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Total Phenolic Content, Cytotoxic, Antioxidant, Thrombolytic, Membrane Stabilizing And Antimicrobial Activities of *Eupatorium Ayapana* Vent.

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Abstract: *In the present study, the Kupchan fractions of methanolic extract of Eupatorium ayapana were investigated for total phenolic content, in vitro cytotoxic, antioxidant, thrombolytic, membrane stabilizing and antimicrobial activities. The highest phenolic content was found in carbon tetrachloride soluble partitionate (1.203 mg of GAE / gm of extractives). In the Brine shrimp lethality bioassay, methanol extract revealed the highest activity (LC50 = 0.149 µg/mL) whereas, in thrombolytic activity test, pet-ether soluble partitionate revealed significant value with 15.75 % clot lyses. Among all fractions, the Pet-ether soluble fraction demonstrated highest antioxidant activity (IC50 = 1.67 µg/ml) and in the assay for membrane stabilizing activity, the carbon tetrachloride soluble fraction displayed significant result with 57.61 % and 55.13 % RBC hemolysis in hypotonic solution and heat-induced conditions. Carbon tetrachloride soluble extract exhibited good to excellent inhibitory activity towards microbial growth with average zone of inhibition of 14-29 mm for each species. This is the first report of cytotoxic, DPPH scavenging, thrombolytic and membrane stabilizing activities of different fractions of this plant.*

Keywords: *antimicrobial, antioxidant, cytotoxic, Eupatorium ayapana, membrane stabilizing, thrombolytic*

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I. Introduction

According to world health organization almost 80 % of the world population presently uses herbal medicine for their primary health care [1]. During the last few decades, advances in the identification of new bioactive compounds from plants have renewed the popularity of herbal medicines [2]. In fact, due to belief that 'Green Medicine' is safe, effective and reliable than synthetic drugs, many pharmaceutical companies are interested in in plant derived drugs [3]. Various pharmaceuticals have been started in preparing various form of medicine using secondary metabolites from medicinal plants [4]. Hence, screening medicinal plants for promising bioactive metabolites to discover novel drug is a necessity [5]. *Eupatorium ayapana* Vent. (Fam.: Compositae) is an erect annual herb which has folkloric usages such as in dyspepsia, hemorrhage, edema, ulcers, stomatitis, cardiac debility, skin diseases, poison bites, asthma, bronchitis and general debility. It was reported that methanol extract of *Eupatorium ayapana* has both hepatoprotective and antioxidant property [6] and its petroleum ether fraction has high antimicrobial property than methanol fraction [7]. However, as other species of Eupatorium like *E. triplinervis* have been reported to exhibit antinociceptive, neurobehavioral, antioxidant activities [8], it was presumed that *Eupatorium ayapana* may have these properties. In this study we have investigated to find potential fractions of *Eupatorium ayapana* that contain bioactive metabolites.

II. Materials and Methods

General experimental procedure

Rotary evaporator was used for concentrating extracts. All other chemicals, solvents and reagents were of analytical grade.

Plant materials

The leaves of *Eupatorium ayapana* were collected from National Botanical Garden of Bangladesh, Mirpur, Dhaka.

Extraction

The leaves were sun dried and then oven dried for 24 hours. Dried leaves were powered by grinding machine. The powered materials (500g) were extracted with methanol at room temperature for 10 days accompanying occasional shaking and stirring. The whole mixture was then filtered and evaporated to get crude extract. A partition of the crude extract was done by Kupchan protocol which is modified by VanWagenen et al. [9] to afford methanol, petroleum ether, chloroform, carbon tetrachloride, methanol and aqueous soluble materials.

Total phenolic content

Total phenolic content of leaf of estimated as described by Singleton and Rossi [10], and modified by Kim et al[11]. One ml aliquot of extracts or standard solution of gallic acid of varying concentrations (10, 20, 30, 40 and 50 μg/mL) was added in a volumetric flask containing 9 mL of water. One milliliter of Folin-Ciocalteu's reagent was added and vortexed. After 5 min, 10 mL of 7 % sodium carbonate was added and incubated for 90 min at room temperature. After that, the absorbance was determined at 750 nm against the reagent blank. A reagent blank was prepared using distilled water. The amount of phenolic compound in the extract was determined from the standard curve made from varying concentrations (10, 20, 30, 40, 50 μg/mL) of gallic acid (R^2=0.973). The total phenolic content of the plant was expressed as g Gallic acid equivalent (GAE)/100 g dry weight of sample. All samples were analyzed in triplicates.

