PROTECTIVE AND THERAPEUTIC EFFICACY OF DIFFERENT EXTRACTS OF PLUMBAGO ZEYLANICA LINN ROOT AGAINST CCl₄ INDUCED CHRONIC LIVER INJURY BY ATTENUATING OXIDATIVE STRESS AND SUPPRESSING INFLAMMATION

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ABSTRACT

The constituents in the Plumbago zeylanica (Pz) roots are credited with various therapeutic potentials in Indian traditional medicine. However, very little is known about the antioxidant effects of various extracts (hexane, dichloromethane and methanol) of Pz. This study was designed to investigate the antioxidant activities of these Pz extracts. The highest antioxidant effect was shown by the methanolic extract of Pz (MEPz), which was further tested for its anti-inflammatory effect against carbon tetrachloride (CCl₄) induced rat liver inflammation and its anti-proliferative effect on HepG2 cells was also determined. Chronic liver inflammation in male Wistar rats was induced with intraperitoneal injection of CCl₄. Increased inflammatory markers, serum C-reactive protein, myeloperoxidase activities and liver marker enzymes in CCl₄-induced group were significantly reverted to near normal levels upon 15 days oral treatment with MEPz. Reactive oxygen species generation was markedly attenuated and the antioxidant levels were restored upon MEPz treatment. Also, liver histology highly correlated with the biochemical observations. Overall, co-treatment with MEPz prevented CCl₄ induced rat liver damage hence MEPz could be used as a potential anti-inflammatory agent in the early stages of liver diseases.

KEYWORDS: Inflammation; Carbon tetrachloride; Oxidative stress; Antioxidant; Plumbago zeylanica.
INTRODUCTION

Inflammation is an immediate response of the body to tissue injury caused by microbial infection and other noxious stimuli. Chronic inflammation represents a major pathological basis for tumor development. Oxygen is a vital element for life and cells use oxygen to generate energy, while mitochondria produce free radicals. These by-products include reactive oxygen species (ROS) and reactive nitrogen species (RNS) and have a strong affinity towards biological macromolecules viz carbohydrates, lipids, proteins and nucleic acids. These molecules are capable of causing damage to the cells and tissue during conditions like infection, inflammation, other disorders viz neurodegenerative diseases like Alzheimer’s disease, aging, cardiovascular disease and cancer.

Liver is the primary organ where CCl₄ exert its toxic action through a free radical-mediated mechanism which is widely investigated in rodents. In liver, CCl₄ is biotransformed by cytochrome P450 to hepatotoxic trichloromethyl free radical (CCl₃•), that in turn reacts with oxygen to form peroxy radical (CCl₃OO•). This peroxy radical thus formed oxidizes polyunsaturated fatty acids, thereby initiating lipid peroxidation of ER membrane, as a result of which, the activities of enzymes associated with it are inhibited and an imbalance between the synthesis and degradation of lipids is also exhibited. The increasing awareness about the side effects of allopathic drugs turned the attention of researchers towards plant- based drugs. Several compounds have been studied as hepatic protectors, which belong to a group called phytochemicals, in the form of antioxidants like flavonoids and other phenolic compounds. Previous studies performed in this laboratory have also showed beneficial effects of bioactive compounds against chemical-induced liver diseases.

*Plumbago zeylanica* (Pz) (Plumbaginaceae), widely used in traditional medicine for the treatment of inflammation, is native to South Eastern Asia and is commonly known as Chitrak. It exhibits various medicinal properties like anticancer, antioxidant, antimicrobial, radiosensitizing, antifertility. Plumbagin (a napthoquinone, Fig 1), the active principle of Pz, is primarily responsible for these pharmacological effects. Earlier studies in this laboratory determined the apoptosis inducing efficacy of plumbagin on colon cancer cells, protective role of Pz against cyclophosphamide induced genotoxicity in experimental animals. However, no study has been done on the antioxidant potential of different extracts (Hexane, Dichloro methane and Methanol) obtained from Pz. Therefore, the study was aimed to investigate the antioxidant activities of these extracts *in vitro* and to
determine the anti-proliferative effect of these extracts in HepG2 cells. Also, the anti-inflammatory effect of these extracts against CCl₄-induced liver inflammation was determined.

MATERIALS AND METHODS

Plant material collection and extraction
The root of Pz was collected from Kuttiyadi, Calicut district, Kerala. It was authenticated by a taxonomist from the Department of Botany, Presidency College, Chennai, (PARC/2012/1257). The root extracts were prepared via sequential extraction. First the roots were washed well to remove any impurities, shade dried, ground to fine powder and sieved. The root powder of Pz was subjected to sequential extraction in order to identify a compound with potential antioxidant and anti-inflammatory activity. The extraction steps were followed according to the protocol.[24]

Chemicals
Plumbagin, 3-(4,5-dimethyl2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), diethyl pyrocarbonate, CCl₄ were purchased from Sigma-Aldrich Chemicals (USA). Dulbecco’s Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were obtained from Invitrogen (Grand Island, NY). Agarose, TRI reagent, random hexamer primer, deoxy nucleoside triphosphate (dNTP), Moloney murine leukemia virus (M-MLV) reverse transcriptase (RT) enzyme with buffer and PCR ready master mix (2X) were purchased from GeNei™ (Bangalore, India). All the other chemicals and reagents used were of analytical grade.

