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COMPARATIVE PHYTOCHEMICAL & PHARMACOLOGICAL STUDY ON ENHYDRA FLUCTUANS, ALTERNANTHERA PHILOXEROIDES AND CHENOPODIUM ALBUM

Sarker Ramproshad[1], Banani Mondal[1], Milon Mondal[2], Shaikh Jamal Uddin[1], Md. Golam Hossain[1]

[1]Pharmacy Discipline, Khulna University, Khulna- 9208, Bangladesh.

[2]Department of Pharmacy, Jahangirnagar University, Savar, Dhaka-1342, Bangladesh.

Email address: ramproshad131135@gmail.com

Abstract

Edible herbs for traditional medicine possess large amount of components which can be used to treat many diseases. Extracts from three edible herbs namely *Enhydra fluctuans, Alternanthera philoxeroides* and *Chenopodium album* have been investigated for the antioxidant, antibacterial, anti- nociceptive and anti-inflammatory activities using different in-vitro and in-vivo assay models. After acute toxicity test, the anti- nociceptive activity was evaluated by acetic acid induced writhing and formalin-induced paw licking method. Then anti-inflammatory activity was performed by xylene induced ear edema and cotton pellet granuloma model while the antioxidant activity was evaluated using DPPH, Nitric oxide, Hydrogen peroxide and Hydroxyl radical scavenging assay. The antibacterial activity was done by disk diffusion method. *E. fluctuans* extract showed higher DPPH, Nitric oxide, Hydrogen peroxide and Hydroxyl radical scavenging activities than *A. philoxeroides* and *C. album*. Besides, total phenol, total flavonoid and total antioxidant contents are higher in *E. fluctuans* than other two herbs. In ferric reducing and reducing power capacity assays, extracts exhibited dose dependent reducing capacity but *E. fluctuans* showed markedly response. *E. fluctuans* extract showed significant antibacterial activity against all tested microorganisms with more potent activity against *Shigella sonnei* (10.04mm). In anti- nociceptive activities, *E. fluctuans* (500 mg/kg) inhibited 67.19% writhing, 65.42% formalin-induced paw licking. In the case of anti-inflammatory activities, *E. fluctuans* (500 mg/kg) showed 67.16% inhibition in xylene induced ear edema which was the highest among the tested herbs, and 73.70% inhibition in cotton pellet granuloma model where the extract of *E. fluctuans* showed highest % inhibition among the three extracts. The results demonstrated that all the herbs tested in this study especially *E. fluctuans* can be used as a potential source for the above mentioned bioactivities and could be a source for novel lead compound.

Keywords: *Enhydra fluctuans, Alternanthera philoxeroides, Chenopodium album, Antioxidant, Antibacterial, Anti-nociceptive and Anti-inflammatory*.

Introduction

Edible herbs have been used as natural remedies for curing many physiological disorders which are in great demand both in developed and developing countries. Edible herbs provide the minerals like sodium, potassium, magnesium, iron, calcium, phosphorus etc. to maintain healthy life. Free radicals are one of the major barriers to maintain healthy life. By mopping up the free radicals, antioxidants prevent radicals induced disorders. Recently food scientists and nutrition specialists have agreed that food antioxidants contribute to the maintenance of good health.[1] Herbs remain an important recourse to combat serious diseases in the world. With the continuous use of antibiotics, microorganism have become resistant.[2] Therefore, there is a need to develop alternative antibacterial drugs for the treatment of infectious diseases; one approach is to screen local edible herbs for possible antimicrobial properties. Research to discover other alternatives to treat pain is crucial. Medicinal herbs have been used for centuries for therapeutic purposes. Many of these herbs with analgesic activity had been used without any adverse effects. Many medicinal edible herbs have shown to exhibit potent anti-inflammatory effect in the treatment of inflammation by using various models. In this research, alkaloids, carbohydrates, glycosides, flavonoids, saponins, steroids and tannins were identified in *E. fluctuans, A. philoxeroides* and *C. album* through phytochemical evaluation. *E. fluctuans* (Family:Asteraceae) is an annual herb and used in ascites, dropsy and anasarca. Leaf paste is applied over head as a cooling agent and around the inflamed brest to reduce inflammation.[3] *A. philoxeroides* (Family: Amaranthaceae) is used against coughing up blood, hematuria, cold and pyrexia, measles, encephalitis B, stranguria with turbid urine, eczema, anthracia and furunculosis. Whereas, *C. album* (Family: Chenopodiaceae) is widely introduced and improves the appetite, acts as anthelmintic, laxative, diuretic and tonic. It is also useful in biliousness, abdominal pain and eye diseases. It is used in the form of pot herb in piles. The finely powdered leaves are used as a dusting powder about the external genitalia in children.[4]

Materials & Methods

2.1. Chemicals, reagents and equipments

Folin-Ciocalteu reagent, Methanol, Ethanol, Sodium Phosphate (Na_3PO_4) and Ammonium molybdate were purchased from Merck, Germany. Sodium carbonate, Potassium Acetate and Concentrated H_2SO_4 (98 %), Cupric Chloride, Neocaproin, Ammonium acetate Buffer (pH 7.0), were purchased from Merck (India) Limited. Gallic acid, Quercetin and 1, 1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma Chemicals, USA. Aluminium Chloride and Ascorbic acid were purchased from SD Fine Chem. Ltd., Biosar, India. Sodium Nitroprusside was purchased from Ranbaxy lab, Mohali, India. Griess reagent(1% sulphanilamide, 0.1%naphthylethylenediamine dichloride and 3% phosphoric acid) was purchased from Rouch-light, Suffolk, England. Potassium Ferricyanide was purchased from May and Becker, Dagenham, UK. Trichloro Acetic acid and Ferric Chloride were purchased from Fine Chemical, India. Diclofenac Na was obtained from Beximco Pharmaceuticals Ltd., Bangladesh. Heparin inj. was purchased from Rotexmedica, Germany. Spectrophotometer (Shimadzu UV PC-1600) was purchased from Japan. All reagents and chemicals used were of analytical grade.

