Evaluation of Hepatoprotective and antioxidant potential of methanolic extract of *Polyalthiya longifolia* fruits: An *In-vitro* and *In-vivo* approach

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ABSTRACT

The aim of the study was to investigate the hepatoprotective and antioxidant potential of methanolic extract of *Polyalthiya longifolia* fruits (MEPL) by *in-vitro* and *in-vivo* methods.In the invitro study, Freshly isolated rat primary hepatocytes and HepG2 cells were exposed with CCl_4 along with/without various concentrations of MEPL(125,250,500 µg/kg) and the effects were studied.In the *in-vivo* studies, CCl_4 intoxication method was used and aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin and total proteins were estimated and supported with histopathological studies.MEPL (125,250,500 µg/kg) treated animals increased the percentage of viability in both primary hepatocytes (p<0.001) and in HepG2 cells (p<0.01) where as in the invivo studies,MEPL produced significant hepatoprotective effect by decreasing the elevated serum enzymes, bilirubin, LPO and significantly increased the levels of glutathione (GSH), catalase (CAT) and super oxide dismutase (SOD) .Moreover, *in-vitro* antioxidant studies revealed that MEPL scavenged free radicals and maximum percentage of inhibition was 62% at 800µg/mL. In addition to this, treatment with MEPL fruits showed a dose dependent reversal of histopathological changes. Based on the results, we conclude that, MEPL possesses hepatoprotective and antioxidant activity against CCl_4 intoxication in both *in-vitro* and *in-vivo* methods through its free radical scavenging and antioxidant properties.

INTRODUCTION

In our body, liver is the main organ for various metabolic reactions including oxidation, sulfation, acetylation, hydrolysis and conjugation reactions. It has an important role in the maintenance, performance and regulating homeostasis mechanism of the body. Injury to liver and damage to the hepatic parenchyma cells were always proved to be associated with distortion of different metabolic functions of liver (Ward and Daly, 1999).Etiologically various infectious agents including viruses and different hepatotoxins along with environmental pollutants are thought to be responsible for causing different types of liver damage and hepatic injuries (Law and Brunt, 2010; Stickel and Schuppan, 2007).

The pathophysiological role of free radicals and oxidative stress in liver damage, acute and chronic hepatic injury

were clearly observed in recent studies on free radicals research (Feijoo *et al.,* 2010;Chandan *et al.,*2008). The mechanism of actions of potent hepatotoxins such as CCl_4, Paracetamol etc, also indicated the role of oxidative stress and free radicals in the pathophysiology of hepatic injury**.** Excess production of reactive oxygen species (ROS) along with significant decrease of antioxidant defence in these pathological conditions impairs and alters various cellular functions through the processes of lipid peroxidation.Accordingly, effects of antioxidants or free radical scavengers have been widely tested for the prevention and treatment of acute and chronic liver damages (Stehbens, 2003; Jaeschke, 2003; McDonough, 2003; Jaeschke, 1998). Free radicals mainly act by attacking the unsaturated fatty acids in the cell membranes causing membrane lipid peroxidation which is a hallmark sign of hepatotoxicity, and damaging membrane protein which finally triggers the cell inactivation and death (Martin and Grotewiel, 2006; Kataki *et al.,* 2012). Available data from earlier studies also indicated the beneficial effects of antioxidants specifically, for prevention and treatment of acute and chronic liver

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injury (Gupta etal.,2004;Kukongviriyapan *et al.,*2003). Therefore, antioxidants can be used to reverse the harmful and pathological actions caused by free radicals. The antioxidants in use are either derived from plants as natural origin or prepared synthetically. Due to possibility of carcinogenic effect, synthetic antioxidants are not preferred much (Kukongviriyapan *et al.,*2003). Therefore, many folklore remedies from natural origin are tested for its antioxidant and hepatoprotective potential on liver damage by using various animal models like Carbon tetrachloride (CCl_4) induced hepatotoxicity (Rubinstein, 1962 ; Suja *et al.,* 2002). *Polyalthia longifolia* (Family: *Annonaceae*) is a lofty evergreen tree found in India and Sri Lanka, commonly planted for its effectiveness in alleviating noise pollution. (Raghunathan and Mitra ,1982). The extract of stem bark of the plant and the alkaloids isolated from it has been reported for anti-bacterial and anti-fungal activities (Hasan *et al.,*1988). Its aqueous extract stimulates the isolated ileum and uterus, depresses heart rate, decreases blood pressure and respiration rate in experimental animals (Achari and Lal, 1952). The crude extracts of the seeds of the plant have also showed remarkable anti-bacterial activities (Sayeed *et al.,*1995) and plants of *annonaceae* family are said to contain antitumor and anticancer active principles (Chakrabarti and Mukherjee,1968;Chen *et al.,* 2000). In this study, we have evaluated the hepatoprotective and antioxidant potential of methanolicextract of *polyalthiya longifolia* fruits (MEPL) against CCl_4 intoxication by *in-vitro* and *in-vivo* methods.

