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Original Research Article

Hepatoprotective activity of *Macrothelypteris torresiana* (Gaudich.) aerial parts against CCl_4-induced hepatotoxicity in rodents and analysis of polyphenolic compounds by HPTLC

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ABSTRACT

Macrothelypteris torresiana is a fern species belonging to the family Thelypteridaceae. The present study was conducted to evaluate hepatoprotective potential of ethanol extract from *M. torresiana* aerial parts (EEMTAP) and detect the polyphenolic compounds present in the extract using high performance thin layer chromatography (HPTLC). Hepatoprotective potential of EEMTAP were tested at doses of 300 and 600 mg/kg, per os (p.o.), on Wistar albino rats. The extract and silymarin treated animal groups showed significant decrease in activities of different biochemical parameters like serum glutamic oxaloacetic transaminase (SGOT), serum glutamate-pyruvate transaminase (SGPT), and alkaline phosphatase (ALP), which were elevated by carbon tetrachloride (CCl_4) intoxication. The levels of total bilirubin and total protein along with the liver weight were also restored to normalcy by EEMTAP and silymarin treatment. After CCl_4 administration, the levels of hepatic antioxidant enzymes such as glutathione (GSH) and catalase (CAT) were decreased whereas the level of hepatic lipid peroxidation (LPO) was elevated. The levels of these hepatic antioxidant enzymes were also brought to normalcy by EEMTAP and silymarin treatment. Histological studies supported the biochemical findings, and treatment with EEMTAP at doses of 300 and 600 mg/kg, p.o. was found to be effective in restoring CCl_4-induced hepatotoxicity in rats. A simple HPTLC analysis was conducted for the detection of polyphenolic compounds in EEMTAP, and the result revealed the presence of caffeic acid as phenolic acid and quercetin as flavonoid. The proposed HPTLC method is simple and concise and provides a good resolution of caffeic acid and quercetin from other constituents present in EEMTAP.

1. Introduction

Macrothelypteris torresiana (Gaudich.), syn. *Lastrea torresiana* Moore (family: Thelypteridaceae), is a species of fern which is native to tropical and subtropical region of the world. It is a robust fern with a short creeping rhizome [1,2]. In traditional medicine *M. torresiana* leaves and roots have a wide range of reputed medicinal applications. The aerial parts are used by the tribes of Pakistan, India and China for treatment of fever, pain and granulation, healing and reducing odour in chronic skin ulcer and inflammation [3]. It is also used in Chinese folk medicine for the treatment of edema for patients suffering from kidney problems [4]. Only a few pharmacological properties have been reported from this plant such as renoprotective potential via ameliorating oxidative stress and proinflammatory activities [4], in vitro and in vivo antitumor activities [5], wound healing properties [3], analgesic, antipyretic and anti-inflammation activities [6]. A few phyto-constituents are reported from this fern species such as 5,7-dihydroxy-2-(1,2-isopropyldioxy-4-oxocyclohex-5-enyl)-chromen-4-one, a novel flavonoid isolated from the root [7], 2-(cis-1,2-dihydroxy-4-oxo-cyclohex-5-enyl)–5,7-dihydroxy-chromone, 2-(trans-1,4-dihydroxy-cyclohexyl)–5,7-dihydroxy-chromone, protoapigenin, apigenin, kaempferol and quercetin [8]. An analytical technique for the simultaneous determination of phytochemical constituents was developed by Xiong et al. [9] using liquid chromatographic method with ultra-violet detector and successfully quatified the presence of protoapigenone, protoapigenin 4'-*O*-β-D-glucoside, apigenin 4'-*O*-β-D-glucoside, and apigenin.

Tissue damage is caused by excessive production of free radicles

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[10]. Liver is known to be the major organ involved in the detoxification of xenobiotics, and is thus the main target of tissue injury produced by these chemicals and their metabolites. Reactive oxygen species produce deleterious effects on membrane lipids of the cellular components, thereby producing peroxidation of lipids which leads to cell death [11]. Liver is also responsible for regulating homeostasis in the body and is involved with almost all the biochemical pathways related to growth, nutrient supply, maintenance of immunity and reproduction [12]. Hence, prevention of hepatotoxic damage is of great concern. Phenolic acids and flavonoids possess diverse biological activities including antioxidant and hepatoprotective properties. Recently it has been considered that polyphenolic compounds are great antioxidants and proved to be more effective than vitamin C, E and carotenoids [13]. Several flavonoids such as catechin, apigenin, quercetin, naringenin, rutin, and venoruton are also reported to ameliorate hepatotoxic effects [14].

High performance thin layer chromatography (HPTLC) is considered more sophisticated, efficient and reliable than thin layer chromatography (TLC) with the latest technical developments for quality assessment and evaluation of botanical materials [15]. Moreover, in HPTLC fingerprinting technique, many samples of divergent nature can be run in a single analysis with simultaneous processing of the sample and standard [16,17].

Literature available from all possible scientific sources reveals that there is only one analytical method developed for the estimation of important phyto-constituents from this fern species, and also there is no scientific proof of the plant's use in liver protection. Therefore, the present study deals with hepatoprotective activity of ethanol extract from *M. torresiana* aerial parts (EEMTAP) on Wistar albino rats and detection of polyphenolic compounds present in the extract by HPTLC fingerprinting analysis.

2. Materials and methods

2.1. Chemicals and reagents

Standards for HPTLC analysis such as gallic acid, caffeic acid and quercetin were purchased from Natural Remedies Pvt. Ltd. (Bangalore, India). Silymarin was obtained from Micro Labs Ltd. (Bangalore, India). High performance liquid chromatography (HPLC) grade methanol was purchased from Merk India Ltd. (Mumbai, India). All other chemicals and reagents were of analytical grades and were purchased from Sisco Research Laboratories Pvt. Ltd. (Mumbai, India) and Merk India Ltd. (Mumbai, India). Diagnostic kits for the estimation of biochemical parameters were purchased commercially (Span Diagnostics Ltd., Surat, India).

2.2. Plant material

The aerial parts of the plant *M. torresiana* were collected from in and around East Godavari dist., Andhra Pradesh, India and authenticated by Prof. K. Madhava Chetty, Department of Botany, Sri Venkateswara University, Tirupati, Andhra Pradesh, India. A voucher specimen has been kept in our research laboratory for further reference.

