ANTINOCICEPTIVE, ANTIDIARRHOEAL AND CYTOTOXIC ACTIVITIES OF *RHIZOPHORA MUCRONATA* LAMK.

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Summary

The ethanol extract of dried leaves of *Rhizophora mucronata* lamk. (Family–Rhizophoraceae) was investigated for its possible antinociceptive, antidiarrhoeal and cytotoxic activities in animal models. The extract produced significant (P<0.001) writhing inhibition in acetic acid-induced writhing in mice at the oral dose of 250 and 500 mg/kg of body weight comparable to the standard drug diclofenac sodium at the dose of 25 mg/kg of body weight. The extract showed antidiarrhoeal activity on castor oil induced diarrhoea in mice, it increased mean latent period and decreased the frequency of defecation significantly (P<0.001, P<0.01) at the oral dose of 500 mg/kg of body weight comparable to the standard drug Loperamide at the dose of 50 mg/kg of body weight. The crude ethanolic extract also produced the most prominent cytotoxic activity against brine shrimp *Artemia salina* (LC_{50} = 40 µg/ml and LC_{90} = 80 µg/ml). The obtained results provide a support for the use of this plant in traditional medicine and its further investigation.

Key Words: Antinociceptive activity, antidiarrhoeal activity, cytotoxic activity, *Rhizophora mucronata* lamk.

Introduction

Rhizophora mucronata lamk. (English Name: True Mangrove, Asiatic Mangrove, Mangrove; Family: Rhizophoraceae; Synonym: *Rhizophora latifolia* Miq., *Rhizophora macrorrhiza* Griffith.) locally known as 'Jhana or Jhana Gorjone' in Bangladesh. It is also known as Pyoo (Burmese), Bakßuan (Filipino), Bakau Bakau Hitam (Indonesian), Dyankar (Javanese), Bakau Jangkar (Malay), Phangka (Thai), Duoc Bop (Vietnamese), Lengayong (Brunei), Doeum Prasak (Cambodia), Mangoro (Papua New Guinea) and Belukap (Singapore). It is a much branched large shrub or moderate sized tree, up to 10 m tall, supported on adventitious prop roots from stem and branches with reddish brown bark distributed throughout largest mangrove forest in Bangladesh. It is native to tropical and subtropical coastal areas from the African east coast, throughout Asia to Australia and to most islands of the eastern Pacific Ocean; closely allied with Atlantic–East Pacific red mangroves whose ranges naturally overlap only in a small number of southern Pacific islands. It is also found in Mozambique, Madagascar, Pacific Islands, Islands of the Indian Ocean, Indonesia, Philippines and South Pacific Islands as far as the Tonga group. In 1922 this species was introduced into Hawaii and is naturalized there[1-2].

Leaves of *Rhizophora mucronata* are poulticed armored fish injuries. Different communities use it for different purposes such as Indo-Chinese use the roots for angina and hemorrhage, Malayans use old leaves and roots for childbirth, Burmese use the bark for bloody urine, Chinese and Japanese for diarrhoea[2].

Rhizophora mucronata helps to maintain marine life and balances the ecosystem. Traditionally the bark of this plant has been used to treat heamaturia, diabetes[3], diarrhea[4] and inflammation[5]. The plant was used in leather industry because of its rich tannin content. The polysaccharides have been reported for anti-HIV activity[6]. Phytochemical analysis of the bark is found to contain terpenoids, sterols, saponins , flavanoids and phenol acid[4].

From the existing information it is evident that the plant may possess some important biological activities. The main objective of this study was to evaluate the antinociceptive, antidiarrhoeal and cytotoxic activities of the ethanol extract of dried leaves of *Rhizophora mucronata* lamk.

Materials and Methods

Plant Material

Leaves of *Rhizophora mucronata* lamk. were collected from the Sundarbans at Sathkhira range, Sathkhira, Bangladesh in January 2009 and were authenticated by the experts at National Herbarium (Accession Number: 34179). After collection, leaves were sun dried for several days to remove moisture. After drying, the dried leaves were ground into course powder by 'Hammer' mill. The powder of dried leaves was then extracted by hot extraction process using ethanol as solvent. Each time 50 gm powdered material was extracted with 200 ml of solvent in a soxhlet extraction apparatus. The extraction was carried out until the process was completed. After the extraction, the extract was poured in a 1000 ml beaker and evaporated the solvent by using a rotary evaporator (Bibby RE200, Sterilin Ltd., U.K.) to get the crude extract. And this crude ethanolic extract was used for all phytochemical and pharmacological screening.

