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Research Article

PHYTOCHEMICAL SCREENING AND ANALGESIC, ANTI-BACTERIAL AND CYTOTOXIC ACTIVITY EVALUATION OF ETHANOL EXTRACT OF *PITHCELLOBIUM DULCE* (ROXB.) BENTH LEAF

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ABSTRACT

Introduction: *Pithcellobium dulce* (Roxb.) benth, tree 5-18 m high, with pendulous branches, short, sharp stipular spines. It belongs to Fabaceae family. This plant's accession number is 31,388 as per Bangladesh national herbarium.

Methods: For the investigation of its phytochemical and biological activities, the concentrated ethanolic extract of the leaf were extracted.

Objective: This crude extract was tested for the presence of various chemical groups and alkaloid, flavonoids, saponin and reducing sugar were identified. The traditional medicinal use of this plant prompted us to check analgesic, anti-bacterial, anti-diarrheal and cytotoxic effects.

Result and Discussion: The ethanol extract of the leaf produced 29.4% and 61.54% protection or writhing inhibition in mice at orally dosage of 250 mg/kg ($p<0.001$) and 500 mg/kg ($p<0.001$) body weight of mice, respectively. This was comparable to the standard drug diclofenac sodium at the dose of 25 mg/kg of body weight. This extract increased mean latent period and decreased the frequency of defecation significantly at the oral dose of 500 mg/kg body weight, which is also comparable to standard loperamide at dose of 50 mg/kg body weight. This leaf extract also showed antimicrobial activity only against shigella dyst-1 among 10 bacterial species culture. In addition, it also showed cytotoxic effect-50% (LC_{50}) mortality of Brine Shrimp nauplii occurred at 20 µg/ml and 90% (LC_{90}) mortality at 80 µg/ml ethanolic leaves crude extract.

Conclusion: Most of the medicinal plants of Bangladesh have more or less biological activity. It was a preliminary study; further study can be done for the fulfillment of this experiment based on different extract concentration.

Keywords: Reagent, Dulce, Extract, Benth Leaf.

INTRODUCTION

Plants play an important role in healthcare for about 80% of the world's population. The phytochemicals derived from plant sources possess a complex of chemicals with unique biological activity. The presence of diverse bioactive metabolites such as carbohydrates, saponins, flavonoids, tannins, etc. in plants has formed the therapeutic basis of herbal medication. Thus, emphasis is given on the biological screening of medicinal plants for further exploration of their active constituents. In Bangladesh the plant is traditionally used for various purposes e.g., analgesic, anti-diarrheal, tranquilizing action [1]. The number of plants with medicinal properties included in the material medica of traditional medicine in this subcontinent at present stands at about 2000 (Chopra *et al*., 1958) [16]. More than 500 of such medicinal plants have so far been enlisted as growing in Bangladesh (Yusuf *et al.*, 1994). About 33% of drugs produced in the developed countries are derived from plants 60% of the modern medicinal products are of natural origin and 6 (Goldstein, 1974). For the year 1960, it was revealed that over 300 million drug prescriptions were of natural origin, mostly of antibiotics (Gosselin, 1962). At a glance more than 80% of the present-day medicine are directly and indirectly are obtained from plants [12]. Surprisingly only 15% from this large quantity were investigated pharmacologically (Fransworth *et al.*, 1976).