2.2. Plant material identification and preparation of the extract

Whole plant of *E. fluctuans, A. philoxeroides* and *C. album* were collected in May 2013 from Khulna, Bangladesh and identified by the experts of Bangladesh National Herbarium, Mirpur, Dhaka where voucher specimen were deposited for each sample (*E. fluctuans*(DACB:36566) *A. philoxeroides* (DACB: 36567) and *C. album* (DACB: 36568)). The herbs were dried in a hot air oven at moderate temperature (not more than 50°C) to make suitable for grinding purpose. After drying, herbs were ground to coarse powder separately with a mechanical grinder (Grinding Mill). Plant materials used for extraction were 500 g. The powdered materials were extracted by Soxhlet apparatus at 65°C temperature using 500 mL methanol as a solvent. The filtrates obtained were dried at temperature of 40±2°C using rotary evaporator to have gummy concentrate of the crude extracts. Each extract was preserved in suitable container in cold and dry place with appropriate labeling. Extraction

yield was obtained from *E. fluctuans, A. philoxeroides* and *C. album* were 11.34, 8.67 and 7.23g respectively.

2.3. Phytochemical screening

The methanol extracts of *E. fluctuans*, *A. philoxeroides* and *C. album* were undergone phytochemical group tests to detect the presence of major phytochemical constituents like alkaloids, carbohydrates, glycosides, flavonoids, saponins, steroids and tannins.[5]

2.4. DPPH free radical scavenging assay

DPPH scavenging activity of the extract was measured according to a reported method.[6] Briefly, in different test tubes 1 mL of each extracts or standard ascorbic acid of different concentration (800, 400, 200, 100, 50, 25, 12.5, 6.25 µg/mL) solutions were taken and 2 mL of 0.004 % DPPH solution in the solvent was added to each test tube to make the final volume 3 mL. After 30 min, the absorbance was measured at 517 nm. The percentage (%) inhibition activity was calculated from the following equation: $\{(A_0 - A_1)/A_0\}\times100$, Where, A_0 is the absorbance of the control, and A_1 is the absorbance of the extract/standard. IC_{50} was calculated by using regression analysis.

2.5. Nitric oxide scavenging capacity assay

The Nitric Oxide Scavenging Capacity of extract was determined by the established method.[7] After adding extracts in varying concentrations (160, 80, 40, 20, 10 µg/mL), Sodium nitroprusside (5mM) in phosphate buffer was added to each test tube to make volume up to 1.5ml. Solutions were incubated at 25°C for 30 minutes and then 1.5ml of Griess reagent was added to each test tube. The absorbance was measured at 546 nm. The percentage (%) inhibition was calculated by following equation $\{(A_0 - A_1)/A_0\}\times100$; where, A_0 is the absorbance of the control and A_1 is the absorbance of the extract or standard. IC_{50} was calculated by linear regression method.

2.6. Hydrogen peroxide radical scavenging assay

The ability of the extracts to scavenge hydrogen peroxide was determined by Nabavi *et al.*, 2009.[8] Different concentrations (12.5, 25, 50, 100, 200, 500 µg/mL) of extracts and standard (ascorbic acid) were prepared in distilled water. Then these were added to a hydrogen peroxide solution (6 ml, 40 mM). 1 ml of each mixture was taken into test tube and 3 ml of phosphate buffer solution was added to each mixture. A blank solution containing phosphate buffer without hydrogen peroxide was prepared. The absorbance of hydrogen peroxide at 230 nm was determined after ten minutes. The percentage of hydrogen peroxide scavenging by the extracts and standard compound was calculated as follows: % Scavenged of $[H_2O_2] = [(A_0 - A_1)/A_0] \times 100$, Where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the sample of extract and standard. IC_{50} was calculated by linear regression method.

2.7. Hydroxyl radical scavenging assay

Hydroxyl radical scavenging activity of the extract was measured by the published method.[9] 0.5 mL 2-deoxy-2-ribose solution (2.8 mM) was mixed with 12.5 µL of different concentrations (12.5, 25, 50, 100, 200, 500 µg/mL) of sample extracts or standard (ascorbic acid). Then 1 mL of 200 µM $FeCl_3$, 1 mL of 1.04 mM EDTA, 0.5 mL of 1 Mm H_2O_2 and 0.5 mL of 1 mM of ascorbic acid were added to prepare the reaction mixture. After an incubation period of 1 hour at 37°C, 3.75 mL of 2.8 % TCA was added to the reaction mixture. 3.75 mL of 1 % TBA was added and kept at 100°C for 20 mins. The absorbance was measured at 530 nm. The hydroxyl radical scavenging activity was calculated by the following equation: % of OH radical scavenging activity = $[(A_0 - A_1)/A_0] \times 100$, Where, A_0 was the absorbance of the blank and A_1 was the absorbance of sample and standard. IC_{50} was calculated by linear regression method.

2.8. Determination of total phenolic content

Total phenolic content was determined by a reported method.[10] 1.0 mL of each plant extracts or standard of different concentration (50, 100, 150, 200, 250 mg/mL) solution were taken in test tubes and 5 mL of Folin ciocalteu (Diluted 10 fold) reagent solution was added to the test tubes. 4 mL of Sodium carbonate solution was added into the test tubes. The test tubes were incubated for 30 minutes at 20°C (standard) and 1 hour at 200°C (extracts). The absorbance was measured at 765 nm. Standard curve was prepared and total phenol value was expressed in terms of Gallic acid equivalent.

2.9. Determination of flavonoid content

Total flavonoid was determined using the aluminum chloride colorimetric method.[11] 1.0 mL of each extracts (200 µg/mL) or standard of different concentration (100, 50, 25, 12.5, 6.25 µg/mL) solution was taken in test tube and 3 mL of methanol was added. Then 200 µL of 10 % aluminum chloride solution was added into the same test tube, followed by the addition of 200 µL of 1 M potassium acetate solution. Finally, 5.6 mL of distilled water was mixed with the reaction mixture and then incubated for 30 min at room temperature and absorbance was measured at 415 nm. The total flavonoid content in methanol extracts were expressed in mg/g quercetin equivalent (QE).

