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Assessment of Anti-Oxidant and Hepatoprotective Potential of Euphorbia Milii against Carbon Tetrachloride Induced Hepatic Damage in Experimental Rats

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A B S T R A C T

The plant *Euphorbia milii* (*E. milii*) is used as a folk medicine for the treatment of hepatitis, warts and cancer. However, the anti-oxidant and hepatoprotective activities of *E.milii* remain largely unknown. The present study is aimed to evaluate the anti-oxidant and hepatoprotective potential of *E.milii* by using various *in vitro* and *in vivo* models. Initially, two crude extracts, hydro-alcoholic and methanolic, were prepared using sonication method. The anti-oxidant assays were performed to find out the most potent crude extract, which was fractionated into five sub-fractions including *n*-hexane, chloroform, ethyl acetate, *n*-butanol and water. To investigate the most potent fraction, the anti-oxidant assays were employed. Among these fractions, ethyl acetate fraction (EMEtOAc) showed the most potent anti-oxidant effects. To prove the *in vivo* relevance a CCl_4-induced hepatotoxicity model was employed. EMEtOAc fraction significantly inhibited the increased-level of enzymes (AST, ALT and ALP) and bilirubin in serum, and reversed the CCl_4-induced lipid peroxidation and nitric oxide levels in liver tissues. Further, protective effect of EMEtOAc fraction was demonstrated by preventing the CCl_4-induced histopathological changes in liver tissues. Our study suggests that EMEtOAc fraction has the potent anti-oxidant and hepatoprotective effects, and may be a promising treatment against a variety of hepatic disorders.

Keywords: *Euphorbia milii*, Anti-oxidant, Hepatoprotective.

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INTRODUCTION

The liver is a vital organ involved in various physiological functions such as secretion of bile, storage of vitamins, and metabolism of carbohydrates, fats, and proteins (UROKO et al., 2021). The liver is also responsible for detoxifying xenobiotics or drugs, however exposure to these exogenous chemicals may produce cellular injuries by inducing oxidative stress and lipid peroxidation which generate free radicals, and are associated with various liver disorders including fibrosis, cirrhosis, and cancer (Fry, 2011; Reed, 2009). Despite the tremendous advancement in the modern era of hepatology, the availability of limited and less efficacious medicines rendering the need to find out new therapeutic options for the management of hepatic disorders (Khan et al., 2017).

Free radicals are highly reactive and unstable atoms or molecules usually have unpaired electrons (Finkel and Holbrook, 2000). Oxygen and Nitrogen based free radicals

mainly including hydroxyl radical ($^{\cdot}OH$), hydrogen peroxide (H_2O_2), and nitric oxide ($NO^{\cdot}$), are usually produced in smaller amounts during many metabolic functions of the body. Since reactive oxygen species (ROS) under physiological levels are not a threat to the body, excess production of these free radicals might be a cause for the depletion of intracellular antioxidant enzymes which lead to several diseases. The xenobiotics including drugs, environmental toxins, and other chemicals can harm the hepatocytes by releasing free radicals leading to the development of acute and chronic hepatic disorders (Sun et al., 2001).

Carbon tetrachloride (CCl_4), is a well-reported hepatotoxic chemical, mainly used in scientific research to design an experimental model of acute hepatotoxicity in animals to evaluate the hepatoprotective activity of newly developed compounds and plant extracts (Liu et al., 2010). CCl_4-induced hepatotoxicity triggers similar liver injuries in the rodents as induced by the different hepatotoxins in humans(Muriel, 2007). In the liver, CCl_4 is metabolized by cytochrome P450 into highly reactive species including trichloromethyl peroxy ($CCl_3OO^{\cdot}$) and trichloromethyl ($CCl_3^{\cdot}$) radicals. Both these free radicles are involved to initiate lipid peroxidation and deterioration of cellular proteins, which may lead to subsequent damage to the hepatocyte membranes and the release of intracellular enzymes such as aspartate aminotransferase (AST), alkaline phosphatase (ALP), and alanine aminotransferase (ALT) into the serum. In response to these processes, calcium pumps are inactivated with calcium influx which may cause subsequent death of hepatocytes. Furthermore, the release of various inflammatory mediators by macrophages is also responsible to potentiate CCl_4-induced hepatic damage (Basu, 2003).

Naturally occurring medicinal plants are highly enriched with flavonoids and polyphenolic compounds that have the potential to counteract the deleterious effects of ROS-mediated tissue injuries and provide protection by scavenging the free radicals and reforming the level of endogenous antioxidant enzymes (Akram et al., 2016b; Terao, 2009). The plant *Euphorbia milii (E. milii)* genus Euphorbia, belongs to the family Euphorbiaceae, a medicinal plant (Yadav et al., 2006), native to Madagascar and Philippine, widely distributed in China, India, and Pakistan (Islam et al., 2015). Ethnopharmacological evidence indicates that *E. milii* used as a folk medicine for the treatment of warts, cancer and hepatitis (Leet et al., 1982). Moreover, it has been reported that *E. milii* possesses anti-inflammatory, analgesic, antipyretic, anxiolytic and sedative activities (Rauf et al., 2014). Despite the emerging evidence for ethnopharmacological uses of this plant, the detailed anti-oxidant activity, and hepatoprotective potential have not been studied yet.

In this study, we investigated the anti-oxidant potential of *E. milii* total extracts and their sub-fractions. Among the five sub-fractions, the ethyl acetate (EMEtOAc) fraction showed the most potent anti-oxidant activity. Further, EMEtOAc fraction showed excellent hepatoprotective potential in CCl_4-induced toxicity model in rats. It suggests that EMEtOAc fraction can provide a very important clue for the development of new therapeutic candidates for hepatic disorders.

MATERIALS AND METHODS

2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), trichloroacetic acid (TCA), carboxy methyl cellulose (CMC), and silymarin were purchased from Sigma-Aldrich (Steinheim, Germany). Sulphanilamide and N-(1-naphthyl)ethylenediamine (NED) were obtained from Dae-Jung Chemicals (Seoul, Korea). Sodium nitroprusside (SNP), L-ascorbic acid, gallic acid, and thiobarbituric acid (TBA) were procured from Merck (Darmstadt, Germany). Carbon tetrachloride (CCl_4) was purchased from BDH (Poole, England).

Plant material and extraction

The fresh leaves of *E. milii* were collected from a botanical nursery at Hyderabad, Pakistan in April 2017. The plant was authenticated, and a voucher specimen was deposited in the herbarium of the Institute of plant sciences, University of Sindh, Jamshoro (Reference No. 20104). Initially the coarse powder of dried leaves was extracted with methanol and methanol: water (1:1; v/v) by sonication for 15 min at room temperature. The extract solutions were concentrated by using a rotary evaporator (Buchi, Switzerland) under reduced pressure to yield crude total extracts including methanolic (EMME) and hydro-alcoholic (EMHAE). For fractionation, the total extract was suspended in distilled water and successively partitioned with *n*-hexane, chloroform, ethyl acetate, and *n*-butanol. Each fraction was vaporized to dryness under reduced pressure and stored at 4 °C until further study.