2.3. Preparation of the extract

The collected plant materials were gently washed in tap water to remove dirt and then they were shade dried in the laboratory under room temperature (24 ± 2 °C) for 3–4 weeks. After complete drying, the dried plant materials were pulverized by using a mechanical grinder followed by sieving (sieve no. 40) to obtain a coarse powder. The powdered plant material (500 g) was extracted with 1.5 L of ethanol (90%, v/v) for 48 h using a Soxhlet extractor. The extract obtained was evaporated under vacuum using rotary evaporator (Evator, Media Instrument Mfg. Co., Mumbai, India) and concentrated to obtain a dark greenish residue (yield 10.68%, m/m) which was kept in the refrigerator at 4 °C.

The percentage yield of EEMTAP was calculated by the following formula

$$\text{Yield\%} = \frac{\text{Weight of dry extract obtained(g)}}{\text{Weight of plant material before extraction(g)}} \times 100$$

2.4. Preliminary phytochemical tests

Preliminary phytochemical studies of EEMTAP were performed for determination of major phytochemical constituents like alkaloids, carbohydrates, proteins, tannins, sterols, triterpenoids, saponins, and flavonoids using standard procedures [16,18].

2.4.1. Test for alkaloids

The dry crude extract was dissolved in 2 M Hydrochloric acid. The mixture was filtered and the filtrate was divided into three equal portions. Mayer's test: The first portion was treated with a few drops of Mayer's reagent; appearance of buff coloured precipitate proves the presence of alkaloids. Dragondorff's test: A few drops of Dragondorff's reagent were added in second portion where appearance of orange brown precipitate confirms the presence of alkaloids. Wagner's test: The third portion was treated with a few drops of Wagner's reagent, formation of reddish brown precipitate proves the presence of alkaloids in the test extract.

2.4.2. Test for carbohydrates

The test extracts were divided into three portions and kept in a test tube. Molisch's test: To the first portion, 10% alcoholic solution of α-napthol was added. The mixture was shaken well and a few drops of concentrated sulphuric acid were added along the side of the test tube. Appearance of a violet coloured ring at the junction of the two liquids confirms the presence of carbohydrates. Fehling's test: The second portion was treated with 2 mL of Fehling's solution A and 2 mL of Fehling's solution B and boiled. Formation of brick red precipitate confirms presence of reducing sugars. Benedict's test: The third portion was treated with 5 mL of Benedict's reagent and boiled on a water bath. Formation of brick red precipitate at the bottom of the test tube shows the presence of monosaccharides.

2.4.3. Test for proteins and amino acids

The test extracts were divided into four portions and kept in a test tube. Biuret test: The first portion was treated with 2 mL of 10% sodium hydroxide solution and 2–3 drops of 1% copper sulphate solution and mixed. Appearance of violet or purple colour confirms presence of proteins. Ninhydrin test: The second portion was treated with 0.5 mL of ninhydrin solution and boiled for 2 min and cooled. Appearance of blue colour confirms presence of proteins. Xanthoproteic test: To the third portion, 1 mL of concentrated nitric acid was added, then boiled and cooled. 40% sodium hydroxide solution was added to the mixture drop by drop. Appearance of coloured solution indicates presence of proteins. Millon's test: The forth portion was treated with 2 mL of Millon's reagent, then boiled and cooled. To the mixture a few drops of sodium nitrite solution were added. Appearance of red precipitate or colour indicates presence of proteins.

2.4.4. Test for tannins and phenolic compounds

Ferric chloride test: The test extract was treated with 1% (m/m) solution of ferric chloride. Appearance of blue/green/brown colour confirms the presence of tannins and phenolic compounds.

2.4.5. Test for steroids and sterols

The test extracts were divided into two portions and kept in test

tubes. Liberman Burchard test: The first portion (2 mL of test extract solution in chloroform) was treated with a few drops of acetic anhydride and mixed well. 1 mL of Conc. H_2SO_4 was added from side of the test tube. A reddish brown ring is formed at the junction of two layers, which confirms the presence of sterols and steroids. Salkowski's test: The second portion (5 mL of test extract solution in chloroform) was treated with an equal volume of concentrated sulphuric acid which was added gently along the sides of the test tube. The upper chloroform layer and the lower acid layer were observed. The acid layer developed a yellow colour with a green fluorescence and the chloroform layer gave a play of sundry colours first from bluish red to gradually violet red in the presence of sterols and steroids.

2.4.6. Test for triterpenoids

Sulphuric acid test: About 300 mg of extract was mixed with 5 mL chloroform and warmed for 30 min. The chloroform solution was then treated with a few drops of concentrated sulphuric acid and mixed properly. The appearance of red colour indicates the presence of triterpenes.

2.4.7. Test for saponins

Foam test: The test extract of about 300 mg was boiled with 5 mL of distilled water for 2 min. Then the mixture was cooled and mixed vigorously and left idle for 3 min. The formation of frothing indicates the presence of saponins.

2.4.8. Test for flavonoids

The test extracts were divided into three portions and kept in a test tube. Shinoda test: To the first portion a piece of magnesium ribbon and a few drops of concentrated hydrochloric acid were added. A pink/magenta colour develops which indicates presence of flavonoids. Ferric chloride test: The second portion was treated with a few drops of neutral ferric chloride solution. Appearance of a blackish green colour indicates the presence of flavonoids. Lead acetate test: The third portion was treated with a few drops of 10% lead acetate solution. Appearance of yellow precipitate proves the presence of flavonoids in the extract.

2.5. Experimental animals and housing conditions

Swiss albino mice (20–25 g) of either sex were used for acute toxicity evaluation and Wistar albino rats (150–250 g) both male and female were used to evaluate hepatoprotective potential. The animals were kept for at least one week in the animal house at GITAM Institute of Pharmacy, GITAM University, Visakhapatnam, Andhra Pradesh, prior to testing and housed in clean polypropylene cages with optimum light, temperature and humidity (light/dark cycles (12/12 h), Temp: 25 ± 2 °C, and 75% relative humidity) and fed with commercially pelleted rat diet (M/s Hindustan Lever Ltd., Mumbai) and water *ad libitum*.

2.6. Ethical approval

All experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) of GITAM Institute of Pharmacy, Visakhapatnam, Andhra Pradesh, India (Regd. No.1287/ac/09/CPCSEA and protocol No: IAEC/GIP-1307/B Pharm/IP/SM-HV/11/2012-13). Experiments were performed according to the guide for the care and use of laboratory animals.

