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Evaluation of Analgesic, Antidiarrhoeal and Cytotoxic Activities of Ethanolic Extract of *Bacopa monnieri* (L)

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Authors' contributions

This work was carried out in collaboration between all authors. HH designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. MSIH and SKD managed the analyses of the study. AH and AA managed the literature searches. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aims: The crude ethanolic leaf extract of *Bacopa monnieri* (L) Penn. (family: Scrophulariaceae) was evaluated for its possible phytochemical nature (group determination of plant constituent) and selected pharmacological activities (analgesic, antidiarrhoeal and cytotoxic activity) growing in Bangladesh.
Methodology: The antinociceptive activity was evaluated by acetic acid induced writhing model, antidiarrhoeal activity by castor oil induced diarrheal method and cytotoxicity by brine shrimp lethality bioassay.
Results: Phytochemical analysis of the ethanolic extract of *B. monnieri* indicated the presence of reducing sugar, tannins, steroid, alkaloid, saponin and gum types of compounds. The ethanolic extract of *B. monnieri* has effect on acetic acid induced writhing in mice. At the dose of 250 mg/kg and 500 mg/kg of body weight, the extract produced 36.69% and 59.17% writhing inhibition in test animals respectively. The results were statistically significant (p <0.01 and p <0.001) and was comparable to the standard

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drug Diclofenac Na, which showed 72.78% at a dose of 25 mg/kg weight. The ethanolic extract of *B. monnieri* has effect on castor oil induced diarrhea in mice. The result showed that the extract decreased the mean number of defecation which were 35.42 % and 47.92 % (p <0.001) at the doses of 250mg/kg and 500mg/kg respectively. The latent period for the extract treated group was increased (p <0.01) as compared to control group. The ethanolic of extract of *B. monnieri* showed significant toxicity to the brine shrimp nauplii. The concentrations of crude extract for 50% mortality (LC_{50}) and 90% mortality (LC_{90}) were 40 µg/mL and 150 µg/mL respectively.

Conclusion: Therefore, the obtained results tend to suggest the antinociceptive, antidiarrhoeal and cytotoxic activities of crude ethanolic extract of *Bacopa monnieri* leaves and thus provide the scientific basis for the traditional uses of this plant part as a remedy for pain and diarrhoea.

Keywords: Bacopa monnieri; analgesi; antidiarrhoeal; cytotoxic activity.

1. INTRODUCTION

Bacopa monnieri (L) Penn. (family: Scrophulariaceae) commonly known in both India and Bangladesh as 'Brahmi' is an ancient and renowned medicinal plant with legendary reputation as a memory vitalizer (Anonymous, 1988) in the traditional system of medicine (Ayurveda). Brahmi is classified as medhya rasayana, is drug that is supposed to counteract the effect of mental stress and improved the intelligence and memory function.

Brahmi is found to be effective in the case of anxiety and neurosis (Singh et al., 1997). It possesses anti-inflammatory, analgesic and anti-pyretic activity (Agrawal, 1993; Vohora et al., 1997). It is also used to treat asthma, insanity epilepsy, enlargement of spleen, snake bite, rheumatism, leprosy, eczema, ring worm and as a diuretic and cardiotonic (Basu et al., 1944). In a recent study, *B. monnieri* was placed second in the priority list of the most important Indian medicinal plants evaluated on the basis of medicinal importance commercial value and potential for further research and development (Anonymous, 1998).

Since no literature is currently available to substantiate antinociceptive, antidiarrheal and cytotoxic activities from ethanolic extract of *B*. *monnieri*, therefore the present study was designed to investigate the antinociceptive antidiarrheal and cytotoxic activities of crude ethanolic extract.

2. MATERIALS AND METHODS

2.1 Collection and Identification of Plant Materials

B. monnieri (leaves) were collected from Karamjal, Sundarban Khulna, Bangladesh in August, 2011. The collected samples were then identified by Sarder Nasir Uddin, Senior Scientific Officer, Bangladesh National Herbarium, Mirpur, Dhaka, Bangladesh. A specimen copy was deposited to Bangladesh National Herbarium for identification and the accession number was DACB-32706.

2.2 Preparation of Ethanolic Extract

The plant materials (leaves) of *B. monnieri* were freed from any of the foreign materials. Then the plants were air-dried under shed temperature followed by drying in an electric oven at 40ºC. The dried plant materials were then ground into powder. About 400g of powdered material was soaked in 1300 ml of 95% ethanol in a well sealed, flat-bottomed glass container for 14 days accompanying occasional shaking and stirring. The whole mixture then underwent a coarse filtration by a piece of clean, white cotton material. Then it was filtered through whatman filter paper (Bibby RE200, Sterilin Ltd., UK) and concentrated with rotary evaporator at bath temperature not exceeding 40˚C to have gummy concentrate of extract (yield approx. 2.77%).

