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Anti-diarrheal, Anxiolytic, Antimicrobial and Membrane Stabilizing Activities of *Vernonia cinerea* Less:A Medicinal Plant of Bangladesh

Md. Khairul Bashar[1], Mohammed Ibrahim[1], Irin Sultana[1], Md. Ruhul Kuddus[2], Ridwan Bin Rashid[3], Mohammad Abdur Rashid[2*]

1. Department of Pharmacy, Southern University Bangladesh, Chittagong, Bangladesh
2. Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Dhaka, Dhaka-1000, Bangladesh
3. Department of Microbiology, University of Dhaka, Dhaka-1000, Bangladesh

ABSTRACT

In this study, the crude methanol extract of whole plant of *Vernonia cinerea* Less. has been investigated for anti-diarrheal, anxiolytic, antimicrobial and membrane stabilizing activities. Test for anti-diarrheal activity was carried out by castor oil-induced diarrhea in mice. The anxiolytic activity was examined in mice by using the hole-cross test and open field-tests (OFT). The preliminary antimicrobial activity was determined at 500 µg/disc by the disc diffusion method against a number of Gram positive and Gram negative bacteria and fungi. RBC membrane stabilization method was used for the evaluation of *in-vitro* anti-inflammatory activity. The crude methanol extract of *V. cinerea* showed significant and dose-dependent anti-diarrheal activity in castor oil-induced diarrhea in mice. The results showed that the crude extract significantly increased the number of hole crossing as compared to control in hole cross tests. In open field test, the extract showed significant increase in number of square crossed. The efficacy of the extract (200-400 mg/kg b.w.) was compared with standard anxiolytic drug diazepam (1 mg/kg). Furthermore, the plant extract, at 500 µg/disc exhibited moderate antimicrobial activity against *Blastomyces dermatitidis* (zone of inhibition = 17.7 mm) and *Shigella dysenteriae* (zone of inhibition = 14.0 mm). In red blood cell stability test, the sample at 0.5 and 1 mg/ml inhibited the heat-induced haemolysis of RBCs by 15.45% and 27.73%, respectively whereas standard acetyl salicylic acid (ASA) demonstrated 71.36% inhibition of haemolysis. The obtained results provide supports for the use of this plant in traditional medicine but further pharmacologic studies are required to isolate and identify the bioactive molecules.

Keywords: *Vernonia cinerea*, Anti-diarrheal, Anxiolytic, Antimicrobial, Membrane Stabilization

*Corresponding Author Email: rashidma@du.ac.bd
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INTRODUCTION

The use of natural products is growing all over the world especially in the developing countries such as Bangladesh, India, China, and the Middle East. The chemical diversity of plants has made them an ideal source for the isolation of bioactive organic compounds[1]. A large number of poor people in Bangladesh has no access to modern medical services. Most of them are usually dependent upon the traditional practitioners for their health problems.

The plants of the genus *Vernonia* (Asteraceae) are widely distributed in most tropical and subtropical countries, and have long been used in traditional medicine to treat various types of diseases. *Vernonia cinerea* (Bengali name- kukshim) is a terrestrial annual erect herb. It grows up to 80 cm high. It can be found roadside in open waste places, dry grassy sites and in perennial crops during plantation[2]. It is located especially in different Asian countries such as Bangladesh, India and Nepal. This herb has been used to treat a number of disorders including inflammation, malaria, fever, worms, pain, diuresis, cancer, abortion, and various gastro-intestinal disorders[3] and is also used in smoking cessation, cough, fever, malaria, urinary calculi, arthritis[4] and leprosy[5]. A quick literature survey revealed that the plant possesses antimicrobial[6], antibacterial, anti-inflammatory, analgesic, antipyretic[7], anti-flatulent, antispasmodic[8] and anti-diuretic properties[9].

As part of our ongoing efforts to study the medicinal plants of Bangladesh[10-12], we evaluated the anti-diarrheal, anxiolytic, antimicrobial and membrane stabilizing activities of *V. cinerea* Less. as well as to find out the logical evidence for its folk uses.

MATERIALS AND METHODS

Collection of plant materials

The whole plant of *V. cinerea* was collected from the Bhatiyari and Pahartoli, Chittagong, Bangladesh in June, 2012. The plant was identified by the experts of Bangladesh Forest Research Institute, Chittagong where voucher specimen has been deposited.

Drying and grinding

After collection, the whole plant was washed with running tap water. The clean plant was dried at a temperature not exceeding 50 ºC. The dry materials were ground to coarse powder with the help of a grinder and kept in airtight container and stored in a cool and dark place until extraction was commenced.