P. dulce (Roxb.) benth. belongs to family fabaceae, mostly grown in India and Bangladesh. These are usually street trees and used for ornament because of its handsome foliage and curious pods. The local name is "Khai Babla" in Bangladesh. The tress is 5-18 m high, with pendulous branches and with short and sharp stipular spines. Leaves are evenly 2 pinnate, 4-8 cm long. Flowers are white and dense head, 1 cm in diameter. Pods are turgid, twisted and spiral, 10-18 cm long, and seeds are 6-8 cm, the edible and a whitish pulpy aril. The plants usually prefer tropical and sub-tropical climate areas where rainfall is between 450 and 1650 per-year. It is drought resistant and grows from sea level-up to 1800 m in clay, dry and wet soil and sands (Edwards, R., 1998). Chemical investigation reports that presence of steroids, saponins, triterpene oligoglycosides such as mixture of oleanolic acid and echinocystic acid glycosides, lipids, phospholipids, glycosides, glycolipids, and polysaccharides has been present in the seeds [8]. A lysozyme has been isolated, purified and identified from *P. dulce* seeds with antifungal activity (3). Three acylated saponins and a related compound are found in *P. dulce* (Suzaki *et al*., 1997). Traditionally, the bark is used as an astringent in dysentery, fever, dermatitis and eye inflammation. The leaves possess emollient, anti-diabetic, anti-leprotic, anti-convulsant and antibacterial including anti-tubercular property [5]. In addition, the stem bark and leaves of *P. dulce* have antifungal and also possess a-glycosidase inhibitory activity [3,4]. It was reported; antioxidant and free radical scavenging activity of extracts of *P. dulce* seeds were identified [2]. Usually, the tender leaf paste is mixed with the seeds powder of *P. dulce* and is given orally in empty stomach to cure diabetes [7]. This plant has also venom neutralizing property [6].

However, it is a matter of regret that there is a dearth of scientific reports on this plant properties based on traditional use such as anti-diabetic, analgesic, antibacterial, anti-diarrheal activity despite of its widely usage as medicinal plant. Due to the availability of this genus in Bangladesh and depending on the tenability of laboratory facilities,

the present study was designed to evaluate the analgesic, antibacterial, anti-diarrheal and cytotoxic effects. The objective of the study is to investigate the scientific basis of the traditional use of this plant, *P. dulce*.

METHODS

Collection of plant material

Leaf of *P. dulce* was collected from Khulna University Campus, Bangladesh, during the month of October, 2013. The plant material was authenticated by the expert of Bangladesh national Herbarium. The pants accession number is 31,388. Freshly collected leaves were cleaned to remove adhering dust, and any adulteration was strictly prohibited. Then the collected leaves were dried under shade for 2 weeks. The dried samples were powdered with a suitable grinder to make powder and stored in an air tight container and cool, dark and dry place for solvent extraction and analysis.

Extraction of plant material

The air dried powdered leaf material (250 g) of *P. dulce* was successively tays accompanying occasional shaking and stirring. The whole mixer was then underwent a coarse filtration by a piece of clean, white cotton material. Then it was filtered through Whatman filter paper. The filtrate thus obtained was concentrated in the open air. The ethanol extract was evaporated by rotary vacuum evaporator and then it rendered gummy concentrated (12 g) extract. The extract was stored in the refrigerator for subsequent analysis.

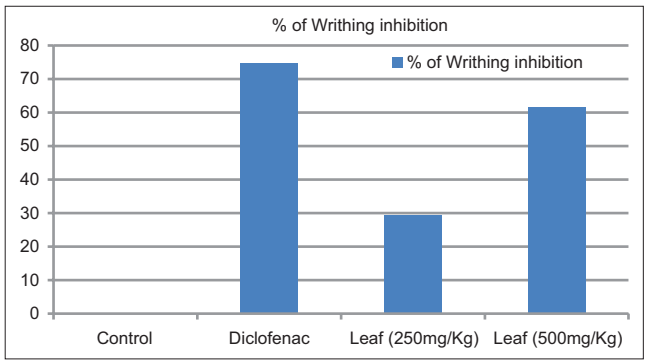

Fig. 1: Percent of writhing inhibition of ethanolic extract of the leaf of *Pithecellobium dulce* (Roxb.) Benth on acetic acid-induced writhing of mice