2.10. Determination of total antioxidant capacity

Total antioxidant capacity was determined using the phosphomolybdenum method.[12] In different test tubes 300 µL (200 µg/mL) of each extracts or standard of different concentration (200, 100, 50, 25, 5 µg/mL) solutions were taken and 3 mL of reagent solution was added into each of the test tubes and then incubated at 95°C for 90 min. The absorbance was measured at 695 nm. The antioxidant activity was expressed as mg/g ascorbic acid equivalent (AAE).

2.11. Ferric reducing antioxidant power assay

Ferric Reducing Antioxidant Power Assay of extract was determined by established method.[13] Plant extracts (0.15 ml) and standard solutions (0.15 ml) of different concentration (2.00, 1.00, 0.50, 0.25 0.12 0.06 µg/mL) were allowed to react with 2.85 ml of FRAP solution for 30 min in the dark condition. After 30 minutes, absorbance was taken at 593 nm.

2.12. Reducing power capacity assessment

Reducing power capacity assessment of the extracts was determined using reported method.[14] 2.0 ml of each extract or standard of different concentration (2.00, 1.00, 0.50, 0.25 0.12 0.06 µg/mL) were taken and 2.5 ml of 1% potassium ferricyanide was added into each test tube. The test tubes were incubated for 10 min at 50° C and 2.5 mL of tri-chloroacetic acid (10%) was added. The mixtures were centrifuged at 3000 rpm for 10 min and 2.5 ml supernatant solution were withdrawn from each of the mixtures and mixed with 2.5 mL of distilled water. Then 0.5 mL of ferric chloride ($FeCl_3$), 0.1% solution was added. The absorbance was measured at 700 nm.

2.13. Antibacterial activity

Antibacterial activity of methanol extracts was determined by disc diffusion method.[15] Both gram positive (*Shigella dysenteriae, Escherichia coli, Shigella flexneri, Shigella sonnei, Vibrio cholera, Shigella boydii* and *Pseudomonus spp.*) and gram-negative (*Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus pyogens, Streptococcus agalactiae* and *Enterococcus faecalis*) bacterial strains were taken for the test. These organisms were collected from the Microbiology Lab. of Pharmacy Discipline, Khulna University, Khulna. In an aseptic condition, the test organisms were transferred from the subculture to 5 mL of nutrient broth contained in screw-capped test tubes using a transfer loop. Extracts of 250 and 500 µg/disc concentrations were used for this investigation. Standard disc of Kanamycin (30 µg/disc) and blank discs were used as positive and negative control, respectively. 100 µL of bacterial cell suspension was spread throughout the plates by spread plate method, using sterile 'L' shaped spreader. Then the discs were placed in the agar plates and kept in a refrigerator at 4°C for about 24 hours upside down to allow sufficient diffusion of the materials from the discs to the surrounding agar medium. The plates were then inverted and kept in an incubator at 37°C for 24 h. After incubation, the antibacterial activities of the test materials were determined by measuring the diameter of the zones of inhibition in millimeter with a transparent scale.

2.14. Animal's experimental setup

Sprague Dawley female rats of 120–140 g and Swiss albino female mice of 25–30 g were collected from Pharmacology Laboratory, Department of Pharmacy, Jahangirnagar University and were acclimatized to normal laboratory conditions for one week prior to study and were assessed to pellet diet and water ad libitum. Temperature of facility was 25 ± 5°C and light/darkness alternated 12 hours apart. The animals were divided into five groups of five animals each. The study was conducted following the approval by the Institutional Animal Ethical Committee of, Bangladesh.

2.15. Acute toxicity study

According to the OECD guideline, mice were divided into five groups of ten animals each. Different doses (250, 500, 1000, 2000, and 4000 mg/kg) of methanol extract were administered by stomach tube. Then the animals were observed for general signs of toxicity.

2.16. Anti-nociceptive activity evaluation

2.16.1. Acetic acid induced writhing

A reported method was employed for this test.[16] Eight groups of mice (5 mice in each group) were pretreated with control, Diclofenac Na (100 mg/kg) and the extract (250 and 500 mg/kg). Forty-five minutes later each mouse was injected i.p. with 0.7% acetic acid at a dose of 10 mL/kg body weight. The number of writhing responses was recorded for each animal during a subsequent 5 min period after 15 min i.p. administration of acetic acid. The percentage inhibition of writhing was calculated using the following formula: % Inhibition= [(1– No. of Writhing (Drug/Standard))/ No. of Writhing (Control)]× 100.

2.16.2. Formalin-induced paw licking

An established method was employed for this test.[17] Mice were divided into 8 groups of 5 animals each. Group 1, the control group received normal water, p.o., group 2, the standard group received Diclofenac (100 mg/kg) and other 6 groups received the extracts at 250 and 500 mg/kg doses. After 1 hr. drug administration 2.7% formalin was injected into the dorsal surface of the left hind paw. The time spent licking the injected paw was recorded. Animals were observed for the 5 min post formalin (acute phase) and for 5 min starting at 20 min post formalin injection (delayed phase).

2.17. Anti-inflammatory activity evaluation

2.17.1. Xylene induced ear edema in mice

The xylene induced ear edema test was performed by a published method.[18] The tested samples including Diclofenac Na (100 mg/kg) as a positive control and extracts (250 and 500 mg/kg) were given orally to the mice. One hour later, each animal received 20 μL of xylene on the anterior and posterior surfaces of the right ear lobe. The left ear was considered as control. Mice were sacrificed one hour after xylene application and circular sections were taken, using a cork borer with a diameter of 3 mm, and weighed. The weight of edema was considered as the difference between weight of ear treated with xylene (right ear) and the weight of ear without xylene treatment (left ear). The percentage inhibition was calculated by the following formula: % Inhibition = (1 – Weight of Edema (Drug/Standard)/Weight of Edema (Control)) × 100.

