EVALUATION OF HEPATOPROTECTIVE ACTIVITY OF LEAF EXTRACT OF PHYLLANTHUS EMBLICA AND MORINGA OLEIFERA ON CARBON TETRACHLORIDE INDUCED HEPATIC DAMAGE IN ALBINO RATS

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ABSTRACT

Phyllanthus emblica and Moringa oleifera have been used in Ayurveda for treatment of hepatic disorders. Most of the pharmacological studies are largely focused on their fruits while the rest of the parts remain less investigated. Therefore, we aimed to investigate the hepatoprotective effect of the petroleum ether extract of Phyllanthus emblica (PELPE) and Moringa oleifera (PELMO) leaves on carbon tetrachloride (CCl4) induced hepatotoxicity model in Albino rats. 54 male albino rats were divided into nine groups. Mice orally received saline, 100, 200, or 300 mg/kg b.w of PELPE /PELMO, 300 mg/kg b.w of PELPE+ PELMO respectively for fourteen days. Treatment groups were challenged with single dose of 3 ml/kg b.w of CCl4. Exposure of CCl4 elicited severe reduction in serum antioxidant enzymes. Hepatoprotection of PELPE and PELMO are evidenced by restoration of ALT, AST, Total protein, Superoxide dismutase, Catalase, Glutathione peroxidase and further confirmed by Liver histopathological studies. Presence of α–Tocopherol, β-Amyrin of M. oleifera and octadecanoic acid, methyl ester, gamma sitosterol of P emblica may be responsible for the bioactivity of these extracts. PELPE and PELMO possess significant hepatoprotective activities against CCl4 induced hepatotoxicity in rats and the extracts might be effective in management of liver damage.

KEYWORDS: Antioxidants, CCl4, Hepato protective activity, Phyllanthus emblica, Moringa oleifera, and Histopathology.
INTRODUCTION
Liver is the largest internal organ of the human body, which performs more than 500 different functions, and all those are necessary to our life (Redmond, 2009). Some of the major functions of liver are digestion, storage, synthesis, filtration, regulation and detoxification. Hence it is obvious that any defect in the functions of liver or its activities would be disastrous to the body. Industrial toxins like carbon tetrachloride, trichloroethylene, yellow phosphorus, and pharmacological compounds used in medical therapy like paracetamol, nimesulide, antitubercular drugs like rifampicin, isoniazid are the chief causative agents of liver injury which enter the body through inhalation, ingestion, or oral administrations. Some hepatotoxic drugs can generate metabolic intermediate or free-radicals that cause membrane lipid peroxidation resulting in liver cell injury (Gupta, 2008). Hepatotoxic chemicals produce reactive species which forms covalent bond with the tissue lipids and damage liver cells. Production of the reactive species are evident in tissue thiol depletion, lipid peroxidation, plasma membrane damage etc., culminating into severe hepatic injury. Generally the smooth ER of liver protects against the hazards of harmful drugs and chemicals by metabolizing the toxic substances from the portal circulation and makes them suitable for elimination. Excessive exposure to any hazardous chemical will generate very high free radicals which overwhelm the natural defensive mechanism leading to hepatic damage and cause jaundice, cirrhosis and fatty liver (Gupta et al., 2009).

Inspite of the tremendous advances made in allopathic medicine, no efficient hepatoprotective medicine is available. The effectiveness of synthetic drugs available for the treatment of liver diseases is contradictory and has greater chance of side effects (Manoj et al., 2013). Plant drugs are known to play a vital role in the management of liver diseases. There are numerous plants and polyherbal formulations claimed to have hepatoprotective activities. People are looking for the traditional systems of medicine in support of suitable drug preparation to stimulate liver functions or to regenerate the hepatic cells (Rajesh and Latha, 2001).

