Effect of Mallotus Philippensis Muell.-Arg leaves against hepatotoxicity of Carbon tetrachloride in rats


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ABSTRACT
Liver Toxicity is a major health problem of worldwide proportions. Herbal medicines derived from plant extracts are being increasingly utilized to treat a wide variety of clinical diseases. In the present study MEMP leaves is used to screen the hepatoprotective activity. Hepatotoxicity was induced in experimental animals by administration of carbon tetra chloride (CCl4) (25ml/kg, i.p.). Silymarin (25 mg/kg, p.o.) was used as the standard. Functional parameters like onset of sleep and duration of sleep, Biochemical Parameters like serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), serum alkaline phosphatase (SALP), total bilirubin and direct bilirubin were measured. Cytotoxicity of CCl4 was estimated by quantitating the release of malondialdehyde (MDA). The activity of tissue antioxidant enzymes namely super oxide dismutase (SOD), catalase (CAT), and the level of total protein (TP) were also measured. Histopathological evaluation of liver sections was also done. CCl4 administration in rats elevated the levels of SGPT, SGOT, SALP and bilirubin. Administration of MEMP significantly (P<0.001) prevented this increase. The activity of anti-oxidant enzymes in carbon tetrachloride (CCl4) group was decreased and these enzyme levels were significantly (p<0.001) increased in Mallotus philippensis leaves groups. Histopathological studies revealed that the concurrent administration of MEMP with CCl4 exhibited protection of liver tissue, which further evidenced the above results. The study confirmed the hepatoprotective activity of MEMP, which may be attributed to its antioxidant property.

Keywords: Mallotus philippensis, Hepatoprotective effect, Antioxidants, Carbon tetrachloride

INTRODUCTION
Humans are continuously exposed to different kinds of chemicals such as food additives, industrial chemicals, pesticides and other undesirable contaminants in the air, food and soil.[1] Most of these chemicals induce a free radical-mediated lipid peroxidation leading to disruption of biomembranes and dysfunction of cells and tissues.[2] Therefore lipid peroxidation is a crucial step in the pathogenesis of free radical-related diseases including inflammatory injury and hepatic dysfunctions.[3, 4] It is also thought that antioxidants play a significant role in protecting living organism from the toxic effect of various chemicals by preventing free radical formation.[5]

The liver is a vital organ of paramount importance involved in the maintenance of metabolic functions and detoxification from the exogenous and endogenous challenges like xenobiotics, drugs, viral infections and chronic alcoholism. During all such exposures to the above mentioned challenges the natural protective mechanisms of the liver are overpowered, the result is hepatic injury. Liver damage is always associated with cellular necrosis, increase in tissue lipid peroxidation and depletion in the tissue GSH levels. In addition serum levels of many biochemical markers like SGOT, SGPT, triglycerides, cholesterol, bilirubin, alkaline phosphatase, are elevated [6, 7].

CCl4 is an extensively used xenobiotic to induce lipid peroxidation and toxicity.[8] It is well established that CCl4 is metabolized in the liver to highly reactive trichloromethyl radical which initiate free radical-mediated lipid peroxidation of the cytoplasmic membrane phospholipids and causes functional and
morphological changes in the cell membrane leading to accumulation of lipid-derived oxidants causing liver injury.[9, 10] It also induces hydropic degeneration, centrilobular necrosis, fatty changes, cirrhosis and hepatoma.[11, 12] CCl4-induced damage also produces alteration in the antioxidant status of the tissues, which is manifested by abnormal histopathological changes.[13] Several studies have previously demonstrated that antioxidants prevent CCl4 toxicity particularly hepatotoxicity, by inhibiting lipid peroxidation and increasing antioxidant enzyme activities.[14, 15]

Inspite of phenomenal growth of modern medicine, there are no synthetic drugs available for the treatment of hepatic disorders. However there are several herbs/herbal formulations possess beneficial activity in treating hepatic disorders. In one of our field surveys we found that a widely grown plant *Mallotus philippensis* Muell.-Arg possess hepatoprotective property. *Mallotus philippensis* (kamala tree) is a small to medium-sized monoecious tree, up to 25m tall of the family Euphorbiaceae.[16] It was found that this plant contains flavonoids, glycosides, tannins, phenolics, and triterpenes. There are reports showed that kamala possess antiallergic, anti oxidative activity, antitherpetic activity, antifungal activity, antimicrobial properties and free radical scavenger.[17] However there are no scientific bases or reports in the modern literature regarding its usefulness as hepatoprotective agent. Thus the present study was conducted to evaluate the hepatoprotective activity of the MEMP by using CCl4-induced hepatic injury in rats.