2.7. Acute toxicity studies

The acute toxicity studies were conducted as per the OECD guidelines 423 [19], with slight modifications [20,21], where the limit test dose of 3000 mg/kg, p.o., was used. The animals were divided into four groups where group I (control group) received only vehicle (3 mL/kg, p.o.), whereas groups II, III and IV separately received 100, 2000 and 3000 mg/kg, p.o., of the test extract respectively in a similar manner. Immediately after dosing, the animals were closely observed for the initial 4 h after the administration and then once daily during the following days. The general behavioural changes closely observed were mainly hyperactivity, ataxia, convulsions, salivation, tremors, diarrhoea, lethargy, sleep and coma. They were then kept under observation up to 14 days after drug administration to observe any mortality. One-fifth and one-tenth of the maximum tolerated dose of EEMTAP (600 and 300 mg/kg, body weight, p.o.) was selected for hepatoprotective study.

2.8. Assessment of hepatoprotective activity

2.8.1. Experimental groups

Hepatoprotective effect was evaluated against CCl_4 induced hepatotoxicity according to standard procedures [22]. Animals were divided into five groups (n=6). Group I (normal control) animals received a single dose of water (25 mL/kg, p.o.) daily for 7 days and received a single dose of 8 mL/kg olive oil through intraperitoneal (i.p.) injection on 7th day. Group II (CCl_4 intoxicated) animals received 0.2% CCl_4 in olive oil (8 mL/kg, i.p.) on 7th day intraperitoneally and were also administered with a single dose of water (25 mL/kg, p.o.) once daily for 7 days. Group III animals were treated with standard drug silymarin (100 mg/kg, p.o.) once daily for 7 days whereas groups IV and V animals were treated with EEMTAP at doses of 300 and 600 mg/kg, p.o., dissolved in 2% gum acacia once daily for 7 days respectively. Similarly groups III to V animals were simultaneously treated with 0.2% CCl_4 in olive oil (8 mL/kg, i.p.) on 7th day after 1 h of administration of silymarin and EEMTAP. After 24 h of treatment, blood from all animals was collected by retro-orbital puncture and after that the animals were sacrificed. Blood was allowed to clot and centrifugation was performed at 3500 rpm for 15 min at 4 °C to separate the serum which was used for the assay of biochemical marker enzyme. Liver tissue samples were taken from the left liver lobe and cut into two pieces. One piece was fixed in 10% formalin solution for 24 h for pathological examination; the other piece was used in assessment of lipid peroxidation assay.

2.8.2. Biochemical estimations

Serum separated by centrifugation was used to determine the biochemical parameters like serum glutamic oxaloacetic transaminase (SGOT), serum glutamate-pyruvate transaminase (SGPT), alkaline phosphatase (ALP), total bilirubin and total protein using commercially available kits (Span Diagnostics Ltd., Surat, India). Liver weight was also determined by recording the weight of liver with respect to body weight per 100 g.

2.8.3. Assay for hepatic antioxidant activities

2.8.3.1. Lipid peroxidation (LPO). Liver tissue (900 mg) was taken from each experimental animal and then washed in normal saline and soaked in filter paper. Homogenization of the tissues was done using 3.0 mL (0.15 M) of Tris-HCl buffer (pH 7.4) and then centrifuged for 1 h at 3000 rpm at 4 °C. The resultant supernatant was collected and estimated for LPO. LPO was determined by measuring the amounts of malondialdehyde (MDA) produced primarily, which was expressed as nM/g liver tissue homogenate [23].

2.8.3.2. Glutathione (GSH) content. Liver homogenate (30%, m/v) was prepared in 0.15 M Tris-HCl buffer (pH 7.4) and proteins were precipitated by adding trichloroacetic acid. The following samples were centrifuged for 1 h at 15000 rpm at 4 °C. The supernatant obtained was used for determination of GSH content which was expressed in M/mg liver tissue homogenate [24].

2.8.3.3. Catalase (CAT) activity. Homogenization of 900 mg of liver tissue was done using M/15 phosphate buffer (pH 7.0) in ice and then centrifuged for 1 h at 30000 rpm at 4 °C and the resulting supernatant was collected and CAT activity was measured. An aliquot of the collected liver supernatant (10 µL) was brought in a quartz cuvette, and the reaction was started by the addition of freshly prepared 30 mM hydrogen peroxide (H_2O_2) in 50 mM phosphate buffer (pH 7.0). The rate of H_2O_2 decomposition was measured spectrophotometrically by recording the absorbance at 240 nm during 120 s using a Shimadzu UV–Vis spectrophotometer. CAT activity was expressed in U/mg liver tissue homogenate. One unit (U) of CAT activity was defined as the amount of extract needed to decompose 1 µmol of H_2O_2 per min [24].

2.8.4. Histological observation

Fixatives such as picric acid, formaldehyde and 40% glacial acetic acid were used to fix the washed liver for 24 h, and the liver was then dehydrated with alcohol, cleaned and embedded in paraffin (melting point 58–60 °C). 3–5 µm sections of the liver tissue were cut and stained with haematoxylin and eosin (HE). After staining they were observed under photomicroscope and the following morphological changes like hepatic cell necrosis, ballooning degeneration, fatty changes or inflammatory cell infiltration and congestion were observed [25].

2.9. Statistical analysis

The data obtained in the studies were subjected to one way of analysis of variance (ANOVA) for determining the significant difference. The inter group significance was analyzed using Dunnet's *t*-test. A p-value < 0.05 was considered to be significant. All the values were expressed as mean ± SE.

2.10. HPTLC fingerprint profile

HPTLC studies were carried out according to the standard methods [26,27]. The HPTLC fingerprint technique was used to detect possible phenolic acids and flavonoids in EEMTAP.

2.10.1. Sample preparation and stationary phase

EEMTAP was dissolved in methanol which was used for sample. Silica gel 60F 254 HPTLC plates (E. MERCK KGaA) of size (10.0 cm×10.0 cm) were used as the stationary phase.

2.10.2. Sample application

Different applied volumes of reference standards gallic and caffeic acid such as 4, 5, 6 and 7 µL were used for phenolic acid detection and 2, 3, 4 and 5 µL of reference standard quercetin were used for detection of flavonoids. The reference standard along with 10 µL of the extract was applied as a band on the plates using syringe (100 µL), with inert gas flow providing a delivery speed of 150 nL per second. The syringe was mounted on a CAMAG linomat V sample applicator attached to CAMAG HPTLC system and was programmed through WIN CATS software. The length of the applied band was kept at 8 mm.