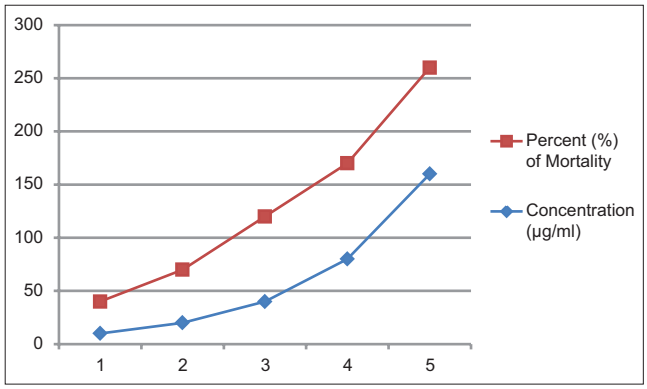

Fig. 2: LC_{50} and LC_{90} of *Pithecellobium dulce* (Roxb.) Benth

Chemical group test

Testing of different chemical groups present in the extract, represent the preliminary phytochemical studies. The performed chemical group test was completed as per Evans, 1983; Walls, 1985 and Plummer, 1985 described methods. In each test 10% (w/v) ethanol extract solution taken (Evans, 1983; Walls, 1985 and Plummer, 1985).

Chemicals, medicines and reagents

Mayer's reagent, Hager's reagent, Dragendroff's reagent, Fehling's solution A and B, benedicts reagent, Molish reagent, 10% of lead acetate solution, sodium carbonate, ammonia, sodium hydroxide, bromine solution, diclofenac sodium, loperamide and Liebermann–Burchard reagent were used for identifying different chemical groups. All other chemicals and solvents used are of analytical grade. These were collected from BCSIR as a gift and purchased from local chemical and pharmaceutical companies.

Determination of steroids

Liebermann–Burchard test

1 ml solution of ethanol extract was taken and then added Liebermann–Burchard reagent. If reddish purple color was produced, which indicated the presence of steroids.

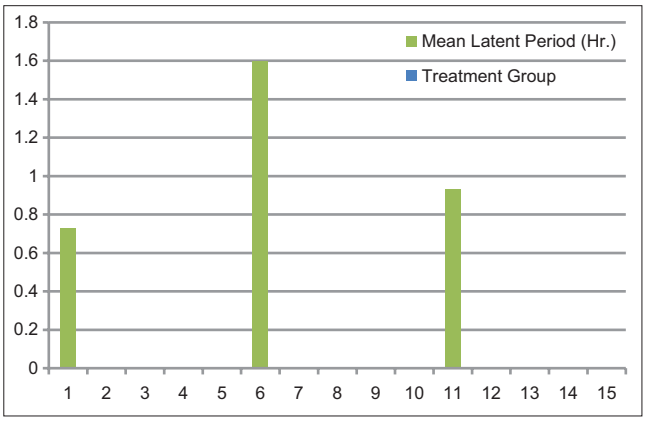

Fig. 3: Effect of crude extract of leaves of *Pithecellobium dulce* (Roxb.) Benth. on the latent period of castor oil-induced diarrheal episode in mice. Each bar represents mean latent period

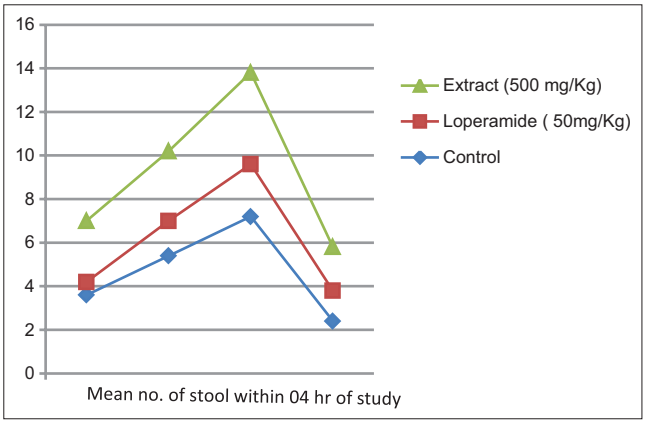

Fig. 4: Effect of loperamide and crude extract of leaves of *Pithecellobium dulce* (Roxb.) Benth. on castor oil-induced diarrhea in mice through 4 hrs of observation period

Table 1: Results of different group tests

Extract	Steroid	Alkaloid	Flavonoid	Reducing sugar	Tannin	Gum	Saponin
Ethanolic extract of leaf	–	+	+	+	–	–	+

+: Present, –: Absent

Sulfuric acid test

1 ml solution of ethanol extract was taken and then added 1 ml of sulfuric acid. If ethanol layer showed reddish brown color and acid layer showed fluorescence, which indicated the presence of steroids.

