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ORIGINAL ARTICLE

Evaluation of Toxicological, Diuretic, and Laxative Properties of Ethanol Extract from *Macrothelypteris Torresiana* (Gaudich) Aerial Parts with *In silico* Docking Studies of Polyphenolic Compounds on Carbonic Anhydrase II: An Enzyme Target for Diuretic Activity

Sumanta Mondal, Naresh Panigrahi, Purab Sancheti, Ruchi Tirkey, Prasenjit Mondal[1], Sara Almas, Venu Kola[1]

Department of Pharmaceutical Chemistry, Institute of Pharmacy, GITAM (Deemed to be University), Visakhapatnam, Andhra Pradesh, [1]Department of Pharmacy, Vaageswari College of Pharmacy, Karimnagar, Telangana, India

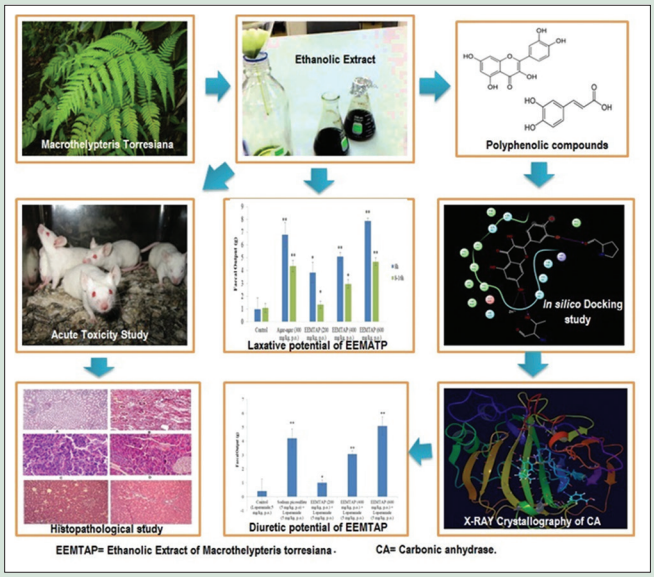
ABSTRACT

Background: *Macrothelypteris torresiana* (Gaudich) is a species of fern having a wide range of reputed medicinal properties for the treatment of inflammation, fever, renal failure, stomach problems, etc. **Objective:** The present investigation focused on the evaluation of toxicity profile and diuretic and laxative activities of ethanol extract from *M. torresiana* aerial parts (EEMTAP), with *in silico* docking studies of polyphenolic compounds on carbonic anhydrase (CA)-II, an enzyme target for diuretic activity. **Materials and Methods:** Acute and subacute toxicity was performed according to the Organization for Economic Co-operation and Development guidelines. EEMTAP at doses of 200, 400, and 600 mg/kg, p.o., employed for the assessment of diuretic and laxative activities with loperamide-induced constipation in Wistar albino rats. Furosemide (10 mg/kg, p.o.), agar-agar (300 mg/kg, p.o.), and sodium picosulfate (5 mg/kg, p.o) were used as reference standards, respectively, for activity comparison. During saluretic activity study, total urine volume, body weight before and after the experiment, and urinary levels of Na^+, K^+ (by flame photometry), and Cl^- (by titrimetry) were estimated. Polyphenolic compounds such as caffeic acid and quercetin were successfully detected through chromatographic method of EEMTAP, and to rationalize the results obtained in diuretic activities, we carried out docking studies of the natural phenolic compounds against CA-II enzyme co-complexed with furosemide (Protein Data Bank ID: 1Z9Y CA-II in complex with furosemide as sulfonamide inhibitor). **Results:** In acute toxicity study, no mortality was observed at 2000 mg/kg, p.o., and in subacute toxicity study, the extract-treated group did not show any significant changes in body weight and organ weights. The hematological and biochemical parameters did not show any significant changes in the sample-treated groups when compared with the control group animals. The laxative activity of the extract was found to be in a dose-dependent increase in fecal output of rats at selected dose levels; similarly, EEMTAP significantly increased the urinary output as well as urinary electrolyte concentration in a dose-dependent manner. The molecular docking studies of phenolic compounds (caffeic acid and quercetin) into the binding site of CA II enzyme reveals that these analogues are having more favourable interaction when compared to the furosemide with better docking scores and hydrogen bonding interactions. **Conclusion:** The result demonstrated that the EEMTAP possesses a reasonable safety profile and shows promising diuretic and laxative activities in a dose-dependent manner.

Key words: Diuretic, *in silico* docking studies, laxative, *Macrothelypteris torresiana,* toxicity

SUMMARY

- *Macrothelypteris torresiana* (Gaudich) is a species of fern focused on the evaluation of toxicity profile, diuretic and laxative activities of ethanol extract, with in silico docking studies of polyphenolic compounds on carbonic anhydrase (CA)-II, an enzyme target for diuretic activity. The result demonstrated that the ethanol extract from *M. torresiana* aerial parts possesses a reasonable safety profile and shows promising diuretic and laxative activities in a dose-dependent manner, simultaneously in molecular docking studies with phenolic compounds (caffeic acid and quercetin) into the binding cavity of CA II enzyme showed the analogues having more favourable interaction than furosemide with better docking scores and hydrogen bonding interactions.



Abbreviations Used: EEMTAP: Ethanol Extract from *M. Torresiana* Aerial Parts; CA-I: *Carbonic Anhydrase* I; CA-II: *Carbonic Anhydrase* II; hCA-II: Human Carbonic Anhydrase-II; OECD: Organisation for Economic Co-operation and Development; PDB: Protein Data Bank.