2.17.2. Cotton Pellet Induced Granuloma Formation in Rat

The cotton pellet induced granuloma method was performed by reported method.[19] Sterilized cotton pellets (40±1mg) were impregnated subcutaneously on both side of the abdomen of the animal, under ketamine anesthesia and sterile technique. Extracts at doses of 250 and 500 mg/kg were administered orally to test animals, in a once-daily dosage regimen for 7 days; the control group received vehicle only. Diclofenac (100 mg/kg) was used as a reference drug. The rats were sacrificed on the 8th day and the cotton was removed and dried at 60°C for 24hrs, and dry cotton weight was recorded. The weight difference of dry cotton and the cotton before implantation is considered as weight of granuloma formed. The percentage inhibition of granuloma formation was calculated by the following formula: % Inhibition= (1–Weight of Granuloma (Drug/Standard)/Weight of Granuloma (Control)) × 100.

2.18. Statistical Analysis

The results were expressed as the mean ± SEM (standard error of mean). The results were statistically analyzed using one way ANOVA followed by Bonferroni, LSD and Dunnett's multiple comparison tests. $P < 0.05$ and $P < 0.001$ was considered as statistically significant. Statistical programs used were GRAPHPAD PRISM (version 6.00; GraphPad Software Inc., San Diego, CA, USA).

Results

3.1. Phytochemical screening

In *Table* 1, the colorimetric phytochemical screening displays that methanolic extracts of *E. fluctuans* (EFMe), *A. philoxeroides* (APMe) and *C. album* (CAMe) showed the positive test indications for alkaloids, flavanoids and tannin. *A. philoxeroides*

and *C. album* exhibited a little extent of presence of carbohydrates. However, all extracts showed negative test results for steroids.

3.2. Antioxidant activity evaluation

Methanolic extract of *E. fluctuans, A. philoxeroides, C. album* were found to exert DPPH Radical Scavenging Activity in a decreasing order of EFMe > CAMe >APMe (*Table* 2). IC_{50} (µg/ml) for *E. fluctuans, C. album* and *A. philoxeroides* were 91.80 ± 0.26, 114.33 ± 2.40, 443.38 ± 0.38 respectively against Ascorbic Acid (Standard), which IC_{50} (µg/ml) is 13.91 ± 0.08.

In comparison, extract of *E. fluctuans* showed IC_{50} (µg/ml) 165.99 ± 0.58, greatest nitric oxide radical scavenging activity among all where *A. philoxeroides* and *C. album* demonstrated IC_{50} (µg/ml) values 228.11 ± 0.61 and 492.97 ± 0.11 respectively. But less than Ascorbic acid (Standard) which IC_{50} (µg/ml) value was 49.15 ± 0.13. All values were presented in *Table* 2.

In *Table* 2, extract of *E. fluctuans* exhibited a significant IC_{50} (µg/ml) value: 42.21 ± 0.31 hydrogen peroxide radical scavenging, which was near about the reference compound Ascorbic acid which IC_{50} (µg/ml) value was 12.57 ± 0.21 but markedly differed with *A. philoxeroides*, IC_{50} (µg/ml) value was 210.85 ± 0.41 and *C. album* IC_{50} (µg/ml) value was 193.01 ± 1.07.

From *Table* 2, hydroxyl radical scavenging activity of *E. fluctuans*, IC_{50} (µg/ml) value 78.08± 0.79, appeared to be closed to standard compound ascorbic acid which exerted IC_{50} (µg/ml) value 15.78± 0.06 in comparison to *A. philoxeroides* (IC_{50} (µg/ml) value151.80± 0.15) and *C. album* (IC_{50} (µg/ml) value 128.35± 0.21). The magnitude of IC_{50} (µg/ml) for the extracts can be ranked up as decreasing order as follows EFMe > CAMe >APMe.

Total phenolic content were presented in *Table* 3. Extracts of both *E. fluctuans* (111.48 ± 0.29 mg/gm, GAE) and *A. philoxeroides* (109.25 ± 0.43 mg/gm, GAE) was found nearly similar but higher than *C. album* (72.34 ± 0.12 mg/gm, GAE).

Extracts of *E. fluctuans* demonstrated a remarkable higher value 225.13 ± 0.16 mg/g of total flavonoid content whereas other two extracts of *A. philoxeroides* and *C. album* showed values 78.52 ± 0.22 mg/g and 22.08 ± 0.86 mg/g respectively which were given in *Table* 3.

In *Table* 3, the magnitude of total antioxidant capacity of extracts of both *A. philoxeroides* and *C. album* exhibited closer, 30.53 ± 0.30 mg/g and 31.22 ±0.58 mg/g respectively, less than *E. fluctuans* which value was 58.63 ± 0.19 mg/g.

Unlike total phenol content and total flavonoid content, total tannin content of the extracts didn't vary such a great extent. The values of total tannin content of extracts of *E. fluctuans, A. philoxeroides* and *C. album* were 20.65 ± 0.29 mg/g, 15.16 ± 0.09 mg/g, 22.34 ± 3.95 mg/g respectively (*Table* 3).

The absorbance of standard compound ascorbic acid and *E. fluctuans* raised markedly with increased concentration which were seen in Figure 1. *E. fluctuans* followed the curve line of ascorbic acid and exhibited better response than those of *A. philoxeroides* and *C. album*. (Fig. 1)

In Figure 2, *E. fluctuans*, *A. philoxeroides* and *C. album* showed moderate responses in reducing power capacity assessment contrast with reference standard compound ascorbic acid. (Fig. 2)

3.3. Antibacterial activity evaluation

The ratio of EFMe 500 µg to standard kanamycin 30 µg was found to provide an outstanding result of zone diameter (mm) of inhibition against the experimental bacterial strains in such extent that 21:29 for *Staphylococcus aureus*, 20:24 for *Staphylococcus epidermidis*, 25:29 for *Streptococcus agalactiae*, 21:29for Vibrio cholera, 20:28 for *Shigella dysenteriae*, 25: 34 for Escherichia coli, 17:18 for *Shigella boydii*, 10:8 for *Shigella sonnei*. CAMe 500 also carried out moderate response against few bacterial strains however APMe 250 and APMe 500 exhibited almost nil activity (*Table* 4).