2.10.3. Development of chromatogram

The plate for phenolic acids was developed in toluene: ethyl acetate: formic acid (4.5:3:0.2, v/v/v) and the plate for flavonoids in toluene: ethyl acetate: methanol: formic acid (4:8:0.5:0.2, v/v/v/v) and 10 mL of mobile phase was used per chromatography run. The linear ascending development was carried out in a (20 cm×10 cm) twin trough glass chamber saturated with the mobile phase.

2.10.4. Detection of spots and photodocumentation

The developed plate was dried by hot air with the help of a hair dryer at a temp of 60 °C to evaporate solvents from the plate. The plate was scanned using D2 lamp at 280 nm in CAMAG TLC Scanner 3. The R_f values and fingerprint data were recorded by WIN CATS software.

3. Results

3.1. Preliminary phytochemical analysis of EEMTAP

Preliminary phytochemical screening of EEMTAP revealed the presence of sterols, flavonoids, saponins, reducing sugar, triterpenoids, tannins and phenolic compounds (Table 1).

3.2. Acute oral toxicity studies

No mortality or morbidity was observed in the animals through the 14-day period following single oral administration. Morphological characteristics (fur, skin, eyes and nose) appeared normal. No tremors, convulsions, salivation, diarrhoea, lethargy or unusual behaviours such as self-mutilation and walking backward were observed. Gait and posture, reactivity to sensory stimuli, and grip strength were all normal. There was no significant difference in body weights between control and treatment groups. Food and water intake showed daily fluctuations within the range of control animals. This indicates that EEMTAP was safe up to a single dose of 3000 mg/kg, p.o., in experimental mice.

3.3. Hepatoprotective evaluation

3.3.1. Effects of EEMTAP on serum marker enzymes (SGPT, SGOT and ALP), total protein, total bilirubin and liver weight

As indicated from the results (Figs. 1A and B), CCl_4 intoxicated animals showed an increase in the activities of SGPT, SGOT, and ALP and the content of total bilirubin when compared to the normal control group. Treatment of animals with EEMTAP at the doses of 300 and 600 mg/kg, p.o., or silymarin 100 mg/kg, p.o., significantly decreased the levels of serum marker enzymes (SGPT, SGOT and ALP) and total bilirubin compared to the CCl_4 intoxicated group. Whereas total

Table 1
Preliminary phytochemical tests to identify the presence of various phytochemicals in ethanol extract of *Macrothelypteris torresiana* aerial parts.

Sl no.	Phytochemicals	Tests performed	Inference
1	Alkaloids	Mayer's test	–
		Dragondorff's test	–
		Wagner's test	–
2	Carbohydrates	Molisch's test	+
		Fehling's test	+
		Benedict's test	+
3	Proteins and amino acids	Biuret test	–
		Ninhydrin test	–
		Xanthoproteic test	–
		Millon's test	–
4	Tannins and phenolic compounds	Ferric chloride test	+
5	Steroids and sterols	Liberman Burchard test	+
		Salkowski's test	+
6	Triterpenoids	Sulphuric acid test	+
7	Saponins	Foam test	+
8	Flavonoids	Shinoda test	+
		Ferric chloride test	+
		Lead acetate test	+

(–) Absent, (+) Present.

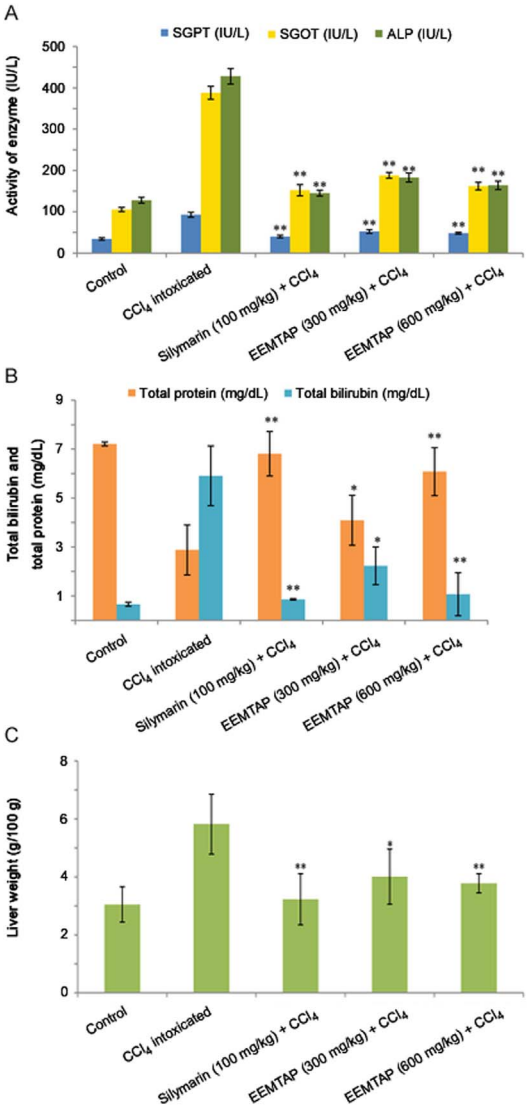

Fig. 1. Effects of EEMTAP on different biochemical parameters. (A) Serum marker enzymes (SGPT, SGOT and ALP), (B) total protein and total bilirubin and (C) liver weight with respect to body weight per 100 g. Values are expressed as mean ± S.E. (n=6). All columns are significant using ANOVA; $^{**}P < 0.01$ when compared to CCl_4 group; Dunnet's t-test.

protein level was decreased in the CCl_4 intoxicated group, which was significantly restored to normal level when the animals were treated with EEMTAP at the doses of 300 and 600 mg/kg, p.o., or silymarin 100 mg/kg, p.o., (Fig. 1B). Liver weight was increased in CCl_4 intoxicated group compared to normal control group but it significantly regained its normal size when the animals were treated with EEMTAP at the doses of 300 and 600 mg/kg, p.o., or silymarin 100 mg/kg, p.o., (Fig. 1C).