ABTS Radical Cation Decolorization assay

The ABTS assay was based on the method as described previously (Syed et al., 2016). The $ABTS^+$ working solution was prepared by reaction of ABTS (7 mM) with potassium persulfate (2.45 mM) in dark at room temperature for 14-16 hours. Before evaluating the anti-oxidant activities of the

plant extracts, $ABTS^+$ solution was diluted to an absorbance of 0.700 ± 0.05 at 735 nm. Then 2.7 ml of diluted $ABTS^+$ solution was incubated with 0.3 ml of different concentrations of plant extracts or positive control ascorbic acid for 15 min at room temperature before measuring the absorbance at 735 nm. The antioxidant capacity was determined using the following formula, and IC_{50} values were calculated by linear regression analysis.

$$ABTS\ Radical\ (\%) = \left[\frac{As}{Ao}\right] \text{x } 100$$

Where A_S is the absorbance of the test sample and A_0 is the absorbance of control.

DPPH radical scavenging assay

DPPH free radical scavenging activity was assessed using a previously described method (Talaz et al., 2009). Briefly, 1 ml of 0.3 mM DPPH reagent was mixed with 2 ml solution of the plant extracts or ascorbic acid at varying concentrations. The mixture was incubated in dark at room temperature for 30 min, and the absorbance was measured at 518 nm. The scavenging ability was determined by using the following equation, and the IC_{50} values were calculated by linear regression analysis.

$$DPPH\ Radical\ (\%) = \left[\frac{As}{Ao}\right] \text{x } 100$$

Where A_S is the absorbance of the test sample and A_0 is the absorbance of control.

Thiobarbituric acid reactive species (TBARS) assay

To evaluate the inhibitory effect of the extracts on the formation of malondialdehyde (MDA), TBARS assay was performed according to the previously described method (Awah et al., 2010). The egg yolk (500 µl; 10% v/v in PBS) was mixed with 100 µl of various concentrations of the extracts, and volume was made up to 1.0 ml with distilled water. Then, 50 µl of $FeS0_4$ (0.075 M) and 20 µl L- ascorbic acid (0.1 M) were added and mixed. The mixture was incubated for 1 h at 37 ºC to induce lipid peroxidation. Thereafter, 1.5 ml of TBA reagent (3 g TBA, 120 g TCA, and 10.4 ml 70% $HClO_4$ in 800 distilled water) and 0.2 ml of EDTA (0.1 M) were added in each sample and heated for 30 min at 95 ^oC. After cooling, the mixture was centrifuged for 10 min at 3,000 rpm and the absorbance of the supernatant was measured at 532 nm. The level of MDA was calculated by using the following formula, and IC_{50} values were determined using linear regression analysis.

$$MDA\ level\ (\%) = \left[\frac{As}{Ao}\right] \text{x } 100$$

Where A_S is the absorbance of the test sample and A_0 is the absorbance of control.

Nitric Oxide (NO) radical scavenging assay

NO radical scavenging ability of the extracts was determined according to the method previously described (Awah et al., 2010). Briefly, 0.5 ml of 10 mM SNP in PBS (pH 7.4) was added to 1 ml of the extracts or gallic acid at different concentrations, and the resulting mixtures were incubated at 37°C for 60 minutes. Then an equal volume of the freshly prepared Griess reagent was added to the mixture and incubated for 15 min before measuring the absorbance at 546 nm. The level of NO free radical was calculated using the following equation, and IC_{50} values were determined by linear regression analysis.

$$NO\ radical\ (\%) = \left[\frac{As}{Ao}\right] \text{x } 100$$

Where, A_S is the absorbance of the test sample and A_0 is the absorbance of the control.

Qualitative phytochemical screening

Qualitative analysis of the extracts was carried out for the active constituents including flavonoids, alkaloids, tannins, carbohydrates, saponins, glycosides and terpenoids using standard procedures (Sofowora, 1996; Trease and Evans, 1989).

Quantitative phytochemical screening

Total phenolic content

Total phenolic content of the crude extracts and fractions was determined using Folin–Ciocalteu method as previously described (Baba and Malik, 2015). Briefly, 200 µl of the samples (1 mg/ml) were diluted with water up to 3 ml, and mixed with 0.5 ml of Folin–Ciocalteu reagent followed by incubation for 8 min. Then 2 ml of sodium carbonate (20% w/v) was added to the mixture and allowed to incubate for 1 hr in dark, and the absorbance of the resulting mixture was taken at 650 nm. The standard calibration curve of gallic acid was prepared, and total phenolic content was expressed as mg of gallic acid equivalent per gram of the extract.

Total flavonoid content

Total flavonoid content of the crude extracts and fractions was determined using the aluminum chloride colorimetric method as previously described (Baba and Malik, 2015). Briefly, 1 ml of the samples in methanol was diluted with water up to 5 ml, and then 0.3 ml of $AlCl_3$ (10%) and 0.3 ml of $NaNO_2$ (5%) solutions were added. The mixture was incubated for 6 min at room temperature, and then 2 ml of 1 M NaOH was added, and the total volume was made up to 10 ml with distilled water. The absorbance of mixture was taken at 510 nm after 15 min of incubation. The standard calibration curve of quercetin was prepared, and total flavonoid content was expressed as mg of quercetin equivalent per gram of the extract.

Experimental animals

Adult Sprague Dawley (SD) rats, weighing about 180-200 gm were purchased from S.S.J company (Karachi, Pakistan). All the animals were kept under the defined standard laboratory conditions and were fed with standard diet such as pellet chow and water *ad libitum*. The handling and care of animals were carried out according to the National Institute of Health (NIH) Guide for the care and use of laboratory animals (NIH, 2011). The experimental protocols were approved by the ethical committee at the Faculty of Pharmacy, University of Sindh Jamshoro Pakistan (Approval No. D.Ph/2019-02).

Acute toxicity study

To determine the toxic effects of EMEtOAc fraction, the acute toxicity study was performed according to the method described by Organization for Economic Co-operation and Development (OECD) guidelines 423. Adult non-pregnant SD female rats were divided into five groups (*n*=5) and kept on fasting overnight prior to experiment but water *ad libitum*. Group I (control) was given 0.5% CMC through per oral (p.o). Groups II-V were administered a single dose of EMEtOAc fraction at different concentrations (5, 50, 300, and 2000 mg/kg) suspended in 0.5% CMC through p.o. To observe the acute signs of toxicity, the animals were examined continuously for 4 h, and thereafter every 24 hourly for 14 days in a blinded fashion. After 14 days, the blood samples were collected for analysis of hematological toxicities. Then animals were sacrificed, and the vital organs including liver, heart, and kidneys were collected, weighed, and subjected for histopathological examination.