In vitro antioxidant activity and anti-arhritic evaluation
Free radical scavenging activity was assessed according to the method described by[25] for DPPH assay; reducing power by[26]; nitric oxide scavenging activity by Griess reaction [27]; inhibition of protein denaturation by[28]; proteinase inhibitory action by.[29] The total soluble phenols present in the extracts were also determined by the method of[30] using Folin Ciocalteu reagent.

Cell culture and extract treatment
HepG2, (human liver carcinoma cells) obtained from NCCS, Pune was used in this study. The cells were grown in DMEM medium supplemented with 10% FBS, 100 U/ml of penicillin and 100 μg/ml of streptomycin at 37°C in a 5% CO₂ incubator as described by.[31]
A 100 mM solution of plumbagin was prepared in dimethyl sulfoxide (DMSO), stored as small aliquots at -20°C and then diluted as needed in cell culture medium.

**Cell cytotoxicity assays**
The effect of MEPz on the viability of HepG2 was assessed by MTT and LDH leakage assay. Plumbagin was used as a standard control. HepG2 cells were seeded at a concentration of 5 × 10^5 cells/well in 96-well plates and incubated at 37°C in 5% CO₂ for 24 h. Once the cells reached 70% confluency, media with different concentrations of MEPz along with Plumbagin (1-10 μg) were added and incubated at 37°C in 5% CO₂ for 24 and 48 h. The samples of medium alone served as blank and DMSO alone served as control. After 24 and 48 h, the percentage of growth inhibition (IC₅₀) was determined.

**Isolation of RNA and Reverse Transcription-PCR (RT-PCR)**
Total RNA was isolated from HepG2 cells using TRI reagent and the RNA obtained was then reverse transcribed to cDNA and PCR was performed according to standard protocol.

**Animals and experimental grouping**
Male Wistar rats weighing 150-200g were procured from Kings Institute of Preventive Medicine, Chennai, India. The animals were maintained as per the principles and guidelines of the Institutional Ethics Committee (IAEC No. 01/084/09). The rats were randomly divided into four groups with each group consisting of 6 animals for a study period of 15 days as follows: Group I - control rats fed with standard pellet diet and *ad libitum*. Group II - drug control rats received MEPz (250 mg/kg BW), orally for 15 days. Group III – induced rats received CCl₄ in olive oil (1.195g/kg BW) intraperitoneally, three times a week for two weeks. Group IV – co-treated rats received MEPz (250 mg/kg BW) along with CCl₄ (1.195g/kg BW) intraperitoneally, three times a week for two weeks. At the end of the experimental period, the control and experimental rats were euthanized and the liver tissue & blood samples were collected and used for various biochemical assays.

**Biochemical parameters**
Hemoglobin concentration, red blood cell count (RBC), platelet count, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and white blood cell count (WBC) were analysed by Beckman Coulter (Ac.T 5diff CP). PCV was determined by micro hematocrit centrifuge. The activities of alkaline phosphatase (ALP), aspartate transaminase (AST), alanine transaminase (ALT),
lactate dehydrogenase (LDH), γ-glutamyl transpeptidase (γ GT) and bilirubin were assayed in the serum.\[36-40\] The activities of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione trasferase (GST) were estimated in liver tissue homogenate.\[41-44\] The activity staining of SOD and CAT was carried out in the liver samples that are sepearted by Native-PAGE according to the method of\[45,46\] respectively. The levels of lipid peroxidation products were also estimated in the liver tissue.\[47\] Serum concentration of CRP was measured by an enzymatic colorimetric method of\[48\] using a commercial enzymatic kit (Bio-Diagnostic, Cairo, Egypt). Activity of MPO was measured according to the method.\[49\]

**Histological analysis**

Small pieces of liver tissues were initially fixed in 10% buffered formalin\[50\], dehydrated in ethanol (50 to 100%), cleared in xylene and embedded in paraffin. Liver sections of 4µm thickness were prepared and stained with Haematoxylin and Eosin (H&E) for microscopic observation.

**STATISTICAL ANALYSIS**

The data obtained were evaluated with SPSS/10 software. Hypothesis testing methods included one way analysis of variance (ANOVA) followed by least significant difference [LSD] test. p values of less than 0.05 were considered to indicate statistical significance. All these results were expressed as mean ± S.D for six animals in each group and significant variations were compared as follows. Group III was compared with group I and group IV was compared with group III.