MATERIALS AND METHODS

Chemical*s*

All chemicals and solvents used were obtained from S.D. Fine Chemicals,Ranbaxy laboratories Ltd., Punjab, Sigma Fine Chemicals, Mumbai and Hi media Laboratories, Mumbai, India. For various biochemical   estimations, assay kits were procured from E. Merck Ltd., M.I.D.C., Taloja.The human liver derived HepG2 cell line was obtained from, cell culture department, KMCH, Coimbatore, India.

Plant material

Polyalthiya longifolia fruits were collected from Irumbulikurichi, Ariyalur district, Tamilnadu, India and authenticated by G.V.S Murthy, botanical survey of India (BSI), southern circle, Coimbatore, Tamilnadu, India (BSI/SC/5/23/11-12/Tech-1759).

Preparation of methanolic extrct of *Polyalthiya longifolia* fruits

The chopped and shade dried fruits were powdered and passed through a 40-mesh sieve which was then subjected to single extraction with methanol in a Soxhlet apparatus. The solvent from the methanolic extract was completely removed and concentrated to dryness at 40°c under reduced pressure in a rotary vacuum evaporator. The *Polyalthiya longifolia* fruits yielded brown semisolid residue of methanolic extract, weighing 9.0% w/w with respect to the dried starting material (Trease and Evans, 2005).

Preparation of Suspensions

Methanolic extract of *polyalthiya longifolia* (MEPL) was dissolved in DMSO and the volume was made upto 10 ml to obtain a stock solution of 1mg/ml and stored at-20^0C prior to use. Further dilutions were made to obtain different concentrations (125,250,500µg/kg).

Experimental animals

The experiments were carried out on adult male albino rats (150-180g). They were housed in a quite environment with a temperature of 25 ± 1^0C and fed with standard rat feed with water ad libitum, except during the test period. The experimental protocols were approved by institutional animal ethics committee (IAEC/KMCRET/Pharm/06/2011) and conducted according to the CPCSEA guidelines for the use and care of experimental animals, New Delhi, India.

Acute toxicity studies

Acute oral toxicity study was performed according to the OECD guidelines for the testing of chemicals, Test No. 423 (OECD, 2001; acute oral toxicity acute toxic class method. Wistar rats (n= 3) of either sex were selected by a random sampling technique for the acute toxicity study. The animals were fasted overnight prior to the experiment and maintained under standard laboratory conditions. Extract was administrated orally in increasing dose up to 2000mg/kg.

Preparation of drug solution
Hepatotoxin and test substances

For *in-vitro* studies, CCl_4 (1%), was used to produce submaximal toxicity on isolated rat hepatocytes. The test solution MEPL was administered at dose levels of 125,250 and 500 μg/mL and Sylimarin was used as a positive control.For *in-vivo* studies,liver damage was induced by administration of 30% CCl_4 suspended in olive oil (1ml/kg.b.w,i.p) and   MEPL (200,400mg/kg) was suspended in 1% carboxy methyl cellulose, for oral administration (Tasaduq *et al.,*2003). Sylimarin was administered at a dose of 200 mg/kg in 1% carboxy methyl cellulose solution.

In-vitro hepatoprotective activity
Isolation and culture of rat hepatocytes

The livers were isolated under aseptic conditions and placed   in   HEPES   (N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid) buffer I containing HEPES (0.01 mol/L), NaCl (0.142 mol/L) and KCl (0.0067 mol/L), pH 7.4. The isolated livers were cut into small pieces and incubated with a second buffer containing HEPES (0.1 mol/L), NaCl (0.0667 mol/L),KCl (0.0067 mol/L) and 0.5% Collagenase type IV , pH 7.6, for about 45 min at 37℃ with constant shaking in an incubator. Hepatocytes were obtained after filtration through a nylon mesh (250 μ) followed by cold centrifugation (4℃, 200 rpm for 2 min, thrice) and suspended in HEPES buffer I. The viability of the hepatocytes

was assessed by trypan blue (0.2%) exclusion method. The isolated hepatocytes were cultured in Ham's F12 medium, supplemented with 10% newborn calf serum, antibiotics, 10^{-6} dexamethasone and 10^{-8} bovine insulin. The cell suspension was incubated at 37°C for 30 min in a humidified incubator under 5% CO_2. Twenty four hours after the establishment of the monolayer of hepatocytes, the medium was decanted and the culture was washed with HEPES buffer-I and finally the hepatocytes were suspended in Buffer-I. The hepatic cytotoxicity was induced with CCl_4 (1%) Triplicate hepatocyte suspensions (0.1mL) from different cultures were distributed into various culture tubes and labelled as per the study design (Seglen, 1976).