*Phyllanthus emblica* Linn (Syn. *Emblica officinalis* Gaertn.) is commonly known as “Amla”, belonging to the family Euphorbiaceae. It is the most important medicinal plant in the Indian traditional system of medicine, the Ayurveda. Various plant parts show antidiabetic, hypolipidemic, antibacterial, antioxidant, antiulcerogenic, hepatoprotective, gastroprotective, and chemopreventive properties (Krishnaveni and Mirunalini, 2010). Several parts of the
plant are used to treat a variety of diseases, but the most important is the fruit. The dynamic ingredients that have significant pharmacological action in *P. Emblica* are vitamin C, phenolic compounds, including hydrolyzable tannins, proanthocyanidins, flavanoids, flavonols, and compounds belonging to other phenolic groups etc. (E. Singh *et al.* 2011, Renuka *et al.* 2017). The tannins and flavonoids present in *Phyllanthus emblica* fruit extract contain very powerful antioxidant and hepatoprotective properties (Bhattacharya *et al.*, 2000 and Rajak *et al.* 2004).

*Moringa oleifera* Lam (MO) (Family: Moringaceae), commonly known as drumstick tree or horseradish tree. MO has been claimed in traditional literature to be valuable against a wide variety of diseases. Indian Materia Medica describes the use of roots of *M. oleifera* in the treatment of a number of ailments, including asthma, gout, lumbago, rheumatism, enlarged spleen or liver and internal deep seated inflammations (Fuglie, 1999). MO leaves are rich sources of antioxidant due to the the presence of various phytochemicals such as polyphenolics, carotenoids, α-tocopherol, ascorbic acid, and several amino acids (Omodanisi *et al.*, 2017). In recent decades, the extracts of leaves, seeds and roots of *M. oleifera* have been extensively studied for many potential uses including anti-tumour, hepatoprotective, anti atherosclerotic effect (Al-Said *et al.*, 2012, Jyothi *et al.*, 2017), and antioxidant (Santos *et al.*, 2012). There are no elaborate reports on treatment of diabetes and cancer using moringa (Gopalakrishnan *et al.*, 2016).

Considering the folkloric claims and reports, the present study attempt to evaluate the hepatoprotective potential and antioxidant activities of the crude petroleum ether leaf extract of *P. emblica* and *M. oleifera* by CCl₄-induced stress model in male wister rats.

**MATERIALS AND METHODS**

**Preparation of plant extracts**

The fresh leaves of *P. emblica* and *M. oleifera* used for the present studies were collected from Algar kovil hills, Madurai district, Tamil nadu, India. The selected plants were identified, and authenticated by Dr. P. Jayaraman, Institute of Herbal Botany, Plant Anatomy Research Centre (PARC/2011/ 2085 and PARC/2011/ 2086), West Tambaram, Chennai, Tamil nadu, India. The leaves were shade dried at room temperature to avoid loss of essential oil. The dried material was the pulverized separately into coarse powder with the aid of grinding machine. The resulting powder was then used for extraction. CCl₄ was purchased from S.D-Fine Chemicals Ltd., Mumbai, India. All other chemicals used were of analytical...
grade and were obtained from Glaxo Laboratories, Mumbai, India, and Sisco Research Laboratories, Mumbai, India. About 200gm of grinded plant material was subjected to soxhlet extraction ($60^\circ$C) employing petroleum ether as solvent. The solvent was evaporated at $60^\circ$C to obtain the extract. The dried crude residue of petroleum ether extracts was used for the study.

**Experimental animals**

Normal healthy Male Wister albino rats weighing 180-200g were maintained in standard environmental conditions. The experimental animals were housed (6 per cage) in clean polyethylene cages with sawdust-covered floors. All the animals were accustomed for 7 days to the lab condition and were maintained on standard conditions 45-55% relatively humidity and 12 hour light-dark cycle. Throughout the period of the experiment, the rats had free access to commercial standard commercial pellet diet and water *ad libitum*. All animals received human care in compliance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). Before performing the experiment, the ethical clearance was obtained from Institutional animal ethical clearance committee (CPCSEA NO.659/02/A).

