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Phytochemical Screening and Pharmacological Activities of *Entada Scandens* seeds

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Summary. *Entada scandens (E. scandens)* (family. Mimosaceae) is a widely used medicinal plant has been traditionally used by the folklore medicinal practitioners of Bangladesh to treat pain, cancer, gastrointestinal disorders where antinociceptive, cytotoxic and anti-diarrheal medications are implicated. Therefore, phytochemical groups and antinociceptive, cytotoxic, and anti-diarrheal activities of ethanol extract of seed of *E. scandens* were investigated by using acetic acid induced writhing model in mice, brine shrimp lethality bioassay and castor oil induced diarrheal model in mice. Phytochemical study of the extract indicated the presence of alkaloids, glycosides, tannins, flavonoids and saponins. At the doses of 250 and 500 mg/kg body weight, the extract showed a significant antinociceptive activity showing 60.61 and 72.73% inhibition respectively ($P<0.001$) comparable to that produced by Diclofenac Na (80.30%) used as standard drug. The extract showed significant toxicity in the brine shrimp lethality bioassay (LC_{50}. 20µg/ml & LC_{90}. 80µg/ml). While evaluating anti-diarrheal activity, the extract inhibited the mean number of defecation which were 13.21% ($P<0.01$) and 22.64 % ($P<0.001$) at the doses of 250 and 500mg/kg respectively. The latent period for the extract treated group was ($p<0.01$) increased as compared to control group. In addition, antimicrobial study was carried out by disc diffusion assay, but no significant inhibition was found against *Escherichia coli*, *Pseudomonas aureus*, *Plesiomonas shigelloides, Salmonella typhi, S. paratyphi, Shigella dysenteriae, S. flexneri, S. boydii, S. sonnei, Proteus vulgaris, Enterococcus faecalis, Staphylococcus saprophyticus, S. aureus, S. epidermidis* and *Streptococcus pyogenes*. The study tends to suggest the antinociceptive, cytotoxic and antidiarrheal activities of the crude ethanol extract of the seed of *E. scandens* and justify its use in folkloric remedies.

Industrial relevance. Medicinal plants can form an excellent source for derivation of lead compounds or newer drugs. The knowledge base of folk medicinal practitioners can in this instance form an invaluable source on which further scientific studies may be based, for the folk medicinal practices of the Kavirajes date back to centuries ago. They also have proved to be a rich source of new active compounds which are less toxic and less costly when compared to the synthetic drugs. The present study will help the industry to produce herbal drug with less side effect, economically affordable and more effective in the treatment of pain, diarrhea and inflammation processes. Finally the phytochemical screening from the plant would be effective drug for the antinociceptive, cytotoxic and antidiarrheal activities of the crude ethanol extract of the seed of *E. scandens*.

Keywords. *Entada scandens;* Phytochemical; Antinociceptive; Anti-diarrheal; Cytotoxic.

INTRODUCTION

Entada scandens auct. non. (L) Benth. (Synonym. *E. monostachya* DC, *E. rheedii* Spreng, *Mimosa entada* Linn.) belonging to Mimosaceae (or Mimosoideae), a subfamily of Leguminosae, locally known as Gila Lata (Bangla), Giley Ludi (Chakma) is a large woody climber (Joshi SG, 2000). The plant is found in Africa, Tropical Asia, Australia and in the small part of the Pacific Island. In Bangladesh, the species is commonly occurring in hilly tracts of Sylhet and Chittagong. The plant is also grown in the Asam in India where the seed of the plant is known as Mokori ghila. The plant has been used by the folkloric medicinal practitioners for long. The plant is used in the treatment of skin ulcer (cancer), snake bite, stomach disorders and ureterolithiasis (Uddin NS, 2006). The seed of the plant is used to treat fever, dysentery and rheumatism (Barukial J and Sarmah JN, 2011). Seeds are used in pains of the loins, in debility and in glandular swelling. They are given internally as an emetic. The kernel of the seed is employed by the people living in the hills as febrifuge (Kirtikar KR and Basu BD, 2006).

In a previous study, a potent kunitz type trypsin inhibitor was reported by Lingaraju MH *et al.* (Lingaraju MH and Gowda LR, 2008) and the determination of xenopus index and haemolytic index in seeds of *E. scandens* Benth was reported by Marthe Blyberg (Blyberg M, 1960). Janardhanan *et al.* reported the chemical composition and antinutritional factors (Janardhanan K and

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Nalini K, 1991). Gedeon J reported the presence of saponins and sapogenins (Gedeon J, 1954). Sasipriya G *et al.* reported the effect of different processing methods on antioxidant activity in the seed kernel (Sasipriya G and Siddhuraju P, 2012). Gautam B *et al.* reported the bioactive compounds, antioxidant activity and type II diabetes-related enzyme inhibition properties of the seed of the plant (Gautam B *et al.*, 2012). Vadivel *et al.* reported the total free phenolic content of the wild legume of the plant (Vadivel *et al.*, 2011). Dinesh Kumar *et al.* reported the potential antifertility agents in the plant (Dinesh Kumar *et al.*, 2012).