CCl_4-induced hepatotoxicity in animals

To evaluate the hepatoprotective potential of EMEtOAc fraction, CCl_4-induced hepatotoxicity model was used as described previously (Tseng et al., 2014). SD rats were divided randomly into 5 groups (*n*=5). Group I: vehicle, group II: vehicle + CCl_4, group III: 100 mg/kg of EMEtOAc fraction + CCl_4, group IV: 500 mg/kg of EMEtOAc fraction + CCl_4, and group V: 100 mg/kg of silymarin. The rats were pretreated with vehicle or two doses of EMEtOAc fraction or silymarin by intra gastric route once daily for 4 days. On Day 4, group I received corn oil (2.5 ml/kg), and groups II to V received 2.5 ml/kg of 20% CCl_4 in corn oil by intra peritoneal (i.p) route after 1 h of treatment. On day 5, the blood samples were collected, and serum levels of alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and bilirubin were determined using standard kits (Human, Germany) according to manufacturer's instructions. For histopathological analysis, after fixation of liver tissues in formalin they were embedded in paraffin and cross sections (6 µm) were stained with hematoxylin and eosin (H&E). The liver tissues were observed for histopathological changes as described previously (Knodell et al., 1981) in a blinded manner. To determine the tissue lipid peroxidation, a previously described method was followed (El-Sayed et al., 2015). The liver tissue samples were homogenized in 0.1 M phosphate buffer (pH 7.4) and centrifuged for 15 min at 4 ^oC. The supernatant layer was heated with TBA reagent at 95^oC for 30 min, and the absorbance of resulting pink product was measured at 532 nm.

For determination of NO level in liver tissue, total nitrite (NO^{2-}) level was measured according to a previously described protocol (Sun et al., 2001). The liver tissue was homogenized in normal saline and centrifuged for 30 min at 1000 g, and the supernatants were mixed with 30% $ZnSO_4$ solution to precipitate the proteins. The deproteinized samples were mixed by vortexing with copper coated cadmium granules in glycine buffer (pH 9.7) to convert nitrates (NO^{3-}) to NO^{2-}. The total NO^{2-} level was measured using Griess reaction as described above.

Statistical analysis

The means and standard deviations (S.D) were calculated for all the groups. To determine the statistically significant differences from the control group, the results were subjected to either student paired t-test or one-way ANOVA (ANalysis Of VAriance) with post-hoc Tukey's test. Statistical analysis was performed using SPSS software, and a p value < 0.05 was considered significant.

RESULTS

Anti-oxidant activities of *E.milii* plant extracts in various *in vitro* assays

Initially, we prepared two crude total extracts including EMME and EMHAE. To identify the most potent total extract, we employed various *in vitro* methods including ABTS, DPPH, NO, and lipid per-oxidation assays. Among these total extracts, EMHAE most potently produced anti-oxidant activities in all the assays (Figure 1; Table 1). Therefore, EMHAE was successively fractionated into five fractions including n-Hexane, $CHCl_3$, n-BuOH, EtOAc and water (Figure 2). These fractions were tested for their anti-oxidant activities using various in vitro assays and found that EtOAc fraction was the most potent among all the fractions in reducing the oxidative stress (Figure 3; Table 1). Therefore, we selected EMEtOAc fraction for further studies.

Table 1: Anti-oxidant effects of crude total extracts and EMHAE fractions in various *in vitro* assays.

Sample extracts	IC_{50} (µg/ml)			
	ABTS	DPPH	NO	TBARS
EMME	73.3±0.6	105.8±9.2	572.3±85.9	715±67.1
EMHAE	52.2±0.4	30.0±0.8	141.6±4.1	312.6±4.3
n-Hex	171.3±27.5	219.6±17.2	86.3±3.1	105.8±2.8
$CHCl_3$	154.9±4.5	147.1±3.3	91.5±2.2	84.5±2.1
EtOAc	14.9±2.0	20.9±2.7	43.7±0.8	45.3±0.9
n-BuOH	37.4±1.3	42.6±5.5	71.5±2.2	69.2±1.1
H_2O	59.9±2.4	81.8±2.1	95.9±6.2	152.5±12.9

The data was represented as mean ± S.D (n=3). EMME, *E. milii* methanolic extract; EMHAE, *E. milii* hydro-alcoholic extract; n-Hex, n-hexane fraction; $CHCl_3$, chloroform fraction; EtOAc, ethyl acetate fraction; n-BuOH, n-butanol fraction; H_2O, water fraction.