2.3 Test Animals and Drug

Young Swiss-albino mice either sex, 3-4 weeks of age, weighing 20 -25 g, were used for in vivo pharmacological screening. Mice were purchased from the Animal Research Branch of the International Centre for Diarrhoeal Disease and Research, Bangladesh (ICDDR, B). They were housed in standard environmental conditions and fed with rodent diet and water ad libitum. All experimental protocols were in compliance with BCSIR Ethics Committee on Research in Animals as well as internationally accepted principles for laboratory animal use and care.

The standard drug Diclofenac Na and Loperamide were purchased from Square Pharmaceuticals Ltd, Bangladesh. Tween 80, acetic acid, ferric chloride and potassium dichromate were of analytical grade purchased from Merck (Darmstadt, Germany).

2.4 Phytochemical Screening

The freshly prepared crude extract was qualitatively tested for the presence of chemical constituents, by using the following reagents and chemicals, for example, alkaloids were identified by the dragendorff's reagent, flavonoids with the use of Mg and HCl, tannins with ferric chloride and potassium dichromate solutions, and steroids with Libermann-Burchard reagent. Reducing sugars with benedict's reagent (Ghani, 1998; Evans, 1989; Harborne, 1984).

2.5 Analgesic Activity

The Analgesic activity of the crude ethanolic extract of *B. monnieri* was studied using acetic acid induced writhing model in mice (Whittle, 1964; Ahmed et al., 2004). The animals were divided into control, positive control and test groups with five mice in each group. The animals of test groups received test substance at the dose of 250 and 500 mg/kg body weight. Positive control group was administered with Diclofenac Na (standard drug) at the dose of 25 mg/kg body weight and control group was treated with 1% Tween 80 in water at the dose of 10 ml/kg body weight. Test samples, standard drug and vehicle were administered orally 30 min before intraperitoneal administration of 0.7% acetic acid. After an interval of 15 min, the mice were observed writhing (constriction of abdomen, turning of trunk and extension of hind legs) for 5 min.

2.6 Antidiarrhoeal Activity

Antidiarrhoeal activity of extract of *B. monnieri* was tested by using castor oil induced method in mice (Shoba et al., 2001; Hemayet et al., 2012). Twenty Swiss albino mice were randomly divided into four groups (n=5). Control group received 1% Tween 80 in water of 2ml/mice, positive control group received loperamide 50 mg/kg body weight as standard drug and test groups received the extracts at the doses of 250 mg and 500 mg/kg body weight. Mice were housed in separate cages having paper placed below for collection of fecal matters. Diarrhea was induced in the mice by oral administration of castor oil (1.0 ml/mice). Extract and drugs were given orally 1 hr before the administration of castor oil. The time for first excretion of feces and the total number of fecal output by the animals were recorded. Normal stool was considered as numerical value 1 and watery stool as numerical value 2. Percent inhibition of defecation in mice was calculated by using the following equation: % inhibition = {(Mo–M)/Mo} x100; where, Mo = Mean defecation of control and M = Mean defecation of test sample.

2.7 Assay for Brine Shrimp Lethality

In vitro lethality bioassay of the ethanolic extract of *B. monnieri* was exploited to detect cytotoxicity following the method described by Meyer et al., 2012. Brine shrimp eggs were placed in seawater (3.8% w/v sea salt in distilled water) and incubated at 24-28^{o}C in front of a lamp. Eggs were hatched within 48 hours providing large number of larvae (nauplii). A solution of 50 µg/µl of the extract was prepared by using Dimethyl sulfoxide (DMSO). 18 clean test tubes were taken. 12 of these were used for the samples at six concentrations (duplicate of each concentration) and 6 for control test. Then with the help of micropipette specific volume (1, 2, 4, 8, 16, 32 µl) of samples were transferred from the stock solutions to each test tube. Afterwards, the volume of each test tube adjusted up to 10 ml with seawater to get final sample concentrations of 5, 10, 20, 40, 80 and 160 µg/ml respectively. In the test tubes taken for the control same volumes of DMSO (as in the sample test tubes) were taken. With the help of a Pasteur pipette 10 living nauplii were kept to each of the test tubes. Alive nauplii were counted after 16 hours and the lethal concentrations (LC_{50} and LC_{90}) were calculated. The plot of percent mortality versus log concentration of the extract produced an approximate linear correlation between them graphically. From the graph the concentration at which 50% and 90% mortality (LC_{50} and LC_{90}) of brine shrimp nauplii occurred were obtained.