Determination of alkaloid

Mayer's test

A volume of 2 ml of extract and 0.2 ml of dilute hydrochloride acid were taken in a test tube. Then 1 ml of Mayer's reagent was added. If yellow color precipitate was formed, which indicates the presence of alkaloid.

Dragendroff's test

A volume of 2 ml of extract and 0.2 ml of dilute hydrochloride acid were taken in a test tube. Then 1 ml of Dragendroff's reagent was added. If orange-brown color precipitate was formed, which indicates the presence of alkaloid.

Wagner's test

A volume of 2 ml of extract and 0.2 ml of dilute hydrochloride acid were taken in a test-tube. Then 1 ml of Wagner's reagent was added. If reddish brown color precipitate was formed, which indicates the presence of alkaloid.

Hager's test

A volume of 2 ml of extract and 0.2 ml of dilute hydrochloride acid were taken in a test tube. Then 1 ml of Hager's reagent was added. If yellowish color precipitate was formed, which indicated the presence of alkaloid.

Table 2: The effects of crude extract of the leaf of *Pithecellobium dulce* (Roxb.) Benth at the dose of 250 mg/kg and 500 mg/kg body weight on acetic acid-induced writhing of mice

Group (dose)	Number of mice	Body weight (g)	Extract dose (ml)	AA (dose)	Writhing count
Control (10 ml/kg)	1	28	N/A	0.28	30
	2	24	N/A	0.24	32
	3	20	N/A	0.2	28
	4	26	N/A	0.26	29
	5	24	N/A	0.24	24
Diclofenac sodium (25 mg/kg)	1	20	0.2	0.2	8
	2	23	0.23	0.23	6
	3	24	0.24	0.24	9
	4	21	0.21	0.21	6
	5	22	0.22	0.22	7
Leaf of *Pithecellobium dulce* (250 mg/kg)	1	23	0.23	0.23	23
	2	25	0.25	0.25	20
	3	31	0.31	0.31	18
	4	23	0.23	0.23	21
	5	25	0.25	0.25	19
Leaf of *Pithecellobium dulce* (500 mg/kg)	1	30	0.3	0.3	13
	2	27	0.27	0.27	11
	3	31	0.31	0.31	9
	4	25	0.25	0.25	10
	5	24	0.24	0.24	12

AA: Acetic acid

Determination of saponins

A volume of 1 ml solution of ethanol extract was taken and then diluted with distilled water to 20 ml and shaken in a graduated cylinder for 15 minutes. 1 cm layer of foam indicated the presence of saponin.

Determination of gums

A volume of 5 ml solution of ethanol extract was taken and added molish reagent and sulfuric acid. If a red-violet ring was produced at the junction of two liquids, which indicated the presence of gums.

Determination of reducing sugar

Benedict's test

A volume of 0.5 ml of aqueous extract of the plant material was taken in a test tube. 5 ml of Benedict's reagent solution was added and boiled for 5 minutes and allowed to cool spontaneously. If a red color precipitate of cuprous oxide was formed indicating the presence of reducing sugar.

Fehling's test

A volume of 2 ml of aqueous extract of the plant material was added with 1 ml of equal volume of Fehling's solution A and B and boiled for few minutes. If a red or brick red color precipitate was formed which indicated the presence of reducing sugar.

Alpha napthol test or ring test

A volume of 5 ml solution of ethanol extract was taken, and two drops of freshly prepared alpha napthol solution were added. Then 1 ml of sulfuric acid was added on the side of the test tube. A violet colored ring was formed at the junction of two liquids in the presence of a reducing sugar.

Determination of tannin

Ferric chloride test

A volume of 5 ml solution of ethanol extract was taken in a test tube and then 1% of ferric chloride solution was added. Greenish black precipitate was the determinant for the presence of tannins.