Study design

Groups I	-	Normal control-Treated with DMSO 30%
Group II	-	Toxicant CCl_4 (1%)
Group III	-	MEPL 125 µg/mL +CCl_4 (1%)
Group IV	-	MEPL 250 µg/mL + CCl_4 (1%)
Groop V	-	MEPL 500 µg/mL + CCl_4 (1%)
Groop VI	-	Sylimarin (250 µg) + CCl_4 (1%)

Assessment of hepatoprotective activity-invitro

The effect of MEPL on liver protection was determined by measuring an increase in the percentage of viability of cells by MEPL, compared with the control and toxicant groups. Reversal of toxin induced elevations in the level of marker enzymes was also considered to assess hepatoprotective activity. After 1 hr of CCl_4 challenge, the levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT) (Bergmeyer *et al.,* 1978) and alkaline phosphatase (ALP) (King, 1965), total bilirubin (Malloy and Evelyn, 1937), total proteins (Lowry *et al.,* 1951; Kiso *et al.,* 1983) in the medium were measured as an indication of hepatocytes necrosis using diagnostic Kits procured from Ecoline, E. Merck Ltd, using an auto analyser (Table 1).

Hepatoprotective study in HepG2 Cell line

HepG2 Cell lines are suitable for *in-vitro* model system for the study of polarized human hepatocytes. HepG2 Cell line with proper culture conditions displays robust morphological and functional differentiation with a controlled formation of apical and basolateral cell surface domain in models. The HepG2 cells were exposed to toxicant containing 1% CCl_4 along with /without MEPL extract of different concentrations or the medium alone is considered as control. At the end of the period, cytotoxicity was assessed by estimating the viability of the HepG2 cells by the MTT reduction assay (Hu *et al.,* 1999).

In-vivo hepatoprotective activity

Thirty albino wistar rats of either sex, weighing about 150-180 g were divided into 5 groups of six animals each. Group I animals received CMC (0.3%) and served as control. Group II animals served as CCl_4 treated control. Group III and IV received MEPL at a dose of 200 and 400 mg/kg respectively. Group V

animals received standard Sylimarin 200 mg/kg. The animals were treated for 7 days as per the study design mentioned above and on the 7th day after one hour of dosing, the toxicant 30% of CCl_4 (1 ml/kg i.p.) was administered to all the groups except Group I. After 24 h, the animals were anesthetized and blood was collected from retro orbital plexus for the assessment of various enzyme activities. The blood was centrifuged at 2000 rpm for 10 min. The serum was separated and analyzed for various biochemical estimations such as aspartate aminotransferase (AST), alanine aminotransferase (ALT) (Bergmeyer *et al.,*1978) and alkaline phosphatase (ALP) (King,1965), total bilirubin (Malloy & Evelyn,1937) , total proteins (Lowry *et al.,*1951;Kiso *et al.,* 1983) by using diagnostic kits. (Table 1).

The animals were sacrificed later and the liver was perfused and excised. Part of the liver was stored in 10% formalin saline for histopathological studies. The remaining portion was rinsed in ice cold normal saline, followed by 0.15 M Tris-HCl (pH 7.4) blotted dry and weighed. A 10 % w/v of homogenate was prepared in 0.15 M Tris-HCl buffer and processed for the estimation of lipid peroxidation. A part of homogenate after precipitating proteins with Trichloro acetic acid (TCA) was used for estimation of glutathione. The rest of the homogenate was centrifuged at 1500 rpm for 15 min at 4°C. The supernatant thus obtained was used for the estimation of SOD and CAT activity.

Determination of Reduced Glutathione (GSH)

To measure the reduced glutathione (GSH) 0.2 ml of tissue homogenate was mixed with 1.8 ml of EDTA solution. To this 3.0 ml precipitating reagent (1.67 g of met phosphoric acid, 0.2 g of EDTA disodium salt, 30 g sodium chloride in 1 L of distilled water) was added, mixed thoroughly and kept for 5 min before centrifugation. To 2.0 ml of the supernatant, 4.0 ml of 0.3-M disodium hydrogen phosphate solution and 1.0 ml of DTNB (5,5-dithio bis 2-nitro benzoic acid) reagent were added and was read at 412 nm(Ellman,1959).