3.3.2. Effects of EEMTAP on antioxidant enzymes (LPO, GSH and CAT)

The result showed that there was an increase in level of LPO in CCl_4 intoxicated group. Treatment of the animals with EEMTAP at doses of 300 and 600 mg/kg, p.o., and silymarin (100 mg/kg, p.o.) significantly ($P < 0.01$) decreased the level of LPO compared to CCl_4 intoxicated group (Fig. 2A). The levels of GSH and CAT decreased in CCl_4 intoxicated group when compared to control group but after treatment of animals with EEMTAP (300 and 600 mg/kg, p.o.) or silymarin (100 mg/kg, p.o.) there was a significant rise in levels of GSH and CAT compared to CCl_4 intoxicated group (Figs. 2B and C).

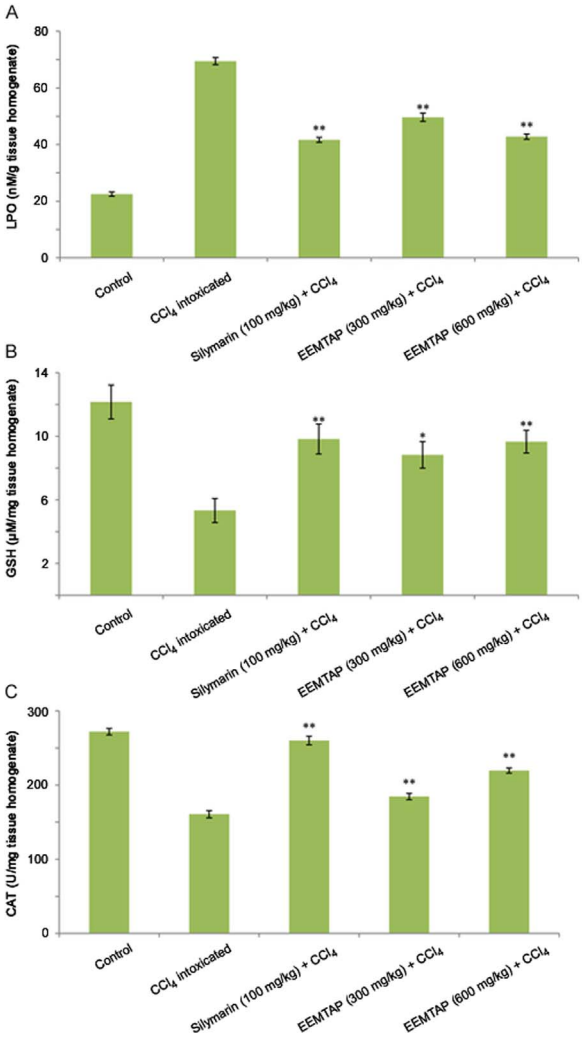

Fig. 2. Effects of EEMTAP on hepatic antioxidant enzymes. (A) Lipid peroxidase (LPO), (B) glutathione (GSH) and (C) catalase (CAT). Values are expressed as mean ± S.E. (n=6). All columns are significant using ANOVA; $^{**}P < 0.01$ when compared to CCl_4 group; Dunnet's t-test.

3.3.3. Histopathological observations

In normal control group, no abnormal appearance or histopathological changes were observed in the liver (Fig. 3A). When compared to the normal tissues, CCl_4 administration caused hepatic damage in rat liver, as demonstrated by hepatic cell necrosis, ballooning degeneration, fatty changes or inflammatory cell infiltration (Fig. 3B). Treatment with silymarin almost restored the normal architecture of liver (Fig. 3C) whereas treatment with EEMTAP decreased the abnormality of liver architecture caused by CCl_4 and restored the altered histopathological changes in a dose-dependant manner (Figs. 3D and E).

3.4. HPTLC fingerprinting analysis

3.4.1. Detection of phenolic acids

HPTLC fingerprinting of EEMTAP revealed ten peaks whereas six peaks were revealed in HPTLC fingerprinting of the reference standards gallic acid and caffeic acid. The result showed that one spot has R_f value similar to that of standard caffeic acid (Tables 2 and 3) (Figs. 4 and 5). Thus caffeic acid was detected in EEMTAP. This is the first report of the presence of caffeic acid in this fern species.

3.4.2. Detection of flavonoids

HPTLC fingerprinting of EEMTAP revealed eleven peaks whereas

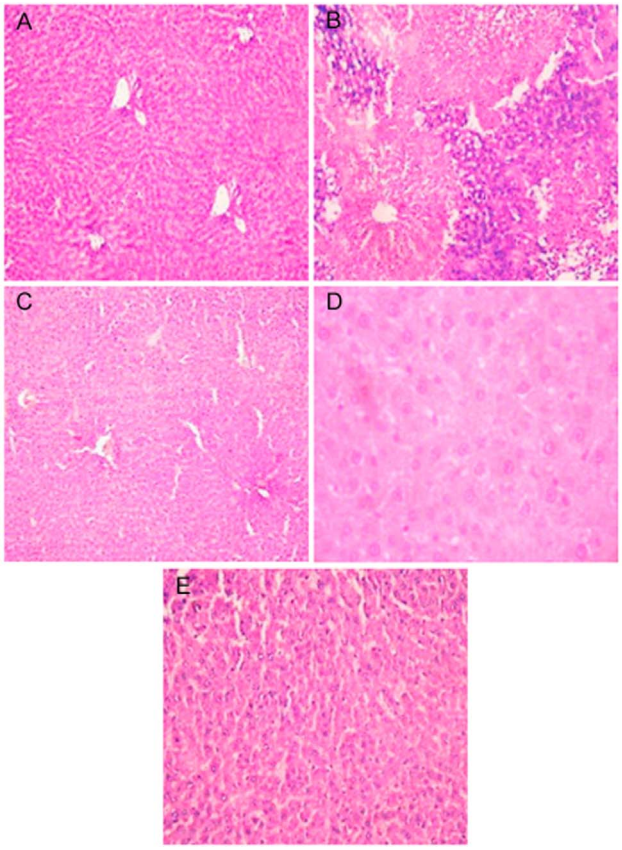

Fig. 3. Paraffin sections of liver stained by haematoxylin and eosin for histopathological changes. (A) Liver section of control group showing normal architecture of normal liver histology. (B) Hepatotoxic liver after treatment of CCl_4 showing hepatic cell necrosis, ballooning degeneration, fatty changes or inflammatory cell infiltration. (C) Liver section treated with CCl_4+silymarin (100 mg/kg) preserving almost the normal structure of the hepatocytes. (D) Liver section treated with CCl_4 and EEMTAP (300 mg/kg) showing mild hepatic cell necrosis and infiltration of inflammatory cells. (E) Liver section treated with CCl_4 and EEMTAP (600 mg/kg) showing liver restoring to normalcy with little hepatic damage.

five peaks were revealed in HPTLC fingerprinting of the reference standard quercetin. The result showed that there was one spot which showed R_f value similar to that of standard quercetin (Tables 4 and 5) (Figs. 6 and 7). Thus quercetin was detected in EEMTAP.