Soxhlet extraction

Exactly 130 gm of powdered material was extracted with 700 ml of methanol (99.98%) with a

Soxhlet apparatus (Quickfit, England). The extract was concentrated with a rotary evaporator (Heidolph, Germany) under reduced temperature and pressure to provide a gummy residue (yield 16.70%).

Drugs and chemicals

All the chemicals and solvents used in this study were of analytical grade and purchased from Merck, Germany. Standard drugs such as loperamide, diazepam, ciprofloxacin, fluconazole and acetyl salicylic acid were obtained from Square Pharmaceuticals Ltd.

Experimental animals

For the experiment *Swiss albino* mice of either sex, 6-7 weeks of age, weighing between 25-30 g, were collected from the Animal Resource Branch of the International Centre for Diarrheal Disease and Research, Bangladesh (ICDDR,B). Animals were maintained under standard environmental conditions temperature: (27.0 ± 1.0 °C), relative humidity: 55-65% and 12 hr light/12 hr dark cycle and had free access to feed and water *ad libitum*. Appropriate measures were taken to minimize pain or discomfort of animals and the animals were acclimatized to laboratory condition for one week prior to experiments and details of animal care should be provided. All protocols for animal experiment were approved by the institutional animal ethical committee[13].

Test for anti-diarrheal activity

This test was carried out by castor oil-induced diarrhea in mice[14]. Young *Swiss albino* mice, average weight of 25-30 g were employed in the experiment. The animals were divided into negative control, positive control and two test groups containing seven mice in each. Control group received 1% Tween-80 (10 ml/kg, p.o). The positive control group received loperamide (3 mg/kg, p.o.) while the test groups received the crude extract (100 and 200 mg/kg b.w.) orally. Acute diarrhea was produced by oral administration 0.4 ml castor oil in each mouse. Then the latency period and diarrheic secretion were counted for 4 hr.

Test for anxiolytic activity

Treatment schedule

The anxiolytic activity was examined by using the hole cross test and open field-tests (OFT). The animals were divided into four groups, with each group consisting of seven mice. First group received normal saline, second group received diazepam (1mg/kg), third and fourth groups received plant extract at 200 and 400 mg/kg b.w. p.o. respectively.

Hole cross test

The hole board is a white painted wooden board (30 cm × 20 cm ×14 cm) with 16 holes (each of

diameter 3 cm) evenly distributed on the base of box. The test groups received the crude extract at the dose of 200 and 400 mg/kg b.w. orally whereas the control group received saline and positive control group received diazepam (2mg/kg, i.p.). The number of passages of a mouse through the hole from one chamber to the other was counted for a period of 5 min for 30 min after oral administration of both doses of the test drug[15].

Open field test

The open field test is one of the tests used to observe general motor activity, exploratory behavior and measures of anxiety. The open field area was made of plain wood and consisted of a square area (45 cm × 45 cm × 20 cm). The floor had a square sheet of wood (45 cm × 45 cm) with the surface divided into sixteen small squares. Mice were divided into four groups of 7 mice and treated similarly as described in hole cross test. About 30 min after treatment, mice of both the control and treated groups were placed individually in the center of the open field and behavioral activities were recorded for five minutes. Subsequently, hand operated counters and stopwatches were used to score the following behavioral parameters for a period of five minutes: (1) the number of entries and time spent in the centre, (2) periphery and corners of the field, (3) the number of crossings (number of square floor units entered) as a measure of distance traveled, (4) rearing (number of times the animal stood on hind legs) and (5) assisted rearing (forepaws touching the walls of the apparatus) [15].

Test for antimicrobial activity

The preliminary antimicrobial activity of the extractives was determined at 500 µg/disc by the disc diffusion method[16] against a number of Gram positive and Gram negative bacteria and fungi. The bacterial and fungal strains used in this experiment were collected from the Microbiology Lab., Department of Pharmacy, BGC Trust University, Chittagong, Bangladesh. The test organisms were maintained on nutrient agar slopes and were sub-cultured. Here, standard ciprofloxacin and fluconazole disc were used as reference standards.

