg/kg/10 mL)</td>
<td>0.633 ± 0.033***</td>
<td>42.333 ± 9.062*</td>
<td>47.667 ± 4.631***</td>
<td>162.000 ± 7.572***</td>
<td>3.100 ± 0.306**</td>
<td>5.833 ± 0.441***</td>
</tr>
<tr>
<td>MO (50 mg/kg)</td>
<td>0.567 ± 0.033***</td>
<td>42.667 ± 1.764*</td>
<td>67.333 ± 11.624***</td>
<td>180.000 ± 5.774***</td>
<td>3.400 ± 0.306*</td>
<td>5.900 ± 0.289***</td>
</tr>
<tr>
<td>MO (100 mg/kg)</td>
<td>0.600 ± 0.05***</td>
<td>30.333 ± 2.906*</td>
<td>48.667 ± 1.333***</td>
<td>160.333 ± 4.333***</td>
<td>3.233 ± 0.033**</td>
<td>6.367 ± 0.176***</td>
</tr>
</tbody>
</table>

Results are expressed as the means ± SEM (n = 4), where, ns = (p > 0.05), * = (p < 0.05), ** = (p < 0.01), *** = (p < 0.001) vs CCl₄ (1
similar to that caused by standard drug, silymarin. The results clearly depict that 50 mg/kg showed more significant reduction in biochemical parameters than 100 mg/kg (Table 1). This may be owing to genetic variations or saturation of receptors at high dose might have occurred, resulting in lesser response.

Similarly, in carbon tetrachloride induced hepatotoxicity model, it was perceived that CCl₄ alone caused an increase in levels of marker enzymes (Table 2). This is an evidence of causing hepatotoxicity as compared to normal animals. However, administration of 50 mg/kg of MO extract produced highly significant reduction (p < 0.001) in levels of SGPT, ALP, total protein and total bilirubin. The extract also caused a significant (p < 0.05) reduction in levels of SGOT and albumin. The activity of MO was similar to that of standard drug i.e., silymarin (Table 2). Though, 100 mg/kg showed more significant reduction in levels of SGOT, SGPT, ALP and albumin than 50 mg/kg. In CCl₄ intoxicated model, the methanolic extract of MO caused a reduction in levels of bilirubin and total protein in dose independent manner i.e., with 50 mg/kg, more significant results were produced. This might be because of genetic variations or receptor/enzyme saturation, as we keep on increasing the dose of drug to observe its biological outcome, a dose is reached that saturates the system, which means that further increasing the dose is meaningless, as the compound will start to display a dose-independent behavior.

In addition, histopathological studies have also supported biochemical analysis as illustrated in Figures 1, 2. Photomicrographs reveal that in normal control group (Fig. 1, 2A), liver sections had healthy set of hepatocytes and also, hepatocytes and portal vein with normal shape and intact cell nuclei were found. The liver tissue of mice treated with standard drug silymarin (Fig. 1, 2C) presented no inflammatory or necrotic changes. The porta hepatis and central veins were also identified and hepatocytes were arranged in single plates. Livers of PCM intoxicated mice (Fig. 1B), showed severe histopathological changes such as, fatty changes in hepatocytes, ballooning degeneration, necrotized areas, hepatocytes

![Histopathological changes in liver following administration of Melilotus officinalis in paracetamol treated mice: A) Normal control group, B) PCM intoxicated group, C) Silymarin treated group, D) MO (50 mg/kg) + PCM group, E) MO (100 mg/kg) + PCM group](image-url)
infiltrated by inflammatory cells and mild portal inflammation. Liver sections from mice pretreated with MO at 50 mg/kg and 100 mg/kg (Fig. 1D, 1E), prevented histopathological changes associated with hepatotoxicity induced by acetaminophen and no inflammation, necrosis, granuloma or malignancy was seen. However, it was found that in the liver tissues of mice treated with 50 mg/kg of extract and PCM, the hepatocytes were arranged in single plates while, central vein and porta hepatis were also prominent.

In the liver sections of CCl₄ treated group (Fig. 2B), severe histopathological changes were observed like, severe hyperemia around the central vein and wide vacuolar degeneration of hepatocytes. Mice pretreated with MO (Fig. 2 D,E), exhibited marked improvements in the liver histopathology in CCl₄ treated mice. Also, the damage produced in hepatocytes was lesser as compared to animals treated with CCl₄ alone. The histopathological studies of liver sections treated with 50 mg/kg of plant extract + CCl₄ revealed hepatoprotective activity in the absence of sinusoidal dilatation and necrosis, which was almost similar to standard silymarin group. Conclusively, supporting the findings of biochemical analysis, histologic examination also proved that hepatoprotection was more significant at 50 mg/kg than 100 mg/kg of plant extract. Thus, the aforementioned biochemical and histopathological outcomes ascertain the protective role of *Melilotus officinalis* against hepatic damage induced by acetaminophen and CCl₄.

**DISCUSSION**

Liver diseases are worldwide problem. Conventional drugs used in treatment of liver diseases are sometimes inadequate and offer serious adverse effects. Therefore, herbal medicines are in great demand in developed as well as developing countries for primary healthcare because of their wide biological/medicinal activities with higher safe-
ty margins and lesser costs. Several hundred plants have been examined for use in a wide variety of liver disorders (15). Though, the use of these herbal plants into modern therapy necessitates their pharmacological assessment on scientific basis. So, the current study was conducted to evaluate hepatoprotective effect of methanolic extract of *Melilotus officinalis* in paracetamol- and CCl₄-induced hepatotoxic mice.