**RESULTS**

**EXPERIMENT I: Potency of Plumbago zeylanica as free radical scavenger**

Table 1 shows the total phenolic content, expressed as gallic acid equivalence, of hexane, dichloromethane (DCM) and methanolic extract of Pz. The methanolic fraction of the extract exhibited the highest concentration of total phenolic content followed by DCM and hexane extract. It was found that the scavenging activity increased with increase in concentration of the plant extract and the highest scavenging was observed with methanolic extract at three different concentrations, i.e. 46.42% ± 3.5 at 100µg/ml; 50.51% ± 4.5 at 200µg/ml and 68.63% ± 5.8 at 300µg/ml which was followed by DCM and hexane extracts (Table 2). The scavenging effect of these extracts on the DPPH radical was in the order Methanol> DCM > Hexane.
Table 1: Total phenolic content of *Plumbago zeylanica* linn root extract.

<table>
<thead>
<tr>
<th>Solvents</th>
<th>Total Phenol (mg/g Gallic acid equivalence)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane</td>
<td>50.14±4.1</td>
</tr>
<tr>
<td>DCM</td>
<td>76.30±5.6</td>
</tr>
<tr>
<td>Methanol</td>
<td>192.25±10.4</td>
</tr>
</tbody>
</table>

The extracts of *Plumbago zeylanica* Linn from the three solvents (hexane, DCM and methanol) are given and the total phenolic content was expressed in mg/g Gallic acid equivalence.

Table 2: Free radical scavenging activity of hexane, dichloromethyl and methanolic extract of *Plumbago zeylanica* linn compared with Plumbagin.

<table>
<thead>
<tr>
<th>Extract</th>
<th>Concentration µg</th>
<th>DPPH Radical Scavenging Assay (%)</th>
<th>Reducing Power Assay (%)</th>
<th>Nitric oxide Scavenging Assay (%)</th>
<th>Inhibition of Protein Denaturation (%)</th>
<th>Proteinase Inhibitory Action (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane</td>
<td>100</td>
<td>26.21 ± 2.10</td>
<td>24.36 ± 2.00</td>
<td>23.35 ± 2.00</td>
<td>32.38 ± 2.60</td>
<td>20.24 ± 1.90</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>33.31 ± 3.20</td>
<td>33.45 ± 3.10</td>
<td>32.48 ± 2.90</td>
<td>38.43 ± 3.00</td>
<td>26.42 ± 2.10</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>42.45 ± 4.50</td>
<td>40.38 ± 4.10</td>
<td>38.36 ± 3.40</td>
<td>51.34 ± 4.20</td>
<td>35.32 ± 3.00</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>28.38 ± 2.50</td>
<td>25.24 ± 2.10</td>
<td>23.39 ± 2.20</td>
<td>35.61 ± 2.90</td>
<td>26.72 ± 2.00</td>
</tr>
<tr>
<td>DCM</td>
<td>200</td>
<td>42.29 ± 3.90</td>
<td>36.39 ± 3.20</td>
<td>32.54 ± 3.10</td>
<td>40.73 ± 3.50</td>
<td>37.56 ± 3.10</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>48.61 ± 4.50</td>
<td>45.41 ± 4.10</td>
<td>40.36 ± 3.50</td>
<td>56.28 ± 4.90</td>
<td>48.62 ± 4.10</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>46.42 ± 3.50</td>
<td>33.47 ± 3.10</td>
<td>28.43 ± 2.20</td>
<td>40.82 ± 3.30</td>
<td>28.54 ± 2.20</td>
</tr>
<tr>
<td>Methanol</td>
<td>200</td>
<td>50.51 ± 4.50</td>
<td>45.30 ± 4.00</td>
<td>38.48 ± 3.00</td>
<td>50.53 ± 4.20</td>
<td>35.62 ± 2.80</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>68.63 ± 5.80</td>
<td>52.41 ± 4.70</td>
<td>46.54 ± 4.10</td>
<td>65.71 ± 5.20</td>
<td>58.68 ± 4.90</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>42.55 ± 3.50</td>
<td>45.38 ± 4.00</td>
<td>45.41 ± 3.80</td>
<td>43.42 ± 3.50</td>
<td>31.39 ± 2.50</td>
</tr>
<tr>
<td>Plumbagin</td>
<td>200</td>
<td>58.25 ± 5.10</td>
<td>60.65 ± 5.50</td>
<td>53.58 ± 4.30</td>
<td>55.54 ± 4.50</td>
<td>45.48 ± 3.20</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>75.38 ± 6.70</td>
<td>78.53 ± 6.90</td>
<td>69.63 ± 6.50</td>
<td>70.63 ± 6.20</td>
<td>65.72 ± 5.30</td>
</tr>
</tbody>
</table>

*In vitro* antioxidant assays viz DPPH radical scavenging, reducing power, NO scavenging assays and *in vitro* anti-arthritic activities viz protein denaturation and protease inhibitory action of hexane, DCM and methanolic extract of *Plumbago zeylanica* at three different
concentrations are compared with the standard plumbagin. Concentrations are expressed in µg.

The reductive capability of the extract was compared to the standard plumbagin (Table 2). The reducing power of the extracts was increased with increasing concentrations. At all the tested concentrations, the methanolic extract showed higher activity which was comparable to that of the plumbagin when compared to DCM and hexane extract. The results obtained showed that scavenging of NO by the extracts was increased in a dose dependent manner. Table 2 illustrates a significant decrease in the NO radical due to the scavenging ability of the extracts. The methanol extract showed maximum activity compared to other two extracts.