Since no literature is currently available to substantiate phytochemical, antinociceptive, cytotoxic and antidiarrheal activities of the ethanol extract of *Entada scandens* seeds, therefore the present study is a part of our on-going pharmacological screening of selected Bangladeshi medicinal plants (Ahmed F *et al.*, 2008a; Ahmed F *et al.*, 2010a; Ahmed F *et al.*, 2010b; Ahmed F *et al.*, 2008b; Faisal KS *et al.*, 2012; Naheed M *et al.*, 2012; Nayeem AA *et al.*, 2011; Rahman M *et al.*, 2010; Sadhu SK *et al.*, 2007a; Sadhu SK *et al.*, 2008; Sadhu SK *et al.*, 2007b; Siraj MA *et al*., 2012a; Siraj MA *et al*., 2013b; Khatum A *et al*., 2013) and designed to provide scientific evidence for its use as a traditional folk remedy by investigating the antinociceptive, cytotoxic and antidiarrheal activities that also confirm its use as pain killer, use against skin ulcer and cancer and other pathological conditions.

Figure 1. Seeds of *Entada scandens*

MATERIALS AND METHODS

Collection and identification of plant materials. *Entada scandens* was collected from Rangamati in the month of December 2011. The plant was mounted on paper and the species was taxonomically confirmed by Sarder Nasir Uddin, Principle Scientific Officer, Bangladesh National Herbarium (BNH), Mirpur, Dhaka. The voucher specimen of the plant has been deposited and preserved in BNH library for further collection and reference and an accession no was provided as DACB-34179.

Preparation of ethanol extract. The seeds of *Entada scandens* were freed from any of the foreign materials. Then the seed were chopped and air-dried under shed temperature followed by air drying. The dried plant materials were then ground into powder with the help of a suitable grinder (Capacitor start motor, Wuhu motor factory, China). About 250g of powdered material was taken in a clean, flat-bottomed glass container and soaked in 1000ml of 80% ethanol. The container with its contents was sealed and kept for a period of 15 days accompanying occasional shaking and stirring. The whole mixture then underwent a coarse filtration through a piece of clean, white cotton material. Then it was filtered through Whatman filter paper (Bibby RE200, Sterilin Ltd., UK) which was concentrated under air and dried. It rendered a 15.4 g gummy concentrate (yield approx. 6.16.0%) and was designated as crude ethanol extract.

Test for different chemical groups. The crude ethanolic extract was subjected for phytochemical study using standard methods (Rahman M *et al*., 2010) for its different chemical groups as alkaloids, flavonoids, gums, reducing sugars, saponins, steroids and tannins. In each test 10% (w/v) solution of the extract in ethanol was taken.

Test Animals & Drug. Young Swiss-albino mice of 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 at animal house of Pharmacy Discipline, Khulna University and fed with rodent diet and water ad libitum. All experimental protocols were in compliance with Khulna University Ethics Committee on Research of Animals as well as internationally accepted principles for laboratory animal use and care. The standard drug diclofenac Na was used for this study and collected from Square Pharmaceuticals Ltd, Bangladesh.

Bacterial strains. Bacterial strains were collected from the Microbiology Laboratory of International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), Dhaka, Bangladesh. Test organisms include Gram negative bacteria- *Escherichia coli*, *Pseudomonas aureus*, *Plesiomonas shigelloides, Salmonella typhi, Salmonella paratyphi, Shigella dysenteriae, Shigella flexneri, Shigella boydii, Shigella sonnei, Proteus vulgaris;* Gram positive bacteria- *Enterococcus faecalis, Staphylococcus saprophyticus, Staphylococcus aureus, Streptococcus pyogenes; Staphylococcus epidermidis.*

Antinociceptive activity. The antinociceptive activity of the crude ethanolic extract of *Entada scandens* was studied using acetic acid induced writhing model in mice (Nayeem AA *et al.*, 2011). 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 doses 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 vehicle control group was treated with 1% Tween 80 in water at the dose of 10ml/kg body weight. Test samples, standard drug and control vehicle were administered orally 30 min before intraperitoneal administration of 0.7% acetic acid. After an interval of 15 min, the mice were observed for writhing (constriction of abdomen, turning of trunk and extension of hind legs) for 5 min.

