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PHYTOCHEMICAL AND PHARMACOLOGICAL STUDIES OF ETHANOLIC EXTRACT FROM THE LEAF OF MANGROVE PLANT *PHOENIX PALUDOSA* ROXB.

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The objective of this study was to evaluate the phytochemical constituents and pharmacological activity of the crude ethanolic extract of the leaves of Phoenix paludosa, collected from the sundarbans of Sathkhira range (specifically from Koromjol), Bangladesh. Phytochemical analyses were done by chemical group test. Antidiarrhoeal activities were assessed by castor oil-induced diarrhoea models in mice. Analgesic activities were studied using the model of acetic acid-induced writhing in mice, cytotoxic activity by brine shrimp lethality bioassay and antioxidant activity was measured by 1,1-diphenyl-2picrylhydrazyl (DPPH) methods. Steroids, glycosides, tannins, flavonoid and gums were found in the group tests. The extract exhibited moderate cytotoxicity with LC_{50} of 6.67 µg/mL and LC_{90} of 34.81 µg/mL and also showed statistically significant ($p=0.009$) analgesic effect. In antidiarrhoeal test, the extract increased the latent period significantly ($p<0.05$) and reduced the total number of passing liquid stool at all doses. When tested for in vitro antioxidant activity against stable DPPH, it was found to exhibit prominent free radical scavenging effect. The results obtained in this study suggested that P. paludosa has antidiarrhoeal and analgesic activities with moderate cytotoxic effect. It also has prominent antioxidant effect. These activity may be due to the presence of steroid, flavonoids and saponins in the extract.

Keywords: *Phoenix paludosa, Analgesic, Antidiarrhoeal, Antioxidant, Ethanolic extract, Cytotoxic*

INTRODUCTION

The importance of medicinal plants in traditional health care practice and in providing clues to new areas of drug research and biodiversity conservation is now well recognised. About 80% of the world's population relies on the use of traditional medicine, which is predominantly based on plant material (WHO 1993). Scientific studies on a number of medicinal plants indicated that promising phytochemical compounds can be developed for many health problems (Gupta 1994). Herbal drugs have gained importance in recent years because of their efficacy and cost effectiveness. These drugs are invariably single plant extracts or fractions or mixtures of fractions/extracts from different plants, which

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have been carefully standardised for their safety and efficacy (Subramoniam and Pushpangadan 1999). Several plants of mangrove forest are used for medical purposes e.g. *Acanthus ebracteatus* Vahl for chronic wound (Sujamnong 1979), the bark of *Rhizophora apiculata* Blume for diarrhoea and wound (School of Thai Traditional Medicine 1981), and the bark of *Rhizophora mucronata* for diarrhoea (Phongboonrod 1976). The plants in the mangrove forest showed the potential as a source of antioxidant and cancer chemoprevention agents (Bunyapraphatsara *et al.* 2003).

Phoenix paludosa (mangrove date palm) is a species of flowering plant in the palm family, indigenous to coastal regions of India, Bangladesh, Thailand, Peninsular Malaysia and Sumatra. Traditionally, *P. paludosa* is used as antipyretic and antiinflammatory agent in the early days in various regions (Saronika 2002). *P. paludosa* fresh shoot has quinone reductase induction activity but no antioxidant activity (Bunyapraphatsara *et al.* 2003). Upon significant literature survey it was found that only a few research work has been performed on *P. paludosa* Roxb. and no work has been performed on the leaf of this plant to investigate their medicinal use in our locality. Hence, the present study was taken up to investigate the antidiarrhoeal, antioxidant, analgesic and cytotoxic properties of the leaves of *P. paludosa* Roxb. and to find out the chemical group present in the active plant parts.

METHODS

Animals

Young Swiss-albino mice aged 4–5 weeks with body weight (BW) of 22–30 g were used for the experiment. The mice were purchased from the animal house of Jahangirnagar University, Bangladesh. The animals were housed in stainless steel cages at room temperature **(**28±2°C) and 12/12 h light dark cycle. All animals were fed with standard pellet diet (Vital Feed Ltd. Jessore, Bangladesh) and water *ad libitum*. The study was approved by the University Teaching and Research Ethics Committee of the University of Chittagong (Ref. 12–2009). All animal experiments were carried out in accordance with Local Ethnical Committee Acts.