Inhibition of protein denaturation and proteinase inhibitory action were evident (Table 2). The maximum percentage inhibition of these parameters was observed as 65.71% ± 5.2, 70.63% ± 6.2, at 300 µg/ml in case of methanolic extract and plumbagin, respectively, whereas comparatively less inhibition of protein denaturation and proteinase activity were found on DCM and hexane extract treatment at the same concentration.

**Figure 1: Chemical structure of Plumbagin.**

**EXPERIMENT II: Potency of* Plumbago zeylanica* as an anti-proliferative agent**

The effect of MEPz on the viability of HepG2 cells was evaluated by MTT assay and LDH leakage assay (Fig 2 (a) and 2 (b)). MEPz significantly affected the cell viability and caused LDH leakage into the culture medium in a concentration and time dependent manner with an IC<sub>50</sub> value of 5µg for MEPz and 4µg for Plumbagin (standard).
Figure 2: (a) Effect of MEPz and Plumbagin on cell viability in cultured HepG2 cells: Cultured HepG2 cells were treated with MEPz and Plumbagin at different concentrations (1, 2, 3, 4, 5, 6, 7, 8, 9, 10 μg for 24 and 48 h. From the MTT assay, the estimated IC$_{50}$ value of MEPz and Plumbagin in cultured HepG2 cells were found to be 5 μg and 4 μg respectively. Each point represents the mean ± SD of three different experiments.

(b) Effect of MEPz and plumbagin on LDH leakage in cultured HepG2 cells. Cultured HepG2 cells were treated with MEPz and Plumbagin at different concentrations (1, 2, 3, 4, 5, 6, 7, 8, 9, 10 μg for 24 and 48 h. LDH leakage was presented in (%). Each point represents the mean ± SD of three different experiments.

Because of the central role of COX-2 in causing inflammation, which is considered as a common mechanism in various diseases including cancer, measurement of its mRNA level was done along with plumbagin. In MEPz treated cells, a marked reduction in the expression levels for COX-2 was observed when compared to untreated cells (Fig 3).
Figure 3: Effect of MEPz and plumbagin on COX 2 mRNA level in cultured HepG2 cells determined by RT-PCR analysis.

Lane 1 — Untreated HepG2 cells, lane 2 — Plumbagin treated HepG2 cells for 24 h, and lane 3 — Plumbagin treated HepG2 cells for 48 h, lane 4 — MEPz treated HepG2 cells for 24 h, lane 5 — MEPz treated HepG2 cells for 48 h. β-actin served as loading control. Intensity of bands was quantified by densitometry using ImageJ software (NIH).

EXPERIMENT III: Potency of Plumbago zeylanica as an anti-inflammatory agent

Rats in the control group were active and the response to stimulus was quick when compared to CCl₄ induced rats which showed sluggish behaviour. The rats in CCl₄ induced group showed significant reduction in the body weight (p< 0.001) and liver weight (p< 0.05) whereas treatment with MEPz markedly ameliorated these differences (Fig 4 (a) and 4 (b)).