Correspondence:
Dr. Sumanta Mondal,
Department of Pharmaceutical Chemistry,
GITAM (Deemed to be University),
Visakhapatnam - 530 045, Andhra Pradesh, India.
E-mail: mondalresearch@gmail.com
DOI: 10.4103/pr.pr_16_18

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INTRODUCTION

Day-by-day medicinal plants are becoming beneficial to humans as they have several bioactive compounds to cure various diseases but due to the potential toxicity of these phytoconstituents have not been well established.[1] There is very little scientific documentation on the safety and efficacy of herbal drugs to the increase in number of its users which raised concerns regarding toxicity and detrimental effects of these herbal medications. Thus, there is a need to evaluate the safety and efficacy of these plants thoroughly to maximize their benefits for humans.[2]

Diuretics are medicines that increase the rate of urine flow and sodium excretion and control the volume and/or composition of body fluids in various clinical situations such as cirrhosis, hypertension, heart failure, nephrotic syndrome, and renal failure.[2] Constipation is very common and often chronic gastrointestinal disorder with a tendency to cause discomfort which affects normal life. Constipation not only causes perturbation but also causes vomiting, abdominal distension, restlessness, perforation, and gut obstruction; in extreme cases, it may be associated with fatal pulmonary embolism or aspiration. Treatment of constipation with classic drugs is often insufficient, leaving patients with inadequate relief of bloating and other symptoms, which has prompted to develop better drugs for the treatment of constipation.[3,4]

Macrothelypteris torresiana (Gaudich) Ching, syn. *Lastrea torresiana* Moore (family: Thelypteridaceae) is a species of fern which is of indigenous origin to tropical and subtropical region of the world. It is a robust fern with a short-creeping rhizome.[4-6] Conventionally, the whole plants have a wide range of reputed medicinal application. The aerial parts of *M. torresiana* are used by the ethnic group of Pakistan, India, and China for the treatment of pyrexia, unpleasant physical sensation caused by illness or injury, stomach problems, healing and reducing odor in chronic skin ulcer, diuretics, uterine hemorrhage, and inflammation.[5-7] Han Chinese used *M. torresiana* for the treatment of edema for patient suffering from kidney problems.[8] Only a few phytochemical and pharmacological properties have been reported on this plant, including the renoprotective potential of *M. torresiana* through ameliorating oxidative stress and pro-inflammatory activities,[8] *in vitro* and *in vivo* antitumor activities,[9] hepatoprotective activity,[10] wound healing properties,[7] and nociceptive, antipyretic, and anti-inflammation activities.[11] A novel flavonoid was isolated from the root and the structure was identified as 5,7-dihydroxy-2-(1,2-isopropyldioxy-4-oxocyclohex-5-enyl)-chromen-4-one,[12] along with four known flavonoids: protoapigenin, apigenin, kaempferol, and quercetin.[13] An analytical technique for the simultaneous determination of phytochemical constituents was developed using chromatographic method and successfully quantified the presence of apigenin 4'-O-β-D-glucoside, apigenin, protoapigenin 4'-O-β-D-glucoside, protoapigenone,[14] caffeic acid as phenolic acid, and quercetin as flavonoid.[10]

Literature available from all possible scientific sources revealed very little research work on this selected fern species, whereas tribes claim that *M. torresiana* was used in the treatment of various diseases and ailments including constipation and stomach problems, although there is no inbuilt scientific proof in support of the utility of this species. Simultaneously, several natural phenols showed human carbonic anhydrase-II (hCA-II) inhibitory effects, in the same range as the clinically used sulfonamide and acetazolamide, and might be used as leads for generating enzyme inhibitors possibly targeting other CA isoforms that have not been yet assayed for their interactions with such agents.[15] Thus, the present study explored the details of toxicity and diuretic and laxative properties of ethanol extract from *M. torresiana* aerial parts (EEMTAP) using different experimental animal models and as the major polyphenolic compounds such as caffeic acid and quercetin successfully detected through chromatographic method from EEMTAP,[10] thereby *in silico* docking studies were carried out over major polyphenolic compounds on CA-II, an enzyme target for diuretic activity.

MATERIALS AND METHODS

Chemicals and reagents

Furosemide, loperamide, sodium picosulfate, and normal saline were obtained as a gift samples from GITAM Institute of Medical Science and Research and Pharmacy, Andhra Pradesh, India. All other chemicals and reagents such as Tween-80, formalin, potassium chromate, silver nitrate, and agar-agar were purchased from Sisco Research Laboratories Pvt. Ltd. (Mumbai, India) and Merck India Ltd. (Mumbai, India). Diagnostic kits for the estimation of biochemical parameters were purchased commercially (Span Diagnostics Ltd., Surat, India).

Plant material

The aerial parts of the plant *M. torresiana* were collected from in and around East Godavari district, Andhra Pradesh, India, and authenticated by Dr. K. Madhava Chetty, Professor, Department of Botany, Sri Venkateswara University, Tirupati, Andhra Pradesh, India. A voucher specimen (specimen no: SVU/MT7/GIP/2013) has been kept in our research laboratory for further reference. The collected materials were washed with water and shade dried for 1 week. The dried aerial parts were pulverized using a mechanical grinder to obtain a coarse powder.

Preparation of the extract

The powdered plant material (500 g) was extracted with 1.5 L of ethanol (90% v/v) for 24 h using a Soxhlet extractor. The extract obtained was evaporated under vacuum to remove the solvent completely and concentrated to obtain a dark greenish semisolid residue and percentage yield of EEMTAP was 2.13%.

Preliminary phytochemical tests

Preliminary phytochemical studies of EEMTAP were performed for determination of major phytochemical constituents such as alkaloids, carbohydrates, proteins, tannins, sterols, triterpenoids, saponins, and flavonoids using standard procedures.[10-16]

Test for alkaloids

The dry crude extract was dissolved in 2 N 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-colored precipitate proves the presence of alkaloids. Dragendorff's test: Few drops of Dragendorff'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 few drops of Wagner's reagent. Formation of reddish-brown precipitate proves the presence of alkaloids in the test extract.

Test for carbohydrates

The test extract was divided into three portions and kept in a test tube. Molisch's test: To the first portion, 10% alcoholic solution of α-naphthol was added. The mixture was shaken well and few drops of concentrated sulfuric acid were added along the side of the test tube. Appearance of a violet-colored 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 the 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.