**Acute Oral Toxicity studies (LD$_{50}$)**

The acute toxicity study was determined for petroleum ether leaf extracts of *Phyllanthus emblica* and *Moringa oleifera*, According to the guidelines of Organization for Economic Cooperation & Development (OECD guideline No. 423, 2002) in Male Wister rats. The animals of same age groups were kept fasting for overnight and provided only with water, after which a single dose of the plant extracts (PELPE/ PELMO) was administered orally up to the highest single dose of 2000 mg/kg body weight to estimate the exact LD$_{50}$ (Median Lethal Dose). Animals were observed individually during first one hour continuously for toxic symptoms. The rats were then observed periodically during 48 hours with special attention given during first 4 hr (short-term toxicity) and daily, thereafter observed for total of 14 days (short-term toxicity) for any mortality or behavioral changes (Chandan *et al.*, 2007). There was no mortality recorded. Therefore the herbal drugs should be free from toxicity.

**In vivo Hepatoprotective Study**

Total of 54 rats (48 CCl$_4$ hepatotoxicity induced rats and 6 normal control rats) were taken and divided into 9 groups of 6 rats each. Group I was considered ‘Normal control’, where rats received saline (3ml/kg body wt.). Group II was considered ‘Toxin control’, where animals
received oral administration of 3 ml of CCl₄ with olive oil mixture (1:1 ratio)/kg b.w. of rats on the first day. Group III to V received CCl₄ as in group-II followed by oral dose of 3ml/kg body weight petroleum ether leaf extracts of *Phyllanthus emblica* (PELPE) at concentration of 100, 200 and 300 mg/kg body weight for 14 consecutive days respectively. Group VI to VIII received CCl₄ as in group-II, Liver injured rats were given orally the petroleum ether leaf extracts of *Moringa oleifera* (PELMO) at the dose of 100, 200 and 300 mg/kg body weight for 14 consecutive days respectively. And Group IX received CCl₄ followed by Oral dose of 3ml/kg body weight PELPE and PELMO mixture at concentration of 300 mg/kg body weight for 14 consecutive days respectively.

**Assessment of Liver function tests**

**Biochemical parameters**

At the end of the experimental period (on 16th day), the animals were fasted and sacrificed 48 hr after the last injection of CCl₄ under mild diethyl ether anesthesia. Blood samples were collected and transferred in centrifuge tubes. Serum was collected, allowed to clot for 3-4 hrs at RT and then separated by centrifugation at 1500 rpm at 4°C for 10 min, and preserved at -20°C for evaluation of biochemical parameters. Liver homogenate was used for the assay of antioxidant parameters.

**Assessment of Liver damage (Antioxidant Assay)**

Liver damage was assessed by the estimation of biochemical parameters such as serum Aspartate transaminase (AST/SGOT), Alanine transaminase (ALT/SGPT) by NADH Kinetic UV liquid method (Reitman S, and Frankel, 1957), Total bilirubin by 3, 5-dichlorophenyldiazonium tetrafluoroborate (DPD) method and serum total protein were estimated by Biuret colorimetric method as per standard procedure prescribed by the manufacturer’s instruction manual provided in the kit. The absorbance of all the biochemical parameters was measured in a UV-VIS Spectrophotometer (Shimadzu, Japan).

The liver homogenate was used to assay antioxidant parameters such as Superoxide dismutase (Kakkar et al., 1984), Catalase (Luck, 1974), Glutathione peroxidase (Rotruck et al., 1973) and reduced glutathione content (Moron et al., 1979).