Potassium dichromate test

A volume of 5 ml solution of ethanol extract was taken in a test tube and then 10% of potassium dichromate solution was added. A yellow precipitate was the determinant for the presence of tannins.

Lead acetate test

A volume of 5 ml solution of ethanol extract was taken in a test tube and then 10% of lead acetate solution was added. A yellow precipitate was the determinant for the presence of tannins.

Aqueous bromine solution test

A volume of 5 ml solution of ethanol extract was taken in a test tube and then 1 ml aqueous bromine solution was added. A brown precipitate was the determinant for the presence of tannins.

Determination of flavonoid content

Determination of flavonoid content was done in a few methods. A few drops of concentrated hydrochloride acid were added to a small amount of an alcoholic extract of the plant material. Immediate development of a red color indicates the presence of flavonoids. This was also analyzed

Table 3: Statistical evaluation of the results

Animal group	Writhing count					Mean	Percentage of writhing	SD	SE	Percentage of writhing inhibition	t-test
Control	30	32	28	29	24	28.6	100	2.653	1.33	0	N/A
Diclofenac	8	6	9	6	7	7.2	25.17	1.166	0.583	74.82	p<0.001 (14.74)
Leaf (250 mg/kg)	23	20	18	21	19	20.2	70.6	1.72	0.86	29.4	p<0.001 (5.306)
Leaf (500 mg/kg)	12	11	10	9	12	11	38.46	1.414	0.707	61.54	p<0.001 (11.69)

SD: Standard deviation, SE: Standard error

by other three means. 10 ml of solution of the extract hydrolyzed with 10% sulfuric acid was divided into three portions: (1) 1 ml dilute ammonia solution was added in one portion. Greenish yellow indicated the presence of flavonoids, (2) 1 ml dilute sodium carbonate solution was added in one portion. Pale yellow indicated the presence of flavonoids, (3) 1 ml dilute sodium hydroxide solution was added in one portion. A yellow indicated the presence of flavonoids.

Pharmacological evaluation

Analgesic activity

Preparation of sample and doses used

To prepare the suspension, the extract was weighed in a beaker and added a small amount of tween 80 with distilled water to it for maintaining the desired volume of sample and triturated properly. This was reserved in a separate container to be used in pharmacological experiment. For the pharmacological experiment the drug (extract) dose was maintained 250 mg/kg and 500 mg/kg body weight.

Experimental animal

Young Swiss-Albino mice aged 4-5 weeks, average weight 20-25 g were used for the experiment. The mice were purchased from animal branch of International Center for Disease and Research, Bangladesh. They were kept in standard condition, in cages having dimensions of 30 cm × 20 cm × 13 cm and soft wood shavings were employed as bedding in the cages. The animals were provided with standard laboratory food, tap water, and libitium. And they were maintained at natural day-night cycle. A group of equal number of mice as the drug treated group was simultaneously employed in the experiment.

Acetic acid (AA) induced writhing test

The peripheral analgesic activity of the extract was evaluated using AA-induced writhing inhibition method in mice. In this method, mice were randomly divided into three groups, each consisting of 05 animals. For this experiment, each group received a particular treatment, i.e. control, positive control and sample extract. Muscular contraction was induced by the intraperitoneal injection of 0.7% AA The test preparations were administered orally 30 minutes before the intraperitoneal injection of AA. Mice were cased individually to count number of writhes (painful muscular contraction) after 5 minutes of AA injection for 15 minutes. The average number of writhes and the percent protection were calculated and then compared between the animals of the experimental groups and the animals of the control group. Full writhing was not always accomplished by the animals; the animals started to give writhing, but they did not complete it. This incomplete writhing was considered as half-writhing. Accordingly, two half-writhings were taken as one full writhing. Analgesic activity was expressed as writhing inhibition (%) and was calculated for each animal using the following formula: Writhing inhibition (%) = {(Wc – Ws)/Wc} 100 Where, Wc is the mean number of writhings of the control and Ws is the mean number of writhings of the test sample [19,20].