3.4. Anti-nociceptive activity evaluation

Acetic acid induced writhing assay result in the *Table* 5 exhibited dose dependent distinct inhibition (%) in the case of EFMe and CAMe. EFMe 250 (mg/kg) was found to cause 37.5% inhibition of writhing responses whereas EFMe 500 (mg/kg) displayed notable 67.19% inhibition which was as similar as standard compound Diclofenac Na (73.44% inhibition). CAMe 500 (mg/kg) also exerts moderate inhibition (34.36%) against control group. (Table 5)

Alike acetic acid induced writhing assay, both EFMe and CAMe responded meaningfully inhibition in the case of Formalin-Induced Paw Licking, which were demonstrated in *Table* 6. EFMe 500 (mg/kg) demonstrated for the first five minutes 51.34% inhibition and for the second five minutes 65.42% inhibition against reference standard compound, Diclofenac Na 60.95% and 76.06% respectively. CAMe 250 (mg/kg) showed moderate level of inhibition against control group.

3.5. Anti-inflammatory activity evaluation

Table 7 showed the result of xylene induced ear edema test where EFMe 250 (mg/kg) responded 52.45%, EFMe 500 (mg/kg) responded 67.16% and CAMe 500 (mg/kg) responded 44.12% inhibition which is a very good response of inhibition against xylene induced ear edema comparable to reference standard compound, Diclofenac Na (84.80%). (Table 7)

Anti-inflammatory activity was evaluated by cotton pellet-induced granuloma formation test in rat. The result were showed in *Table* 8. EFMe 500 (mg/kg) carried out an excellent result of inhibition (73.70%) against cotton pellet-induced granuloma formation comparable to reference standard compound Diclofenac Na (85.99%). EFMe 250 (mg/kg) and CAMe 500 also exhibited noteworthy responses. (Table 8)

Discussion

Antioxidants are substances which can significantly delay or prevent the oxidation of an oxidisable substrate existing in low concentrations compared to the substrate.[20] The antioxidants are two types according to their size, the first one; the small molecule antioxidants, such as vitamin C, vitamin E, carotenoids, and glutathione (GSH), neutralize the ROS in a process called radical scavenging and carry them away, and the other one; large molecule antioxidants are enzymes (SOD, CAT, and GSHPx) and sacrificial proteins (albumin) that absorb ROS and prevent them from attacking other essential proteins.[21]

Initial phytochemical screening study suggested that methanolic extract of *E. fluctuans, A. philoxeroides* and *C. album* contained alkaloids, flavanoids, tannins and a little extent of glycosides and carbohydrates etc. Phenolics and flavonoids or combination of phenolics have been found as great antioxidants and proved to exhibit more activity than pure individual compound.[22] The proposed antioxidant properties of phenolic and flavonoid compounds are mediated by the following mechanisms: (1) scavenging radical species such as ROS/ reactive nitrogen species (RNS); (2) suppressing ROS/RNS formation by inhibiting some enzymes or chelating trace metals involved in free radical production; (3) up regulating or protecting antioxidant defense.[23]

During the investigation, there found significant level of total phenolic content of both EFMe and APMe nearly similar but higher than CAMe. The value of total flavonoid content also provided attractive observation for EFMe. Unlike total phenol content and total flavonoid content, total tannin content of the extracts didn't vary such a great extent. The values of total tannin content of EFMe, APMe, CAMe were nearly similar. It assumed that EFMe, APMe and CAMe would follow one of reaction mechanisms stated above for phenolic and flavonoid compounds. In regards of radical scavenging activity, [22] showed that phenolic compounds (POH) have phenolic hydroxyl groups and extended conjugated aromatic system for free radical scavenging activities. They act as free radical acceptors and chain breakers interfering with the oxidation of lipids and other molecules by rapid donation of a hydrogen atom to radicals (R): $R + POH \rightarrow RH + PO^{\cdot}$

Due to resonance and reacting with other free radicals, the phenoxy radical intermediates ($PO^{\cdot}$) are act as terminators of propagation route of free radicals.[22]

$PO^{\cdot} + R^{\cdot} \rightarrow POR$

Hence, different kinds of radical scavenging activities such as DPPH radical scavenging activity, Nitric Oxide radical scavenging activity, Hydrogen Peroxide radical scavenging and Hydroxyl radical scavenging activity of extracts are very important for determining the mode of antioxidant activity of the extracts. EFMe showed extra ordinary results for these test while two other extracts APMe and CAMe exhibited moderate response. The absorbance of EFMe for reducing power capacity followed the curve line of standard compound ascorbic acid and

exhibited better response than those of APMe and CAMe. Above data of test results indicated that EFMe acted predominantly as antioxidant through scavenging radical species and up regulating or protecting antioxidant defense and APMe and CAMe probably performed their antioxidant activity through suppressing ROS/RNS formation by inhibiting some enzymes or chelating trace metals involved in free radical production as they contained higher amount of tannins relatively to phenolic and flavonoids contents of EFMe because tannins predominantly perform antioxidant activity through suppressing ROS/RNS formation by inhibiting some enzymes or chelating trace metals involved in free radical production.[24]

Flavonoids are synthesized by plants in response to microbial infection, which exhibit antimicrobial activity by inhibiting nucleic acid biosynthesis and other metabolic processes and spore germination of plant pathogens and phenolic compounds has also been reported to be responsible for their antimicrobial activity by inhibiting hydrolytic enzymes (proteases) or other interactions that inactivate microbial adhesins, cell envelope transport proteins and non-specific interactions with carbohydrates.[25] Tannins are being successfully used as feed additives in poultry to control diseases and to improve animal performance. The most accepted mechanism of tannins as antimicrobial has been reported to modulate of the gut microbiota which composition influences the intestinal environment and the development and responses of the host immune system against pathogenic and non-pathogenic antigens.[26] The result of EFMe 500 µg was found more outstanding for zone diameter (mm) of inhibition against the experimental gram positive and gram negative bacterial strains such as Staphylococcus aureus, *Staphylococcus epidermidis, Streptococcus agalactiae, Escherichia coli, Vibrio cholera, Shigella dysenteriae, Shigella boydii*, than EFMe 250µg. It is clearly indicating that EFMe 500 µg contains more phenolic, flavonoids and tannins that's why EFMe 500 µg exerted such strong antimicrobial activity. Surprising matter is that EFMe 500 µg carried out better response for Shigella sonnei than standard kanamycin. CAMe 500 µg also provided moderate response against few bacterial strains however APMe 250 µg and APMe 500 µg exhibited almost nil activity.