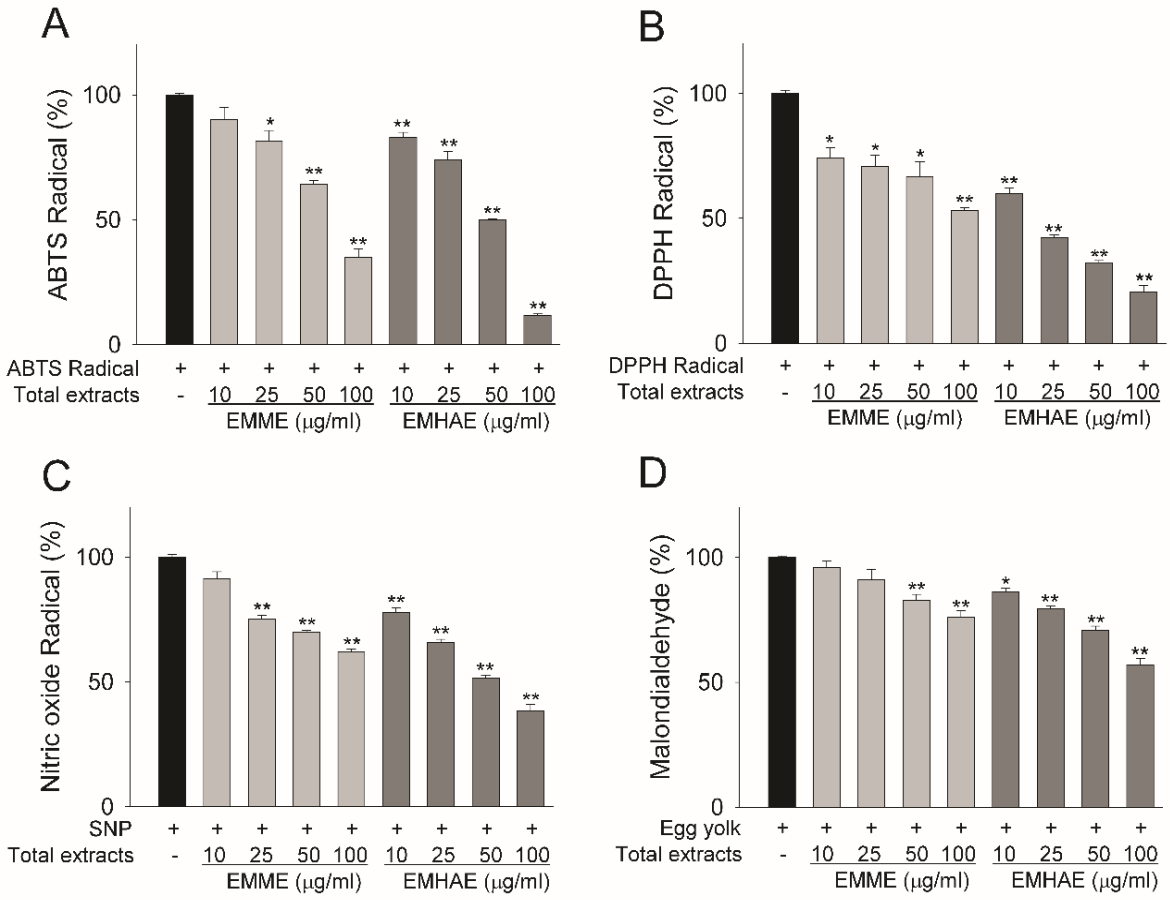

Figure 1: Anti-oxidant effect of crude total extracts of *E. milii* using various *in vitro* assays.
(A) ABTS free radical scavenging activity, (B) DPPH free radical scavenging activity, (C) Nitric oxide radical scavenging activity, and (D) lipid peroxidation assay. Data are presented as mean ± S.D (n=3). The significance of data was determined using paired t-test. $^{*}p < 0.05$; $^{**}p < 0.01$ vs. control group. ABTS, 2,2′-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid); DPPH, 2,2-diphenyl-1-picrylhydrazyl; EMME, *Euphorbia milii* methanolic extract; EMHAE, *Euphorbia milii* hydro-alcoholic extract; SNP, sodium nitroprusside.

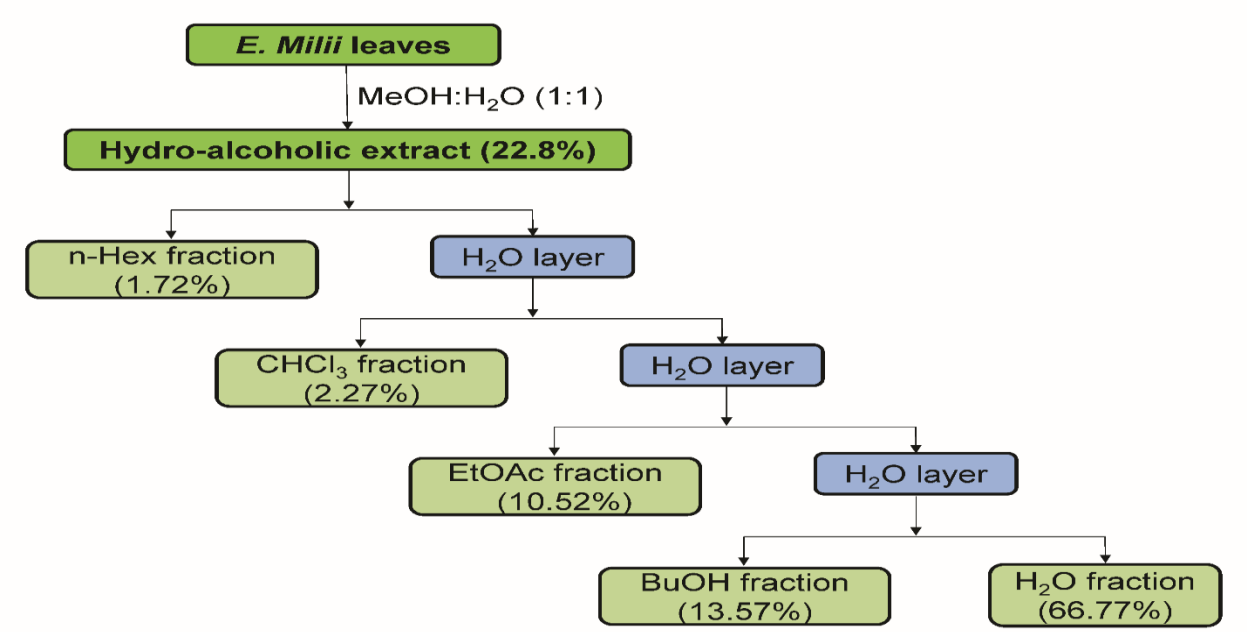

Figure 2: Representative scheme for fractionation of hydro-alcoholic extract of *Euphorbia milii*.
The total extract of *Euphorbia milii* was successively partitioned using various solvents, and the percentage yields were also calculated.