Screening of anti-microbial activity

Anti-microbial screening can be done in three different ways - Diffusion method, bioautographic method and disc diffusion method. The assay was performed using the popular disc diffusion method. The assay was performed with two types of discs-sample discs, standard discs. Seven pathogenic bacteria were used as test organisms for antibacterial activity of dried sample extract. The bacterial strains were collected from BCSIR Chittagong, Bangladesh. 500 µg/disc of the sample extract were used to observe the anti-microbial activity of the plant extract and compared with the standard kanamycin (30 µg/disc). The test organisms were inoculated on 15 ml previously sterilized nutrient agar media, mixed thoroughly and transferred immediately to the sterile petri dish in an aseptic condition using a sterile loop under laminar air flow. Prepared sample and standard solutions were applied to the corresponding petri dish. The plates were incubated for overnight at 37°C approximately for 14-16 hrs. After proper incubation, clear zone of inhibition around the point of application of sample solution were measured which is expressed in millimeter (mm). Nutrient agar media was prepared by adding water to a dehydrated product that contained all ingredients (Pelczar *et al.*, 1986) [13,14].

Cytotoxic test using Brine Shrimp lethality bioassay

Brine Shrimp lethality bioassay was used for testing cytotoxic potential of the extract. The eggs of Brine Shrimp (*Artemia salina* Leach) were collected and hatched in a tank at a temperature around 37°C with continuous oxygen supply. Approximately 40 hrs were allowed to hatch and mature the nauplii. Stock solutions of the sample were prepared by dissolving required amount of extract in a specific volume of pure dimethyl sulfoxide (DMSO). 20 clean test tubes were taken, 10 of which were for sample in five concentrations (two test tubes for each concentration) and 10 test tubes for control test. 5 ml of seawater was given to each of the test tubes. Then specific volume (2, 4, 8, 16, and 32 µg/ml) of sample was transferred from the stock solution to the test tubes to get final sample concentrations of 10, 20, 40, 80 and 160 µg/ml with the help of micro pipette. The concentration of DMSO in these test tubes did not exceed 40 µl/4 ml. In the control test tubes, same volumes of DMSO (as in the sample test tubes) were taken. With the help of a Pasteur pipette, 10 living nauplii were put to each of the test tubes. After 24 hrs, the vials were observed, and the number of nauplii survived in each vial was counted. After that, the percentage of lethality of Brine Shrimp nauplii was calculated for each concentration of the extract [11,12].

Table 4: *In vitro* anti-microbial activity of ethanol extract of *Pithecellobium dulce* (Roxb.) Benth

Bacterial strains	Diameter of zone inhibition (mm)	
	Kanamycin (30 µg/disc)	Ethanol extract (500 µg/disc)
Gram-positive		
Staphylococcus saprophyticus	21	0
Staphylococcus aureus	23	0
Staphylococcus epidermidis	25	0
Gram-negative		
Shigella boydii	24	0
Salmonella typhi	25	0
Pseudomonas	22	0
Shigella dyst-1	23	7
Plesiomonas	24	0
Shigella sonnie	24	0
Shigella flees	24	0

Table 5: Result of brine shrimp lethality bioassay of 80% ethanolic extract

Test sample	Concentrated (µg/ml)	Log (concentrated)	Number of alive Shrimp	Percent of mortality	LC_{50} (µg/ml)	LC_{50} (µg/ml)
80% ethanolic extract	10	1	7	30	20	80
	20	1.3	5	50		
	40	1.6	2	80		
	80	1.9	1	90		
	160	2.2	0	100		

Table 6: Effect of *Pithecellobium dulce* (Roxb.) Benth. on the latent period of castor oil-induced diarrheal episode in mice

Group (dose)	Number of mice	Mean latent period (Hr.)	SD	SE	t-test (p value)
Control	1 2 3 4 5	0.728±0.070	0.14	0.07	N/A
Loperamide (50 mg/kg)	1 2 3 4 5	1.598±0.265	0.53	0.265	3.175<0.05
Extract 500 mg/kg	1 2 3 4 5	0.932±0.047	0.094	0.047	2.41<0.1