Animals

For antinociceptive and antidiarrhoeal activity study, young Swiss-albino mice of either sex, weighing 20 - 25 g, purchased from the Animal Research Branch of the International Centre for Diarrhoeal Disease and Research, Bangladesh (ICDDR, B), were used. After purchase, the animals were kept at animal house of Pharmacy Discipline, Khulna University, for adaptation under standard laboratory conditions (relative humidity 55-65%, room temperature 25.0±2.0°C and 12h light-dark cycle) and fed with standard diets and had free access to tap water. The experimental met the national guidelines on the proper care and use of animals. The Institutional Animal Ethics Committee (IAEC) approved the experimental protocol. All the experiments were conducted on an isolated and noiseless condition.

Drugs

Diclofenac sodium (Opsonin Chemical Industries Ltd, Bangladesh), Loperamide (Square Pharmaceuticals Ltd., Bangladesh).

Preliminary Phytochemical Analysis
The ethanol extract of dried leaves of *Rhizophora mucronata* lamk. was subjected to a preliminary phytochemical screening for major chemical groups. In each test, 10% (w/v) solution of the extract in ethanol was used unless otherwise specified in individual test[1, 7].

Tests for Reducing Sugar
Benedict's Test: 0.5 ml of the extract was placed in a test tube and then 5 ml Benedict's solution was added to it, boiled for 5 min and allowed to cool spontaneously.

Fehling's Test (Standard Test): 2 ml of the extract was added in 1 ml of a mixture of equal volumes of Fehling's solutions A and B, and was boiled for few min.

Tests for Tannins
Ferric Chloride Test: 5 ml of the extract was placed in a test tube and then 1 ml of 5% Ferric chloride solution was added to it.

Potassium dichromate test: 5 ml of the extract was placed in a test tube and then 1 ml of 10% potassium dichromate solution was added.

Test for Flavonoids
A few drops of concentrated hydrochloric acid were added to 5 ml of the extract.

Test for Saponins
1 ml of the extract was placed in a graduated cylinder and was diluted to 20 ml with distilled water and shaken gently for 15 min.

Test for Gums
5 ml of the extract was placed in a test tube and then Molish's reagent and sulphuric acid were added to it.

Tests for Steroids
Libermann-Burchard test: 1 ml of the extract was placed in a test tube and then 2 ml Libermann-Burchard reagent was added to it.

Sulphuric acid test: 1 ml of the extract was placed in a test tube and 1 ml sulphuric acid was added to it.

Tests for Alkaloids
Mayer's test: 2 ml of the extract and 0.2 ml of dilute hydrochloric acid were taken in a test tube and 1ml of Mayer's reagent was added to it.

Dragendroff's test: 2 ml of the extract and 0.2 ml of dilute hydrochloric acid were placed in a test tube and then 1 ml Dragendroff's reagent was added.

Wagner's test: 2 ml of the extract and 0.2 ml of dilute hydrochloric acid were placed in a test tube. Then 1 ml of iodine solution (Wagner's reagent) was added.

Hager's test: 2 ml solution of the extract and 0.2 ml of dilute hydrochloric acid were placed in a test tube. Then 1 ml of picric acid solution (Hager's reagent) was added.

Tests for Glycosides
A small amount of extract was taken in 1 ml water. Then few drops of aqueous sodium hydroxide were added. Yellow precipitate is considered as an indication for the presence of glycosides.

In another test, a small amount of extract was taken in 1 ml water and boiled with 5 ml Fehling's solution in a boiling water bath. Brick-red precipitate is considered as an indication for the presence of glycosides.

In another test, a small amount of extract was boiled with few drops of dilute sulfuric acid, neutralized with sodium hydroxide solution and boiled with 5 ml Fehling's solution in a boiling water bath. Brick red precipitate is considered as an indication for the presence of glycosides.

Pharmacological Studies

Antinociceptive Activity
Antinociceptive activity of the ethanolic extract of dried leaves of *Rhizophora mucronata* lamk. was tested using the model of acetic acid induced writhing in mice[8-9]. The experimental animals were randomly divided into four groups, each consisting of ten animals. Group I was treated as 'control' which received 1% (v/v) Tween-80 solution in water; group II was treated as 'positive control' and was given the standard drug diclofenac sodium at dose of 25 mg/kg of body weight; group III and group IV were test groups and were treated with ethanolic extracts of dried leaves of *Rhizophora mucronata* lamk. at dose of 250 and 500 mg/kg of body weight respectively. Control vehicle, standard drug and the ethanolic extracts were administered orally 30 min prior to the intra-peritoneal injection of 0.7 % acetic acid, then after an interval of 15 min, the number of writhes (squirms) was counted for 5 min.