Pain typically involves a noxious stimulus or event that activates nociceptors in the body's tissues that convey signals to the central nervous system, where they are processed and generate multiple responses, including the "unpleasant sensory and emotional experience" central to the IASP definition. Formalin induced pain model is a biphasic anti-nociceptive procedure which was performed to understand the mechanism of analgesia of the extracts whether they act as like as the centrally acting drug through inhibiting both phase I and phase II while the peripherally acting drugs can only inhibit phase II of formalin induced pain model.[27] In this investigation, it was noticed that both EFMe and CAMe responded meaningfully inhibition specially EFMe 500 (mg/kg) demonstrated for the first five minutes 51.34% inhibition and for the second five minutes 65.42% inhibition against reference standard compound Diclofenac Na 60.95% and 76.06% respectively, indicating that the extracts caused the analgesic effect through central mechanism. Acetic acid application through intra-peritoneal route induces abdominal constrictions due to the release of arachidonic acid, which is the precursor for synthesis of prostaglandin via the cyclooxygenase (COX) enzyme. It is also responsible for stimulating the nociceptive neurons sensitive to non-steroidal anti-inflammatory drugs (NSAIDs) and opioids.[28] In acetic acid-induced writhing assay, a dose-dependent antinociceptive activity of the extracts was found. EFMe 500 (mg/kg) displayed notable 67.19% inhibition which was as same as standard compound Diclofenac Na (73.44% inhibition). CAMe 500 (mg/kg) also exerts moderate inhibition against control group. So, inhibiting acetic acid-induced pain, the extracts play role as antinociceptive agents by blocking prostaglandin synthesis, can be used as alternative natural antinociceptive agents.

Inflammation is a local response of body defense system to inhibit the spread of infection, causes triggers in inflammatory mediators such as TNF-α, interleukins and prostaglandins and consequently characterized by formation of edema, leucocytes infiltration and granuloma formation, tissue injury and repair.[29] Prostaglandins are generated by cyclooxygenase COX-1 and COX-2 pathway of

arachidonic acid metabolism. Anti-inflammatory agents are capable of inhibiting this pathway of prostaglandin synthesis.[30]

Due to side effects of immune suppressants, NSAIDs, corticosteroids and histamines, [31] natural sources having anti-inflammatory activity convey significant demand in research [32] showed that ethyl acetate extract of Anacardium occidentale which is rich of phenolic and polyphenolic compounds like (gallic acid, catechin, epicatechin and epigallocatechin able to inhibit inflammatory mediators such as PGE and Bradykinin, inhibition of cell migration to the site of inflammation, and its interference in levels of pro-inflammatory cytokines (TNF-α and IL-1) demonstrated the antioxidant and anti-inflammatory properties of flavonoids.[33] This may be contribute to the activity of scavenging free radicals and inhibition of arachidonic acid synthesis due to presence of a phenolic ring and hydroxyl groups in the structure of these compounds.

Moreover, flavones and catechins seem to play more effective role in protecting the body against reactive oxygen species and to inhibit degranulation of neutrophils and reduce complement activation.[32] Xylene is a chemical compound which causes acute inflammation manifesting severe vasodilation and edematous changes of skin.[34] EFMe 250 (mg/kg), EFMe 500 (mg/kg) and CAMe 500 (mg/kg) showed a very good response of inhibition of Xylene Induced Ear Edema in rat model comparable to reference standard compound Diclofenac Na. EFMe 500 (mg/kg) carried out an excellent result of inhibition (73.70%) against Cotton Pellet-Induced Granuloma Formation in rat model comparable to reference standard compound Diclofenac Na (85.99%). EFMe 250 (mg/kg) and CAMe 500 also exhibited noteworthy responses. These studies provided significant anti- inflammatory activity of EFMe and CAMe due to presence of highly phenolics and flavonoids content.

Conclusion

In the present investigation, methanol extract of *E. fluctuans* better antioxidant properties than other two herbs. It provides an outstanding result of zone diameter (mm) of inhibition against the experimental bacterial strains. In anti-nociceptive activity evaluation, *E. fluctuans* displayed notable % inhibition which was as similar as standard compound Diclofenac Na. This herb also carried out an excellent result of inhibition against xylene induced ear edema and cotton pellet-induced granuloma formation comparable to reference standard compound. Other two herbs extracts showed moderate activities. There is every possibility to suggest that activity may be due to the presence of different phytochemicals.

Conflict of interest

The authors have no conflict of interest to declare.

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Table 1: Phytochemical constituents identified in the methanol extracts of *E. fluctuans, A. philoxeroides* and *C. album* (++= strongly Presence; += Presence; -= Absence)

Phytochemicals	**Name of the test**	**Observed changes**	**Result**		
			E. fluctuans	***A. philoxeroides***	***C. album***
Alkaloids	Mayer's test	Creamy white precipitate	++	+	+
	Hager's test	Yellow crystalline precipitate	++	++	-
	Wagner's test	Brown or deep brown precipitate	+	+	+
	Dragendorff's test	Orange or orange-red precipitate	++	++	++
Carbohydrates	Molisch's test	A red or reddish violet ring is formed at the junction of two layer and on shaking a dark purple solution is formed	-	+	+
Glycosides	General test	Yellow color	++	-	-
	Test for glucoside	Production of brick-red precipitation (carried out with the hydrolyzed extract)	-	-	-
Flavonoids	General test	Red color	++	+	+
Saponins	Frothing test	Formation of stable foam	+	-	-
Steroids	Libermann–Burchard's test	Greenish color	_	_	_
Tannins	Lead acetate test	A yellow or red precipitate	+	++	+