Values for t-test (n=5), p<0.1 versus control. SD: Standard deviation, SE: Standard error

Table 7: Effect of *Pithecellobium dulce* (Roxb.) Benth. on castor oil-induced diarrhea in mice

Group (dose)	Period of study (hr)	Mean latent period±SE	Mean number of stool	SE	t-test (p value)
Control	1	0.728±0.070	3.6	0.4301	N/A
	2		5.4	0.6964	N/A
	3		7.2	0.406	N/A
	4		2.4	0.533	N/A
Positive control (loperamide)	1	1.598±0.265	0.6	0.187	6.3967[a]
	2		1.6	0.43	4.64[a]
	3		2.4	0.5099	7.365[b]
	4		1.4	0.3674	1.54[d]
Extract of *Pithecellobium dulce* (500 mg/kg)	1	0.932±0.047	2.8	0.463	1.266[e]
	2		3.2	0.604	2.3866[c]
	3		4.2	0.604	4.1225[a]
	4		2	0.353	0.6257[c]

Values for t-test (n=5), [a]p<0.01, [b]p<0.001, [c]p<0.05, [d]p<0.2, [e]p<0.3, [f]p<0.5 versus control, Student's t-test. SE: Standard error

Anti-diarrheal activity

Sample preparation

To prepare the suspension of test sample at the doses of 500 mg/kg body weight was taken and triturated in an unidirectional manner by the addition of a small amount of tween-80. After proper mixing distilled water was added slowly, and final volume was maintained 5.0 ml.

Test design

Anti-diarrheal activity of the ethanol extract of *P. dulce* leaves was tested using the model of castor oil-induced diarrhea in mice. According to the method, mice were randomly divided into three groups and fasted overnight before the experiment. Each group received a particular treatment, i.e. control (1% v/v tween-80 in normal saline, 0.5 ml/mice), positive control (loperamide, 50 mg/kg body weight), and test samples (500 mg/kg). Mice were fed with the sample 1 hr prior to the oral administration of castor oil at the dose of 0.5 ml each. Individual animals of each group were placed in separate cages having adsorbent paper beneath and examined for the presence of diarrhea every hour in 5 hrs study after castor oil administration. Number of stools or any fluid that stained the adsorbent paper were counted each successive hour during 4 hrs of study and noted for each mouse. The latent period was also counted. At the beginning, each hour a new paper were placed for the old ones (Shoba and Thomas, 2011) [15].

RESULT AND DISCUSSION

Presence of different chemicals

Phytochemical studies showed that alkaloids, flavonoids, saponin and reducing sugars are present in ethanolic extract of *P. dulce* (Roxb.) Benth. However, steroid, tannin, and gum were absent.

Analgesic activity

Each mouse of all groups was observed carefully to count the number of writhing that they had made in 15 minutes. The animal do not always perform full writhing because sometimes animals begin to produce writhing but they do not complete it. This incomplete writhing were taken as half writhing, so two half-writhing were taken as one full writhing. That is why total writhing was halved to convert all writhing to full writhing. That is why the total writhing was halved to convert all writhing to full writhing or real writhing.

Result of statistical analysis

Significance

Control versus diclofenac sodium: Significant (p<0.001). Control versus leaf extracts (250 mg/kg): Significant (p<0.001) and control versus leaf extracts (500 mg/kg): Significant (p<0.001).

Writhing inhibition

Ethanol extract of the leaf of *P. dulce* (Roxb.) Benth produced 29.4% and 61.54% protection or writhing the inhibition in mice at orally doses of 250 mg/kg and 500 mg/kg body weight of mice, respectively.

The results of analgesic activity screening showed that the ethanolic extract of the leaf of *P. dulce* (Roxb.) Benth possess writhing inhibitory activity. It was a preliminary study. Further study can be done for more accurate result with confident.

Antibacterial activity

After proper incubation, the antibacterial activity of the test agent was determined by measuring the diameter of the zone of inhibition in term of millimeter with a slide calipers.