Test for proteins and amino acids

The test extract was 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 sulfate solution and mixed. Appearance of violet or purple color confirms the 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 color confirms presence of proteins. Xanthoproteic test: To the third portion, 1 mL of concentrated nitric acid was added, then boiled, and cooled. About 40% sodium hydroxide solution was added to the mixture drop by drop. Appearance of colored solution indicates the presence of proteins. Millon's test: The fourth portion was treated with 2 mL of Millon's reagent, then boiled, and cooled. To the mixture, few drops of sodium nitrite solution were added. Appearance of red precipitate or color indicates presence of proteins.

Test for tannins and phenolic compounds

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

Test for steroids and sterols

The test extract were divided into two portions and kept in test tubes. Liebermann–Burchard test: The first portion (2 mL test extract solution in chloroform) was treated with few drops of acetic anhydride and mixed well. About 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 test extract solution in chloroform) was treated with an equal volume of concentrated sulfuric acid was added gently along the sides of the test tube. The upper chloroform layer and the lower acid layer were observed. The acid layer develops a yellow color with a green fluorescence, and the chloroform layer gives a play of sundry colors first from bluish red to gradually violet red in the presence of sterols and steroids.

Test for triterpenoids

Sulfuric 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 sulfuric acid and mixed properly. The appearance of red color indicates the presence of triterpenes.

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 is cooled and mixed vigorously and left idle for 3 min. The formation of frothing indicates the presence of saponins.

Test for flavonoids

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

Animals

The animal studies were conducted according to the guidance of the Institutional Animal Ethical Committee (IAEC), and care of the experimental animals was taken according to the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines. Swiss albino mice (18–25 g) of either sex were selected for acute toxicity studies, and male Wistar rats (150–250 g) were selected for diuretic and subacute toxicity studies, whereas Wistar rats of either sex were selected for laxative studies. All experimental protocols were approved by the IAEC of GITAM Institute of Pharmacy, Visakhapatnam, Andhra Pradesh, India (Regd. No. 1287/ac/09/CPCSEA).

Toxicological study

Acute toxicity study

Acute toxicity studies of EEMTAP were carried out in Swiss albino mice of either sex as per the Organization for Economic Co-operation and Development (OECD) Guideline 423 (Annex 2d).[17] Five mice were used for the acute toxicity study. The starting dose for the main study was set as 2000 mg/kg, p.o., used. Before oral administration of single dose of the test samples, the mice were deprived of food for 3 h, and after dosing of EEMTAP, all mice were observed continuously for the first 4 h for any behavioral change, symptoms of toxicity, and mortality. Then, they were kept under observation up to 14 days.[10,18]

Subacute toxicity studies

Subacute toxicity studies for 14 days were done according to the OECD Guideline 407,[19] with slight modifications.[20,21] Male Wistar albino rats were randomly assigned into two groups (n = 6/group) where Group I received 1% v/v Tween-80 in normal saline (3 ml/kg body weight, p.o.) which serves as the normal control and Group II received EEMTAP at a dose level (600 mg/kg body weight, p.o.; extract suspended in 1% v/v Tween-80 in normal saline). All rats were treated twice daily for 14 days and were observed twice daily for clinical signs and physiological and behavioral changes. Body weight, food intake, and water intake were monitored. On the 15th day, the animals were anesthetized with pentobarbital sodium 35 mg/kg body weight, i.p., and blood samples were collected by retro-orbital puncture into heparinized and nonheparinized tubes for hematological and biochemical studies. The hematological and biochemical parameters were correlated with the normal range of clinical laboratory parameters for Wistar albino rats.[22,23]

Hematological analysis

The heparinized blood samples were used for the analysis of hematological parameters such as platelet count, hemoglobin count, red blood cell (RBC) count, white blood cell (WBC) count, and differential count (neutrophils, lymphocytes, eosinophils, monocytes, and basophils).[24,25]

Biochemical analysis

For biochemical analysis, serum was separated from nonheparinized blood, and parameters such as serum glutamic-oxaloacetic transaminase, serum glutamate-pyruvate transaminase, alkaline phosphatase, total bilirubin, total protein, albumin, serum creatinine, blood urea, total cholesterol, triglyceride, and glucose content were assayed using commercial kits.[26-35]

Evaluation of body weight and organ weight

The evaluation of body weight of the control and treated animals was performed to check for possible toxicity. Macroscopic analysis of target organs of control and treated animals was done to evaluate any abnormalities in weight, texture, and shape for determination of possible

toxic effects.[23,36] The major targeted organs include rat kidney, pancreas, and liver.

Histopathological studies

Histopathological studies were performed on organ samples of kidney, pancreas, and liver. After euthanasia, all animals were autopsied, and the major organs such as pancreas and kidney were surgically taken out and were fixed in 20% formalin in normal saline. Sections of 5 µm were obtained on a rotary microtome, and then, the material was stained by hematoxylin and eosin.[37] The sections were then analyzed microscopically for pathological examinations.

Diuretic activity

The assessment of diuretic activity was carried out as described in Lipschitz *et al.*, 1943,[38] and Mondal *et al.*, 2009.[39] Thirty male albino rats (150–200 g) deprived of food and water for 18 h before the experiment and divided randomly into five groups of six rats in each. The first group of animals serving as control received normal saline (25 ml/kg, p.o.), the second group received furosemide (5 mg/kg, p.o.) in saline;[40] Groups III, IV, and V received EEMTAP at doses of 200, 400, and 600 mg/kg, p.o., in a similar manner. Immediately, after administration, the animals were placed in metabolic cages (2/cage), especially designed to separate urine and feces kept at 20°C ± 0.5°C. The volume of urine collected was measured at the end of 5 h. During this period, no food and water were made available to animals. The parameters taken were the body weight before and after test period, total urine volume, and concentration of sodium (Na^+), potassium (K^+), and chloride ions (Cl^-) in the urine. Na^+ and K^+ ion concentrations were determined by flame photometer, and Cl^- ion concentration were estimated by titration (Volhard's method) with silver nitrate solution (N/50) using three drops of 5% potassium chromate solution as indicator.[41-43]