Antidiarrhoeal Activity
Antidiarrhoeal activity of the ethanolic extract of dried leaves of *Rhizophora mucronata* lamk. was tested using the model of castor oil-induced diarrhoea in mice[10]. The mice were all screened initially by giving 0.5 ml of castor oil and only those showing diarrhoea were selected for the final experiment. The test animals were randomly chosen and divided into three groups having five mice in each. Group-I was kept as control and received 1% Tween-80 at the dose of 10 ml/kg of body weight; group II was treated as 'positive control' and was given the standard drug loperamide at a dose of 50 mg/kg of body weight; group III was test group and was treated with the extract at a dose of 500 mg/kg of body weight. Control vehicle, standard drug and the extract were administered orally, 1 h prior to the oral administration of castor oil at a dose of 0.5 ml per mouse. Individual animals of each group were placed in separate cages having adsorbent paper beneath and examined for the presence of diarrhoea every hour in five hours study after the castor oil administration. Number of stools or any fluid material that stained the adsorbent paper was counted at each successive hour during the experiment. The latent period of each mouse was also counted. At the beginning of each hour old papers were replaced by the new ones.

Cytotoxicity Test

The brine shrimps used for cytotoxicity test were obtained by hatching 5 mg of eggs of *Artemia salina* in natural seawater after incubation at about 29°C for 48h. The larvae (nauplii) were allowed another 48 h in seawater to ensure survival and maturity before use. Six doses of plant extract (10, 20, 40, 60, 80 and 100 µg/ml) in 5% DMSO and/or seawater were tested. Each extract preparation was dispensed into clean test tubes in 10 ml volumes and tested in duplicates. The concentration of DMSO in the vials was kept below 10 µl/ml. For control, same procedure was followed except test samples. After marking the test tubes properly, 10 living shrimps were added to each of the 20 vials with the help of a Pasteur pipette[11] .The test tube containing the sample and control were then incubated at 29°C for 24 h in a water bath, after which each tube was examined and the surviving nauplii counted. From this, the percentage of mortality was calculated at each concentration.

Statistical Analysis

Student's *t*-test was used to determine a significant difference between the control group and experimental groups.

Results

Chemical Group Test

Results of different chemical group tests on the ethanolic extract of dried leaves of *Rhizophora mucronata* lamk. showed the presence of Steroids, Reducing Sugar, Tannins , Gums, Flavonoids, Glycosides and Saponins (Table 1).

Table 1: Results of different chemical group tests of the extract of dried leaves of *Rhizophora mucronata* lamk.

Extract	Reducing Sugar	Steroids	Alkaloids	Tannins	Gums	Flavonoids	Glycosides	Saponins
Ethanolic extract of dried leaves of *Rhizophora mucronata* lamk.	+	+	-	+	+	+	+	+

Key: + = Presence, - = Absence

Antinociceptive Activity

Table 2 showed the effect of dried leaves of *Rhizophora mucronata* lamk. on acetic acid-induced writhing model in mice. The extract produced about 43.59% and 71.28% writhing inhibition at the dose of 250 and 500 mg/kg of body weight respectively, which were comparable to the standard drug diclofenac sodium where the inhibition was about 83.59% at the dose of 25 mg/kg of body weight (Table 2).

Table 2: Effect of ethanolic extract of dried leaves of *Rhizophora mucronata* lamk. on acetic acid induced writhing in mice

Animal Group / Treatment	Number of writhes (% writhing)	Inhibition (%)
Control 1% tween-80 in water, p.o.	19.5±1.45 (100)	---
Positive control Diclofenac sodium 25 mg/kg, p.o.	3.2±1.12* (16.41)	83.59
Test group-I Ethanolic extract 250 mg/kg, p.o.	11.0±1.48* (56.41)	43.59
Test group-II Ethanolic extract 500 mg/kg, p.o.	5.6±1.91* (28.72)	71.28

Values are expressed as Mean±S.E.M (n=10), *P<0.001, % = Percentage, p.o. = per oral.

Antidiarrhoeal Activity

Antidiarrhoeal activity of the ethanol extract of dried leaves of *Rhizophora mucronata* lamk. was tested by castor oil induced diarrhoea in mice. The extract caused an increase in latent period (2.97 h) i.e. delayed the onset of diarrhoeal episode at the dose of 500 mg/kg of body weight significantly (P<0.001) which was comparable to the standard drug loperamide at the dose of 50 mg/kg of body weight in which the value was 3.25 h (P<0.001) (Table 3a). The extract also decreased the frequency of defecation at the same dose where the mean numbers of stool at the 1st, 2nd, 3rd, 4th and 5th h of study were 1.6, 1.2, 1.2, 1.4 and 1.8 respectively and in standard drug the values were 1.4, 1.0, 1.0, 1.2 and 1.2 respectively (Table 3b).