Plant Materials

The leaves of *P. paludosa* were collected from the sundarbans (Bangladesh part) of Sathkhira range (specifically from Koromjol). The time of collection was in January 2009 during daytime. During collection, any type of adulteration was strictly prohibited. The plants were mounted on paper and the sample was taxonomically identified by the expert authority of Bangladesh National Herbarium, Mirpur and Dhaka (accession no. 34176).

Preparation of Plant Extract

The collected leaves were separated from undesirable materials or plants or plant parts. The leaves were dried by shade drying for 20 days to ensure the active constituents were free from decomposition and also to avoid any photochemical degradation. The leaves were ground into a coarse powder with the help of a suitable grinder. The powder was stored in an airtight container and kept in a cool, dark and dry place until analysis commenced. 170 g of the powdered leaf was extracted with ethanol using soxhlet

apparatus. After extraction, ethanol was removed, firstly by means of a water bath and then in an oven, yielding the extracted compound. The concentrate was designated as crude ethanolic extract of *P. paludosa* leaves.

Phytochemical Screening

The aqueous extract of the plant was subjected to qualitative chemical screening for the identification of the tannins alkaloids and flavonoids using standard procedures (Trease and Evans 1996).

Test for tannins

The aqueous extract (1 mL) was mixed with 10 mL of distilled water and filtered. Ferric chloride reagent (3 drops) was added to the filtrate. A blue-black or green precipitate confirmed the presence of gallic tannins or catechol tannins, respectively.

Test for alkaloids

The aqueous extract (0.2 mL) was stirred and placed in 1% aqueous hydrochloric acid (5 mL) on a steam bath. 1 mL of the filtrate was treated with Mayer's reagent (3 drops) while another portion was similarly treated with Dragendorff's reagent. Turbidity or precipitation with these reagents was considered as evidence for the presence of alkaloids.

Test for flavonoids

A portion of the aqueous extract (2 mL) was heated, and metallic magnesium and concentrated hydrochloric acid (5 drops) were added. A red or orange colouration indicated the presence of flavonoids.

Test for steroids

2 mL of acetic acid was added to 0.2 g of extract. The solution was cooled in ice, and then concentrated sulphuric acid was carefully added. Colour development from violet to blue or bluish-green indicated the presence of a steroidal ring, i.e., a glycone portion of cardiac glycoside.

Test for saponins

1 g of extract was boiled with 5 mL of distilled water and filtered. 3 mL of distilled water was added to the filtrate, and the mixture was shaken vigorously for about 5 minutes. Frothing that persisted upon warming was taken as evidence of the presence of saponins.

Test for glycosides

A small amount of an alcoholic extract of the fresh or dried plant material was placed in 1 mL of water. A few drops of aqueous sodium hydroxide were added. The presence of yellow colour was considered as an indication for the presence of glycosides.

Test for gums

5 mL solution of the extract was taken and then Molish reagent and sulphuric acid were added. Red violet ring produced at the junction of two liquids indicated the presence of gums.

Acetic Acid-induced Writhing in Mice

Analgesic activity of the ethanolic extract of *P. paludosa* leaves were tested using the model of acetic acid-induced writhing in mice (Whittle 1964; Ahmed *et al.* 2004). Experimental animals were randomly selected and divided into four groups. Each group consisted of five mice. Group-I received distilled water only, groups-II (positive control) received diclofenac intraperitoneally at a dose of 50 mg/kg BW, groups-III and IV received the extract orally at 250 and 500 mg/kg BW, respectively. 45 min interval was allowed to ensure proper absorption of the orally administered substances and 30 min for intraperitoneally administered diclofenac. 0.25 mL acetic acid solution (0.7%, 10 mL/kg) was administered intraperitoneally to every mouse. The number of abdominal constrictions (writhing) and stretching with a jerk of the hind limb was counted after 15 min of acetic acid injection. The response of the extract and diclofenac treated groups were compared with those of the control group (distilled water). Percentage protection against writhing movement (% inhibition of writhing was taken as an index of analgesia) was calculated as follows:

$$\text{Percentage inhibition} = \text{Wt (control)} - \text{Wt (test group)} / \text{Wt (control)};$$

Wt = mean number of writhing.