Evaluation of cytotoxicity

Cytotoxicity of the different extracts of Eupatorium ayapana was determined by Brine shrimp lethality bioassay [12, 13, 14]. As a solvent and negative control dimethyl sulfoxide was used and anticancer drug vincristine sulphate was used as positive control. In this experiment, 4 mg of each test sample was dissolved in DMSO and varying concentrations (400, 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.5625 and 0.78125 μg/mL) was made by serial dilution technique. DMSO solutions were then applied against Artemia salina for 24h to calculate percent mortality. The median lethal concentration (LC_{50}) of the test samples was obtained from the value of percentage of the shrimps killed against the logarithm of the sample concentration.

DPPH scavenging activity

DPPH scavenging activity of different extracts was determined by the method developed by Brand-Williams et al [15]. Here, as reference standard,Tert-butyl-1-hydroxytoluene (BHT) and ascorbic acid (ASA) were used. In this study, 2.0 mL of a methanol solution of the sample (extractives/control) of varying concentrations (500, 250, 125, 62.5, 31.25, 15.625, 7.813, 3.906, 1.953 and 0.977 μg/mL) were mixed with 3.0 mL of DPPH methanol solution (20 μg/mL). Keeping at room temperature in dark place, after 30 min reaction period, the absorbance of different test samples were recorded at 517 nm against methanol as blank with a UV-visible spectrophotometer. Inhibition of free radical DPPH in percent (I%) was calculated as follows:

$(I\%) = (1 - A_{sample}/A_{blank})$ X 100

Where, A_{blank} is the absorbance of the control reaction (containing all reagents except the test material). IC_{50} was calculated from the graph plotted inhibition percentage against test sample concentration.

Thrombolytic activity

In vitro thrombolytic activity of extracts was measured using established method [16]. Distilled water was used as negative control and Lyophilized Altepase (Streptokinase) was used as positive control, respectively. Venous blood was incubated in preweighed sterile microcentrifuge tubes (1 mL/tube) at 37°C for 45 min to form clot. After that, the serum was completely removed and for each tube clot weight was determined. 100 μL aqueous solution of different extracts was added separately to each microcentrifuge tube containing preweighed clot. All the tubes were then incubated at 37 °C for 90 min so that clot lyses may occur. With the following equation, Percent of clot lyses were determined

% of clot lysis = (wt of released clot /clot wt) × 100

Membrane stabilizing activity

In hypotonic and heat induced conditions, membrane stabilizing activity was studied by established method [17].

Hypotonic solution induced hemolysis

The extracts and acetyl salicylic acid which is used as standard were taken in different centrifuge tubes. Each tube contained 0.50 mL erythrocyte (RBC) suspension, 5 mL hypotonic solution (50 mM NaCl) and 10

mM sodium phosphate buffer. These mixtures were incubated for 10 min at room temperature and then centrifuged for 10 min. After completion of centrifugation, the soluble supernatant of each tube was decanted and filtered. The absorbance for each tube was measured. Then the percentage inhibition of hemolysis value was determined. % Inhibition of hemolysis = 100 × {(OD1 OD2)/ OD1}
Where, OD1 = Optical density of hypotonic buffered solution (control) and OD2 = Optical density of test sample in hypotonic solution.

Heat induced hemolysis

Portions (5mL) isotonic buffer containing 1.0 mg/mL of an extract was put into two duplicate sets of centrifuge tubes. The vehicle, in the amount was added to another tube as control. 30 µL erythrocyte suspension (30 µl) was added to each tube. After that, they were mixed gently by inversion. One pair of the tubes was incubated for 20 min in a water bath maintain 54 ºC whereas the other pair was maintained at 0-5 ºC in an ice bath. After centrifuging the reaction mixtures for 3 min at 1300 rpm, the absorbance of the supernatants were measured at 540 nm. Acetyl salicylic acid (ASA) was used as a reference standard.

The percentage inhibition or, acceleration of hemolysis was determined as follows:
% Inhibition of hemolysis = 100 × [1 – (OD2OD1/ OD3OD1)]
Where, OD1 = test sample unheated, OD2 = test sample heated & OD3 = control sample heated

Antimicrobial screening

Antimicrobial activity of the extractives was evaluated against 5 Gram positive bacteria (*Bacillus cereus, Bacillus megaterium, Bacillus subtilis, Staphylococcus aureus* and *Sarcina lutea*), 8 Gram negative bacteria (*Escherichia coli, Pseudomonas aeruginosa, Salmonella paratyphi, Salmonella typhi, Shigell boydii, Shigella dysenteriae, Vibrio mimicus* and *Vibrio parahemolyticus*) by the disc diffusion method [18]. The bacterial strains were collected as pure cultures from the Institute of Nutrition and Food Sciences (INFS), University of Dhaka, Bangladesh. Sterile blank discs, 400 µg/disc concentration for each extract and standard antibiotic discs (Ciprofloxacin) were used. The zone of inhibition was measured in mm with a digital slide calipers [18, 19].