**MATERIALS AND METHODS**

**Plant collection and authentication:** The leaves of *Mallotus philippensis* were collected from Nilgiris, Ooty, Tamilnadu and was authenticated by Field Botanist Dr. Rajan S.

**Preparation of extract**
The leaves were shade-dried at room temperature and the MEMP was obtained by extracting with 1500 mL of methanol for 7 days, using soxhlet apparatus. The extract was concentrated and dried using rotary flash evaporator in vacuum at <40 ºC. It was stored in refrigerator at <10 ºC.

The plant materials (leaves) were dried for several days and powdered with the help of an electric grinder. The course material was extracted successively with methanol and the plant marc was finally macerated with distilled water. The extract was dried at 50°C in a water bath. The percentage yield of extract was 1.5 % w/w respectively.

**Phytochemical screening**
A preliminary phytochemical screening was carried out to detect the presence of various phytoconstituents.

**Experimental animals**
The study was carried out on mixed sex of Wistar albino rats (150–200g). The rats were procured from Sri Raghavendra enterprises, Bangalore, Karnataka. Rats were fed with a standard pellet and water ad libitum. The rats were kept in standard environmental conditions (temperature 25–28°C and 12h light/12h dark cycle). The study has got the clearance from the Institutional Animal Ethical Committee (IAEC) (Approval no. DSCP/M Pharm Col/IAEC/24/09-10) of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

**Acute toxicity studies**
Acute oral toxicity (AOT) of MEMP leaves were determined using nulliparous, non-pregnant female mice. The animals were fasted for 3 h prior to the experiment and were administered with single dose of extract dissolved in 1% sodium carboxymethylcellulose and observed for mortality for up to 24 h (short term toxicity). Based on the short-term toxicity, the dose of the next animal was determined as per OECD7 guideline 425. All the animals were also observed for long-term toxicity (14 days) studies.

**Drugs and Chemicals**
Silymarin was purchased from Micro labs Tamilnadu India, KH2PO4 (Qualigens fine Chemicals, Mumbai. Lot No:18986711), K2HPO4 (Leochem, Bangalore. Lot No- 125176, P-2V829). Chem. Kits for SGOT, SGPT, SALP, Bilirubin and Protein estimation (Coral clinical systems, Verna Goa, India) and Heparin (HEP-5, Gland Pharma Ltd, Hyderabad. Batch No.UJ918).
**Induction of hepatic injury**
Experimental protocol was based on previously reported studies.[18] *Mallotus philippensis* (100 and 200mg/kg b/w) and standard hepatoprotective drug silymarin 25mg/kg were prepared in 1% sodium carboxymethylcellulose (CMC). Rats were divided into six groups of 6 animals each.

Group A (control), Group B (CCl₄ treated), Group C (CCl₄+Silymarin (25 mg/kg p.o)), Groups D (CCl₄+MEMP leaves (100mg/ kg) and Group E (CCl₄+ MEMP leaves (200mg/kg). For the first seven days of study Group A & B were fed with normal lab feed and water. Group C animals were treated with Silymarin (25mg/kg) and Group D & E animals were treated orally with MEMP leaves (100mg/kg & 200mg/kg) respectively for seven days. On the seventh and eighth day animals of Group B, C, D & E were administered orally with a single dose of CCl₄ with 5% acacia mixture (600mg/kg/ day). On ninth day, thiopentone sodium (40 mg/kg, i.p) was injected and the sleeping time was recorded in all the animals.