Laxative activity

Method I

The test was performed according to method of Mondal *et al.*, 2009.[39] Rats of either sex fasted for 12 h before the experiment but with water provided *ad libitum*. The animals were divided into five groups of six in each. The first group of animals serving as control was administered orally with vehicle (1% v/v Tween-80 in normal saline, 2 ml, p.o.), the second group received reference standard agar-agar (300 mg/kg, p.o.) in saline, and Groups III, IV, and V received EEMTAP (200, 400, and 600 mg/kg, p.o.) in a similar manner. Immediately, after dosing, the animals were separately placed in cages suitable for collection of feces. After 8 h drug administration, the feces were collected and weighed. Thereafter, food and water were given to all rats, and fecal outputs were again weighed after a period of 16 h.

Method II

Laxative activity on loperamide-induced constipation in rats was performed according to method of Saito *et al.*, 2002[44] and Kim *et al.*, 2017.[45] Rats of either sex were placed individually in cages lined with clean filter paper, allowed to fast for 18 h, and divided into five groups of six animals each. Group I received vehicle 1%, v/v Tween-80 (2 ml, p.o.), Group II received standard drug sodium picosulfate (5 mg/kg, p.o; dissolve in normal saline), Groups III, IV, and V received EEMTAP at doses of 200, 400, and 600 mg/kg, p.o., in a similar manner. After 1 h treatment, all the group animals received loperamide (5 mg/kg, p.o.) by oral gavage. The fecal production (total number of normal as well as wet feces) in all five groups was monitored for 8 h.

Molecular docking studies

Docking is the process of fitting of the ligand into the receptor which helps the scientists to understand and predict the enzyme-ligand interactions *in vivo*.[46] To rationalize the results obtained in diuretic activities, we carried out docking studies of the natural phenolic compounds against CA enzyme co-complexed with furosemide (Protein Data Bank [PDB] ID: 1Z9Y CA-II in complex with furosemide as sulfonamide inhibitor). CAs are metalloenzymes[47] containing one zinc ion (Zn^{2+}) per polypeptide chain, whose main physiological function is to catalyze the reversible hydration of carbon dioxide to bicarbonate anion and proton ($CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+$).[48,49] The metal ion is critical for catalysis, as the apoenzyme is devoid of any catalytic activity.[50]

Computational method

Computational studies were carried out using Maestro version 10.2 (Cambridge, Suite 2230, MA 02142, USA) installed in a single machine running on a Intel® Core™ i5 Processor 2.20 GHz with 4 GB RAM and 1 TB hard disk with Windows 7 as the operating system.

Protein preparation

The three-dimensional structure of the enzyme for this study was downloaded from the PDB (code; PDB ID: 1Z9Y hCA-II in complex with furosemide as sulfonamide inhibitor). The enzyme structure was refined and checked for any missing atoms, bonds, loops, and contacts. All residues excluding ligand molecules and water molecules were deleted manually. After assigning charge and protonation state, finally, energy minimization was done using OPLS2005 force field.

A grid area was generated around the binding site of the receptor by the Glide grid generation wizard, by manually defining the co-crystallized ligand (furosemide), which determines the position and size of the active site and set up Glide constraints for docking the ligands.

Ligand preparation

The major polyphenolic compounds such as caffeic acid and quercetin successfully detected through chromatographic method of the EEMTAP[10] are taken as ligand structures were drawn and energy minimization was carried using MM2 force field of ChemOffice 2004 version (PerkinElmer, OHIO, Suite 2423, Akron, Ohio 44311, United States) and saved in MDL mole format. Now, the MDL mole files were converted to Sybyl Mol2 using the Open Babel program. Using

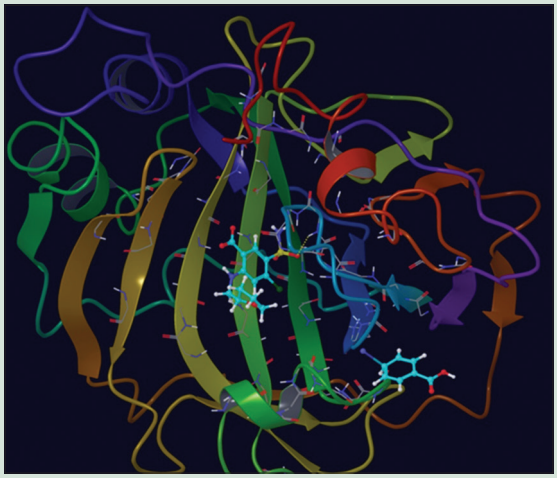

Figure 1: X-ray crystal structure of human carbonic anhydrase-II in co-complex with furosemide (PDB ID: 1Z9Y)

the LigPrep (ligand preparation) utility of Glide, these structures were geometry optimized using the Optimized Potentials for Liquid Simulations-2005 (OPLS-2005) force filed with the steepest descent followed by truncated Newton conjugate gradient protocol. Partial atomic charges were computed using the OPLS-2005 force field.

Docking ligands

Docking was performed for phenolic compounds (caffeic acid and quercetin) and the reference compound furosemide using the "extra precision" mode of Glide Program 6.7 (Cambridge, Suite 2230, MA 02142, USA). A grid (active pocket) was prepared with the center defined by the co-crystallized ligand furosemide of PDB ID: 1Z9Y [Figure 1].

The active site of human carbonic anhydrase-II consists of 18 amino acid residues: Asn 67, Gln 92, Leu 141, Phe 131, Val 121, Val 143, Val 207, Leu 198, Trp 209, Glu 106, HIE 119, HIP 64, Thr 199, Thr 200, His 94, His 96, Pro 201, and Pro 202.