2.8 Statistical Analysis

For analgesic and anti-diarrheal determination, data were presented as mean±SEM (Standard Error Mean). Statistical analysis for animal experiment was carried out using one-way ANOVA followed by Dunnett's multiple comparisons. The results obtained were compared with the control group. *p* values < 0.05 were considered to be statistically significant.

3. RESULTS

3.1 Chemical Group Test

Results of different chemical tests on the ethanolic extract of *B. monnieri* showed the presence of reducing sugar, tannins, steroid, alkaloid, saponin and gum (Table 1).

Table 1. Results of different group tests of ethanolic extract of *B. monnieri*

Phytoconstituents	Ethanol extract of *B. monnieri*
Alkaloid	+
Reducing sugars	+
Tannins	+
Gums	+
Flavonoids	-
Saponin	+
Steroid	+

+: Positive result; - : Negative result;

3.2 Analgesic Activity

Table 2 showed the effect of the ethanolic extract of *B. monnieri* on acetic acid induced writhing in mice. At the dose of 250 mg/kg and 500 mg/kg body weight, the extract produced 36.69% and 59.17 %writhing inhibition in test animals respectively. The results were statistically significant (p <0.01 and p <0.001) and was comparable to the standard drug Diclofenac Na, which showed 72.78% at a dose of 25 mg/kg body weight.

Table 2. Effects of the ethanolic extract *B. monnieri* on acetic acid induced writhing of mice (n=5)

Group	Treatment and Dose	Number of writhes (% Writhing)	% Writhing Inhibition
Control	1% tween 80 solution 10 ml/kg, p.o.	16.9± 0.46 (100)	---
Positive Control	Diclofenac Na 25 mg/kg, p.o.	4.6 ± 0.33 ** (27.22)	72.78
Test Group- 1	Et. Extract of *B. monnieri* 250 mg/kg, p.o.	10.7± 1.62 * (63.31)	36.69
Test group- 2	Et. Extract of *B. monnieri* 500 mg/kg, p.o.	6.9 ± 0.37 ** (40.83)	59.17

*Values are expressed as mean±SEM (Standard Error Mean); Et: Ethanolic; *indicates P < 0.01 & **indicates P < 0.001; one-way ANOVA followed by Dunnett's test as compared to control; n = Number of mice; p.o.: per oral.*

3.3 Antidiarrheal Activity

Table 3 showed the effect of the ethanolic extract of *B. monnieri* on Castor oil induced diarrheal method in mice. The result showed that extract reduce the mean number of defecation which were 35.42 % and 47.92 % *(p*<0.001) at the doses of 250 mg/kg and 500 mg/kg respectively. The latent period for the extract treated group was (p <0.01) increased as compared to control group.

Table 3. Antidiarrhoeal activity of the ethanolic extract of *B. monnieri* in castor oil induced diarrheal test method on mice

Sample	Dose	Mean± SEM		% inhibition
		Latent period	Defecation	
1% Tween 80 in water	2 ml/mice, p.o.	0.75±0.10	9.6±0.25	--
Loperamide	50 mg/kg, p.o.	3.09±0.17**	4±0.32**	58.33
Et. Extract *B. monnieri*	250 mg/kg, p.o.	1.31±0.14*	6.20±0.37**	35.42
	500 mg/kg, p.o.	2.63±0.66*	5.0±0.32**	47.92

*Values are expressed as mean±SEM (Standard Error Mean); Et: Ethanolic; * indicates P < 0.01; ** indicates P < 0.001, one-way ANOVA followed by Dunnett's test as compared to control; n = Number of mice; p.o.: per oral.*

3.4 Cytotoxic Activity

In brine shrimp lethality bioassay, the extract showed lethality against the brine shrimp nauplii. It showed different mortality rate at different concentrations. From the plot of percent mortality versus log concentration on the graph paper LC_{50} and LC_{90} were deduced (LC_{50} = 40 µg/ml; LC_{90} = 150 µg/ml) (Table 4).

Table 4. Result of Brine Shrimp lethality bioassay of ethanolic extract of *B. monnieri*

Test Sample	Conc. (µgm/ml)	Log conc.	Avg. no of alive shrimp (sample)	% mortality	LC_{50}	LC_{90}
Et. Extract of *B. monnieri*	5	0.698	10	0	40	150
	10	1	9.5	5		
	20	1.301	9	10		
	40	1.602	5	50		
	80	1.903	2	80		
	160	2.204	0.5	95		