Assay of Super Oxide Dismutase (SOD)

The assay mixture contained 1.2 ml sodium pyrophosphate buffer (pH 8.3, 0.025 mol/L), 0.1ml phenazine methosulphate (186 mM), 0.3 ml NBT (300 mM), 0.2 ml NADH (780 mM), diluted enzyme preparation and water in a total volume of 3 ml. After incubation at 30ºC for 90 sec, the reaction was terminated by the addition of 1.0 ml glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 4.0 ml n-butanol. The colour intensity of the chromogen in the butanol layer was measured at 560 nm against n-butanol and concentration of SOD was expressed as units/mg protein (Kakkar *et al.,* 1984).

Assay of Catalase (CAT)

The liver tissue was homogenized in M/150 phosphate buffer (pH 7.0) at 1–4ºC and centrifuged at 5000 rpm. The reaction mixture contained 0.01 M phosphate buffer (pH 7.0), 2 mM H_2O_2 and the enzyme extract. The estimation was done spectrophotometrically following the decrease in absorbance at

230 nm. The specific activity of catalase was expressed in terms of units/mg protein (Aebi ,1974).

Invitro Antioxidant activity
DPPH radical scavenging activity

The free radical scavenging activity of MEPL fruits was observed spectrophotometrically by measuring the decrease in the absorbance of methanolic solution of DPPH. 1mL extract of MEPL at various concentrations ((25, 50,100,200,400,800µg/mL) were mixed with 1mL of methanolic solution of DPPH (100µM). Similarly a 1mL methanolic solution of ascorbic acid (100 µg/mL) was mixed with 1mL of DPPH solution.

A mixture of 1mL of methanol and 1mL of methnolic solution of DPPH (100 µM) served as control. After mixing, all the solutions were incubated in dark for 20 minutes and absorbance was measured at 517 nm (Blois,1958).The experiments were performed in triplicate and scavenging activity was calculated by using the following formula and expressed as percentage of inhibition.

$$\text{Scavenging \%} = \frac{\text{Absorbance of control -- Absorbance of test}}{\text{Absorbance of control}} \times 100$$

Nitric oxide radical scavenging activity

The nitric oxide radical scavenging activity was measured by using Griess' reagent. 5ml of each extract solutions of different concentrations (25,50,100,200,400,800µg/mL) in standard phosphate buffer solution (pH 7.4) were incubated with 5mL of sodium nitroprusside solution (5mM) in standard phosphate buffer (pH 7.4) at 25°C for 5 hours.

In an identical manner 5mL of ascorbic acid solution (100µg/mL) in standard phosphate buffer solution (pH 7.4) was also incubated with 5mL of sodium nitroprusside solution (5mM) in standard phosphate buffer (pH 7.4).

Control experiments with equivalent amount of buffer and without the extract were also conducted. After incubation, 0.5mL of the incubation mixture was mixed with 0.5 mL of Griess' reagent (Sulphanilamide 1%, O-phosphoric acid 2% and naphthyl ethylene diamine dihydrochloride 0.1%) and the absorbance was measured at 546nm (Green et al.,1982).The experiments were performed in triplicate and scavenging activity was expressed as percentage of inhibition.

Reducing power

2.5mL of solutions of different concentrations of the extract (25,50,100,200,400,800µg/mL) in standard phosphate buffer solution (pH 6.6) were incubated with 2.5mL of potassium ferricyanide solution (1% w/v) at 50°C for 20 min. Similarly ascorbic acid solution (100 µg/mL) was also incubated. After incubation, 2.5mL of 10% trichloro acetic acid solution was added to each tube and the mixture was centrifuged at 650 rpm for 10 minutes. 5mL of the upper layer solution was mixed with 5mL of deionised water and 1mL of ferric chloride solution (1%w/v) and the absorbance was measured at 700 nm (Oyaizu,1986).

Statistical analysis

The statistical analysis was carried out by one-way analysis of variance (ANOVA). The values are represented as mean ± SEM. Comparison of mean values of different groups treated with different dose levels of extracts and positive controls were estimated by Turkey's Multiple Comparison Test. $p < 0.05$ was considered significant.

RESULT AND DISCUSION

Hepatoprotective effect of MEPL on primary rat hepatocytes and HepG2 Cell line

The present study demonstrated the hepatoprotective and antioxidant effects of MEPL against CCl_4 induced liver injury in rats. Rat hepatocytes incubated with CCl_4 resulted in 69% depletion in viability of cells, elevation of AST, ALT, ALP, total bilirubin (P<0.001) and a significant reduction in the level of total proteins (P<0.001).Treatment with MEPL exhibited significant increase in percentage viability on rat hepatocytes and significant restoration of the altered biochemical parameters (Table.1).In the invitro study,HepG2 cells exposed with CCl_4 showed depletion of viability by 81% whereas MEPL received HepG2 cell after exposure to CCl_4 showed a dose dependant increased percentage viability (P<0.001) as shown in Table 2.