Statistical analysis

The data obtained in the studies were subjected to one-way analysis of variance for determining the significant difference. The intergroup significance was analyzed using Dunnett's t-test. A $P < 0.05$ was considered to be statistically significant. All the values were expressed as mean ± standard error of the mean.

RESULTS

Preliminary phytochemical tests

The preliminary phytochemical screening of the EEMTAP contains sterols, flavonoids, saponins, proteins, carbohydrates, tannins, and phenolic compounds. However, alkaloids, cardiac glycosides, and triterpenoids were absent [Table 1].

Acute toxicity study

In acute toxicity study, oral administrations of the EEMTAP at 2000 mg/kg., p.o., did not produce any death and clinical sign of toxicity in mice. The extract induced sedation and mild diuresis with purgation at all tested doses level. 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, which indicates that the EEMTAP was safe to a single dose of 2000 mg/kg body weight and it is indicating that the median lethal dose is higher than the tested dose level. Hence, the one-fifth of the preceding dose, i.e., 400 mg/kg body weight, p.o., was taken as the testing dose for pharmacological evaluation and lower upper dose of 200 and 600 mg/kg body weight, p.o., also tested to find whether there is any dose-dependent pharmacological effect or not.

Subacute toxicity studies

After 14 days of subacute toxicity study, no significant change in body weight was observed between initial and final body weight of the rats treated with EEMTAP (600 mg/kg, p.o.) and control rats [Figure 2]. No mortalities were recorded in rats during 14 days of treatment with EEMTAP. Simultaneously, absence of toxic effect such as no changes in the skin and fur, eyes, respiratory rate, autonomic (salivation,

Table 1: Preliminary phytochemical tests to identify presence of various phytoconstituents in ethanol extract from *Macrothelypteris torresiana* aerial parts

Test groups	Inference
Alkaloids	–
Carbohydrates	+
Cardiac glycosides	–
Proteins and amino acids	+
Tannins and phenolic compounds	+
Steroids and sterols	+
Triterpenoids	–
Saponins	+
Flavonoids	+

–: Absent; +: Present

Table 2: Effects of ethanol extract from *Macrothelypteris torresiana* aerial parts on biochemical parameters in male Wistar rats.

Parameter	Unit	Normal control (3 ml/kg, p.o.)	EEMTAP (600 mg/kg, p.o.)	Normal range[22]
Total bilirubin	mg/dl	0.11±0.02	0.12±0.02	0.05-0.15
Total protein	g/dl	6.2±0.9	6.8±0.6	5.2-7.1
Albumin	g/dl	4.1±0.9	4.3±0.2	3.4-4.8
ALT	IU/L	25.6±3.01	27.5±3.3	18-45
AST	IU/L	78.6±3.85	83.3±2.5	74-143
ALP	IU/L	65.8±4.3	76.2±4.1	62-230
Creatinine	mg/dl	0.41±0.02	0.22±0.01	0.2-0.5
Urea	mg/dl	20.8±1.91	18.8±3.02	12.3-24.6
Total cholesterol	mg/dl	40.2±2.09	38.3±3.3	37-85
Triglyceride	mg/dl	23.6±1.7	21.1±1.3	20-114
Glucose	mg/dl	105.3±2.3	118.2±4.3	70-208

Results are expressed as mean±SE from six observations. ALT: Alanine transaminase; AST: Aspartate transaminase; ALP: Alkaline phosphatase; EEMTAP: Ethanol extract from *Macrothelypteris torresiana* aerial parts; SE: Standard error

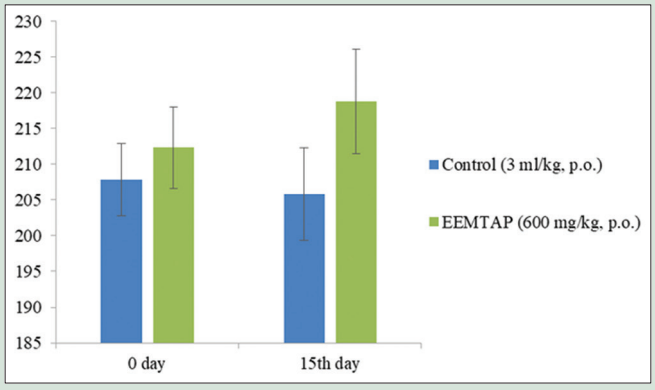

Figure 2: Effects on body weight of rats after treatment with ethanol extract from *Macrothelypteris torresiana* aerial parts

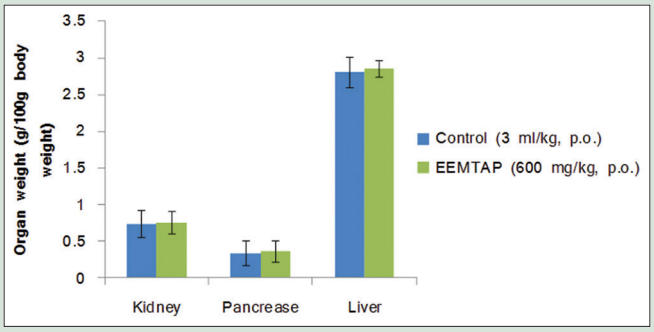

Figure 3: Effects on organ weights of male Wistar rats after oral administration of ethanol extract from *Macrothelypteris torresiana* aerial parts

perspiration and piloerection) and central nervous system (ptosis and drowsiness) effects throughout the experimental period. There was no significant difference between control and EEMTAP-treated groups in organ weight [Figure 3]. In the biochemical parameters evaluated, all parameters remained almost unchanged as nonsignificant variations were observed. All the values of the biochemical parameters for both the control and test groups fall within the normal range, as shown in Table 2. The result concluded that all hematological parameters such as total RBC count, hemoglobin, platelet count, and total WBC count including differential leukocyte count are within normal range in both treated and control groups during the experimental period [Table 3]. Histopathological analysis of kidney, pancreas, and liver of rats was performed on the 15th day after administration with control vehicle and EEMTAP (600 mg/kg, p.o.). There was no strong evidence of acute tubular necrosis and glomerular changes for the extract-treated groups when compared to the observations of the control groups [Figure 4a and b]. Multiple sections of rats' pancreas showed normal architecture in control treated group, whereas in extract-treated group, almost negligible abnormalities were observed in the architecture of both pancreatic acini and islets [Figure 4c and d]. Similarly, multiple sections of the liver showed normal lobular architecture in control treated group. There was also no evidence of bile stasis, granuloma, dysplasia, or malignancy in both the groups [Figure 4e and f].