Table 2: DPPH, NO, Hydrogen peroxide and Hydroxyl radical scavenging assay of different extracts of *E. fluctuans, A. philoxeroides* and *C. album*

Extracts/standard	DPPH (IC_{50} (µg/mL))	NO (IC_{50} (µg/mL))	H_2O_2 (IC_{50} (µg/mL))	•OH (IC_{50} (µg/mL))
Ascorbic acid	13.91 ± 0.08 [a]	49.15 ± 0.13 [a]	12.57 ± 0.21[a]	15.78± 0.06 [a]
E. fluctuans	91.80 ± 0.26 [b]	165.99 ± 0.58 [b]	42.21 ± 0.31[b]	78.08± 0.79 [b]
A. philoxeroides	443.38 ± 0.38 [d]	228.11 ± 0.61 [c]	210.85 ± 0.41[d]	151.80± 0.15 [d]
C. album	114.33 ± 2.40 [c]	492.97 ± 0.11[d]	193.01 ± 1.07[c]	128.35± 0.21 [c]

Values are the mean of triplicate experiments and represented as mean ± SEM. Values in same column with different superscripts are significantly different (p<0.05). One way ANOVA followed by Bonferroni multiple comparisons was performed to analyze data sets

***Table* 3.** Total phenol, total flavonoid, total antioxidant and total tannin of different extracts of *E. fluctuans, A. philoxeroides* and *C. album*

Extracts/standard	Total Phenol (mg/gm, GAE)	Total flavonoid (mg/g, QE)	Total antioxidant capacity (mg/g, AAE)	Total Tannin (mg/gm, GAE)
E. fluctuans	111.48 ± 0.29 [c]	225.13 ± 0.16 [c]	58.63 ± 0.19 [b]	20.65 ± 0.29 [a]
A. philoxeroides	109.25 ± 0.43 [b]	78.52 ± 0.22 [b]	30.53 ± 0.30 [a]	15.16 ± 0.09 [a]
C. album	72.34 ± 0.12 [a]	22.08 ± 0.86 [a]	31.22 ±0.58 [a]	22.34 ± 3.95 [a]

Values are the mean of triplicate experiments and represented as mean ± SEM. Values in same column with different superscripts are significantly different (p<0.05). One way ANOVA followed by Bonferroni multiple comparisons was performed to analyze data sets

Table 4: Antibacterial activity of methanol extracts of *E. fluctuans*, *A. philoxeroides* and *C. album* by disc diffusion method

Bacterial strains	Diameter of zone of inhibition in mm						
	Standard (Kanamycin) 30 µg	*E. fluctuans* 250 µg	*E. fluctuans* 500 µg	*A. Philoxeroides* 250 µg	*A. philoxeroides* 500 µg	*C. album* 250 µg	*C. album* 500 µg
Staphylococcus aureus	29.17 ± 2.67 ^{d}	14.14 ± 0.37 ^{c}	21.75 ± 0.97 ^{c}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	8.32 ± 0.39 ^{b}	14.39± 0.09 ^{c}
Enterococcus faecalis	32.77 ± 2.32 ^{d}	7.56 ± 1.01 ^{b}	14.31 ± 0.91 ^{c}	9.99 ± 0.74 ^{b}	12.99 ± 0.38 ^{b}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}
Staphylococcus epidermidis	24.53 ± 1.76 ^{e}	19.09 ± 0.84 ^{d}	20.24 ± 0.81 ^{d}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	5.59 ± 0.27 ^{b}	8.07 ± 0.03 ^{c}
Streptococcus pyogens	29.60 ± 2.53 ^{d}	11.16 ± 0.78 ^{b}	16.27 ± 0.58 ^{c}	8.40 ± 0.57 ^{b}	11.68 ± 0.95 ^{b}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}
Streptococcus agalactiae	29.04 ± 0.91 ^{d}	12.96 ± 0.76 ^{a}	25.94 ± 1.03 ^{c}	13.08 ± 0.10 ^{a}	12.89 ± 0.93 ^{a}	14.43± 0.21 ^{a}	20.71± 0.14 ^{b}
Vibrio cholera	29.43 ± 0.43 ^{d}	12.65 ± 1.77 ^{b}	21.14 ± 1.66 ^{c}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	13.33± 0.24 ^{b}	20.07± 0.25 ^{c}
Shigella dysenteriae	28.70 ± 0.95 ^{d}	11.50 ± 0.78 ^{b}	20.03 ± 0.67 ^{c}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}
Escherichia coli	34.49 ± 2.07 ^{e}	14.49 ± 0.55 ^{c}	25.04 ± 2.27 ^{d}	4.72 ± 0.35 ^{a}	8.67 ± 0.43 ^{b}	2.97 ± 0.41^{a}	9.48 ± 0.24^{b}
Pseudomonus spp.	28.97 ± 0.47 ^{e}	7.77 ± 0.34 ^{a}	11.66 ± 0.09 ^{c}	12.23 ± 0.54 ^{c}	17.29 ± 0.03 ^{d}	7.46 ± 0.49 ^{a}	9.73 ± 0.18 ^{b}
Shigella flexneri	30.69 ± 0.45 ^{e}	13.33 ± 0.22 ^{c}	15.54 ± 0.06 ^{d}	7.27 ± 0.12 ^{a}	12.76 ± 1.09 ^{c}	9.15 ± 0.45 ^{b}	13.82± 0.79 ^{c}
Shigella boydii	18.25 ± 0 .56 ^{e}	14.14 ± 0.29 ^{d}	17.37 ± 0.03 ^{e}	2.71 ± 0.37 ^{b}	5.21 ± 0.24 ^{c}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}
Shigella sonnei	8.60 ± 0.69 ^{c}	5.45 ± 0.11 ^{b}	10.04 ± 0.35 ^{d}	1.88± 0.42 ^{a}	2.71 ± 0 .34 ^{a}	6.55 ± 0.07 ^{b}	10.84± 0.31 d

Values are presented as mean ± SEM (n = 3). Values with different superscript in each row are significantly different from one another ($p<0.05$). One way ANOVA followed by LSD multiple comparisons was performed to analyze this data set

Table 5: Effect of methanol extracts of *E. fluctuans, A. philoxeroides* and *C. album* in acetic acid writhing test.