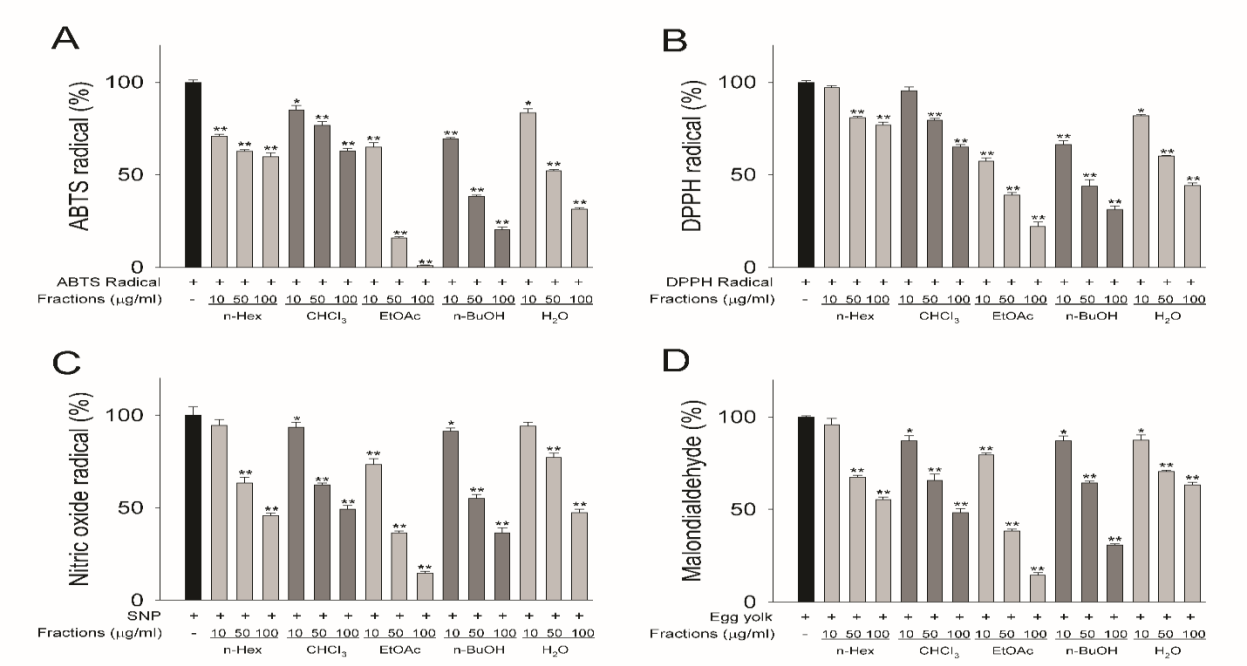

Figure 3: Anti-oxidant effect of EMHAE fractions using various *in vitro* assays.
(A) ABTS free radical scavenging activity, (B) DPPH free radical scavenging activity, (C) Nitric oxide radical scavenging activity, and (D) lipid peroxidation assay. Data are presented as mean ± S.D (n=3). The significance of data was determined using paired t-test. $^{*}p < 0.05$; $^{**}p < 0.01$ vs. control group. ABTS, 2,2′-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid); DPPH, 2,2-diphenyl-1-picrylhydrazyl; SNP, sodium nitroprusside; EMHAE, *Euphorbia milii* hydro-alcoholic extract; n-Hex, n-hexane fraction; $CHCl_3$, chloroform fraction; EtOAc, ethyl acetate fraction; n-BuOH, n-butanol fraction; H_2O, water fraction.

Qualitative and quantitative phytochemical screening

Phytochemical screening of *E. milii* was carried out by using crude total extracts and fractions. The qualitative screening was reported to contain flavonoids, phenols, tannins, terpenoids, saponins, carbohydrates, glycosides, and traces of alkaloids. We also calculated total phenolic and flavonoid contents of crude total extracts and fractions. We found that EMHAE contained a high amount of total phenolic and flavonoid contents when compared with EMME (Table 2). In addition, among all the fractions of EMHAE, the EMEtOAc fraction contained the highest quantities of phenolic and flavonoid contents, correlating its potent anti-oxidant activity.

Table 2: Total phenolic and flavonoid contents of crude total extracts and EMHAE fractions.

Sample extracts	Total phenolic (mg GAE/g extract)	Total flavonoid (mg QE/g extract)
EMME	133.8±11.7	128.5±10.0
EMHAE	233.4±6.5	217.1±14.8
n-Hex	81.1±10.8	88.8±7.6
$CHCl_3$	113.8±7.9	117.3±8.1
EtOAc	348.9±8.1	276.9±4.1
n-BuOH	125.2±4.3	104.6±4.6
H_2O	177.5±13.7	157.1±9.7

The data was represented as mean ± S.D (n=3). GAE, gallic acid equivalent; QE, quercetin equivalent; EMME, *E. milii* methanolic extract; EMHAE, *E. milii* hydro-alcoholic extract; n-Hex, n-hexane fraction; $CHCl_3$, chloroform fraction; EtOAc, ethyl acetate fraction; n-BuOH, n-butanol fraction; H_2O, water fraction

Acute toxicity study

The oral administration of EMEtOAc fraction did not reveal any change in gross behavior or physical appearance such as restlessness, sedation, convulsions, coma, lethargy, salivation, feces consistency, skin/fur effects, and eye color during 14 days of the observation period. No mortality was found at all doses up to 2000 mg/kg of body weight. The body weight and rectal temperature of each animal were taken on Day 1, 7 and 14. The body weight of the animals in each group was increased gradually. We did not find any significant difference in the % change in body weights of the treated animals and control group. The rectal temperature of the treated groups was also not significantly different from the control group on each day observed (Table 3).

Similarly, we did not find any significant difference in the absolute and relative weight of the visceral organs (Heart, liver, and kidneys) of the treated animals when compared to the control group (Table 4). The histological analysis of the visceral organs was also performed after H&E staining. The tissue examination did not reveal any pathological change in any of the organs (Figure 4).

We also evaluated the toxic effects of EMEtOAc fraction on hematological parameters. We did not find any toxic effect of the EMEtOAc fraction on RBCs, WBCs, Hb and random glucose levels (Table 5). All of these levels in treated animals were not significantly different from the control group.

The acute toxicity study did not reveal any pathological change in the animals; therefore, we selected 100 mg/kg and 500 mg/kg as a low and high dose respectively for hepatoprotective studies.

Table 3: Effect of *E. milii* ethyl acetate fraction (EMEtOAc) on change in body weight and rectal temperature of rats in acute toxicity model.

Groups	Change in body weight (%)		Rectal temperature (°F)		
	Day 7	Day 14	Day 1	Day 7	Day 14
Control	5.8±1.7	13.4±2.0	98.5±0.4	97.8±0.5	98.2±0.5
5 mg/kg	5.6±1.5	13.0±3.6	98.0±0.4	98.1±0.5	98.4±0.2
50 mg/kg	5.5±1.6	13.2±2.7	98.0±0.2	98.1±0.3	97.9±0.7
300 mg/kg	5.2±1.8	13.4±2.1	98.7±0.4	98.4±0.6	98.7±0.1
2000 mg/kg	5.8±1.5	13.1±1.0	98.6±0.6	98.0±0.6	98.9±0.1

The data was represented as mean ± S.D (n=5). The significance of data was determined using ANOVA followed by Tukey's test.

Table 4: Effect of *E. milii* ethyl acetate fraction (EMEtOAc) on absolute and relative (organ weight/body weight ×100) weights of visceral organs of rats in acute toxicity model.

Groups	Heart		Liver		Kidneys	
	Absolute (g)	Relative (%)	Absolute (g)	Relative (%)	Absolute (g)	Relative (%)
Control	0.8±0.0	0.3±0.0	9.9±0.09	4.1±0.0	0.6±0.0	0.2±0.0
5 mg/kg	0.8±0.0	0.3±0.0	10.2±0.10	4.3±0.1	0.6±0.0	0.2±0.0
50 mg/kg	0.9±0.0	0.3±0.0	9.9±0.12	4.2±0.1	0.6±0.0	0.2±0.0
300 mg/kg	0.8±0.0	0.3±0.0	9.9±0.06	4.0±0.1	0.7±0.0	0.2±0.0
2000 mg/kg	0.8±0.0	0.3±0.0	10.0±0.11	4.0±0.0	0.6±0.0	0.2±0.0

The data was represented as mean ± S.D (n=5). The significance of the data was determined using ANOVA followed by Tukey's test.

Table 5: Effect of *E. milii* ethyl acetate fraction (EMEtOAc) on hematological parameters of rats in acute toxicity model.