III. Results And Discussion

The amount of total phenolic content differed in different extractives and ranged from 0.154 mg of GAE / gm of extractives to 1.203 mg of GAE / gm of extractives of *E. ayapana* (Table 1). Among all extractives of *E. ayapana* the highest phenolic content was found in carbon tetrachloride soluble partitionate (1.203 mg of GAE / gm of extractives) followed by methanol soluble partitionate (0.833 mg of GAE / gm of extractives). Among all the samples tested for cytotoxicity, methanol soluble fraction revealed the highest activity (LC_{50} = 0.149 µg/mL) as compared to vincristine sulfate (LC_{50} = 0.45 µg/mL) (Table 2).

Table 2 shows the results of DPPH scavenging assay of the extractives and standard tert-butyl-1-hydroxytoluene (BHT). Among all the test samples, the Pet-ether soluble fraction demonstrated highest activity (IC_{50} = 1.67 µg/mL) as compared to BHT (IC_{50} = 27.5 µg/mL). In the in vitro thrombolytic activity test, pet-ether and chloroform soluble partitionate revealed significant thrombolytic activity with 15.75 % and 10.41 % clot lyses, respectively (Table 3). In the assay for membrane stabilizing activity, the carbon tetrachloride soluble fraction displayed the highest % inhibition of hemolysis e.g. 57.61 % and 55.13 % as compared to acetyl salicylic acid 72.79 % and 42.12 % in hypotonic solution- and heat-induced conditions (Table 3).

On the other hand, carbon tetrachloride soluble extract exhibited good to excellent inhibitory activity towards microbial growth with average zone of inhibition of 14-29 mm for each bacteria species as compared to ciprofloxacin (30-50 mm). The highest activity was measured against *Shigella dysenteriae* (29 mm) (Table 4). Phenolic compounds act as antioxidants. The mechanism underlies prevention of the oxidation of Low-Density Lipoproteins (LDL), damage of red blood cells and platelet aggregation [20]. Other than antioxidant property they have multiple biological effects [21]. During 3T3-L1adipocyte differentiation, certain phenolic compounds are responsible for the cell cycle arrest at the G1 phase [22] and this type of compounds also competently induce apoptosis in 3T3-L1 adipocytes by AMPK activating [23, 24]. As total phenolic content of the extract was determined 51.26±0.13 g, presence of phenolic compounds containg antihyperlipidemic agents in crude extract of *E. ayapana* demand further investigations.

In the study, methanol soluble fraction of crude extract showed LC_{50} at a low concentration indicating that the extracts contains cytotoxic agent and may be used to treat cancer. Sometimes, anticancer agents are toxic [25]. As a result, the cytotoxic activity of this extract may be tested against various cancer cell lines as well as normal cell lines. Further investigation could be done to isolate specific anticancer agents.
Synthetic antioxidants such as BHT, butylated hydroxyanisole (BHA), propyl gallate (PG) and tertbutylhydroquinone (TBHQ) used as food additives have carcinogenic effects [26,27]. Therefore, the interest

in antioxidant derived from natural products has greatly increased in recent years [28]. Thus, the purpose of this study was to evaluate different extractives of *E. ayapanaas* new potential sources of natural antioxidants and phenolic compounds. In the investigation, Pet-ether solublefraction revealed the highest antioxidant activity compared to BHT. Though it was previously revealed that methanolic extract has antioxidant property [6]. Further research can be carried out to isolate antioxidant agent from the crude extract of *E. ayapana*.

In developed countries, thromboembolic disorders such as pulmonary emboli, deep vein thrombosis, heart attacks, and strokes are the main causes of mortality and morbidity [29]. Again, the first generation thrombolytic agents such as streptokinase, urokinase show relatively weak substrate specificity and can cause side effects of systemic fibrinolysis and associated bleeding complications [30]. Hence, to develop improved recombinant variants of these drugs, attempts are underway [31]. The present study was also aimed to investigate the thrombolytic activity of different extractives of *E. ayapana*. Pet ether and chloroform soluble extract showed significant thrombolytic activity which may be the justification of the folkloric use of *E. ayapana* in cardiac debility. As erythrocyte cell membrane is similar to lysosomal membrane components, it is extrapolated that the drugs which stabilizes erythrocyte membrane, stabilizes lysosomal membrane [32] and thus interfere with the release and or action of mediators responsible for inflammation like histamine, serotonin, prostaglandins, etc [33]. In the present study, carbon tetrachloride soluble fraction displayed significant inhibition of hemolysis in hypotonic solution- and heat-induced conditions which justifies the folkloric use of skin disease and other diseases related to inflammation. Different extracts of *E. ayapana* were evaluated for antibacterial activities against five gram positive and eight gram negative bacteria. Carbon tetrachloride soluble extract exhibited good to excellent inhibitory activity towards microbial growth with average zone of inhibition of 14-29 mm for each bacteria species as compared to ciprofloxacin (30-50 mm). The highest activity was measured against *Shigella dysenteriae* (29 mm) (Table 4). Previous study had demonstrated that petroleum ether extract showed higher antimicrobial activity than the methanolic extract [7]. So it can be said that *E. ayapana* contain some medium or nonpolar type antimicrobial metabolites. So, further study and phytochemical screening can be done in order to attain antibacterial compounds from this plant.