In-vivo hepatoprotective activity

The liver mainly detoxifies toxic chemicals, drugs and becomes the main target organ for all possible toxic xenobiotics. Carban tetrachloride is one of the most commonly used hepatotoxin in experimental study of liver diseases (Johnson and Kroening, 1998). CCl_4 is biotransformed by cytochrome p-450 in liver to produce highly reactive trichloromethyl free radical leading to loss of integrity of cell membranes and damage of hepatic tissue (Recknogel et al., 1976).This effect was evidenced by increased levels of serum marker enzymes, namely AST, ALT and ALP and total bilirubin which are related to the normal function of hepatic cell (Muriel et al., 1992; Uma Maheswari and Rao, 2005). The same trend was observed in our study which shows elevated levels of serum marker enzymes in CCl4 treated groups. Treatment with MEPL showed a significant restoration of the altered biochemical marker enzymes in a dose dependant manner when compared with normal groups (p<0.01).

This obtained result further indicates its hepatoprotective effects (Table 3). In our study, acute administration of CCl_4 produced a marked elevation of the serum levels of AST,ALT, ALP, (p<0.001), serum bilirubin and total proteins in animals (Group II) when compared with normal control (Group I) confirming cellular breakage and loss of functional integrity of cell membranes in liver. Treatment with MEPL at a dose of 200 mg/kg and 400 mg/kg significantly reduced the elevated levels of ALT, AST, and ALP towards the respective normal value which is an indication of plasma membrane stabilization as well as repair of hepatic tissue damage caused by carbon tetrachloride. Available data from earlier studies states that, stimulation of protein

synthesis accelerates regeneration process and production of liver cells (Nicotera and Orrenius, 1986).MEPL received groups showed increased levels of total protein which indicates its hepatoprotective activity comparable with standard drug Sylimarin. These alterations can be considered as an expression of the functional improvement of hepatocytes, which may be caused by an accelerated regeneration of parenchyma cells.

Serum bilirubin is a breakdown product of haem in red blood cells and regarded as one of the most important clinical and pathophysiological indicator of necrosis of liver tissues. Treatment with MEPL resulted in a significant decrease in total bilirubin levels as compared to CCl_4 treated group (Group II) and this effect was possibly due to stabilization of hepatic cellular membranes by the extract. The results were further confirmed by the histopathological observations. The effective control of alkaline phosphatase (ALP) and bilirubin levels towards normal is good indication suggesting improvement in the secretary mechanism of the hepatic cell.

Effect of MEPL on antioxidant status

An increase in LPO level (from 5.2 ± 0.4 to 15.7 ± 1.2, $p < 0.001$) in liver homogenate suggests enhanced lipid peroxidation which leads to tissue damage and failure of antioxidant defense mechanisms resulting in excessive free radicals. Lipid peroxidaion is a free radical mediated process leads to oxidative detorioration of polyunsaturated lipid.

Most of the tissue damage is considered to be mediated by these free radicals by attacking membranes through peroxidation of polyunsaturated fatty acids. Inhibition of lipid peroxidation to a significant degree is also a predominant mechanism of hepatoprotection. Treatment with MEPL at a dose of 100,200 mg/kg, produced significant (P<0.01) reductions in lipid peroxidation when compared to CCl_4 administered rats which further supports the above said hepatoprotective mechanism.

Glutathione is known to protect the cellular system against toxic effects of lipid peroxidation (Anuradha and Selvam,1993) Decreased level of GSH in the liver tissue on CCl_4 exposure represents its increased utilization due to oxidative stress (Curtis et al., 1972).In the present study, a significant elevation of GSH level (p< 0.05) was observed in the MEPL treated CCl_4 rats which indicate that MEPL can either increase the biosynthesis of GSH or reduce the oxidative stress leading to less degradation of GSH. Suproxide dismutase is also an important indicator of hepatocellular damage in both acute and chronic conditions.

SOD has potential role in scavenging the superoxide anion to form hydrogen peroxide and thus diminishes the toxic effect caused by this radical (Chance et al.,1952).