Test for membrane stabilizing activity

For this study, three clean centrifuge tubes were taken for positive control (acetyl salicylic acid), three for negative control (99.8% methanol) and six for crude methanol extract and 1.0 ml of 10% RBC suspension was added to each tube. Then 1.0 ml methanol and 1.0 ml acetyl salicylic acid were added to the negative control and positive control tubes respectively. On the other hand, for the test group, 1.0 ml of methanol extract (1000 mg/kg) was mixed. The pH (7.4±0.2) of the reaction mixtures was adjusted by phosphate buffer. The tubes were then incubated in water bath and after cooling these were centrifuged at 2500 rpm for 5 min. After filtration the

absorbance of the supernatants were taken at 556 nm. The total inhibition of haemolysis was then calculated by determining the % inhibition of haemolysis[17].

Statistical analysis

Statistical analysis for animal experiment was carried out using one-way ANOVA followed by Dunnet's multiple comparisons. The results obtained were compared with the vehicle group. *p* values <0.05 were considered to be statistically significant compared with the control.

RESULTS AND DISCUSSION

Anti-diarrheal activity

In the castor oil-induced diarrheal experiment in mice, the crude methanol extract of *V. cinerea* at the doses of 100 and 200 mg/kg significantly reduced the total number of feces as well as delayed the onset of diarrhea in a dose dependent manner (Table 1). These results were shown to be statistically significant ($p<0.05$).

Table 1: Effect of crude methanol extract (VCME) on castor oil-induced diarrhea in mice.

Test groups	Mean latent period (min)	% Defecation	% Inhibition of defecation	TNF (240min)
Control	27.67	100	0	68±1.78
Loperamide (3 mg/kg)	73.67	19.12	80.88	13±0.41
VCME (200 mg/kg)	52.33	36.76	63.24	25±1.63
VCME (100 mg/kg)	39.67	45.44	54.46	33±1.24

TNF = Total number of feces; Values are mean ± SEM (n=7); **p<0.0001 by Dunnett's T test for values between the sample and vehicle treated group

Diarrhea results from an imbalance between the absorptive and secretory mechanisms in the intestinal tract, which is accompanied by an excess loss of fluid in the faeces. In some types of Diarrhea, the secretory component predominates, while other types of Diarrhea are characterized by hyper motility. Castor oil causes Diarrhea due to its active metabolite, ricilonic acid[18], which stimulates the peristaltic activity in the small intestine, leading to changes in the electrolyte permeability of the intestinal mucosa. Its action stimulates the release of endogenous prostaglandins[19]. In this study, the crude extract of the plant successfully inhibited the castor oil-induced diarrhea, the extract might have exerted its anti-Diarrheal action by antisecretory mechanism. This was also evident from the reduction of the total number of feces as well as delayed the onset of diarrhea in a dose dependent manner in the experiment. Phytochemical screening revealed the presence of alkaloids, glycosides, sugars, terpenes and flavonoids. Earlier studies have shown that the anti-dysenteric and anti-Diarrheal properties of medicinal plants were due to the presence of tannins, alkaloids, saponins, flavonoids, sterols and/or tri-terpenes and reducing sugars. Hence, tannins, reducing sugars, sterols and/or tri terpenes may be

responsible for the mechanism of action of the anti-Diarrheal activity of *V.cinerea*[20].

Anxiolytic activity

The number of hole crossings was increased notably in case of diazepam treated animals as compared to control animals. The plant extracts at the 200 and 400 mg/kg (p.o) dose showed significant increase in the number of line crossing as compared to control animals as shown in table 2. In the open field test, administration of plant extract in mice showed significant increase in the number of squares crossed during 5 min intervals of test as compared with that of control as shown in table 2.

Table 2: Anxiolytic effect of VCME in mice by hole cross test and open field test.

Treatment	Dose (mg/kg)	Hole cross test	Open field test
		Number of hole crossing	Number of square crossed
Saline	1 ml	25.67 ± 1.47	251.67 ± 2.04
Diazepam	1	3.33 ± 1.08	39.67 ± 1.78
VCME	200	11.67 ± 1.47	127.67 ± 1.78
VCME	400	5.33 ± 0.82	63.33 ± 7.08

All values are mean ±SEM (n=7); *p< 0.1 when compared to control.

Anxiety disorders are due to involvement of GABAergic, serotonergic, involvement. The adrenergic and dopaminergic system have also been shown to play a role in anxiety. BZA have been extensively, used for the last 40 years to treat several forms of anxiety, but due to their unwanted side effects, alternative treatment strategies with favorable side effect profiles. Medicinal plants are a good source to find new remedies for these disorders. Despite the wide spread traditional use of *Vernonia cinerea* for treating various disorders there are no reports of scientific evaluation of its anxiolytic activity. The present work demonstrates that the *V. cinerea* extract had anxiolytic activity in mice by open field and hole cross models[21].