IV. Conclusions

The present study demonstrated that *E. ayapana* can be a very potential source of cytotoxic, thrombolytic, antioxidant, anti-inflammatory and antimicrobial agents. Thus, the authors strongly recommend further studies for the isolation, purification and characterization of bioactive metabolites.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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Table 1. Total phenolic content of different fractions of *E. ayapana*

Test sample	Total phenolic content (mg of GAE / g of extractives)
ME	0.833
CTCSF	1.203
CSF	0.648
PESF	0.154
AQSF	0.278

ME = Crude methanolic extract of *E. ayapana* and CTCSF, CSF, PESF and AQSF were the Carbon tetrachloride, Chloroform, Pet-etherand aqueous soluble fraction of methanolic extract of *E. ayapana*, respectively.

Table 2. LC_{50} and IC_{50} values of standard and *E. ayapana* extractives in brine shrimp lethality bioassay and antioxidant activity test, respectively

Test samples	LC_{50} (µg/ml)	IC_{50} (µg/ml)
VS	0.45	-
BHT	-	27.5
ASA	-	5.8
ME	0.149	26.89
CTCSF	4.302	9.05
CSF	4.2855	181.303
PESF	3.4639	1.67
AQSF	4.8220	661.347

VS = Vincristine sulphate; BHT =tert-butyl-1-hydroxytoluene; ASA = acetyl salicylic acid; ME = Crude methanolic extract of *E. ayapana* and CTCSF, CSF, PESF and AQSF were the Carbon tetrachloride, Chloroform, Pet-ether and aqueous soluble fraction of methanolic extract of *E. ayapana*, respectively.

Table 3. Thrombolytic activity in terms of % of clot lyses and % inhibition of haemolysis (in hypotonic solution- and heat-induced conditions) of erythrocyte membrane by standard and *E. ayapana* extractives

Test samples	% clot lyses	% Inhibition of Haemolysis	
		Hypotonic solution induced (50 mM)	Heat-induced
ASA	-	72.79	42.12
SK	61.5	-	-
ME	2.28	48.15	68.12
PESF	15.75	30.86	47.76
CTCSF	4.46	57.61	55.13
CSF	10.41	35.80	51.68
AQSF	6.83	51.44	59.87

Here, SK = streptokinase, ASA = acetyl salicylic acid; ME = Crude methanolic extract of *E. ayapana* and CTCSF, CSF, PESF and AQSF were the Carbon tetrachloride, Chloroform, Pet-ether and aqueous soluble fraction of methanolic extract of *E. ayapana*, respectively.

Table 4. Antimicrobial activity of *E. ayapana* extractives against gram positive and gram negative bacteria.

Test microorganisms	Diameter of zone of inhibition (mm)					
	ME	PESF	CTCSF	CSF	AQSF	Ciprofloxacin
Gram positive bacteria						
Bacillus cereus	-	-	-	9	-	30
Bacillus megaterium	14	7	22	13	9	50
Bacillus subtilis	13	7	22	16	-	30
Staphylococcus aureus	15	9	21	10	-	35
Sarcina lutea	15	7	22	10	9	30
Gram negative bacteria						
Escherichia coli	15	8	21	20	-	32
Pseudomonas aeruginosa	10	9	22	14	-	30
Salmonella paratyphi	15	8	22	20	-	35
Salmonella typhi	15	9	21	11	-	50
Shigella boydii	15	10	20	14	-	35
Shigella dysenteriae	10	-	29	19	-	35
Vibrio mimicus	14	10	14	17	9	30
Vibrio parahemolyticus	8	7	15	22	-	35

ME = Crude methanolic extract of *E. ayapana* and CTCSF, CSF, PESF and AQSF were the Carbon tetrachloride, Chloroform, Pet-ether and aqueous soluble fraction of methanolic extract of *E. ayapana*, respectively.

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