4. Discussion

CCl_4 is a hepatotoxin used extensively for inducing liver injury in various experimental models to elucidate the mechanisms underlying hepatotoxicity [28]. CCl_4 mediated hepatotoxicity is developed from the biotransformation of CCl_4 by cytochrome P450 2E1 to the trichloromethyl free radical ($^{\bullet}CCl_3$). Conversion of this free radical to a highly reactive species ($CCl_3O_2^{\bullet}$) is done by reaction with oxygen. Trichloromethylperoxy radical binds covalently to cellular macromolecules and leads to a chain reaction of polyunsaturated fatty acids in the cytoplasmic membrane phospholipids, causing functional and morphological changes to the cell membrane, and finally, cell necrosis [29].

Hepatocytic damage is characterised by different hepatic marker enzymes (SGPT, SGOT and ALP) and the levels of total bilirubin and total protein. When liver cells are damaged, these enzymes leak into the bloodstream from liver tissue and produce markedly elevated serum levels [30]. Both SGOT and SGPT are associated with liver parenchymal cells. SGPT is found predominantly in the liver with negligible quantities found in heart, kidneys and skeletal muscles, whereas SGOT is found in liver, cardiac muscles, skeletal muscles, brain, kidney and red blood cells. Thus SGPT is a more specific indicator of liver intoxication as levels of SGOT may also be increased in diseases affecting other organs [31]. On the other hand, serum ALP and bilirubin levels are related to the functions of hepatic cell. Elevation in level of serum ALP is due to increased synthesis, in presence of increased biliary pressure. Our experiment showed that rats intoxicated with CCL_4 develop a significant liver necrosis which was evidenced by increased activities of hepatic marker enzymes (SGPT, SGOT and ALP) and the levels of total bilirubin, whereas levels of total protein were decreased due to liver injury. The result of this study showed that after administration with EEMTAP the activities of the serum marker enzymes (SGPT, SGOT and ALP) and the levels of total bilirubin and total protein were restored to normal level, thus indicating that EEMTAP preserved the structural integrity of hepatocellular

Table 2
HPTLC chromatogram of reference standards Gallic acid and Caffeic acid.

Peak	Start Rf	Start height	Max Rf	Max height	Max height (%)	End Rf	End height	Area	Peak area (%)	Assigned substance
1	−0.06	1.0	−0.04	18.9	2.07	−0.02	0.7	269.2	1.15	Unknown
2	−0.02	0.7	−0.00	73.9	8.11	0.02	8.9	1102.7	4.71	Unknown
3	0.11	4.8	0.16	300.9	32.99	0.19	0.0	7140.9	30.52	Gallic acid
4	0.24	3.6	0.31	414.8	45.50	0.35	1.2	9924.6	42.42	Caffeic acid
5	0.63	1.0	0.70	60.1	6.59	0.73	47.8	2950.7	12.61	Unknown
6	0.76	42.6	0.77	43.1	4.73	0.87	0.2	2007.8	8.58	Unknown

Table 3
HPTLC chromatogram of EEMTAP for detection of phenolic acids.

Peak	Start Rf	Start height	Max Rf	Max height	Max height (%)	End Rf	End height	Area	Peak area (%)	Assigned substance
1	−0.06	1.2	−0.05	28.8	1.66	−0.03	13.3	379.4	0.68	Unknown
2	−0.03	13.4	−0.01	794.0	45.83	0.05	142.6	21080.2	37.79	Unknown
3	0.06	142.6	0.07	162.2	9.36	0.16	33.1	6407.1	11.48	Unknown
4	0.23	27.2	0.27	42.2	2.43	0.28	41.1	1113.2	2.00	Unknown
5	0.28	40.9	0.33	306.2	17.68	0.39	41.9	12067.0	21.63	Caffeic acid
6	0.39	42.4	0.42	177.1	10.22	0.46	20.1	4276.5	7.67	Unknown
7	0.46	20.2	0.47	21.8	1.26	0.51	9.0	669.8	1.20	Unknown
8	0.58	1.7	0.62	36.4	2.10	0.65	18.5	1074.4	1.93	Unknown
9	0.65	18.8	0.69	65.5	3.78	0.71	61.8	2263.0	4.06	Unknown
10	0.73	61.6	0.79	98.3	5.68	0.86	9.5	6456.6	11.57	Unknown

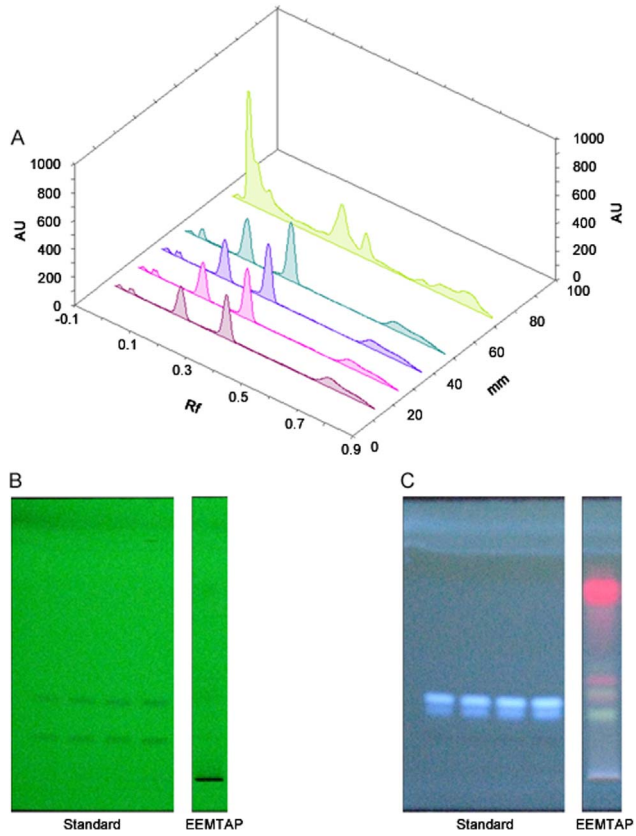

Fig. 4. (A) 3D display of HPTLC chromatogram of EEMTAP for detection of phenolic acids. (B) HPTLC chromatographic plate for phenolic acids detection in EEMTAP viewed at 254 nm. (C) HPTLC chromatographic plate for phenolic acids detection in EEMTAP viewed at 366 nm.

components and protected the liver from the harmful effect of this hepatotoxin.