Groups	RBCs ($\times10^6$/µL)	WBCs ($\times10^3$/µL)	Hb (g/dL)	Glucose (mg/dL)
Control	9.1±0.6	7.0±0.5	11.1±0.7	99.5±0.9
5 mg/kg	9.2±0.7	6.7±0.5	11.7±0.6	98.7±0.9
50 mg/kg	8.5±1.2	6.6±0.5	11.8±0.9	99.2±1.9
300 mg/kg	9.2±0.9	6.7±0.5	11.0±0.6	100.5±1.5
2000 mg/kg	9.24±0.89	7.33±0.17	11.5±0.6	98.9±1.0

The data was represented as mean ± S.D (n=5). The significance of the data was determined using ANOVA followed by Tukey's test.

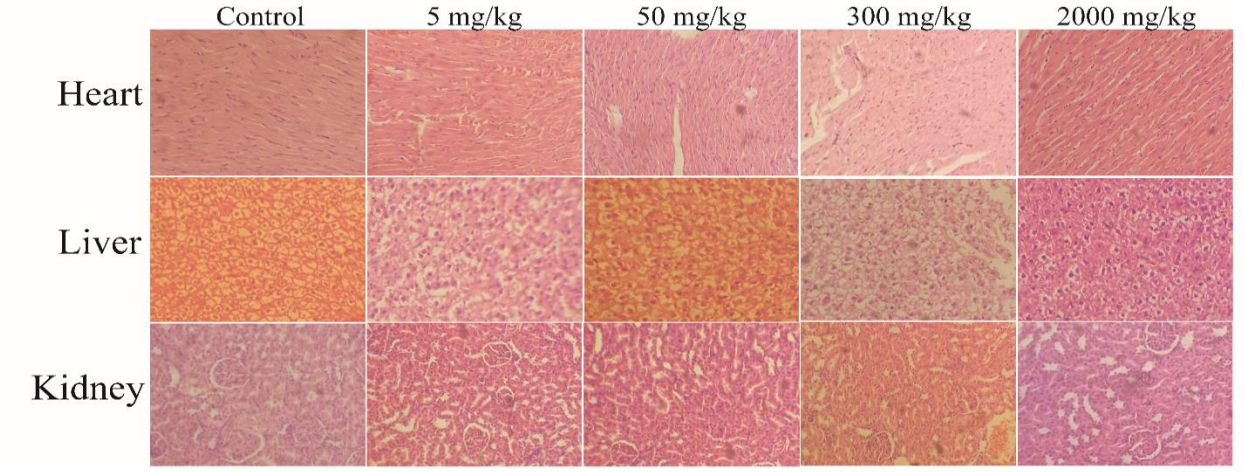

Figure 4: Photo microscopic assessment of heart, liver and kidney sections stained with hematoxylin and eosin (H&E) in the acute toxicity study model.

After 14 days of administration of various doses of EtOAc fraction, the tissue sections of heart, liver and kidney were obtained and stained with hematoxylin and eosin (H&E). Histopathological analysis of all tissues in the treated groups did not reveal any pathological change when compared with a control group.

Hepatoprotective studies

Effect of EMEtOAc fraction on liver enzymes and bilirubin level in CCl_4-induced hepatotoxicity model

To assess the hepatoprotective effect of EMEtOAc fraction, we measured the serum levels of liver-related biochemical parameters including AST, ALT, ALP, and bilirubin (total and direct) 24 hours following CCl_4 intoxication (Figure 5). The results showed that all the biochemical parameters were found to increase significantly in the rats intoxicated with CCl_4 when compared with the control group. However, the administration of EMEtOAc fraction reduced their levels in a dose-dependent manner, which is comparable to the group treated with standard hepatoprotective drug

silymarin.

Effect of EMEtOAc fraction on liver weight in CCl_4-induced hepatotoxicity model

A significant increase in the absolute and relative weight of the liver was observed in CCl_4 treated group when compared to the control group (Table 6). However, the treatment with EMEtOAc fraction and silymarin prevented the utmost liver weight changes.

Effect of EMEtOAc fraction on liver MDA and nitrite levels in CCl_4-induced hepatotoxicity model

The MDA and nitrite levels in liver tissues were measured as indicators of lipid peroxidation and NO level respectively. The data showed that production of MDA and nitrite was increased significantly in CCl_4-intoxicated rats, which were subsequently reduced by EMEtOAc fraction in a dose-dependent manner (Figure 6). In addition, the standard drug silymarin also reversed their levels.

Histopathological analysis

To further assess the protective effect of EMEtOAc fraction against CCl_4-induced toxicity, histological analysis of liver tissue was performed. These observations were in agreement with the results obtained from other biochemical assays. The liver section of control rats showed normal hepatocytes with preserved cytoplasm and prominent nucleus, normal architecture of lobules, and normal central vein (Figure 7). The rats intoxicated with CCl_4 demonstrated significant liver injuries characterized by fatty change, hepatocellular necrosis, loss of cellular boundaries, hydropic change, ballooning degeneration, leucocyte infiltration, and vascular congestion. A significant reduction in the severity of liver tissue injury was found in the animals treated with EMEtOAc fraction or silymarin. Lipidosis, infiltration of inflammatory cells, lobular necrosis, and congestive changes of the central vein were ameliorated significantly in the treated animals when compared with CCl_4-intoxicated animals. Interestingly, the rats treated with a high dose of EMEtOAc fraction (500 mg/kg) or silymarin showed regeneration of hepatocytes and restoration of normal liver architecture. These results demonstrate that EMEtOAc fraction possesses potent hepatoprotective action.