Tests for Antidiarrhoeal Activity

The experiment was performed according to the method described by Shoba and Thomas (2001). Mice were fasted for 24 h and were randomly allocated to 4 groups of 5 animals each. The animals were all screened initially by giving 0.5 mL of castor oil. Only those showing diarrhoea were selected for the final experiment. Group I received distilled water, groups III and IV received the extract orally (250 and 500 mg/kg respectively). Group II was given Loperamide (50 mg/kg, orally) in suspension. After 60 min, each animal was given 0.5 mL of castor oil and was placed in an individual cage, the floor of which was lined with blotting paper which was changed every hour, observed for 6 h and the characteristic diarrhoeal droppings were recorded. The latent period of each mouse were also counted.

Assay for Cytotoxicity of *P. paludosa* Leaf Extract

Assay for brine shrimp lethality

It was used for probable cytotoxic action (Meyer *et al.* 1982; McLaughlin 1991). The eggs of brine shrimp (*Artemia salina* Leach) were collected and hatched in a tank at a temperature around 37°C with constant oxygen supply. Two days were allowed to hatch and mature the nauplii. Stock solution of the sample was prepared by dissolving required amount of

extract in specific volume of pure dimethyl sulfoxide (DMSO). 4 mL of seawater was given to each of the vials. Specific volumes of sample were transferred from the stock solution to the vials to get final sample concentrations of 0.1, 0.5, 1, 10, 20, 40, 60, 80 and 100 µg/mL. In the control vials same volumes of DMSO (as in the sample vials) were taken. With a pasteur pipette, 10 living nauplii were put into each of the vials. After 24 h, the vials were observed and the number of nauplii that survived in each vial was counted. From this, the percentage of lethality of brine shrimp nauplii was calculated for each concentration of the extract.

Assay for Antioxidative Activity of *P. paludosa* Leaf Extract

The antioxidant activity of *P. paludosa* extract was assessed in comparison to standard antioxidant ascorbic acid and vitamin E (Sigma, Germany) on the basis of scavenging effect of the stable 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical activity according to established procedure (Brand-Williams, Cuvelier and Berset 1995). Five different concentrations (1, 5, 10, 20 and 50 µg/mL) of plant extract (0.1 mL) was added to 3 mL of a 0.004% methanol solution of DPPH. Absorbance at 517 nm was determined after 30 min, and the percentage inhibition activity was calculated from [(A0–A1)/A0]x100, where A0 is the absorbance of the control, and A1 is the absorbance of the extract/standard. The inhibition curves were prepared and IC_{50} values were calculated.

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 control group. p values <0.05 were considered to be statistically significant. The concentration producing 50% of the maximum response (LC_{50} or IC_{50}) was obtained by the best visual fit from the plot of the individual experiments.

RESULTS

Phytochemical Screening

P. paludosa leaf revealed the presence of flavonoids, glycosides, tannins, gum and steroid (Table 1).

Table 1: Phytochemical screening of *P. paludosa* leaf extract.

Test	Alkaloid	Flavonoids	Tannins	Gum	Glycosides	Saponin	Steroids
Inference	–	+	+	+	+	–	+

Note: + = present, – = absent.

Analgesic Activity

In the acetic acid-induced writhing test, the ethanolic extract of leaves of *P. paludosa* showed dose dependent decrease in the total number of writhings after 15 min of administration of acetic acid intraperitoneally. The analgesic effect at the dose of 500

mg/kg body weight was significant (p=0.009) and the effect increased with the increase of dose (Table 2).

Table 2: Effect of *P. paludosa* extract on acetic acid induced writhing of mice.

Group (n=5)	Dose (mg/kg BW)	Writhing (mean±SEM)	% writhing	Percentage writhing inhibition
I (Control)	–	27.2±1.93	–	–
II (Diclofenac-Na)	50	6.8±2.13	25.00**	75
III (Et. extract)	250	24.8±1.46	91.18	8.82
IV (Et. extract)	500	18.6±1.60	68.38*	31.61

Notes: *p<0.05, **p<0.001, Et=ethanolic, n=number of rat, BW=body weight, SEM=standard error of mean.

Antidiarrhoeal Activity

In the castor oil-induced antidiarrhoeal model, the extract of leaves of *P. paludosa* significantly increased the latent period in both 250 mg/kg body weight (p=0.020) and 500 mg/kg body weight (p=0.002) doses in the treated animals (Table 3). It was also observed that there was a decreasing trend (p=0.052) in the number of liquid stools during the total observation period at both doses of 250 and 500 mg/kg (Table 4).

Table 3: Effect of extract of leaves of *P. paludosa* on the latent period in castor oil-induced diarrhoeal episode in mice.