In the present study, it was observed that the MEPL caused a significantly increased in the hepatic SOD (p< 0.05) activity of the CCl_4 intoxicated rats. This shows MEPL can reduce reactive free radicals that might cause oxidative damage to the tissues and improve the activities of the hepatic antioxidant enzyme. Increase in the SOD level portraits the repairing mechanism of antioxidant defense system, which plays an important role in hepatoprotection. CAT is a hemeprotein, which catalyzes the reduction of hydrogen peroxides and protects the tissues from highly reactive hydroxyl radicals (Yan and Harding, 1997).This decrease in CAT activity could result from inactivation by glycation of the enzyme (Kono and Fridovich,1982). Further, an increase in the SOD activity may protect CAT against enzyme inactivation by superoxide radical as these radicals have been shown to inactivate CAT (Wu et al., 2003) Thus, the increase in SOD activity in our tudy may indirectly play an important protective role in preserving the activity of CAT.

In-vitro Antioxidant activity

Results from our in-vitro antioxidant studies revealed that MEPL scavenged free radicals in a concentration dependent manner in the models studied. Maximum percentage inhibition of DPPH radicals by MEPLwas about 76% at 800 µg/mL and in nitric oxide radical scavenging model its about 59% at 800µg/mL whereas in reducing power method, MEPL demonstrated dose dependent antioxidant activity comparable with ascorbic acid. (Table.5)

The results observed from the DPPH radical scavenging and nitric oxide radical scavenging method shows, the definite scavenging activity of the MEPL towards DPPH radicals in comparison with ascorbic acid.

Nitric oxide is a free radical produced in the mammalian cells and is involved in regulation of various physiological processes. However excess production of nitric oxide is associated with several diseases (Gibanananda and Sayed ,2002; Ialenti et al.,1993).Administration of MEPL has demonstrated dose dependent radical scavenging activity against NO free radicals and also in reducing power method. In all the methods, scavenging effect of MEPL increases with increasing concentration and maximum antioxidant activity was found at the dose of 800 µg/Ml and above of MEPL.

Table. 1: Effect of MEPL on the biochemical parameters of CCl_4 induced toxicity in isolated rat hepatocytes.

Treatment	Viablecells (%)	ALT (IU/dl)	AST(IU/dl)	ALP (IU/dl)	Total Bilirubin (mg/dl)	Total protein (mg/dl)
Control	92.56±0.73	17.38±0.52	18.42±0.22	36.22±0.67	0.24±0.002	1.24±0.03
CCl_4	31.32±1.41	61.34±1.73 [a]	76.37±1.65 [a]	96.34±2.54 [a]	0.65±0.03 [a]	0.62±0.03 [a]
Sylimarin	85.71±2.11 [b]	18.63±0.46 [b]	23.56± 0.27 [b]	34.33±0.56 [b]	0.28±0.002 [b]	1.17±0.02 [b]
MEPL 125 µg/mL	52.43±2.35 [b]	38.25±0.56 [b]	35.22±0.63 [b]	44.21±0.37 [b]	0.35±0.003 [b]	1.03±0.03 [b]
250 µg/mL	66.45±1.14 [b]	32.47±0.43 [b]	32.44±0.46 [b]	37.48±0.72 [b]	0.33±0.003 [b]	1.07±0.02 [b]
500 µg/mL	79.41±1.12 [b]	27.33±0.56 [b]	27.64±0.43 [b]	34.22±0.55 [b]	0.30±0.00 1 [b]	1.12±0.03 [b]

Values are mean ± SEM: [a]p<0.001 When compared to normal group, [b]p<0.001 When compared to CCl_4 groups.

Table. 2: Hepatoprotective activity of MEPL on CCl₄ intoxicated HepG2 cells.

Treatment	% Viability
Control	100
CCl₄	19.43 ± 2.12^{a}
Sylimarin + CCl₄	89.23 ± 3.24^{b}
MEPL (125 µg/mL) + CCl₄	77.43 ± 3.61^{b}
MEPL (250 µg/mL) + CCl₄	80.67 ± 3.53^{b}
MEPL (500 µg/mL) + CCl₄	84.66 ± 3.45^{b}

Average of 5 determinations (n=5), 4 replicates:
[a] $p<0.001$, When compared to normal; [b]$p<0.01$, when compared to CCl₄ intoxicated cells.