Group	Doses (mg/ kg)	Number of writhing responses	Inhibition (%)
Control	-	12.8 ± 0.55	-
Diclofenac Na	100	3.4 ± 0.12 [b]	73.44
E. fluctuans	250	8.0 ± 0.14 [b]	37.50
	500	4.2 ± 0.06 [b]	67.19
A. philoxeroides	250	12.2 ± 0.22	4.68
	500	11.4 ± 0.42 [a]	10.93
C. album	250	12.0 ± 0.10	6.25
	500	8.4 ± 0.18 [b]	34.36

Values are presented as mean ± SEM (n = 5). One way ANOVA followed by Dunnett's multiple comparisons was performed to analyze this dataset. [a]P<0.05, [b]P<0.001 were considered statistically significant when compared against control.

Table 6: Effect of methanol extracts *of E. fluctuans, A. philoxeroides* and *C. album* in Formalin-Induced Paw Licking test.

Group	Doses (mg/kg)	First 5 min	Inhibition (%)	Second 5 min	Inhibition (%)
Control	-	70.46 ± 0.23	-	58.60 ± 0.36	-
Diclofenac Na	100	27.51 ± 0.39 [b]	60.95	13.88 ± 0.43 [b]	76.06
E. fluctuans	250	44.80 ±1.21 [b]	36.42	31.68 ± 0.38 [b]	45.93
	500	34.28 ± 0.67 [b]	51.34	20.26 ± 0.61[b]	65.42
A. philoxeroides	250	70.28 ± 0.44	0.25	52.80 ± 0.68 [a]	9.89
	500	62.56 ± 0.64 [a]	11.21	49.08 ± 0.98 [a]	16.24
C. album	250	59.00 ± 0.71 [b]	16.26	42.80 ± 0.74 [b]	26.96
	500	52.36 ± 1.11 [b]	25.68	32.56 ± 0.78 [b]	44.44

Values are presented as mean ± SEM (n = 5). One way ANOVA followed by Dunnett's multiple comparisons was performed to analyze this dataset. [a]P< 0.05, [b]P<0.001 were considered statistically significant when compared against control.

Table 7: Effect of methanol extracts of *E. fluctuans, A. philoxeroides* and *C. album* in Xylene Induced Ear Edema Test.

Group	Doses (mg/ kg)	Ear weight difference (mg)	Inhibition (%)
Control	-	2.04 ± 0.05	-
Diclofenac Na	100	0.31 ± 0.01 [b]	84.80
E. fluctuans	250	0.97 ± 0.02 [b]	52.45
	500	0.67 ± 0.01 [b]	67.16
A. philoxeroides	250	2.01± 0.06	1.47
	500	1.96 ± 0.04	3.92
C. album	250	1.86 ± 0.04 [a]	8.82
	500	1.14 ± 0.03 [b]	44.12

Values are presented as mean ± SEM (n = 5). One way ANOVA followed by Dunnett's multiple comparisons was performed to analyze this dataset. [a]P<0.05, [b]P<0.001 were considered statistically significant when compared against control.

Table 8: Effect of methanol extracts of *E. fluctuans*, *A. philoxeroides* and *C. album* in Cotton Pellet-Induced Granuloma Formation test.

Group	Doses (mg/ kg)	Granuloma wt. (mg/mg cotton)	Inhibition (%)
Control		4.64 ±0.09	-
Diclofenac Na	100	0.65 ± 0.02 b	85.99
E. fluctuans	250	2.63 ± 0 .06 b	43.31
	500	1.22 ± 0.05 b	73.70
A. philoxeroides	250	3.36 ± 0.09 b	27.58
	500	2.66 ± 0 .04 b	42.67
C. album	250	4.57 ± 0.09	1.51
	500	2.38 ± 0.32 b	48.70

Values are presented as mean ± SEM (n = 5). One way ANOVA followed by Dunnett's multiple comparisons was performed to analyze this dataset. [a]P<0.05, [b]P<0.001 were considered statistically significant when compared against control.

Figure 1: Ferric Reducing Antioxidant Power Assay of different extracts and standard. Values are presentedas mean ± SD (n = 2).

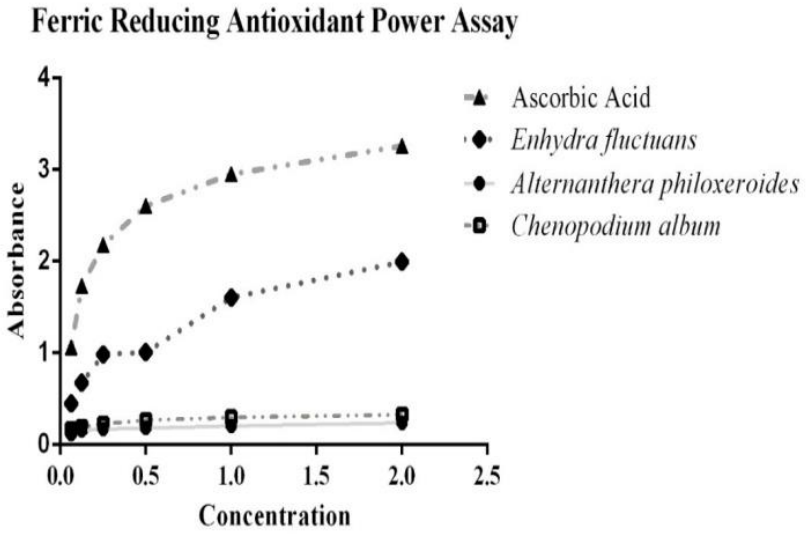

Figure 2: Reducing Power Capacity Assessment of different extracts and standard. Values are presented as mean ± SD (n = 2)