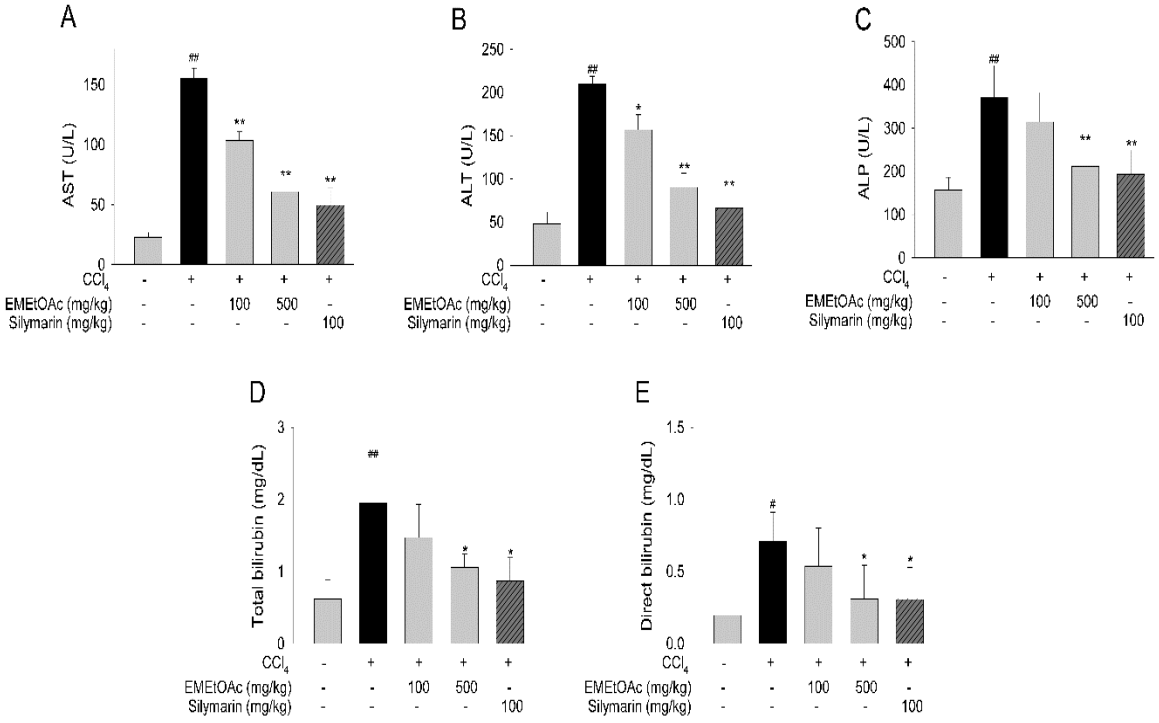

Figure 5: Protective effect of EMEtOAc fraction against CCl_4-induced liver enzymes and bilirubin level in rat model.
After 24 h of CCl_4 administration by intraperitoneal route, the blood sample was collected and serum levels of (A) aspartate aminotransferase (AST), (B) alkaline phosphatase (ALP), (C) alanine aminotransferase (ALT), (D) total bilirubin, and (E) direct bilirubin were measured. The data was represented as mean ± S.D (n=5). The significance of the data was determined using ANOVA followed by Tukey's test (n=5). $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs. control group; $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. CCl_4 group. EMEtOAc, *Euphorbia milii* ethyl acetate fraction.

Table 6: Effect of *E. milii* ethyl acetate fraction (EMEtOAc) on absolute and relative liver weight in CCl4-induced hepatotoxicity rats model.

Groups	Absolute (g)	Relative (%)
Control	8.60±0.10	4.60±0.12
CCl_4	10.46±0.12$^{\#\#}$	5.49±0.15$^{\#\#}$
100 mg/kg EMEtOAc + CCl_4	9.74±0.28**	5.18±0.20*
500 mg/kg EMEtOAc + CCl_4	9.24 ±0.07**	4.82±0.10**
100 mg/kg Silymarin + CCl_4	9.07±0.07**	4.75±0.20**

The data was represented as mean ± S.D (n=5). The significance of the data was determined using ANOVA followed by Tukey's test. $^{\#\#}p < 0.01$ vs. control group; $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. CCl_4 group.

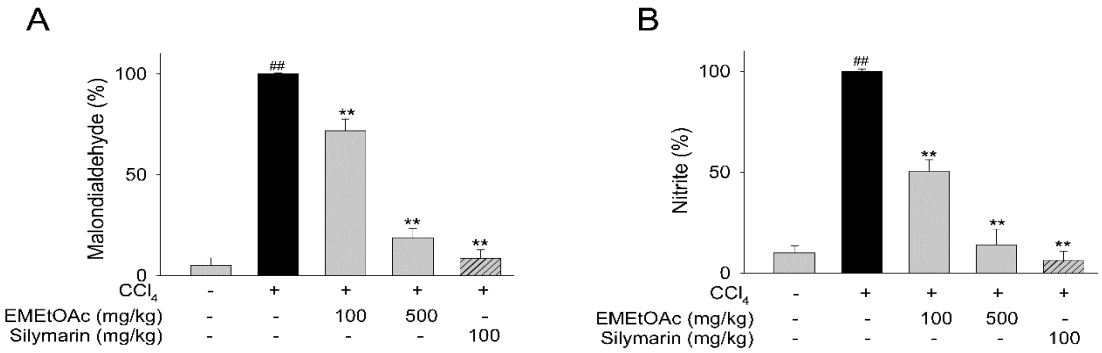

Figure 6: Protective effect of EMEtOAc fraction against CCl_4-induced liver tissue biochemical markers in rat model.
Following 24 h of CCl_4 administration by intraperitoneal route, the rats were sacrificed and livers were collected. The liver tissue was homogenized, and the tissue levels of (A) malondialdehyde, and (B) nitrite in supernatants were measured. The data was represented as mean ± S.D (n=5). The significance of the data was determined using ANOVA followed by Tukey's test (n=5). $^{\#\#}p < 0.01$ vs. control group; $^{**}p < 0.01$ vs. CCl_4 group. EMEtOAc, *Euphorbia milii* ethyl acetate fraction.

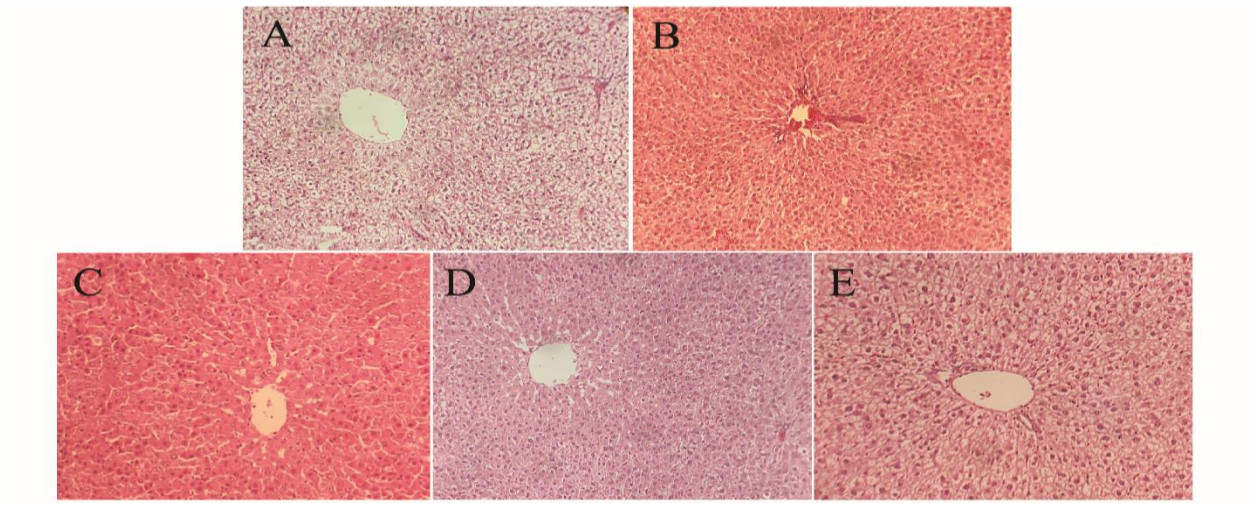

Figure 7: Photo microscopic assessment of liver sections stained with hematoxylin and eosin (H&E) in CCl_4-induced hepatotoxicity model.
Liver section from normal control rats showing normal hepatocytes, central vein and lobules (A). CCl_4-intoxicated rats showing hepatocellular necrosis, inflammatory cell infiltration, and vascular congestion (B). The liver sections from treated groups showing regeneration and restoration of normal liver architecture (C, D, E).