Diuretic activity

In the present study, we can demonstrate that the single dose-response administration of the EEMTAP (200, 400, and 600 mg/kg, p.o.) significantly increased ($P < 0.05$) the volume of urine as well as urinary electrolyte concentration in dose-dependent manner when compared with the reference standard furosemide (5 mg/kg, p.o.). Further, EEMTAP was found to be more effective in enhancing urinary electrolyte concentration for all the three ions tested (Na^+, K^+, and Cl^-) when compared with control group animals. The results are compiled in Table 4.

Table 3: Effects of ethanol extract of *Macrothelypteris torresiana* aerial parts on hematological parameters in male Wistar rats

Parameter	Unit	Normal control (3 ml/kg, p.o.)	EEMTAP (600 mg/kg, p.o.)	Normal range[22]
Platelet	10^3/µl	896±22.03	976±33.6	638-1177
Hemoglobin	g/dl	14.8±0.2	15.1±0.3	13.7-17.6
Red blood cell	10^6/µl	8.23±0.5	8.8±0.8	7.27-9.65
White blood cell	10^3/µl	6.1±0.5	6.3±0.7	1.96-8.25
Neutrophils	10^3/µl	0.71±0.03	0.93±0.03	0.22-1.57
Lymphocytes	10^3/µl	4.33±0.7	4.8±0.7	1.41-7.11
Eosinophils	10^3/µl	0.06±0.01	0.07±0.01	0.01-0.16
Monocytes	10^3/µl	0.08±0.01	0.07±0.01	0.03-0.18
Basophils	10^3/µl	00	0.00	0.0-0.05

Results are expressed as mean±SE from six observations. EEMTAP: Ethanol extract from *Macrothelypteris torresiana* aerial parts; SE: Standard error

Molecular docking results

To investigate the detailed intermolecular interactions, docking studies were carried out between the phenolic compounds (caffeic acid and quercetin) and the target protein CA-II enzyme co-complexed with furosemide (PDB ID: 1Z9Y). The detail results of interactions were shown in Figure 5 and score was obtained by these natural compounds in the following Table 5.

Laxative activity

The laxative activity for Method I, EEMTAP was studied in Wistar albino rats. The activity of the extract was found to be in a dose-dependent increase in fecal output of rats at selected dose levels. EEMTAP at the doses

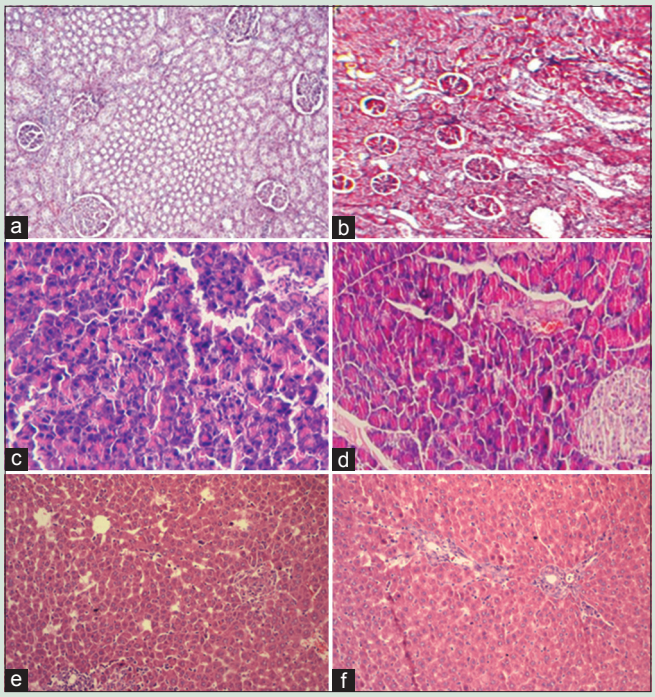

Figure 4: Photomicrographs of kidney, pancreas, and liver histopathology. (a) Section of rat kidney showing normal architecture for normal control group. (b) Section of rat kidney treated with ethanol extract from *Macrothelypteris torresiana* aerial parts (600 mg/kg, p.o.). (c) Section of rat pancreas showing normal architecture for normal control group. (d) Section of rat pancreas treated with ethanol extract from *Macrothelypteris torresiana* aerial parts (600 mg/kg, p.o.). (e) Section of rat liver showing normal architecture for control group. (f) Section of rat liver treated with ethanol extract from *Macrothelypteris torresiana* aerial parts (600 mg/kg, p.o.)

Table 4: Diuretic activity of ethanol extract of *Macrothelypteris torresiana* aerial parts in male Wistar rats

Groups	Treatment	Dose (mg/kg, p.o.)	Urine volume (ml)	Concentration of ions (mmol/l)			Na^+/K^+ ratio
				Na^+	K^+	Cl^-	
I	Control	Vehicle	3.1±0.55	63.33±2.15	74.8±2.33	57.01±4.26	0.86
II	Furosemide	20	12.83±1.33**	126.80±2.14**	63.81±3.21*	75.84±1.45**	1.98
III	EEMTAP	200	7.01±0.71*	97.21±3.02*	67.45±2.98*	61.63±4.05*	1.44
IV	EEMTAP	400	9.66±2.19**	118.16±2.11**	65.11±2.08*	70.23±0.80*	1.81
V	EEMTAP	600	11.6±1.41**	124.06±1.22**	63.05±2.61*	72.22±3.29**	1.96