There was a significant decrease in the levels of Hb, PCV and RBC in CCl₄ induced group (Table 3), while co-treatment with MEPz markedly restored the levels of Hb, PCV and RBC. There was no significant difference in the MCH values calculated in all the groups. The MCV levels were reduced in CCl₄ induced group, whereas a non-significant increase in these levels was observed in MEPz treated group. MCHC level was similar in all the groups, when compared with the control groups. Differential WBC count data showed significant increase (p< 0.001) in the levels of neutrophils in CCl₄ induced group (Table 3). Neutrophil levels were significantly reduced (p< 0.001) in rats that were co-treated with Pz extract.
Table 3: Effect of methanolic extract of *Plumbago zeylanica* linn on hematological parameters in CCl₄ induced hepatic inflammation.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb (g/dl)</td>
<td>13.50 ± 1.10</td>
<td>13.80 ± 1.50</td>
<td>10.10 ± 1.20*#</td>
<td>13.80 ± 1.30#</td>
</tr>
<tr>
<td>PCV (%)</td>
<td>38.20 ± 2.50</td>
<td>38.50 ± 2.60</td>
<td>30.10 ± 3.8as</td>
<td>36.50 ± 3.70as</td>
</tr>
<tr>
<td>RBC (10⁶/µl)</td>
<td>8.20 ± 0.80</td>
<td>8.50 ± 0.80</td>
<td>6.93 ± 0.60*</td>
<td>7.52 ± 0.80*</td>
</tr>
<tr>
<td>TC (10³/µl)</td>
<td>11.40 ± 1.10</td>
<td>11.60 ± 1.30</td>
<td>8.70 ± 1.00as</td>
<td>10.50 ± 1.20b*</td>
</tr>
<tr>
<td>Platelet (10³/µl)</td>
<td>680 ± 50</td>
<td>690 ± 48</td>
<td>532 ± 44as</td>
<td>640 ± 62bs</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>53.00 ± 4.00</td>
<td>54.00 ± 4.30</td>
<td>47.00 ± 4.1as</td>
<td>51.00 ± 3.80b</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>18.20 ± 1.50</td>
<td>19.10 ± 1.70</td>
<td>17.00 ± 1.50b</td>
<td>17.80 ± 1.40b</td>
</tr>
<tr>
<td>MCHC (g/dl)</td>
<td>32.10 ± 2.10</td>
<td>32.10 ± 2.30</td>
<td>33.10 ± 2.80b</td>
<td>32.20 ± 2.70b</td>
</tr>
<tr>
<td>MPV (fl)</td>
<td>6.20 ± 1.00</td>
<td>6.30 ± 1.20</td>
<td>6.00 ± 1.20b</td>
<td>6.30 ± 1.30b</td>
</tr>
<tr>
<td>RBW (%)</td>
<td>9.00 ± 1.10</td>
<td>9.20 ± 1.30</td>
<td>8.90 ± 1.50</td>
<td>9.00 ± 1.60</td>
</tr>
<tr>
<td>Neutrophil (%)</td>
<td>53.00 ± 5.80</td>
<td>53.40 ± 6.00</td>
<td>65.50 ± 6.50as</td>
<td>57.50 ± 5.80as</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>37.00 ± 3.80</td>
<td>36.40 ± 3.90</td>
<td>24.50 ± 3.40#</td>
<td>32.50 ± 3.70as</td>
</tr>
<tr>
<td>Monocytes (%)</td>
<td>7.00 ± 0.83</td>
<td>7.10 ± 0.78</td>
<td>7.00 ± 0.76</td>
<td>7.00 ± 0.70</td>
</tr>
<tr>
<td>Eosinophils (%)</td>
<td>2.00 ± 0.20</td>
<td>2.10 ± 0.20</td>
<td>2.00 ± 0.20b</td>
<td>2.00 ± 0.20b</td>
</tr>
<tr>
<td>Basophils (%)</td>
<td>1.00 ± 0.10</td>
<td>1.00 ± 0.10</td>
<td>1.00 ± 0.10</td>
<td>1.00 ± 0.20</td>
</tr>
</tbody>
</table>

Each value expressed as mean ± S.D for six rats in each group. Statistical significance at *p* < 0.05, $p$ < 0.01, $^a$ *p* < 0.001. a: CCl₄ vs. Control; b: CCl₄ vs. CCl₄ + MEPz. Hb: haemoglobin content, PCV: haematocrit value, RBC: red blood cell count, TC: Total Count, MCV: mean corpuscular volume, MCH: mean cell haemoglobin, MCHC: mean cell haemoglobin concentration, MPV: Mean Platelet Volume, RBW: Red blood cell Distribution Width.
Table 4: Effect of methanolic extract of *Plumbago zeylanica* linn on serum marker enzymes of liver damage (AST, ALT, ALP, LDH, γ-GT) and serum bilirubin on CCl₄ induced liver inflammation

<table>
<thead>
<tr>
<th>Groups</th>
<th>AST</th>
<th>ALT</th>
<th>ALP</th>
<th>LDH</th>
<th>γGT</th>
<th>Serum Bilirubin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>70.12± 8.32</td>
<td>31.45 ± 4.34</td>
<td>100.38 ± 11.55</td>
<td>428.50 ± 43.10</td>
<td>8.60 ± 0.70</td>
<td>0.27 ± 0.02</td>
</tr>
<tr>
<td>MEPz alone</td>
<td>71.42 ± 8.70</td>
<td>32.13 ± 4.60</td>
<td>100.45 ± 11.20</td>
<td>430.73 ± 45.38</td>
<td>8.71 ± 0.80</td>
<td>0.26 ± 0.03</td>
</tr>
<tr>
<td>CCl₄</td>
<td>193.51 ± 20.47⇑#</td>
<td>66.54 ± 6.91⇑#</td>
<td>253.68 ± 28.47⇑#</td>
<td>694.78 ± 61.47⇑#</td>
<td>28.85 ± 2.30⇑#</td>
<td>0.83 ± 0.09⇑#</td>
</tr>
<tr>
<td>CCl₄ + MEPz</td>
<td>98.74 ± 10.52⇑⇑#</td>
<td>48.92 ± 5.41⇑⇑#</td>
<td>145.76 ± 4.83⇑⇑#</td>
<td>493.13 ± 50.28⇑⇑#</td>
<td>9.12 ± 1.10⇑⇑#</td>
<td>0.32 ± 0.02⇑⇑#</td>
</tr>
</tbody>
</table>

Each value expressed as mean ± S.D for six rats in each group. Statistical significance at *p* < 0.001. a: CCl₄ vs. Control; b: CCl₄ vs. CCl₄ + MEPz. Activity is expressed as IU/L for AST, ALT, ALP, LDH, γ-GT and mg/dl for bilirubin.