Table 3a. Effect of the extract of dried leaves of *Rhizophora mucronata* lamk. on castor oil induced diarrhoea in mice (latent period)

Animal Group / Treatment	Dose(/kg, p.o)	Latent Period (h)
Group-I (control) 1% tween-80	10 ml	1.38 ± 0.246
Group-II (positive control) Loperamide	50 mg	3.25 ± 0.226*
Group - III Ethanolic extract	500 mg	2.97 ± 0.275*

Values are expressed as Mean±S.E.M (n=5), *P<0.001, p.o. = per oral.

Table 3b. Effect of the ethanolic extract of dried leaves of *Rhizophora mucronata* lamk. on castor oil induced diarrhoea in mice (Number of stools)

Animal Group/Treatment	Dose (/kg, p.o.)	Period of study (h)	Total number of stool
Group-I (control) 1% tween-80 solution in water	10 ml	1	3.6 ± 0.374
		2	3.4 ± 0.350
		3	3.6 ± 0.403
		4	3.4 ± 0.380
		5	3.8 ± 0.421
Group-II (positive control) Loperamide	50 mg	1	1.4 ± 0.384*
		2	1.0 ± 0.418*
		3	1.0 ± 0.412*
		4	1.2 ± 0.342*
		5	1.2 ± 0.377*
Group-III Ethanolic extract	500 mg	1	1.6 ± 0.431*
		2	1.2 ± 0.460*
		3	1.2 ± 0.441*
		4	1.4 ± 0.342*
		5	1.8 ± 0.364*

Values are expressed as Mean±S.E.M (n=5), **P*<0.01, p.o. = per oral.

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} = 80 µg/ml) (Table 4).

Table 4. Brine shrimp lethality bioassay of the ethanolic extract of dried leaves of *Rhizophora mucronata* lamk.

Test sample	Concentration (µg/ml)	Log (concentration)	Number of alive shrimp	Mortality (%)	LC_{50} (µg/ml)	LC_{90} (µg/ml)
Ethanolic Extract	10	1.00	09	10	40	80
	20	1.30	07	30		
	40	1.60	05	50		
	60	1.77	02	80		
	80	1.90	01	90		
	100	2.00	00	100		

Discussion

Antinociceptive activity of the ethanolic extract of dried leaves of *Rhizophora mucronata* lamk. 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[12]. The extract produced significant writhing inhibition comparable to standard drug diclofenac sodium. Based on this, it could be concluded that it might possess antinociceptive activity.

Antidiarrhoeal activity of the ethanol extract of dried leaves of *Rhizophora mucronata* lamk. was tested using the model of castor oil induced diarrhoea in mice[10]. Castor oil, which is used to induce diarrhoea in mice, mixes with bile and pancreatic enzymes and liberates ricinoleic acid from the triglycerides upon oral administration. Most of the ricinoleic acid remains in the intestine and produces its anti absorptive or secretory effect. The ricinoleic acid thus liberated readily forms ricinoleate salts with sodium and potassium in the lumen of the intestine. The salt formed as such behaves like a soap or surfactant within the gut and at the mucosal surface. Most agreed view is that ricinoleate salts stimulates the intestinal epithelial cell's adenyl cyclase[13] or release prostaglandin[14]. The extract caused an increase in latent period i.e. delayed the onset of diarrhoeal episode and decreased the frequency of defecation as well as the number of stool. On the basis of the result of castor oil induced diarrhoea, it can be concluded that the ethanol extract of dried leaves of *Rhizophora mucronata* lamk. might possess antidiarrhoeal activity.

The cytotoxic activity of the ethanol extract of dried leaves of *Rhizophora mucronata* lamk. was tested by using brine shrimp lethality bioassay. It is a recent development in the bioassay for the bioactive compounds. Brine shrimp lethality bioassay indicates cytotoxicity as well as a wide range of pharmacological activities such as antimicrobial, pesticidal, antitumor etc.[15]. The extract was found to show potent activity against the brine shrimp nauplii. Therefore the positive response obtained in this assay suggests that the extract may contain antitumor, antibacterial or pesticidal compounds.

In conclusion, it could be suggested that the crude ethanolic extract of dried leaves of *Rhizophora mucronata* lamk. might possess antinociceptive, antidiarrhoel and cytotoxic activities. However, further studies comprising of thorough phytochemical investigations of the used plant to find out the active principles and evaluation for these activities using other models are essential to confirm its pharmacological properties.

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