DISUCSSION

In this study, for the first time we demonstrated the detailed antioxidant and hepatoprotective activities of medicinal plant *E. milii* by using various *in vitro* and *in vivo* models. We identified that the hydro-alcoholic extract of *E. milii* produced more potent anti-oxidant activity in various in vitro assays when compared with the methanolic extract. On successive fractionation of the hydro-alcoholic extract of *E. milii* and their further analysis, we found that ethyl acetate fraction of *E. milii* possessed the most potent anti-oxidant activity. Moreover, ethyl acetate fraction was also found to possess potential *in vivo* hepatoprotective activity in CCl_4-induced hepatotoxicity model.

Free radicals are highly unstable molecules that can damage the cells by combining with cellular components to gain stability, and play a crucial role in the development of various hepatic disorders (Paiva et al., 2013). However, the administration of anti-oxidants can stop the free radical chain reaction by donating its electron and convert them into stable molecules, thereby preventing the hepatocytes from injury (Maimaitimin et al., 2018). Natural plants are known as health promoters for their protective effects against free radicals. It has been reported that the plants containing secondary metabolites such as tannins, phenolic acids, and flavonoids possess strong antioxidant activity (Negi et al., 2003). Of note, these natural plants may not be the final drug entities; instead, they can serve as a source of novel drug candidates (Akram et al., 2015). In the present study, we made efforts to find out the potential anti-oxidant and hepatoprotective activities of the plant *E. milii* that may serve as a source of new compounds.

Various in vitro assays are employed to find out the anti-oxidant potential of the test samples. A single experimental procedure cannot conclude the definite anti-oxidant activity of a sample, therefore in our study we employed several free radical scavenging assays. In ABTS decolorization assay, $ABTS^+$ chromophore is produced by the reaction of ABTS with potassium persulfate, which can be reduced by hydrogen donating antioxidant compounds (Saeed et al., 2012). In our study, we found that EMEtOAc fraction most potently inhibited ABTS free radical concentration with an IC_{50} value of 14.9±2.0 µg/ml (Table I). This shows that EMEtOAc fraction contains hydrogen donating anti-oxidant compounds. The electron-donating property of a compound can be identified using DPPH assay. DPPH itself is a free radical having purple color that can accept an electron or hydrogen radical to become a stable molecule giving a yellowish color (Cieśla et al., 2012)(Cieśla *et al.*, 2012). A significant reduction in DPPH free radical concentration by EMEtOAc fraction (IC50: 20.9±2.7 µg/ml) shows that the extract also contains electron-donating anti-oxidant compounds. The excessive amount of nitric oxide in mitochondria of hepatocytes results in the release of reactive nitrogen species (RNS) including nitric oxide ($NO^{\cdot}$) and peroxynitrate ($OONO^{\cdot}$). These RNS have very strong oxidizing property towards various cellular components, and can contribute to hepatocyte cell death and carcinoma (Akram et al., 2016a; Jaeschke et al., 2012). In our study, we demonstrated that EMEtOAc fraction down-regulated the NO free radical concentration in *in vitro* and *in vivo* models (Figure 3 and Table I), suggesting beneficial effects of the extract in preventing the RNS induced liver injuries. In addition, lipid peroxidation is also involved in inducing liver injuries which are initiated by free radicals (Morita et al., 2012). In the present study we found that EMEtOAc fraction potently inhibited lipid peroxidation in *in vitro* and *in vivo* models (Figure 3 and Table I). This suggests that EMEtOAc fraction can produce hepatoprotective effects via inhibiting lipid peroxidation.

CCl_4-induced liver injury is the most extensively studied method for screening of hepatoprotective agents. Mechanistic studies revealed that CCl_4 is metabolized into highly reactive oxymetabolites that play a crucial role to induce oxidative stress and damages to the hepatocellular membrane resulting in elevated levels of AST, ALT, ALP, and bilirubin in serum (Weber et al., 2003). Administration of EMEtOAc fraction significantly attenuated the CCl_4-induced enzyme levels in serum, and interestingly recovery in case of high dose group and silymarin was comparable to the normal control group. This shows that EMEtOAc fraction has the potential to prevent oxidative stress-induced hepatocellular membrane injury. The hepatoprotective potential of EMEtOAc fraction was further confirmed by histopathological analysis. Our results clearly demonstrated that EMEtOAc fraction offers protection against inflammatory, hydropic and necrotic changes induced by CCl_4 intoxication, and maintained normal lobular architecture. Since EMEtOAc fraction prevented oxidative stress-induced liver injuries in *in vivo* model, therefore it may be possible that the mechanism of hepatoprotection by EMEtOAc fraction is due to its potent anti-oxidant activity.

Flavonoids are natural polyphenol compounds, which are recognized to possess potent hepatoprotective effects. The potential hepatoprotective activity of these compounds is due to their anti-oxidant capabilities, thereby quenching the free radicals to prevent damage to hepatocytes (Dhiman et al., 2016). On quantitative phytochemical screening, we found that EMEtOAc fraction contains the highest amount

of phenolic and flavonoid contents (Table II). It suggests that strong anti-oxidant and hepatoprotective activity of EMEtOAc fraction might be related to the high quantity of phenolic and flavonoid compounds. However, further studies are needed to find out the active constituents responsible for hepatoprotection.

CONCLUSION

The present study revealed that the EMEtOAc fraction has potent anti-oxidant and hepatoprotective effects that were proven by various *in vitro* and *in vivo* methods. This may be due to the presence of high quantities of phenolic and flavonoid compounds in the extract. However, isolation of chemical constituents from EMEtOAc fraction and screening of hepatoprotective activity of individual compounds are needed, which may serve as potential therapeutic drugs.

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AUTHORSHIP CONTRIBUTIONS

Concept – F.S., M.A.; Design – M.A., G.K., M.A.G., S.A.S.; Supervision – M.A., G.K.; Resources – J.J., S.B, F.S., M.A.;Materials – A.Z., O.S., S.B., G.K.; Data Collection and/or Processing – F.S., M.A., F.D., S.A.S, S.B.,.; Analysis and/or Interpretation – M.A.,M.W, O.S., A.Z., F.D.; Literature Search – F.S., M.A., M.W, F.D.; Writing – F.S., M.A., J.J, S.B., R.M, ; Critical Reviews – M.A.G., S.A., G.K., O.S., A.Z., F.D, R.M.

CONFLICT OF INTEREST STATEMENT

The authors have declared no conflict of interest.

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