Table. 3: Effect of MEPL on the biochemical parameters of CCl₄ intoxicated rats.

| Treatment	Liver Weight (g)	AST (IU/dl)	ALT (IU/dl)	ALP (IU/dl)	Total Bilirubin (mg/dl)	Total protein (mg/dl)
Control	3.8 ± 0.15	87.34 ± 0.42	48.40 ± 0.22	246.26 ± 0.67	0.44 ± 0.002	7.37 ± 0.03
CCl₄	6.7 ± 0.13^{c}	261.31 ± 8.73^{c}	226.31 ± 8.65^{c}	326.24 ± 9.54^{a}	0.88 ± 0.03^{a}	4.49 ± 0.03^{c}
Sylimarin	5.9 ± 0.17^{b}	118.53 ± 3.45^{b}	73.46 ± 4.27^{b}	234.37 ± 7.56^{b}	0.40 ± 0.002^{b}	6.76 ± 0.02^{b}
MEPL 200 mg/kg	3.7 ± 0.11^{a}	128.25 ± 3.56^{b}	65.25 ± 4.63^{b}	214.11 ± 9.37^{b}	0.37 ± 0.003^{b}	6.083 ± 0.03^{b}
400 mg/kg	5.4 ± 0.18^{b}	122.47 ± 3.43^{b}	62.47 ± 5.46^{b}	217.42 ± 9.72^{b}	0.33 ± 0.003^{b}	6.17 ± 0.02^{b}

Number of animals (n=6) : Values are mean ± SEM: [a] $p<0.01$, [c]$p<0.001$When compared to normal group, [b]$p<0.01$ When compared to CCl₄ groups.

Table. 4: Effect MEPL on the biochemical parameters of CCl₄ intoxicated rats.

Treatment	LPO nmol of MDA/mg of Protein	GSH µg/mg of protein	SOD U/mg protein	CAT U/ mg protein
Control	5.2 ± 0.4	7.49 ± 0.39	146.22 ± 6.21	4.21 ± 0.26
CCl₄	15.7 ± 1.2^{c}	2.59 ± 0.23^{c}	67.29 ± 5.66^{c}	1.89 ± 0.15^{c}
Std Sylimarin	6.3 ± 0.7^{b}	5.64 ± 0.26^{b}	118.34 ± 6.17^{b}	2.24 ± 0.27^{b}
MEPL 200 mg/kg	7.5 ± 0.5^{b}	3.85 ± 0.27^{a}	69.44 ± 5.86^{b}	3.59 ± 0.31^{b}
MEPL 400 mg/kg	7.3 ± 0.7^{b}	4.56 ± 0.19^{b}	78.23 ± 4.21^{b}	3.89 ± 0.34^{b}

Number of animals (n=6): Values are mean ± SEM: [a] $P<0.01$, [c]$P<0.001$When compared to normal group, [b]$P<0.01$ When compared to CCl₄ groups.

Table. 5: Invitro Antioxidant activity of MEPL.

Sample (µg/mL⁻¹)	% Inhibition (Mean± SEM)		Absorbance (Mean± SEM)
	DPPH	NO	Reducing Power
MEPL 800	76.35 ± 1.47	59.54 ± 0.33	0.77 ± 0.03
MEPL 400	61.59 ± 1.63	51.63 ± 0.49	0.77 ± 0.05
MEPL 200	57.59 ± 1.63	49.63 ± 0.49	0.72 ± 0.05
MEPL 100	46.48 ± 1.22	31.65 ± 0.31	0.66 ± 0.08
MEPL 50	24.67 ± 1.31	19.52 ± 0.68	0.36 ± 0.06
MEPL 25	19.51 ± 1.49	09.37 ± 0.19	0.14 ± 0.005
Ascorbic acid 200	82.39 ± 1.61	56.23 ± 0.63	1.25 ± 0.37
Ascorbic acid 100	68.37 ± 2.34	39.36 ± 0.22	0.79 ± 0.06
Ascorbic acid 50	48.57 ± 2.48	29.11 ± 0.71	0.65 ± 0.09
Ascorbic acid 25	35.34 ± 2.56	19.72 ± 0.83	0.59 ± 0.02