Here our research also evaluated the effect of EEMTAP on hepatic antioxidant enzymes such as LPO, GSH and CAT, whose levels were impaired due to oxidative stress caused by CCl_4. An important mechanism involved in the protection against CCl_4-induced liver damage is the inhibition of excessive ROS production. MDA is widely used as a marker of lipid peroxidation and a major parameter for the status of oxidative stress [32]. The hepatic MDA level increases under the enhancement of oxidative stress in a rodent model [33]. Here in our study rats treated with CCl_4 exhibited a significant rise in MDA level (Lipid peroxidation assay) compared to control group. Treatment with EEMPTAP significantly reduced the CCl_4-induced hepatic MDA elevation. Thus EEMTAP provides protective effect against CCl_4-induced liver damage in terms of preventing lipid peroxide formation and blocking oxidative chain reaction. The defence system in the body also includes small molecules such as GSH, vitamin E as well as antioxidant enzymes [34]. The activities of hepatic antioxidants including GSH and CAT were decreased by CCl_4 administration to rats, which is stated in earlier studies [35]. Here our study revealed that treatment with EEMTAP ameliorated the impaired antioxidative defence system in rat livers, as indicated by the restoration of enzymatic activities.

The above inferences were further confirmed by histopathological studies. The result of the histopathology showed that CCl_4 administration caused severe acute liver damage in rats which is characterised by hepatic cell necrosis, ballooning degeneration, fatty changes or inflammatory cell infiltration and other histological manifestations, which were consistent with previous findings [36,37]. After treatment with EEMTAP hepatic injury caused by CCl_4 administration was significantly prevented and the structure of hepatocytes was almost restored to normal. The results of the different biochemical parameters, hepatic

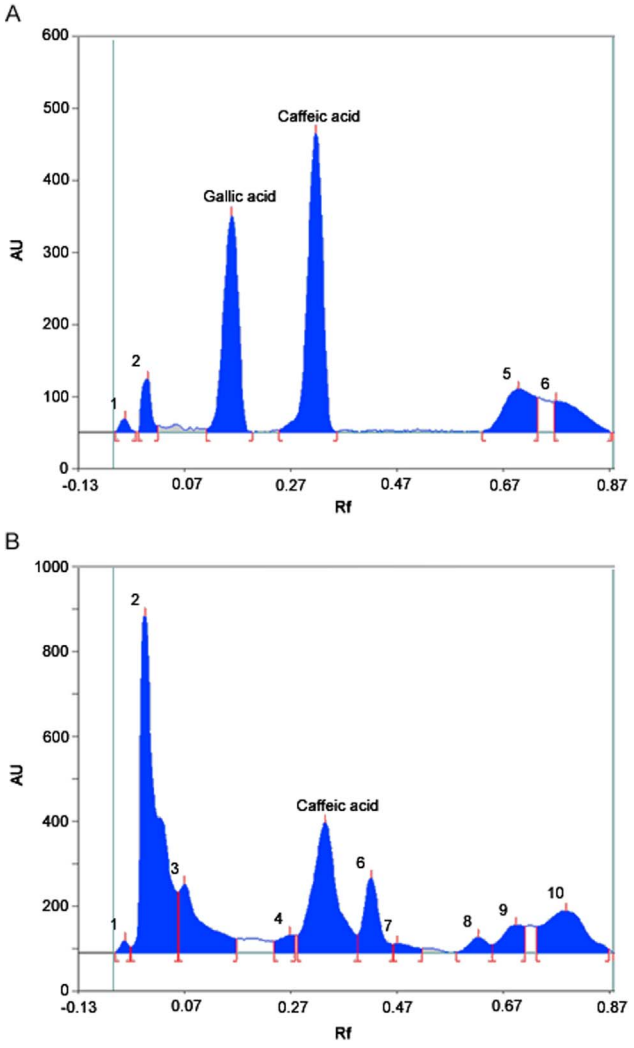

Fig. 5. Peak densitograms of (A) reference standards gallic acid and caffeic acid and (B) EEMTAP showing the presence of caffeic acid.

antioxidant enzymes and the histopathological finding co-related well with each other, which indicated that EEMTAP possesses significant hepatoprotective effect in CCl_4-intoxicated liver injury in rats.

HPTLC is an invaluable quality assessment tool for the evaluation of botanical materials, and it allows for the analysis of a broad number of compounds both efficiently and cost effectively. HPTLC analysis was performed to detect possible polyphenolic compounds in EEMTAP. Analysis of the extract for phenolic acids showed the presence of ten peaks out of which one peak is comparable with standard caffeic acid, whereas in detection of flavonoids, eleven peaks were observed out of which one peak is comparable to standard quercetin. Thus EEMTAP revealed the presence of caffeic acid and quercetin, both of which have been previously reported to possess hepatoprotective properties [38]. Apigenin, a flavonoid derivative, was identified from this fern by Xiong et al. [9] and is reported to possess hepatoprotective properties [14].

The HPTLC analysis method employed in this work resulted in good peak shape and enabled good resolution of caffeic acid and quercetin from other constituents present in the extract. Thus this HPTLC method was successful in detection of polyphenolic compounds such as caffeic acid and quercetin and this is the first study to report the presence of caffeic acid in this plant. Therefore, we can consider that these identified compounds, either individually or synergistically, might be responsible for its hepatoprotective action against CCl_4-induced hepatotoxicity in rats.

Table 4
HPTLC chromatogram of reference standard Quercetin.

Peak	Start Rf	Start height	Max Rf	Max height	Max height (%)	End Rf	End height	Area	Peak area (%)	Assigned substance
1	−0.06	0.0	−0.04	10.6	1.84	−0.03	2.6	105.9	0.45	Unknown
2	−0.03	3.4	−0.03	19.3	3.34	−0.02	2.2	107.8	0.46	Unknown
3	−0.02	0.6	−0.01	33.2	5.74	0.02	3.2	500.9	2.13	Unknown
4	0.47	22.6	0.60	497.4	85.86	0.64	3.2	21828.3	93.02	Quercetin
5	0.74	4.9	0.80	187.0	3.22	0.86	2.7	924.0	3.94	Unknown

Table 5
HPTLC chromatogram of EEMTAP for detection of flavonoids.