Values are expressed as mean±SE (n=6). All columns are significant using ANOVA. *P<0.05, **P<0.01 when compared to control; Dunnett's t-test. EEMTAP: Ethanol extract from *Macrothelypteris torresiana* aerial parts; SE: Standard error; ANOVA: Analysis of variance

of 200, 400, and 600 mg/kg, p. o., increased significantly fecal output of rats compared to control group, and EEMTAP (600 mg/kg, p.o.) was found to be superior to that of the standard drug agar-agar (300 mg/kg, p.o.). The results are compiled in Figure 6. Similarly, the result for Method II in the loperamide-induced constipation, EEMTAP increased the total number of feces in a dose-dependent manner, and the results were statistically significant ($P < 0.05$) when compared with control group animals [Figure 7]. The reduction of the loperamide-induced constipation at 600 mg/kg, p.o., of the EEMTAP treatment was also found to be superior to that of the standard group treatment by 5 mg/kg, p.o., of sodium picosulfate.

Table 5: Summary of docking scores and interactions of phenolic compounds and furosemide with active site of amino acids of carbonic anhydrase-II enzyme

Compound	Docking score	Interactions with amino acids
Caffeic acid (689043)	−8.5	Thr 199
Quercetin (5280343)	−6.2	Thr 199, Pro 201
Furosemide (3440) (co-complexed in crystal)	−6.2	HIE* 119, HIP* 64, Thr 199

*HIE: Neutral histidine protonated at epsilon position; *HIP: Positively charged histidine at both delta and epsilon position. Figures in parenthesis denote PubChem CID. HIP: Positively charged Histidine; CID: Compound ID number

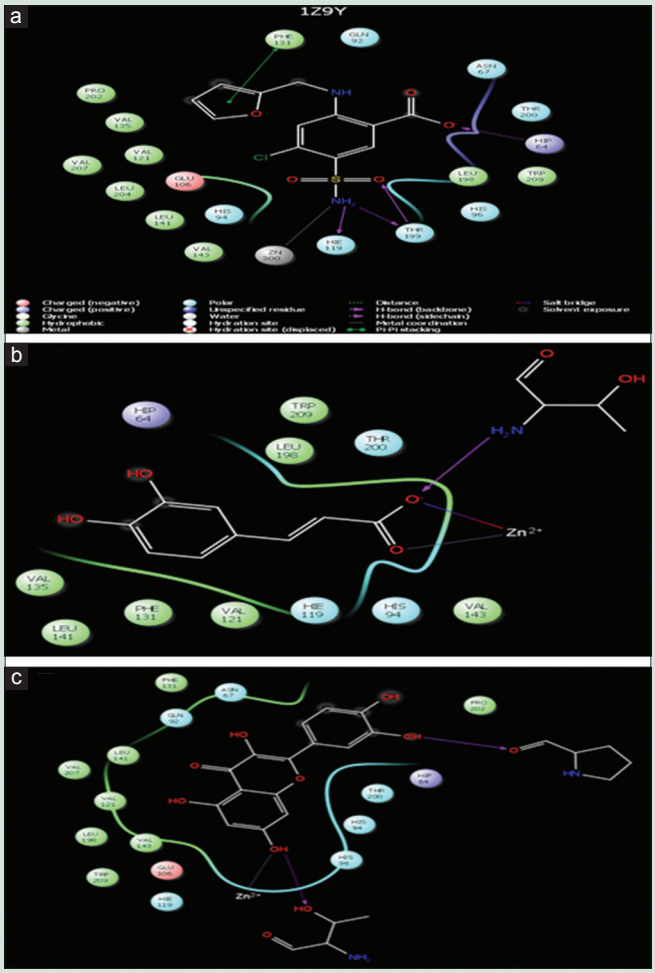

Figure 5: (a) Ligand plot diagram of furosemide showing interaction into the binding sites of carbonic anhydrase-II enzyme (PDB code: 1Z9Y), hydrogen bond (pink-dotted line) with HIE 119, HIP 64, and Thr 199 and pi–pi interaction (green solid line) with Phe 131. (b) Ligand plot diagram of caffeic acid showing interaction into the binding sites of carbonic anhydrase-II enzyme (PDB code: 1Z9Y), hydrogen bond (pink-dotted line) with Thr 199. (c) Ligand plot diagram of quercetin showing interaction into the binding sites of carbonic anhydrase-II enzyme (PDB code: 1Z9Y) and hydrogen bond (pink-dotted line) with Thr 199 and Pro 201

DISCUSSION

A plant may be considered as a biosynthetic laboratory, not only for the chemical compounds such as carbohydrates, proteins, and lipids that are utilized as food by man but also for a multitude of compounds such as glycosides, alkaloids, flavonoids, volatile oils, and saponins that exert a physiological effect. The compounds that are responsible for therapeutic effects are usually the secondary metabolites.[51] A systematic study of a crude drug embraces through consideration of both primary and secondary metabolites derived as a result of plant metabolism. The plant material may be subjected to preliminary phytochemical screening for the detection of various plant constituents. In many countries, herbal medicines and its derivatives have been used as an alternative to allopathic medicines in the treatment of various diseases. Despite the widespread use of herbal medicine for treating various diseases, there has been very few scientific studies conducted on herbals to provide knowledge about their efficacy and safety.[52] Acute toxicity is an initial study on the safety assessment of the drug and also provides us the basis for classification and labeling. It also provides initial information about the mode of toxic action of a substance by which we can fix a dose of a new compound and help in dose determination in animal studies.[23,53] Single-dose oral administration of EEMTAP in Swiss albino mice of either sex did not produce any abnormities in acute toxicity study. Subacute ingestion of EEMTAP produced behavioral change of very low intensity. The body weight and organ weight show very little change when compared with the control. Thus, EEMTAP does not alter much of the behavioral and general morphological changes. The hematopoietic system is considered one of the most sensitive targets for toxic compounds and an important index of physiological and pathological status in man and animal.[54] The hematological profile of rats after treatment with extracts showed values which falls within the normal range values of clinical laboratory parameters. Biochemical parameters were also studied, and they showed very little variation when compared with the control, and they also fall within the normal range of biochemical parameters of rats. This indicates that the subacute administration of EEMTAP is not able to produce toxic effects on the hematological and biochemical profile of rats. Diuretics relieve pulmonary congestion and