Antimicrobial activity

In the disc diffusion test, the zone of inhibition was found within the range of 9.67 to 14 mm. Highest zone of inhibition (14 mm) was observed against *Shigella dysenteriae* (Table 3). But the plant extract showed no activity against *Bacillus cereus, Salmonella* Paratyphi, *Vibrio cholerae, Escherichia coli, Pseudomonas aeruginosa* bacterial species. During the anti-fungal test, the zone of inhibition was found within the range of 13.67 to 17.17 mm. The highest zone of inhibition (17.17 mm) was obtained against *Blastomyces dermatitidis* (Table 3).

Now a days, microorganisms have developed resistance to several antibiotics and this has produced vast clinical problem in the management of infectious diseases. This situation enforced scientists to explore new antimicrobial substances from diverse sources, such as medicinal

plants[22]. The chemical constituents of plants vary depending on the species, variety and part of the plant, with conditions of growth (soil, water and temperature), and with the age of the plant. The phytochemistry also varies according to the geographical regions, season and time of collection and different climatic conditions[23]. In the present investigation the results for zone of inhibition for extracts of *Vernonia cinerea* were found to be promising against *Blastomyces dermatitidis* and *Shigella dysenteriae* (Table 3) Which supports the traditional uses of this plant in folk medicine. which are currently available for evaluation of natural products.

Table 3: Antimicrobial activity of VCME at the dose of 500 µg/disc.

Microorganisms	**Zone of inhibition (MZI±SD) mm**	
	VCME (500 µg/disc)	**Standard (30 µg/disc)**
Gram positive bacteria		**Ciprofloxacin**
Bacillus megaterium	11.0±0.71[a]	20.67±0.41
B. subtilis	11.33±0.82[a]	15.00±0.71
Staphylococcus aureus	10.67±0.41[a]	18.00±1.41
Gram negative bacteria		
Salmonella Typhi	9.67±0.82[a]	15.67±0.41
Shigella dysenteriae	14.0±0.71[b]	15.33±0.82
Fungi		Fluconazole
Aspergillus niger	13.67±0.41[c]	16.5±0.35
Blastomyces dermatitidis	17.17±1.74[d]	19.67±0.54
Candida albicans	13.83±0.74[c]	17.5±0.35
Cryptococcus neoformans	16.33±1.08[c]	18.5±1.06

[a]p<0.005, [b]p<0.05; [c]p<0.001, [d]p<0.5 MZI: Mean zone of inhibition (mm); zone of inhibition under 8 mm were considered as less active and were discarded.

Membrane stabilizing activity

The red blood cell stability test is based on the result that a number of non-steroidal anti-inflammatory agents inhibit heat-induced rupture of erythrocytes, most probably by stabilizing the membrane of the cell. The erythrocyte membrane may be considered as a model of the lysosomal membrane. Agents that can prevent the rupture of the latter, and thereby prevent damage to the tissue caused by the release of the hydrolytic enzymes contained within the lysosome maybe expected to improve some symptoms of inflammation[24]. It has been demonstrated that certain herbal preparations were capable of stabilizing the red blood cell membrane and this may be indicative of their ability to exert anti-inflammatory activity[25].

The crude extract at 0.5 and 1 mg/ml inhibited the heat-induced haemolysis of RBCs by 15.45% and 27.73%, respectively whereas standard acetyl salicylic acid showed 71.36% inhibition of haemolysis (Table 4). The stabilization activity of the plant was found to be moderate. Although the precise mechanism of this membrane stabilization is yet to be elucidated, it is thought that the

plant may possibly inhibit the release of lysosomal content of neutrophils at the site of inflammation[25].

Table 4: *In-vitro* membrane stabilization activity of test sample and controls.

Test groups	Total inhibition of haemolysis
Control	00.00±0.018
ASA (0.1mg/ml)	71.36±0.021[a]
VCME (1mg/ml)	27.73±0.014[a]
VCME (0.5mg/ml)	15.45±0.011[c]

Total inhibition of haemolysis = %IMHLs±SEM, [a]p<0.00001, [b]p<0.0005, [c]p<0.005

CONCLUSION

In conclusion, the methanol extract of *Vernonia cinerea* was proved a natural safe remedy for the treatment of diarrhea, anxiety disorders and inflammation. Our current findings demonstrated scientific rationale for the folk use of the plant as anti-diarrheal, anxiolytic, antimicrobial and anti-inflammatory agent. Nevertheless, the isolation of pure secondary metabolites from the plant will help us further in understanding the mechanism of these activities and identification of lead compounds of clinical utility.

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