The hepatic cells perform metabolic functions and liver has many marker enzymes. In the body, higher concentrations of ALT and AST are found in cytoplasm while AST is found in mitochondria. In case of hepatic injury, transport in liver cells is disturbed causing high concentration of enzymes in serum due to outflow of plasma membrane, which results in efflux of those enzymes into blood. Whenever levels of marker enzymes are increased in blood, it indicates cellular leakage due to loss of functional integrity of cell membranes in liver. In case of hepatotoxicity, liver excretes ALP in bile due to hepatotoxins, which causes defective bile excretion that is observed through high levels of ALP in serum (16).

Paracetamol (PCM) intoxication is a universally recognized model to evaluate probable hepatoprotective activity of plant extracts (17). The hepatotoxic doses of PCM cause depletion of normal hepatic glutathione levels. PCM is metabolized by CYP 450 enzyme system to NAPQI (N-acetyl-p-benzoquinone imine), an alkylating metabolite. NAPQI is irreversibly conjugated with sulphydryl group of glutathione. Thus, glutathione, which is a natural anti-oxidant gets depleted by conjugation. So, NAPQI is highly responsible for toxic effects of PCM to liver. When PCM metabolite is produced in excess amounts, it causes initial hepatic damage and inflammatory mediators are produced such as TNF-α, causing tissue necrosis (18). Besides, it has also been proposed that NAPQI causes the protective effect of protein adducts via its action on proteins and DNA, which finally leads to dysfunctioning, death of liver cells and ultimately hepatic necrosis (19).

CCl₄ is a hepatotoxic agent that undergoes biotransformation in endoplasmic reticulum by CYP 450 and produces trichloromethyl free radical, which on combining with cellular proteins and lipids is converted into trichloromethyl peroxyl radical in the presence of oxygen. The trichloromethyl peroxyl radical in turn causes lipid peroxidation. Hereafter, Ca²⁺ homeostasis is disturbed, ensuing in cell death. CCl₄ increases total bilirubin levels by causing hemolysis due to lipid peroxidation of cell membrane. When aromatase activity of CYP 450 is inhibited by constituents of any phytotherapeutic agent, this cause hepatoprotective activity as liver regeneration is favored (20).

From the results of present investigation, it is clearly demonstrated that, in both paracetamol and carbon tetrachloride induced hepatotoxicity model, there was a significant increase in the levels of liver marker enzymes in case of negative control group. However, methanolic extract of MO (50 mg/kg, 100 mg/kg) attenuated significantly the decreased level of these enzymes and caused a subsequent recovery towards normalization almost like that of silymarin treatment, which is a clear manifestation of anti-hepatotoxic effect of *Melilotus officinalis*. The results of biochemical investigations were also supported by histopathological studies.

In addition, these findings underpin the investigation of Sheikh and Desai (21), who have recently evaluated hepatoprotective and anti-oxidant potential of *Melilotus officinalis* on iron dextran induced hepatotoxicity in Sprague Dawley rats and suggested that hepatoprotective effect of *Melilotus officinalis* was due to its anti-oxidant potential by scavenging free radicals through chelating excess iron as it contains flavonoids and phenolic compounds (21). Phytochemicals formerly reported in *Melilotus officinalis* include melilotin, essential oils, coumarin glycoside and flavonoids (8) and phenolics (gallic acid, catechin, caffeic acid, chlorogenic acid and quercetin) (10).

Moreover, hepatoprotective action of aqueous methanolic extract of *Melilotus officinalis* can be linked with hepatoprotective effect of Phytenol capsule (a new medical preparation containing a mixture of various medicinal plants including *Melilotus officinalis* herb), which had formerly been reported owing to its anti-oxidant properties due to the presence of flavonoids and tannins (22). Likewise, earlier studies have indicated that plants having alkaloids, sterols and flavonoids as active constituents as well as plants with radical scavenging activities have potential to protect liver from toxic effects of hepatotoxins (8). Also, phenolics have been described as efficient scavengers of free radicals because of their molecular structures (23).

The oxidative stress is considered an important cause of liver injury induced by hepatotoxic agents and free radicals, especially reactive oxygen species, are involved in hepatic tissue damage (11). In various studies, antioxidant activity has been emphasized as a mechanism for hepatoprotective potential of herbs. The extracts may produce hepatoprotective effect by neutralizing the effect of free radical species (16, 24, 25). A recent study on *Melilotus officinalis* has revealed strong antioxidant activity...
because of its radical scavenging activity (26). Therefore, it is anticipated that hepatoprotective activity of *Melilotus officinalis* might be attributed to the presence of high content of different groups of phenolic compounds including flavonoids and phenolic acids that have been earlier reported to exhibit strong anti-oxidant and hepatoprotective effects (27,28).

CONCLUSION

Keeping in view the above results and discussion, it is concluded that methanolic extract of *Melilotus officinalis* has potential hepatoprotective activity and it might be due to the presence of flavonoids/phenols in plant extract and also anti-oxidant principles as reported in earlier studies, thus supporting its folkloric claim.

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