Table 5: Effect of methanolic extract of *Plumbago zeylanica* linn on liver antioxidant status and lipid peroxidation in control and experimental groups of rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>SOD</th>
<th>CAT</th>
<th>GPx</th>
<th>GST</th>
<th>GSH</th>
<th>LPO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.63 ± 0.34</td>
<td>49.74 ± 4.50</td>
<td>117.43 ± 14.30</td>
<td>1.53 ± 0.28</td>
<td>5.76 ± 0.83</td>
<td>4.23 ± 0.59</td>
</tr>
<tr>
<td>MEPz alone</td>
<td>2.71 ± 0.37</td>
<td>50.10 ± 4.60</td>
<td>118.34 ± 14.50</td>
<td>1.55 ± 0.27</td>
<td>5.81 ± 0.89</td>
<td>4.38 ± 0.63</td>
</tr>
<tr>
<td>CCl₄</td>
<td>1.48 ± 0.25⇑#</td>
<td>30.92 ± 5.40⇑#</td>
<td>85.66 ± 12.50⇑#</td>
<td>1.12 ± 0.25⇑#</td>
<td>3.72 ± 0.59⇑#</td>
<td>6.97 ± 0.87⇑#</td>
</tr>
<tr>
<td>CCl₄ + MEPz</td>
<td>2.39 ± 0.32⇑⇑#</td>
<td>39.90 ± 5.80⇑⇑#</td>
<td>106.75 ± 14.40⇑⇑#</td>
<td>1.48 ± 0.22⇑⇑#</td>
<td>4.82 ± 0.75⇑⇑#</td>
<td>5.63 ± 0.75⇑⇑#</td>
</tr>
</tbody>
</table>

Each value expressed as mean ± S.D for six rats in each group. Statistical significance at *p*< 0.05, $p$< 0.01, #p < 0.001. a: CCl₄ vs. Control; b: CCl₄ vs. CCl₄ + MEPz. The activity is expressed as 50% inhibition of epinephrine autooxidation for SOD; μmole of hydrogen peroxide decomposed per min per mg of protein for catalase; μmole of GSH oxidized per min per mg of protein for GPx; units per min per mg protein for GST; nmole of TBARS formed per min per mg protein for LPO; nmole per g liver tissue for GSH.
The levels of serum AST, ALT, ALP, LDH, total bilirubin and γGTP were significantly elevated ($p < 0.001$) in CCl$_4$ induced group (Table 4). Co-treatment with MEPz (250 mg/kg BW) remarkably prevented CCl$_4$-induced hepatotoxicity. Table 5 explains the effect of MEPz on liver antioxidant enzymes (SOD, CAT, GPx and GST). The activities of antioxidant enzymes were significantly decreased in CCl$_4$ induced group. Treatment of MEPz was able to restore the altered activities of these enzymes indicating its antioxidant potential. The antioxidant potential of MEPz was further confirmed by Native-PAGE followed by activity staining for SOD and CAT (Fig 5). A profound decrease in expression levels was noted in CCl$_4$ induced group while MEPz co-treatment markedly restored their activities. Also, CCl$_4$ induction significantly ($p < 0.001$) elevated the lipid peroxide levels and decreased the reduced glutathione levels in the rat liver. MPEZ co-treatment markedly ameliorated these changes to near normal levels (Table 5).

![Figure 4: Body weight and relative liver weight of control and experimental groups of rats.](image)

(a) Weight (g) vs. Animal Groups

(b) Liver Weight vs. Animal groups
(a) Body weight of control and experimental groups of rats taken on first, seventh and fifteenth day of the study. Each value was expressed as mean ± S.D for six rats in each group. Statistical significance at *$p < 0.05$*, $^*$*$p < 0.01$. a: CCl$_4$ vs. Control; b: CCl$_4$ vs. CCl$_4$ + MEPz.

(b) Relative liver weight of control and experimental groups of rats taken on day fifteen of the study. Each value was expressed as mean ± S.D for six rats in each group. Statistical significance at *$p < 0.05$*. a: CCl$_4$ vs. Control; b: CCl$_4$ vs. CCl$_4$ + MEPz. Relative liver weight is the average of liver weight at final body weight multiplied by 100.

Figure 5: Expression of superoxide dismutase (SOD) and catalase (CAT) in the liver of experimental animals on 10% Native-Polyacrylamide gel electrophoresis (Native-PAGE). Lane 1: Control rat liver cytosol showing expression of SOD and CAT. Lane 2: Shows SOD and CAT expression comparable to control in the cytosol of MEPz alone administered animals. Lane 3: Cytosolic fraction of CCl$_4$ alone induced rats showing repressed expression of both SOD and CAT with reference to control (Lane 1). Lane 4: Increased expression of SOD and CAT in the liver cytosol of CCl$_4$ + MEPz treated rats when compared with CCl$_4$ alone induced animals (Lane 3).
Figure 6: Effect of methanolic extract of *Plumbago zeylanica* Linn on inflammatory markers namely C- reactive protein (CRP) and myeloperoxidase (MPO).