Hatching of shrimp. Artificial sea water was prepared by dissolving 20 g of NaCl and 18 g of table salt in one liter of distilled water and was filtered off to get a clear solution. A rectangular tank was divided in to two unequal compartments by a porous separator. The larger compartment was darkened while the smaller one was kept illuminated. The eggs of *Artemia salina* were hatched at room temperature (25-30 ºC) for 18-24 h. The larvae (nauplii) were attracted by the light and moved to the smaller compartment through the holes. They were then collected by a Pasteur pipette.

Brine shrimp lethality bioassay. The method of Meyer *et al*. was adopted to study the general toxicity of the extract (Meyer BN *et al*., 1982). The sample was dissolved in DMSO and then transferred to vials to get concentrations of 160, 80, 40, 20, 10 and 5µg/ml in 5 ml artificial sea water with ten nauplii in each vial. The concentration of DMSO did not exceed 0.01% in any of the vial. Control vials containing DMSO in artificial sea water at the same concentration as in test vials were also taken while anticancer drug 5-fluro uracil was used as positive control with the same concentration. After 24 h incubation at room temperature (25-30 ºC), the number of viable naupliis were counted using a magnifying glass.

Antibacterial assay by disc diffusion assay. Sterile blank discs (BBL, Cocksville, USA) were impregnated with test substances at the dose of 400 and 600 µg/disc. These discs, along with positive standard disc (30 µg/disc) (Kanamycin, Oxoid Ltd., UK) and negative control discs were placed in Petri dishes containing the Mueller-Hinton agar medium seeded with the test organisms using sterile transfer loop and kept at 4ºC to facilitate maximum diffusion. The plates were then kept in an incubator (37ºC) to allow the growth of the bacteria. The antibacterial activities of the test samples were determined by measuring the diameter of the zone of inhibition in terms of millimeter (Rahman M *et al.*, 2010).

Antidiarrheal activity. Antidiarrheal activity was tested by using Castor oil induced diarrheal method in mice (Ahmed F *et al.*, 2008; Shoba FG *et al.*, 2001). Twenty Swiss albino mice were randomly divided in to four groups (n=5). Control group received only distilled water (2ml/mice), positive control group received loperamide (50mg/kg body weight) as standard and test groups received the extracts at the doses of 250mg and 500mg/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 (1.0ml/mice). Extract and drugs were given orally 1hr 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 for 4 hours. 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}×100; where, Mo = Mean defecation of control and M = Mean defecation of test sample.

Statistical Analysis. All the in vitro experimental results were given as mean±SEM of three parallel measurements and data were evaluated by using student's t test. Statistical analysis for animal experiment was carried out using one-way ANOVA followed by Dunnet's multiple comparisons. The significant difference between the control group and experimental groups was determined. The results obtained from samples and control group were plotted in standard diagrams and good level of significance were found. P values<0.001 were regarded as significant.

RESULTS

Chemical group test. Results of different chemical tests on the ethanolic extract of *Entada scandens* seed showed the presence of alkaloid, glycoside, tannins, flavonoid and saponins and presented in Table 1.

Table 1. Results of different group tests of ethanolic extract of *Entada scandens* seed.

Phytoconstituents	Ethanol extract of *Entada scandens*
Alkaloid	+
Glycoside	+
Tannins	+
Gums	-
Flavonoids	+
Saponin	+
Steroid	-

Antinociceptive activity. Table 2 showed the effect of the ethanol extract of *Entada scandens* on acetic acid induced writhing in mice. At the dose of 250 & 500 mg/kg of body weight, the extract produced 60.61 & 72.73% writhing inhibition in test animals respectively. The results were statistically significant (P <0.001) and was comparable to the standard drug Diclofenac Na, which showed 80.30% writhing inhibition at a dose of 25 mg/kg weight (Figure 2).