**Assessment of hepatoprotective activity**
The same animals were then anaesthetized using anaesthetic ether, 1hr after complete recovery from thiopentone sodium effect and blood was collected by retro orbital puncture and centrifuged (3000 rpm for 10 min) to obtain serum. The levels of like serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT) [19], serum alkaline phosphatase (SALP) [20], direct and total bilirubin [21], and total proteins [22] were estimated as per the standard procedures prescribed by the manufacturer (Coral Clinical Systems, Verna, Goa, India). Immediately after the collection of blood the animals were euthanized with an over dosage of ether and their livers were removed and small piece of liver tissue was collected and preserved in 10 % formalin solution for histopathological studies. Livers of animals were homogenized with ice-chilled 10 % KCl solution and centrifuged at 2000 rpm for 10 min. The supernatant was collected and the lipid peroxidation was assessed in tissue by measuring the levels of catalase (CAT) [23], thiobarbituric acid reactive substances (TBARS) like malondialdehyde [24] and tissue antioxidant enzymes superoxide-dismutase (SOD) [25].

**Statistical analysis**
Results are presented as Mean ± SEM (n=6), and percentage degree of reversal against hepatotoxin by test. The percentage was calculated by considering enzyme level difference between CCl₄ and normal rats as 100% degree of reversal. Statistical differences at P<0.001 between the groups were analyzed by one-way ANOVA followed by Tukey-Kramer’s multiple comparison test.

**RESULTS**

**Phytochemical study**
Preliminary phytochemical screening revealed presence of carbohydrates, glycosides, phenolic compounds, triterpinoids and flavonoids.

**Acute toxicity studies**
In acute toxicity, no mortality was observed up to a dose level of 2000 mg/kg body weight. As per the ranking system European Economic Community (EEC) for acute oral toxicity, the LD₅₀ dose of 2000 mg/kg and above is categorized as unclassified (EC Directive 83/467/EEC, 1983).

The results presented in Table 1 indicate that standard and MEMP group has been able to significantly (P<0.001) shorten thiopental “sleeping-time” in rats as compared to animals receiving CCl₄ alone.

The activities of various biochemical enzymes in normal, CCl₄ control and treated groups were represented in Table 2. The activities of SGOT, SGPT, SALP, total bilirubin, and direct bilirubin were significantly (P<0.001) increased with a significant decrease in total protein levels in CCl₄ control compared to normal control. The levels of the above enzymes were significantly reversed on treatment with MEMP in a dose-dependent manner. The activity of the MEMP at the dose of 200 mg/kg was comparable to that of the reference drug silymarin.

Results cited in Table 3 clearly reveal increased levels of MDA in Group II (CCl₄ control) compared to the Group I (normal control). Treatment with MEMP significantly prevented this rise in levels. Levels of
antioxidant enzymes SOD and CAT were significantly increased in MEMP treated groups. MEMP (200mg/kg) demonstrated maximum hepatoprotection.

Table 1: Effect of MEMP leaves on Onset of sleep & Duration of sleep in CCl₄ induced hepatotoxic rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Dose</th>
<th>Onset of sleep (sec)</th>
<th>Duration of sleep (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Normal control</td>
<td>10ml/kg, p.o.</td>
<td>177.5±2.5</td>
<td>96.75±3.775</td>
</tr>
<tr>
<td>B</td>
<td>Toxicant Control</td>
<td>CCl₄ - 600 mg/kg, p.o.</td>
<td>65±10.0</td>
<td>225.6±7.68</td>
</tr>
<tr>
<td>C</td>
<td>Standard (Silymarin)</td>
<td>25mg/kg, p.o. + CCl₄</td>
<td>157.5±7.5***</td>
<td>115±2.74***</td>
</tr>
<tr>
<td>D</td>
<td>MEMP</td>
<td>100mg/kg, p.o. + CCl₄</td>
<td>101.5±3.5*</td>
<td>192.83±3.712**</td>
</tr>
<tr>
<td>E</td>
<td>MEMP</td>
<td>200mg/kg, p.o. + CCl₄</td>
<td>130±5.0**</td>
<td>152.33±3.84***</td>
</tr>
</tbody>
</table>