**Hepatotoxicity mediated histopathological studies**

On the 16th day the animals were sacrificed after withdrawal of the blood, liver samples were dissected out from the animals and washed separately with normal saline and processed
separately for dehydration, infiltration and embedding for histopathological observation. The rat liver tissues were fixed with 10% formalin neutral buffer solution for 48 hours and then with bovine solution for 6 hour. Tissue immersed in the fixative to prevent autolysis and bacterial attack. Tissues were dehydrated in increasing grades series of ethyl alcohol (70%, 90% and 100%) and then embedded in paraffin wax. Then wax containing tissue slice were cut with 5 μm thicknesses using rotary microtome. De-paraffinization and hydration of liver tissues was done followed by stained with alum haematoxylin-eosin (HE) dye. Then the sections were examined for the identification of tissue damage under binocular light microscope. (Strate et al., 2005).

**Statistical analysis**

All the experimental values were expressed as Mean ± SEM (Standard Error of Mean) and subjected to analysis of variance using one way analysis of variance (ANOVA) followed by student’s t-test. For the statistical t-tests, p values of less than 0.05 were taken as significant.

**RESULTS**

**Acute oral toxicity studies**

According to OECD guideline 423, Animals treated with test extract of both the plants extracts (PELPE and PELMO) for toxicity studies taking three male Wister rats with starting dose of 500, 1000 and 2000 mg/ kg body weight. The animals were observed for 14 days and were found to be safe. They did not show any sign and symptoms of toxicity and mortality at dose of 2000 mg/ kg body weight. Hence LD$_{50}$ was found to be greater than 2500 mg/kg body weight, in limit test. The testing doses were selected as 100, 200 and 300 mg/ kg body weight respectively.

**Effect of Plant extracts on Hepatoprotective activity**

The levels of serum alanine aminotransferase (ALT/ SGPT), and aspartate amino transferase (AST/ SGOT) activities and total bilirubin were significantly elevated in CCl$_4$ treated groups with simultaneous reduction in the total protein when compared to the normal control rats, indicating liver damage and are presented in Figure 1 (a–d). Administration of PELPE and PELMO (100, 200 and 300 mg/kg) individually and in a combination for 14 days to hepatotoxic bearing rats had produced remarkable protective effect on CCl$_4$-induced hepatotoxicity in a dose dependent manner. The plant extracts decreased the levels of elevated serum enzymes like SGOT, SGPT, and total bilirubin. The levels of total proteins in the plant extract treated animals increased (p<0.05) significantly.
Fig. 1: Serum biochemical parameters in CCl₄ induced hepatotoxicity in rats.

Negative Control, II-CCl₄ treated-Positive Control, III - CCl₄ + PELPE (100µg/ml), IV - CCl₄ + PELPE (200µg/ml), V - CCl₄ + PELPE (300µg/ml), VI - CCl₄ + PELMO (100µg/ml), VII - CCl₄ + PELMO (200µg/ml), VIII - CCl₄ + PELMO (300µg/ml), and IX - CCl₄ + PELPE+ PELMO (300µg/ml).

**Enzymatic and Non-enzymatic Antioxidants in liver tissues**

The activity of enzymic antioxidants, glutathione peroxidase (EC.1.11.1.6), superoxide dismutase (EC 1.15.1.1), catalase (EC 1.11.1.6), and reduced glutathione (GSH) in liver homogenates are presented in Figure 2 (a-d).

**Glutathione level**

Hepatic GSH level decreased significantly on CCl₄ administration compared to control (Table-2). CCl₄ induced depleted GSH level showed significant protection in animals concomitantly administered with PLEPE and PELMO extract. The synergistic effect of PELPE and PELMO at 300µg/ml concentration demonstrated an increased concentration of serum GSH, 5.1 ±3.12 U/L.
Negative Control, II-CCl₄ treated-Positive Control, III - CCl₄ + PELPE (100µg/ml), IV - CCl₄ + PELPE (200µg/ml), V - CCl₄ + PELPE (300µg/ml), VI - CCl₄ + PELMO (100µg/ml), VII - CCl₄ + PELMO (200µg/ml), VIII - CCl₄ + PELMO (300µg/ml), & IX - CCl₄ + PELPE+ PELMO (300µg/ml).