Values represent the mean ± SEM : Number of readings in each group = 3
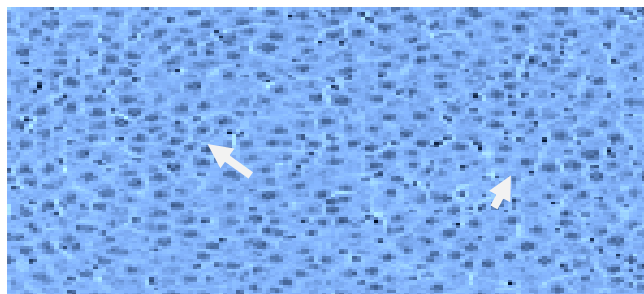
Fig. 1: Shows the liver section of normal control rats with normal histology and normal architecture. (H & E, 100 xs).
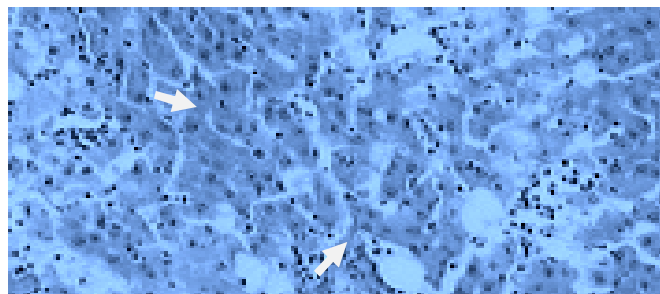
Fig. 2: Shows the liver treated with CCl₄showing necrosis and ballooning like structure in the hepatocytes and fatty vacuole (H & E, 100 x).
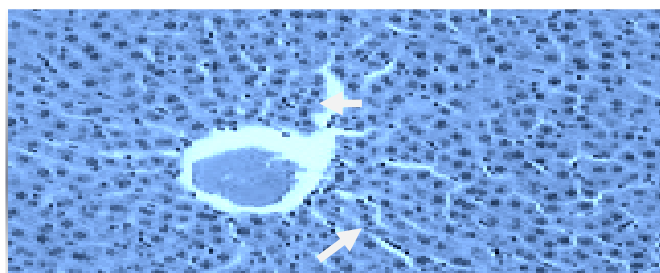
Fig. 3: Section of liver tissue treated with Sylimarin showing normal histology with little ballooning, degeneration of hepatocytes. (H & E,100x).
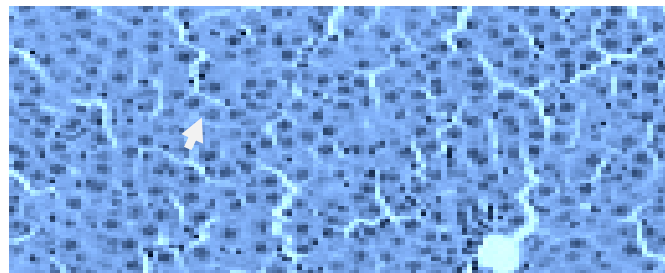
Fig. 4: Liver tissue treated with MEPL 200 showing a normal histology lobular pattern with mild changes (H & E,100x).
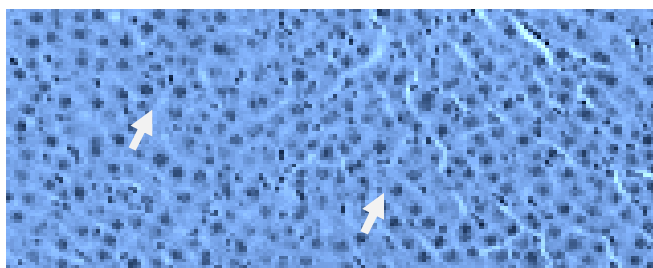
Fig. 5. Liver section treated with MEPL 400 rat showing a normal histology lobular pattern with when compared to normal and Sylimarin received groups. (H & E,100x).

Toxicity test

Experiment was carried out on normal healthy rats. The behaviour of the treated rats appeared normal. No toxic effect was reported at doses up to 4-5 times of effective dose of the methanol extract and there was no death in any of these groups.

Histopathology

In histopathological studies, the normal histological architecture of liver was lost in rats exposed to CCl_4 with the appearance of vacuolated hepatocytes and degenerated nuclei (Figure 2) were observed, while groups that received MEPL 200 mg/kg and 400 mg/kg (Figure 4,5) showed dose dependent protection of liver tissue, which supports the findings of biochemical studies. The ability of a hepatoprotective drug to reduce the injury or to preserve the normal hepatic physiological mechanisms against hepatotoxin, is an index of its protective effects. This histopathological findings further supports the hepatoprotective activity of methanolic extract of of *Polyalthiya longifolia* fruits against CCl_4 induced hepatic injury.

CONCLUSION

Administration of MEPL inhibited the degree of hepatic necrosis and concomitantly decreased the leakage of intracellular enzymes by stabilizing hepatic cellular membranes. Inhibition of lipid peroxidation to a significant degree is also a predominant mechanism of hepatoprotection as suggested by the significant decrease in MDA levels. Increase in the SOD level was also suggestive of repairment of antioxidant defense system, which plays an important role in hepatoprotection. Based upon the results of the present study, it can be concluded that the MEPL of *polyalthiya longifolia* fruits has proven itself as a significant hepatoprotective as well as a considerable antioxidant against CCl_4 intoxication which was further supported by the histopathological results. The *in-vitro* and histopathological studies clearly indicates and supports the hepatoprotective and antioxidant potential of MEPL of *polyalthiya longifolia* fruits against CCl_4 intoxication through its free radical scavenging and antioxidant properties.

CONFLICT OF INTEREST STATEMENT

We declare that there are no conflicts of interest.

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