Table 2. Effects of the ethanolic extract *Entada scandens* 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.	13.20± 0.59 (100)	
Positive Control	Diclofenac Na 25 mg/kg, p.o.	2.6 ± 0.51 * (19.70)	80.30
Test Group- 1	Et. Extract of *E. scandens* 250 mg/kg, p.o.	5.20 ± 0.38 * (39.39)	60.61
Test group- 2	Et. Extract of *E. scandens* 500 mg/kg, p.o.	3.6 ± 0.40 * (27.27)	72.73

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

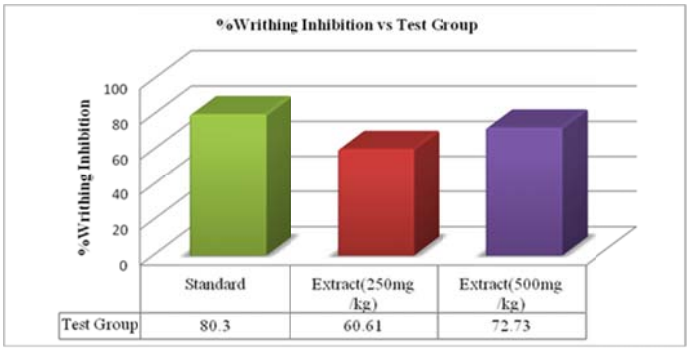

Figure 2. Percent writhing inhibition of acetic acid induced writhing in mice by the extract of *Entada scandens*

Brine shrimp lethality bioassay. In brine shrimp lethality bioassay (Table 3), the extract showed lethality against the brine shrimp nauplii. It showed different mortality rate at different concentrations. For the extract, the number of nauplii died and percent mortality was counted. From the plot of percent mortality versus log concentration (Figure 3), LC_{50} and LC_{90} were deduced (LC_{50}. 20 µg/mL; LC_{90}. 80 µg/mL) while the LC_{50} and LC_{90} of the standard anticancer drug 5-fluro uracil were 4.5 µg/ml and 6.5 µg/mL respectively. DMSO was used as a solvent. Control was used to see whether DMSO had any effect on brine shrimp lethality. The control group of brine shrimp nauplii with and without DMSO exhibited no mortality.

Table 3. Brine shrimp lethality bioassay of the ethanol extract of *Entada scandens*

Test sample	**Conc. (µg/ml)**	**Log of (Conc.)**	**No. of alive shrimp**	**% mortality**	**LC_{50} (µg/ml)**	**LC_{90} (µg/ml)**
Ethanol extract of *Entada scandens*	5	0.69	7	30	20	80
	10	1	6	40		
	20	1.3	5	50		
	40	1.7	3	70		
	80	1.9	1	90		
	160	2.2	0	100		

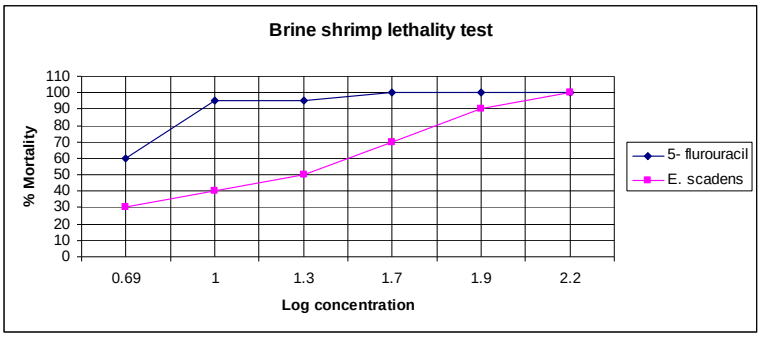

Figure 3. Brine shrimp lethality Test for the ethanol extract of *Entada scandens*

Antibacterial activity. The antibacterial property of the extract was assessed by conventional disc diffusion method using dried extracts of 500 µg/disc against a panel of 16 pathogenic bacterial strains and the results were compared with the activity of the positive control, kanamycin (30 µg/disc). But the extract did not show any significant zone of inhibition against the test organisms.

Antidiarrheal activity. Table 4 showed the effect of the ethanol extract of seed of *Entada scandens* on castor oil induced diarrheal method in mice. The results (Table 4) showed that the extract inhibited mean number of defecation which were 13.21% ($P<0.01$) and 22.64 % ($P<0.001$) at the doses of 250 and 500mg/kg respectively. The latent periods (1.01 and 1.24 hr) for the extract treated group at the doses of 250 and 500mg/kg respectively were ($p<0.01$) increased (Figure 4) as compared to control group (0.65 hr).

Table 4. Antidiarrheal activity of *E. scandens* in castor oil induced diarrheal test method on mice (n=5).