(a) Effect of methanolic extract of *Plumbago zeylanica* Linn on the serum levels of CRP in CCl$_4$ induced hepatic Inflammation. Activity is expressed as mg/l. Each value expressed as mean ± S.D for six rats in each group. Statistical significance at $^{#}p<0.001$. a: CCl$_4$ vs. Control; b: CCl$_4$ vs. CCl$_4$ + MEPz.

(b) Effect of methanolic extract of *Plumbago zeylanica* Linn on MPO level in the liver of CCl$_4$ induced hepatic inflammation. The activity is expressed in U/ mg protein. Each value expressed as mean ± S.D for six rats in each group. Statistical significance at $^{#}p<0.001$. a: CCl$_4$ vs. Control; b: CCl$_4$ vs. CCl$_4$ + MEPz.
Figure 7: Effect of CCl$_4$ and MEPz on liver tissue and its histology in control and experimental groups shown in 40X magnification.

A1 to A4 depict the macroscopic examination of the liver in control and experimental groups. (A1) control and (A2) MEPz alone treated liver showing normal liver morphology without any nodules; (A3) CCl$_4$ induced rat liver showing numerous distinct nodules on their surfaces; (A4) MEPz co-treated rat liver showing significantly minimal liver damage.

Microscopic examination of the liver in control and experimental groups determined by hematoxylin and eosin (HE) staining is presented in B1 to B4. (B1) section of control group showing normal liver cell architecture; (B2) section of MEPz alone treated liver showing a normal liver architecture revealing the non-toxic nature of the drug; (B3) section of CCl$_4$ induced rat liver showing hepatocyte damage and centrilobular congestion (marked by black arrows); whereas animals co-treated with MEPz and subsequently given CCl$_4$ showing a more or less normal liver architecture.
A significant elevation \((p < 0.001)\) in the level of Serum C-reactive protein (CRP) was observed in CCl_4 administered group where the level rose from 1.34± 0.41 to 15.50± 2.9 indicating severe liver damage and treatment with MEPz markedly reverted back to near normal levels (Fig 6(a)). Myeloperoxidase (MPO) activity of liver tissue showed that, intoxication of liver with CCl_4 led to the significant increase \((p < 0.001)\) in MPO level, which on treatment with MEPz was brought down to normal (Fig 6(b)).

Macroscopic examination of rat liver in control and experimental groups were shown in Fig 7 (A1-A4). Control (A1) and MEPz alone treated (A2) liver showed normal morphology without any nodules whereas CCl_4 induced liver (A3) exhibited significant damage with numerous nodules (marked by arrows). MEPz co-treatment (A4) markedly ameliorated the liver damage. Fig 7 (B1-B4) depicts the hematoxylin and eosin (HE) staining of the liver tissue in control and experimental groups. CCl_4-induced liver sections (B3) showed significant hepatocyte damage with centrilobular congestion (marked by black arrows). Liver sections of MEPz co-treated rats (B4) showed mild degree of necrosis, fatty change and lymphocyte infiltration that are comparable to the control liver (B1) and MEPz alone treated liver sections (B2).

**DISCUSSION**

The present study was conducted to investigate the antioxidant and anti-proliferative activities of different extracts of Pz, on HepG2 cells and to demonstrate the anti-inflammatory activities of the extract against CCl_4 induced liver injury. The antioxidant activities of hexane, DCM and methanolic extract of Pz were evaluated in vitro. From the results it was seen that, all the three extract had significant radical scavenging property with the methanolic fraction showing the best effect which was evidenced by (i) highest phenolic content, (ii) increased DPPH scavenging activity, (iii) increased level of reducing activity, (iv) efficient NO scavenging ability and (v) controlled production of auto antigen. A study by Devender et al\(^{[51]}\), documented the presence of alkaloids, flavonoids, glycosides, carbohydrates, saponins, steroids and tannins in the methanolic fraction of Pz. Flavonoids and alkaloids are known to possess free radical scavenging activity.\(^{[52]}\) The best antioxidant potential shown by the MEPz could be due to the presence of these phytochemicals, which would have donated hydrogen atom to the free radical produced and thus made it as a non radical form.
The anti-proliferative effect of MEPz along with the standard Plumbagin was evaluated in liver carcinoma cells (HepG2). The growth inhibitory effect shown by MEPz on HepG2 cells signifies the presence of active components in the methanolic fraction. The LDH release in the culture medium in turn emphasis the occurrence of HepG2 cell death in a concentration and time dependent manner. Also, the effect of MEPz in modulating the expression of the key inflammatory mediator COX-2 was analysed by RT-PCR. COX-2 acts as a representative molecular bridge between inflammation and cancer, is a rate limiting enzyme that is involved in the conversion of arachidonic acid to prostaglandins. The inhibition of COX-2 expression is correlated with an increase in apoptosis in several disease models including cancer.\[53\] The marked decrease in mRNA levels in MEPz treated cells highlights its anti-inflammatory activity which is mainly mediated due to the presence of key phytochemicals present in the methanol extract.