**Antioxidant enzymes**

Exposure to CCl₄ led to a significant depletion of Glutathione Peroxidase, Superoxide dismutase, and Catalase activity compared to the controls which indicates CCl₄ induced oxidative damage of liver. The level of antioxidant enzymes were significantly improved by administration of the PLEPE and PELMO. These plants must have the potential to normalize these enzyme activities in CCl₄ damaged liver. PELMO providing the best effects compared to PLEPE extract but the combined extract showed very effective.

**Histopathological examinations**

The results of histopathological examinations of nine experimental groups (as depicted in Fig.-3) also indicate the hepatoprotective efficacy of PLEPE and PELMO.
Histopathological examinations showed extensive liver injuries, characterized by extensive hepatocellular degeneration/ necrosis, inflammatory cell infiltration, congestion, and sinusoidal dilatation. Oral administration of both the leaf extracts at dose of 100, 200, and 300 mg/kg body weight significantly reduced the toxic effects of CCl$_4$. Synergistic effect of PELPE and PELPO in CCl$_4$ induced rats demonstrated a significant recovery from the degeneration, developed due to CCl$_4$, which is evident from the confluent growth of the hepatic cells with central vein and prominent nucleus.

**DISCUSSION**

In the modern era various plants and individual or combined herbal formulations are used for the treatment of liver diseases. However, in most of the cases, the treatments are not satisfactory, the studies and experimental evaluation were mostly incomplete and insufficient (Kumar *et al.*, 2011). In the present study, the individual as well as combined extract of *Phyllanthus emblica* and *Moringa oleifera* was investigated for hepatoprotective activity in CCl$_4$ induced liver damage. Both PELPE and PELMO have individually and combined demonstrated liver-protecting potentials, reversing the damage induced by CCl$_4$. High level of CCl$_4$ hepatotoxicity of the present study is in concordance with earlier reports by Hamzah (2010). Carbon tetrachloride commonly used model for screening of hepatoprotective drugs.
in experimental studies. CCl$_4$ administration to the healthy albino rats significant increases in transaminases activity (ALT and AST). The enzymes leaking out from damaged liver cells in to circulating blood represent the hepatic membrane damage or necrosis. AST is predominantly found in Liver mitochondria and gets significantly increased during chronic hepatitis and cirrhosis; ALT increased during hepatitis, cirrhosis and obstructive jaundice. CCl$_4$ exposed animals showed increase in serum bilirubin level but decrease in serum total protein content.

The administration of CCl$_4$ have increased the marker enzymes (ALT and AST) and TB from 38.0 U/L, 53.0 U/L and 0.61 mg/dl to 346.3 U/L, 271.5 U/L and 3.97 mg/dl whereas TP decreased from 8.5 to 4.8 gm/dl. Similar results of elevated concentration of AST, ALT, TB and ALP was reported by Adewale et al. (2014) on administration of CCL$_4$ in experimental Sprague Dawley rats, supports our findings. Investigation by Prakash et al. (2008) and Patrick-Iwuanyanwu et al. (2010) also demonstrated a remarkable elevation in the rat’s marker enzyme and Bilirubin in CCl$_4$ administered rats authenticates the hepatotoxicity of CCl$_4$. Total bilirubin and protein levels are related to the secretary function of hepatic cells (Drotman et al., 1978).

Superoxide dismutase (SOD) has been reported as the most sensitive enzymatic index in liver injury. SOD scavenges the superoxide anion to form hydrogen peroxide and thus diminishing the toxic effect caused by this radical. SOD plays an important role in the elimination of ROS derived from the peroxidative process of xenobiotics in liver tissues. CAT is a key component of antioxidant defense system which decomposes hydrogen peroxide and protects the tissues from highly reactive hydroxyl radicals (Chance et al., 1992). The most abundant tripeptide Glutathione (GSH) is a non-enzymatic biological antioxidant against toxic stimuli. In liver, it plays a central role of co-ordinating the body’s antioxidant defense process and maintains membrane proteins. GSH is a substrate for glutathione peroxidase (GPx). SOD, CAT and GSH reported to mutually play a defensive role against ROS (Halliwell and Gutteridge, 2007).