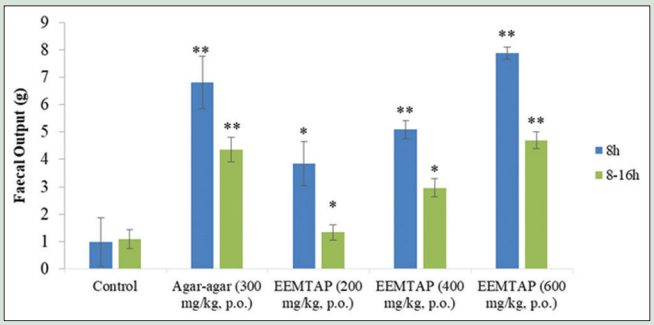

Figure 6: Effect of ethanol extract from *Macrothelypteris torresiana* aerial parts on laxative activity. Values are expressed as mean ± standard error ($n = 6$). All columns are significant using analysis of variance. *$P < 0.05$, **$P < 0.01$ when compared to control; Dunnett's *t*-test

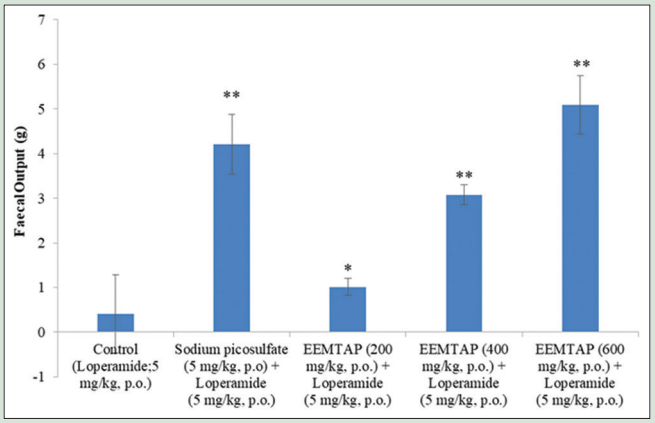

Figure 7: Effect of ethanol extract from *Macrothelypteris torresiana* aerial parts on loperamide-induced constipation in adult Wistar rats. Values are expressed as mean ± standard error ($n = 6$). All columns are significant using analysis of variance. *$P < 0.05$, **$P < 0.01$ when compared to control; Dunnett's *t*-test

peripheral edema and are useful in reducing the syndrome of volume overload, including orthopnea and paroxysmal nocturnal dyspnea. They decrease plasma volume and subsequently venous return to the heart (preload). This decreases cardiac workload, oxygen demand, and plasma volume, thus decreasing blood pressure.[55] Thus, diuretics play an important role in hypertensive patients. We can demonstrate that the EEMTAP significantly increased the urinary output as well as urinary electrolyte concentration at all tested dose level. Further, the EEMTAP was found to be more effective in enhancing urinary electrolyte concentration for all the three ions tested (Na^+, K^+, and Cl^-). The increase in the ratio of concentration of excreted sodium and potassium ions indicates that the extracts increase sodium ion excretion to a greater extent than potassium, which is a very essential requirement of an ideal diuretic with lesser hyperkalemic side effect.[43] The presence of phytoconstituents such as flavonoids, terpenoids, and saponins has been previously found to be responsible for diuretic and laxative activities in plants,[56,57] thereby various phytoconstituents such as protoapigenin, apigenin, kaempferol, quercetin, and caffeic acid were reported from the *M. torresiana* which may be responsible for the observed diuretic and laxative activities.

Plant phenolic compounds are known to display many pharmacological activities. Several natural phenols such as luteolin-5-O-β-glucoside, apigenin, and vicenin showed effective against anhydrase-II (CA-II) and urease using microtiter assays.[58,59] The phenolic compounds and acids had marked, especially CA-I and CA-II inhibitory effects, and might be used as leads for generating CA isoenzyme inhibitors. This class of compounds may lead to isoform-selective inhibitors targeting just one or few of the medicinally relevant CAs, thereby in this study, a combined computational approach was applied to gain insight into the structural basis and selectivity mechanism for the diuretic activity. The molecular docking studies with phenolic compounds into the binding cavity of CA-II enzyme showed the analogs having more favorable interaction than furosemide with better docking scores and hydrogen-bonding interactions because the caffeic acid and quercetin bind more externally within the active site cavity, making contacts with the catalytic zinc ion and with various amino acid residues. Hence, herewith, we can conclude that the promising diuretic activities of the EEMTAP are mainly due to the presence of the phenolic compounds.

Similarly, the EEMTAP significantly accelerated stool frequency and suitable for constipation. The presence of phytoconstituents such as terpenoids, sterols, flavonoids, phenolic compounds, tannins, and alkaloids[19,20] has been previously found to be responsible for laxative activities in plants. Phytochemical screening of the EEMTAP revealed the presence of flavonoids, phenols, and tannins. These constituents may be responsible for the laxative activity.

CONCLUSION

The present research demonstrated that the ethanol extract from *M. torresiana* aerial parts (EEMTAP) possesses promising diuretic and laxative activities in a dose-dependent manner. Acute and subacute toxicity study conducted on this plant showed that it has minimal amount of toxic effect on animals. Thus, we can say that ethanolic extract from *M. torresiana* aerial parts possesses significant diuretic and laxative activities with a reasonable safety profile. Further investigations are required to identify the phytoconstituents which are responsible for the following activities and also study their mechanism of actions.

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Conflicts of interest

There are no conflicts of interest.

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