The antioxidant and anti-proliferative effects attributed by MEPz in experiment I and II would be beneficial in preventing the progression of oxidative stress related diseases such as inflammation. Hence, the anti-inflammatory effect of MEPz was determined in CCl4 induced rat model. The rats induced with CCl4 had severe liver dysfunctions as evidenced by (i) decrease in the body weight and relative liver weight (ii) alterations in haematological parameters (iii) increased activities of serum liver marker enzymes (iv) decreased levels of antioxidant enzymes (v) increased lipid peroxide levels (vi) macroscopic and microscopic alterations in the liver tissue. MEPz markedly reversed these dysfunctions to near normal mainly by attenuating oxidative stress and suppressing inflammation caused by the CCl4 induction.

The decrease in the body weight and relative liver weight observed in the CCl4 induced group is a hallmark feature noticed in various inflammation models.\[54\] Haematological parameters have been associated with health indices and are of diagnostic significance in routine clinical evaluation of the state of health. Intoxication with CCl4 reduced certain haematological parameters viz RBC count, Hb, PCV and MCV values. Mandal et al\[55\], detected a decrease in RBC count along with a decrease in Hb in mice treated with CCl4. In differential count, the observed increase in neutrophils of CCl4 induced rats, may be considered as a defensive mechanism by the immune system which is in line with the report by Patrick-Iwuanyanwu et al.\[56\]. The co-treatment with MEPz was able to increase RBC, Hb, PCV and MCV values and brought down the levels of neutrophils.
An increased level of liver marker enzymes (AST, ALT, ALP, LDH, γ-GT and bilirubin) in serum is highly related to the severity of liver damage. The elevated levels of marker enzymes observed during CCl₄ induction were markedly decreased upon MEPz treatment indicating its inhibitory effect on CCl₄ induced inflammation. MEPz acted mainly by maintaining the membrane integrity and preventing the leakage of these enzymes into serum thus confirming its hepatoprotective efficacy. The free radicals generated as a result of CCl₄ induction largely affected the host antioxidant defense system thereby decreasing the levels of SOD, CAT, GPx, GST and GSH. Membrane damage via lipid peroxidation is known to occur in CCl₄ induced hepatotoxicity. A significant reduction in the TBARS levels upon MEPz treatment is attributed to the free radical scavenging, membrane stabilising and anti-peroxidative activity of the Pz. The antioxidant activity of MEPz against CCl₄ is mainly due to the synergistic action of key compounds in the roots of Pz.

CRP is an acute-phase protein that has been identified as an important biomarker for various inflammatory and degenerative diseases. Recent reports demonstrate that, level of CRP in hepatic cells may be reduced, in a dose-dependent manner, by various phytochemicals present in plants. It is also known that CRP level may be induced by IL-6 by means of NF kappa B activation. The alterations in the intracellular redox state lead to the inhibition of NF kappa B translocation to the nucleus and the synthesis of CRP by liver cells. Hence, it could be stated that, the effect produced by various phytochemicals on CRP level could be mediated by the NF kappa B pathway. Marked elevation of serum CRP to the peak (11.56 fold) during chronic liver inflammation in the present study was brought down to near normal upon MEPz treatment. This could be due to the maintenance of the redox status, inhibiting the NF κB activation and IL-6 and protecting the hepatocyte against oxidative stress. The mechanism behind the host tissue damage involves the reaction of MPO with hydrogen peroxide (H₂O₂, formed by the respiratory burst) in the presence of physiological chloride concentrations. It results in the formation of hypochlorous acid/ hypochlorite (HOCl/OCl) and other oxidizing species. All these events may contribute to tissue damage and finally inflammation by making lipids, DNA and protein amino groups as substrates. When the physiological chloride concentrations are absent, the MPO-H₂O₂ system can also form reactive nitrogen species that may initiate lipid peroxidation or form protein tyrosine residues, another posttranslational modification found in many pathological conditions. Significant reduction in the activity of MPO in MEPz co-treated animals may be due to ameliorating the toxicity of CCl₄ and inhibiting the conversion of the toxicant to highly
reactive free radicals which are responsible for the inflammation and recruitment of neutrophils by the body’s defense mechanism and release of the inflammatory marker MPO.

The histopathological studies performed, provided the direct evidence of the possibility of the extract being able to minimize disruption of structure of hepatocytes and accelerate hepatic regeneration thus decreasing the leakage of AST, ALT and ALP into the circulation. This strong evidence proved the anti-inflammatory effect of MEPz in CCl₄ induced inflammation.

CONCLUSION
The present study revealed that CCl₄ injection caused a marked rise in oxidative stress and degeneration of liver cells. Oral administration of MEPz (250 mg/kg BW) significantly ameliorated the undesirable effects mediated by CCl₄. The mechanism by which MEPz exhibited its anti-inflammatory effect was mainly due to the restoration of antioxidant enzymes and by decreasing lipid peroxidation. The observed pharmacological effect might be due the synergistic effects of different components present in the extract. Further studies are being investigated to elucidate the molecular mechanism involved to prove the efficacy of MEPz as an anti-inflammatory agent.

REFERENCES
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