The antioxidant enzyme analysis of liver homogenate revealed that on CCl$_4$ administration the enzymes SOD, CAT, GPx and GSH decreased from 7.6 U/L, 88.5 U/mg, 16.3 U/L and 5.4 U/L to 4.1 U/L, 44.0 U/mg, 11.2 U/L and 1.6 U/L respectively. Similar decrease in concentration of antioxidant enzymes were reported by Palanivel et al. (2008) in CCl$_4$ induced hepatic damage in rats.
Our study demonstrated that the administration of PELPE and PELMO extracts were able to prevent the degradation of the antioxidant enzymes which was evident from their significant increased concentration and this antioxidant enzyme activity was reflected in the decrease in biomarker enzyme (ALT and AST) and total bilirubin and total protein concentration. Histopathological study of the liver of CCl₄ treated rat’s demonstrated defect ranging from inflammation of hepatocytes, tissue necrosis, congested central vein and fatty degeneration. However histology of sections treated with PELPE and PELMO evidenced that as the concentration of the extract increased there was the reduction and further absence of necrosis, lesser fatty infiltration. Similar results of histological deteriorative effects of CCl₄ intoxication in liver were also improved after treatments with Moringa oleifera extract were demonstrated by El-bakry et al. (2016).

*P. emblica* which is rich in vitamin C, gallic acid, flavonoids, and tannins, protects against hepatotoxicity induced liver injury. Hepatoprotective activity of PE was found to be due to its membrane stabilizing, antioxidative and Cytochrome (CYP) inhibitory effects (Dhuley and Naik, 1997) Moringa plants are rich in compounds containing the simple sugar (rhamnose, glucosinolates), niazimicin, pterygosperm, benzyl isothiocyanate, and a number of vitamins and minerals as well as other more commonly recognized phytochemicals such as carotenoids (Lowell, 1989) which could be the possible reason for its hepatoprotective activity. High level of hepatotoxicity of the present study is consistent with earlier reports by Hamza (2010). The bioactive compounds from the petroleum ether leaf extract of *Moringa oleifera* and *Phyllanthus emblica* using GC-MS analysis and also their HPTLC Fingerprinting profile were reported in our previous studies. Gas Chromatography- Mass Spectrometry (GC-MS) analysis of these plant extracts shows the presence of hepatoprotective compounds namely D. L. α –Tocopherol, Beta- Amyrin in *Moringa oleifera* whereas Octadecanoic acid, Methyl ester, Gamma Sitosterol in *Phyllanthus emblica* (Malliga et al., 2015) might be responsible for the effective bioactivity of these extracts.

**CONCLUSION**

On the basis of biochemical and histological results of this study, it is concluded that the petroleum ether leaf extract of *Phyllanthus emblica* and *Moringa oleifera* have multiple advantageous therapeutic properties viz., antioxidant (preventing the free radical formation and related diseases) hepatoprotective property and thereby effectively reducing the consequences of hepatic cancer without causing any defects to the cells. The presence of
various bioactive phytoconstituents, identified in this study and its synergistic activity would be of greater therapeutic benefits in the management of liver damage and hepatocellular carcinoma. Both the plants demonstrated that they provide an excellent source phytodrugs which may be useful in treating various disease conditions and thus promote the quality of life. However further studies shall be conducted in isolating and purifying the crude phytochemicals obtained in the study into an active ingredient and perform a clinical study in human population to elucidate the mechanism involved in the hepatoprotective nature thereby further developing into an active pharmaceutical formulation.

CONFLICT OF INTEREST
None

REFERENCES