Sample	Dose	Mean± SEM		% inhibition
		Latent period	Defecation	
Distilled water	2ml/mice, p.o.	0.65±0.06	10.6±0.25	--
Loperamide	50mg/kg, p.o.	3.51±0.16**	4±0.32**	62.26
Et. Extract *Entada scandens*	250 mg/kg, p.o.	1.01±0.09*	9.2±0.37*	13.21
	500 mg/kg, p.o.	1.24±0.18*	8.2±0.49**	22.64

Values are expressed as mean±SEM (Standard Error for Mean); Et.. Ethanol; *P<0.01 ; **P< 0.001*; n = Number of mice; p.o.. per oral*

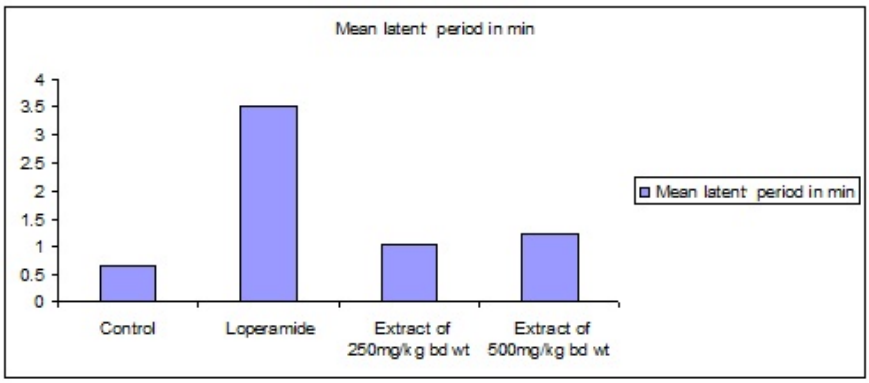

Figure 4. Antidiarrheal activity of the extract of *Entada scandens* seed in castor oil induced diarrheal test

DISCUSSION

The ethanol extract of seed of *Entada scandens* demonstrated the presence of alkaloids, tannins, saponins, flavonoids, and reducing sugar as secondary metabolites with cytotoxic, analgesic and antidiarrhoeal activities. The plant was reported for saponins and sapogenins, free phenolic content and of antioxidant activities (Gedeon J, 1954; Sasipriya G *et al* 2012; Vadivel V *et al*, 2011). Presence of saponins, sapogenins and phenolic contents may indicate the presence of tannins, saponins, flavonoids and other phenolic compounds (Gedeon J, 1954; Siddhuraju P, 2012; Gautam B *et al.*, 2012; Vadivel V *et al.*, 2011).

Analgesic activity of the extract was tested by acetic acid induced writhing model in mice. Acetic acid, which is used to induce writhing, causes algesia by liberation of endogenous substances, which then excite the pain nerve endings. 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 *et al*. and Williamson *et al.* who postulated that acetic acid-induced writhing method is useful technique for the evaluation of peripherally acting analgesic drug (Koster *et al*., 1959; Williamson *et al.*, 1996). With respect to the writhing test, the research group of Derardt *et al.* 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. The extract produced significant writhing inhibition (60.61% at dose 250 mg/kg and 72.73% at dose 500 mg/kg body weight) comparable to standard drug diclofenac sodium (80.3% at dose 25 mg/kg body weight). On the basis of the result of acetic acid induced writhing test, it can be concluded that the ethanol extract of *E. scandens* might possess a peripherally acting antinociceptive activity.

Brine shrimp lethality bioassay is an easy and straight forward bench top screening method for predicting important pharmacological activities like enzyme inhibition, ion channel interference, antimicrobial and cytotoxic activity (Nayeem AA *et al.*, 2011; Rahman M *et al.*, 2010; Meyer BN *et al.*, 1982; Anderson JE *et al.*, 1991). In the present study the extract showed LC_{50} at a low concentration indicating that the extract is significantly potent. Ideally, any agent useful in the treatment of cancer should not be toxic to normal cell. However, in reality, anticancer agents are often toxic to normal cells, particularly towards rapidly growing cells (Priestman T, 2008). It is necessary to test this extract against various cancer cell lines as well as normal cell lines to justify the potential to further investigate this plant for anticancer activity. Further investigation is required to find the responsible compound(s) for the cytotoxic activity observed for *E. scandens*.

Compared to control animals, the extract inhibited significantly the frequency of defecation and reduced greatly the wetness of faecal excretion. As with other laxatives, castor-oil changes the intestinal permeability and the histology (Mascolo N *et al.*, 1993). The findings provide a support for the use of the plant as antidiarrhoeal remedies in Bangladeshi folk medicine.

CONCLUSION

Present study is based on the report of preliminary biological screening of *E. scandens* seed extract. The results are quite promising; support the use of the plant in traditional medicine and demands further investigation. Advanced studies including LC-MS can be carried out to get a bigger picture of the chemical constituents present in the plant. Screening methods applying various cell lines or bacterial enzymes can be carried out to find the underlying mechanism for the observed biological activities. On the basis of the results from above studies, bioassay guided approach can be undertaken to isolate and identify the active component(s).

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