Values are mean ± SEM (n=6) one way ANOVA followed by Tukey-Kramer’s test. Where, * represents mild significant at p<0.05, ** represents moderate significant at p<0.01, *** and represents highly significant at p<0.001.
Table 2. Effect of MEMP on serum parameters in CCl$_4$ induced hepatic damage in rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>SGPT (U/L)</th>
<th>SGOT (U/L)</th>
<th>SALP (mg/dl)</th>
<th>Direct Bilirubin (mg/dl)</th>
<th>Total Bilirubin (mg/dl)</th>
<th>Total Protein (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Normal control 10 ml/kg p.o</td>
<td>28.17±0.325</td>
<td>34.05±4.5</td>
<td>33.0±0.50</td>
<td>0.17±0.0120</td>
<td>0.293±0.029</td>
<td>5.45±0.07</td>
</tr>
<tr>
<td>II</td>
<td>Toxicant control (CCl$_4$ - 600 mg/kg, p.o.)</td>
<td>108±2.50</td>
<td>188.05±2.50</td>
<td>92.95±0.550</td>
<td>1.679±0.099</td>
<td>1.8±0.005</td>
<td>2.89±0.25</td>
</tr>
<tr>
<td>III</td>
<td>Silymarin (25mg/kg, p.o) + Ranitidine</td>
<td>37.5±1.0***</td>
<td>51.5±1.0**</td>
<td>40.0±0.55**</td>
<td>0.2±0.01***</td>
<td>0.46±0.04***</td>
<td>4.86±0.06***</td>
</tr>
<tr>
<td>IV</td>
<td>MEMP(100mg/kg, p.o)+ CCl$_4$</td>
<td>87.5±2.0*</td>
<td>143.75±8.75*</td>
<td>81.2±0.30*</td>
<td>1.26±0.03**</td>
<td>1.33±0.05**</td>
<td>3.52±0.78**</td>
</tr>
<tr>
<td>V</td>
<td>MEMP(200mg/kg, p.o)+ CCl$_4$</td>
<td>53.9±1.30**</td>
<td>88.5±3.0**</td>
<td>51.7±0.25**</td>
<td>0.50±0.04**</td>
<td>0.73±0.11**</td>
<td>4.45±0.12***</td>
</tr>
</tbody>
</table>

Values are mean ± SEM (n=6) one way ANOVA followed by Tukey-Kramer’s test. Where, * represents mild significant at p<0.05, ** represents moderate significant at p< 0.01, *** and represents highly significant at p<0.001.
Fig. 1: Effect of MEMP leaves on Onset of sleep & Duration of sleep in CCl₄ induced hepatotoxic rats.

Fig. 2: Effect of MEMP leaves on SGPT, SGOT, SALP, Direct bilirubin, Total bilirubin & Total Protein levels in CCl₄ induced hepatotoxic rats.
Table 3: Effect of MEMP leaves on Catalase, SOD and Lipid peroxidation in CCl₄ induced hepatotoxic rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Dose</th>
<th>CAT^c (Mean±SEM)</th>
<th>SOD^b (Mean±SEM)</th>
<th>LPO^a (Mean±SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Normal control</td>
<td>10ml/kg, p.o.</td>
<td>91.8±3.412</td>
<td>14.5±0.5774</td>
<td>3.83±0.60</td>
</tr>
<tr>
<td>II</td>
<td>Toxicant Control</td>
<td>CCl₄ -600 mg/kg, p.o.</td>
<td>22.3±0.8819</td>
<td>3.23±0.088</td>
<td>89.26±0.1856</td>
</tr>
<tr>
<td>III</td>
<td>Standard (Silymarin)</td>
<td>25mg/kg, p.o. + CCl₄</td>
<td>83.05±0.622***</td>
<td>11.0±0.352***</td>
<td>6.53±0.202***</td>
</tr>
<tr>
<td>IV</td>
<td>MEMP</td>
<td>100mg/kg, p.o. + CCl₄</td>
<td>36.4±0.585**</td>
<td>5.8±0.152**</td>
<td>7.86±0.03**</td>
</tr>
<tr>
<td>V</td>
<td>MEMP</td>
<td>200mg/kg, p.o. + CCl₄</td>
<td>51.0±1.093***</td>
<td>7.7±0.2517***</td>
<td>7.26±0.0667***</td>
</tr>
</tbody>
</table>

^a= nmole of MDA/mg of protein, ^b= Units/mg of protein
^c= µmole of H₂O₂ consumed/min/mg of protein.

Values are mean ± SEM (n=6) one way ANOVA followed by Tukey-Kramer’s test. Where, * represents mild significant at p<0.05, ** represents moderate significant at p< 0.01, *** and represents highly significant at p<0.001.