4. DISCUSSION

Antinociceptive activity of the ethanolic extract of *B. monnieri* was tested by acetic acid induced writhing model in mice. The peripheral analgesic effect of the plant's extract may be mediated via inhibition of cyclooxygenases and/or lipoxygenases (and other inflammatory mediators), while the central analgesic action of the extract may be mediated through inhibition of central pain receptors. This hypothesis is in consonance with those of Koster (Koster et al., 1959) and Williamson (Williamson et al., 1996) who postulated that acetic acid-induced writhing and hot-plate test methods are useful techniques for the evaluation of peripherally- and centrally-acting analgesic drugs, respectively. With respect to the writhing test, the research group of Deraedt described the quantification of prostaglandins by radioimmunoassay in the peritoneal exudates of rats, obtained after intraperitoneal injection of acetic acid (Derardt et al., 1980). These authors found high levels of prostaglandins PGE_2 and $PGF_{2\alpha}$ during the first 30 min after acetic acid injection. Few sympathetic nervous system mediators are also liberated by acetic acid (Hokanson, 1978). Besides, preliminary phytochemicals screening of *B. monnieri* revealed the presence of alkaloids, glycosides, steroids, reducing sugars, saponins and tannins. Plant materials containing phenols and tannins have been reported to possess analgesic property (Mills and Bone, 2000; Morteza-Semnani et al., 2006). The phytochemicals, particularly the triterpenes, phenolics and

tannins, could be attributed to the antinociceptive activity of *B. monnieri* (Vohora et al., 1997). The ethanolic extract of *B. monnieri* exhibited a significant writhing inhibition in test animals comparable to the standard drug, Diclofenac Na, in a dose dependent manner. Bhaskar and Jagtap (2011) also reported the analgesic activity of aqueous extract of *B. monnieri* at the dose of 160 mg/kg body weight. They also suggested that the aqueous extract of *B. monnieri* worked on the endogenous adrenergic, serotonergic and opioidergic systems for analgesic property. The present study also provides the evidence of antinociceptive activity of the ethanol extract of *B. monnieri* at the dose of 500 mg/kg body weight.

Castor oil or its active component ricinoleic acid induces permeability changes in mucosal fluid and electrolyte transport that results in a hypersecretory response and diarrhoea (Inayathulla et al., 2010). Diarrhoea results from an imbalance between the absorptive and secretory mechanisms in the intestinal tract, accompanied by hurry, resulting in an excess loss of fluid in the feces. In some diarrheal conditions, the secretory component predominants, while other diarrheas are characterized by hypermotility. The use of castor oil induced diarrhoea model in our study is logical because the autacoids and prostaglandins are involved these have been implicated in the causation of diarrhoea in human (Horton, et al., 1968; Greenbargena et al., 1978). The liberation of ricinolic acid from castor oil results in irritation and inflammation of the intestinal mucosa, leading to release of prostaglandins, which stimulate motility and secretion (Pierce et al., 1971).These observations tend to suggest that those extracts at a dose of 500 mg/kg reduced diarrhoea by inhibiting castor oil induced intestinal accumulation of fluid. These results are supported by the previous report on ripen fruits extract of *Rhus javanica* (Tangpu et al., 2004). Previous reports have demonstrated the antidiarrhoeal activity of tannin (Mukherjee et al., 1998), flavonoids (Galvez et al., 1993), alkaloids (Gricilda Shoba et al., 2001), saponins, reducing sugars and sterols and/or terpenes (Otshudi et al., 2000) containing plant extracts. The phytochemical analysis of the extract showed the presence of alkaloids, saponins, sterols and /or terpenes and sugars. Again, tannins and phenolics present in the plant extract are reported to inhibit release of autacoids and prostaglandins, thereby inhibit motility and secretion induced by castor oil (Veiga et al., 2001). Therefore, these constituents might be responsible for the antidiarrhoeal activity of *B. monnieri* ethanol extract.

The cytotoxic activity of the ethanolic extract of *B. monnieri* was tested by using brine shrimp lethality bioassay. It is a recent development in the bioassay for the bioactive compounds. Brine shrimp lethality bioassay indicate cytotoxicity as well as a wide range of pharmacological activities such as antimicrobial, pesticidal and antitumor (Beubler, 1979). The extract was found to show potent activity against brine shrimp nauplii. Previous researches demonstrate that *B. monnieri* saponin fractions have cytotoxic activity for sarcoma-180 cells. It is thought this might be due to *inhibition* of DNA replication in the cancerous cell line of this plant (Elangovan et al., 1995). Therefore the positive response obtained in this assay also provides the evidence of antitumor activity of the ethanol extract of *B. monnieri***.**

5. CONCLUSION

In conclusion it can be revealed that crude ethanolic leaf extract of *B. monnieri* possess significant antinociceptive, antidiarrhoeal and cytotoxicity activities. The potential of this extract as antinociceptive, antidiarrhoeal and cytotoxicity agents may be due to the presence of phytoconstituents like tannins, phenolics and might be responsible for its activity.

However, extensive researches are necessary to search for active principles responsible for these activities.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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