The ethanol extract of *P. dulce* (Roxb.) Benth leaves showed activity against *Shigella* dyst-1 among 10 bacterial species.

Cytotoxic activity

In this bio-assay, the crude extract showed the lethality indicating the biological activity of the compound present in the extract. Test sample showed different mortality rate at different concentrations. The mortality rate of Brine Shrimp was found to be increased in concentration of the sample and plot of percent mortality versus log concentration on the graph paper produced an approximate linear correlation between them. From the graph the concentration at which 50% mortality (LC_{50}) of Brine Shrimp nauplii occurred were obtained by extrapolation. The values were found to be 20 µg/ml for the crude extract. The 90% mortality (LC_{90}) values were 80 µg/ml for the crude extract (Table 4 and Fig. 2).

Anti-diarrheal activity

At the doses of 500 mg/kg body weight the isolated compound, *P. dulce* (Roxb.) Benth compared to the control group, offered about 0.932 (hrs) of the mean latent period for diarrheal episode to ensure an increase latent period compare to control. The mean no. of stool at the 1st, 2nd, 3rd and 4th hr of study were 2.8, 3.2, 4.2 and 2, respectively, which show decrease defecation compare to control at 500 mg/kg body weight.

P. dulce leaves extract showed a marked anti-diarrheal activity in castor oil-induced test. At the dose of 500 mg/kg body weight as compared

with the standard loperamide. This extract caused an increase in a latent period, i.e., delayed the onset of diarrheal episode and decrease in frequency of defecation. T-test of these responses showed that the result is slightly significant compare to positive throughout the observation period. Therefore, we claimed *P. dulce* leaves possess anti-diarrheal activity.

CONCLUSION

Most of the medicinal plants of Bangladesh have more or less biological activity. From this point of view ethanolic extract of *P. dulce* (Roxb.) Benth leaves were taken for analgesic, antibacterial, anti-diarrheal and cytotoxic activity evaluation based on its traditional medicinal use. Phytochemical study showed the presence of alkaloid, flavonoids, saponin and reducing sugar in extract of *P. dulce* (Roxb.) Benth leaves. This extract showed writhing inhibitory activity, antibacterial activity against only *Shigella* dyst-1 among 10 species of bacterial, lethality against the Brine Shrimp nauplii and marked anti-diarrheal activity in castor oil-induced mice. However, further researches are necessary particularly with its purified extraction of phytochemicals. From literature review, In case of microbial activity this leaf has also activity against other microbes such as *Candida albicans*, *Aspergillus niger*, *Penicillium digitatum*, *Rhizopus stolonifer*, *Mycobacterium tuberculosis*. These microorganisms were not available at that moment. It was a preliminary study; further study can be done for the fulfillment of this test in different concentration of extract and gender-based effects on mice.

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APPENDIX FOR STATISTICS AND OTHER FORMULA USED

1. Arithmetic Mean ($\overline{X}$) = $\frac{\Sigma X}{n}$

Where, ΣX = Summation of Observed Value
n = No. of Observation

2. Standard Deviation (SD) = $\sqrt{\frac{\Sigma(X-\overline{X})^2}{n}}$

Where, X = individual Value
$\overline{X}$ = Mean Value
n = No. of Observation

3. Standard Error (SE) = $\frac{SD}{\sqrt{(n-1)}}$

Where, SD = Std. Deviation
n = No. of Observation

4. Student's t-Test

$$t = \frac{M_1 - M_2}{\sqrt{(SE_1)^2 + (SE_2)^2}}$$

Where, M_1 = Mean Value of Group- 1 (Control)
M_2 = Mean Value of Group- 2 (Test)
SE_1 = Std. Error of Group- 1
SE_2 = Std. Error of Group- 2

5. Degree of Freedom = $(n_1 - 1) + (n_2 - 1)$

Where, n1 = Number of observation's of 1st sample
n2 = Number of observation's of 2nd sample

Or n*= (n-1),where, n=Number of mice in that group.

6. % of Writhing = (Mean of Test / Mean of Control) X 100

7. SE for % Writhing = (SE / Control Mean) X 100