The histopathological studies of the liver showed fatty changes, swelling and necrosis with loss of hepatocytes in CCl₄ control rats in comparison with normal control. The MEMP treated groups showed regeneration of hepatocytes, normalization of fatty changes and reduced necrosis of the liver. The silymarin treated group showed almost normalization of fatty accumulation and necrosis.
DISCUSSION
Herbal medicines derived from plant extracts are being increasingly utilized to treat a wide variety of clinical diseases. There is a growing interest in the pharmacological evaluation of various plants used in Indian traditional system of medicine.[26]

The hepatotoxicity induced by CCl₄ is due to its metabolite CCl₃’, a free radical that binds to lipoprotein and leads to peroxidation of lipids of endoplasmic reticulum.[27] The ability of a hepatoprotective drug to reduce the injurious effects or to preserve the normal hepatic physiological mechanisms, which have been disturbed by a hepatotoxin, is the index of its protective effects. Although serum enzyme levels and barbiturate sleeping time are not a direct measure of hepatic injury, they show the status of the liver. The lowering of enzyme level is a definite indication of hepatoprotective action of the drug. Protection of hepatic damage caused by carbon tetrachloride administration was observed by recording SGOT, SGPT, SALP and SBLN levels in treated, toxin control and normal groups because serum transaminases, serum alkaline phosphatase and serum bilirubin have been reported to be sensitive indicators of liver.[28] The disturbance in the transport function of the hepatocytes as a result of hepatic injury causes the leakage of enzymes from cells due to altered permeability of membrane.[29]

CCl₄ produces an experimental damage that histologically resembles viral hepatitis.[30] Toxicity begins with the change in endoplasmic reticulum, which results in the loss of metabolic enzymes located in the intracellular structures.[9] The toxic metabolite CCl₃ radical is produced which is further converted to trichloromethyl peroxo radical by cytochrome P450 2E1 enzyme. This radical binds covalently to the macromolecules and causes peroxidative degradation of cellular membrane leading to the necrosis of hepatocytes. [31] Increase in malondealdehyde levels, as evident in our study, suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanisms to prevent formation of excessive free radicals.[32]
Measurement of protein concentration was mainly used to calculate the level of purity of a specific protein. High doses of CCl₄ cause depletion of total proteins indicating tissue damage which was also evidenced in this study. Treatment with CCl₄ significantly depleted CAT and SOD stores indicating that they were used for the detoxification of toxic metabolites of the drug. The formulation restored the antioxidant enzyme levels significantly and reduced the CCl₄ induced oxidative injury, thus proving its antioxidant potential.[33]

Treatment with MEMP significantly reversed these changes. Moreover, administration of MEMP caused a significant increase in activities of enzymatic antioxidants, SOD and CAT, a non-enzymatic biological antioxidant present in the liver. This shows MEMP can scavenge reactive free radicals that might lessen oxidative damage to the liver tissue and improve the activities of the hepatic antioxidant enzymes.

The histopathological studies are direct evidence of efficacy of drug as protectant. Simultaneous treatment of MEMP with CCl₄ exhibits less damage to the hepatic cells as compared to the rats treated with CCl₄ alone. Histological changes such as steatosis (fatty changes in hepatocytes), inflammatory infiltrations and perivenular fibrosis were observed in CCl₄-treated (toxic) control group. Both the extracts prevented these histological changes, further indicating their hepatoprotective activity. All the histological changes observed were in correlation with the biochemical, antioxidant and functional parameters of the liver.

CONCLUSION
It is concluded that pretreatment with MEMP decreases the CCl₄-induced elevation in biochemical parameters (SGOT, SGPT, SALP, direct bilirubin, total bilirubin and MDA). And reversed the changes in functional parameters and antioxidant parameters. These findings suggest that the MEMP were effective in bringing about functional improvement of hepatocytes. The healing effect of this fraction was also confirmed by histological observations. Our results demonstrated that the possible hepatoprotective mechanisms of the MEMP leaves on CCl₄-induced liver damage in rats might be due to the following effects: (1) inhibiting the cytochrome P450-dependent oxygenase activity; (2) preventing lipid peroxidation; and (3) stabilizing the hepatocyte membrane. The active compounds of Mallotus philippensis leaves, which are responsible for the observed hepatoprotective and antioxidant effects, have not been isolated in this study. Therefore, further studies should be conducted to determine the active compounds that are responsible for the hepatoprotective effects and the mechanisms of action involved in the hepatoprotective effect.

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