Peak	Start Rf	Start height	Max Rf	Max height	Max height (%)	End Rf	End height	Area	Peak area (%)	Assigned substance
1	−0.02	0.8	−0.01	769.2	33.54	0.04	331.8	24409.4	31.26	Unknown
2	0.05	333.1	0.05	338.5	14.76	0.13	119.4	12889.7	16.50	Unknown
3	0.13	118.5	0.14	119.7	5.22	0.18	88.0	3997.4	5.12	Unknown
4	0.18	88.0	0.22	216.7	9.45	0.26	58.5	7292.0	9.34	Unknown
5	0.27	60.3	0.32	107.9	4.70	0.35	54.8	4482.1	5.74	Unknown
6	0.35	55.0	0.37	96.8	4.22	0.44	33.3	4172.5	5.34	Unknown
7	0.50	35.1	0.66	376.5	16.42	0.60	80.0	13864.8	17.75	Quercetin
8	0.60	80.9	0.62	131.2	5.72	0.66	10.4	3459.3	4.43	Unknown
9	0.66	10.6	0.68	13.3	0.58	0.70	1.1	267.8	0.34	Unknown
10	0.70	0.0	0.74	29.0	1.26	0.78	2.9	774.0	0.99	Unknown
11	0.79	0.2	0.84	94.4	4.11	0.87	3.3	2487.3	3.18	Unknown

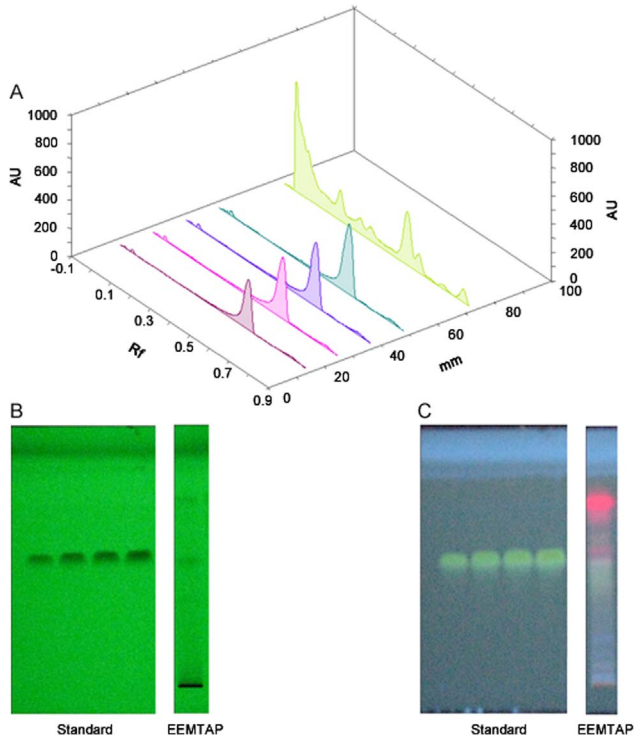

Fig. 6. (A) 3D display of HPTLC chromatogram of EEMTAP for detection of flavonoids. (B) HPTLC chromatographic plate for flavonoid detection in EEMTAP viewed at 254 nm. (C) HPTLC chromatographic plate for flavonoid detection in EEMTAP viewed at 366 nm.

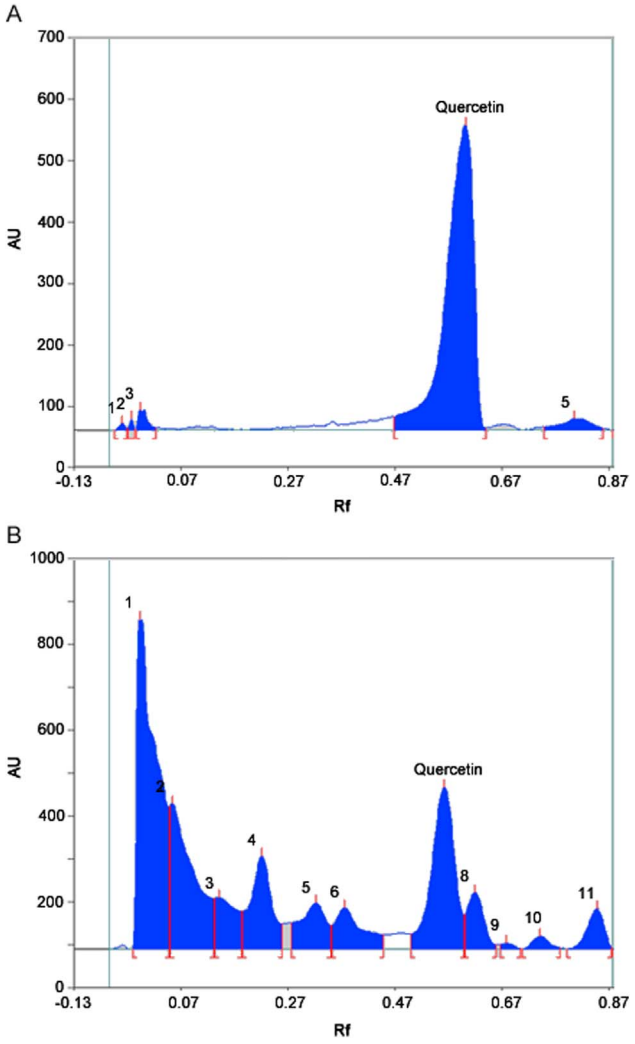

Fig. 7. Peak densitograms of (A) reference standard quercetin and (B) EEMTAP showing the presence of quercetin.

5. Conclusion

The present study demonstrated that the ethanolic extract obtained from the aerial parts of *M. torresiana* (family: Thelypteridaceae) possesses significant hepatoprotective activities against CCl_4-induced hepatotoxicity in Wistar albino rats. Acute toxicity studies on Swiss albino mice revealed that EEMTAP has a reasonable safety profile. The HPTLC fingerprint analysis of EEMTAP showed the presence of caffeic

acid and quercetin. This is the first report of the presence of caffeic acid on this fern species. The hepatoprotective activity of EEMTAP may be due to the presence of caffeic acid and quercetin as they both are reported to possess hepatoprotective effects. Therefore, the study shows that there is a prospective future in the use of plants as a source of natural medicine for curing various diseases due to the presence of medicinally important phyto-constituents in plants.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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