Group (n=5)	Treatment	Dose (mg/kg BW)	Latent period (min; mean±SEM)
I	Distilled water (solvent)	–	13.0±2.21
II	Loperamide	50	45.4±6.01*
III	Ethanol extract	250	25±3.52*
IV	Ethanol extract	500	36±4.48*

Notes: SEM=standard error of mean, n=number of rat, BW=body weight, *p<0.05.

Cytotoxic Activity

In brine shrimp lethality bioassay, test sample showed different mortality rate at different concentrations (Table 5). The mortality rate of brine shrimp was found to increase with the increase in concentration of the sample and the extract of leaves of *P. paludosa* showed significant (p=0.001) toxicity to the brine shrimp nauplii. The concentrations of crude extract for 50% mortality (LC_{50}) and 90% mortality (LC_{90}) were 16.67 µg/mL and 34.81 µg/mL, respectively.

Table 4: Effect of *P. paludosa* leaves extract on liquid defecation in the castor oil-induced diarrhoeal episode in mice.

Type (group)	1st h	2nd h	3rd h	4th h	5th h	6th h
Control (I)	3.00±1.26	3.80±0.37	2.40±0.68	2.40±0.68	1.00±0.45	0.40±0.40
Standard (II)	0.00±0.00	0.00±0.00	0.20±0.20	0.20±0.20	0.00±0.00	0.00±0.00
(*p* value)	(0.077)	(0.001)	(0.014)	(0.014)	(0.089)	(0.374)
250 mg (III)	1.80±0.73	2.80±1.36	1.80±0.73	0.60±0.40	0.20±0.20	0.40±0.24
(*p* value)	(0.436)	(0.598)	(0.684)	(0.052)	(0.141)	(1.000)
500 mg (IV)	1.80±1.11	2.00±1.14	1.00±0.45	0.60±0.40	0.40±0.40	0.20±0.20
(*p* value)	(0.497)	(0.128)	(0.532)	(0.052)	(0.347)	(0.667)

Note: Values are presented as mean±SEM, (n=5).

Table 5: Brine shrimp lethality bioassay of extract of *P. paludosa* leaves.

Extract concentration (µg/mL)	Log concentration	% of mortality	LC_{50} (µg/mL)	LC_{90} (µg/mL)
100	2	100		
80	1.90309	100		
60	1.778151	100		
40	1.60206	100		
20	1.30103	60	16.67	34.81
10	1	30		
1	0	20		
0.1	–0.30103	0		
0.5	–1	0		

Notes: LC_{50} and LC_{90} were determined from 24 h counts using probit analysis method described by FINNEY computer program.

Antioxidant Activity

In the quantitative antioxidant assay using DPPH, the ethanolic extract of *P. paludosa* leaves showed free radical scavenging properties, though the effect was more strong (IC_{50}=7.21 µg/mL) than one of the two standards, tocopherol/vitamin E (IC_{50}>50 µg/mL) but it showed weaker activity than the other standard, ascorbic acid (IC_{50}=3 µg/mL) (Table 6).

DISCUSSION

Phytochemical screening of the ethanol extract of *P. paludosa* leaf reveals the presence of glycosides, saponins, gum, tannins and steroid. These compounds can show extensive pharmacologic and other activities. In the acetic acid induced writhing test, the ethanolic

Table 6: DPPH free radical scavenging activity of ascorbic acid, vitamin E (tocoferol) and *P. paludosa* leaf extract.

Test material	Concentration (µg/mL)	% scavenging activity	IC_{50} (µg/mL)
Ascorbic acid	1	21.86	
	5	86.16	3
	10	96.77	
	20	97.41	
	50	97.28	
Vitamin E	1	16.43	
	5	17.46	>50
	10	23.29	
	20	23.67	
	50	27.17	
P. paludosa extract	1	27.30	
	5	40.75	
	10	60.28	7.21
	20	77.75	
	50	88.87	

extract of leaves of *P. paludosa* showed dose dependent decrease in the total number of writhing. From the observation it can be suggested that the ethanolic extract of the *P. paludosa* leaves has mild analgesic activity. Analgesic activities are commonly exhibited by the non-steroidal antiinflammatory drugs (NSAIDS). These NSAIDs exert antiinflammatory effect principally by inhibiting the synthesis of prostaglandin (Vane 1971), an eicosanoid. Inhibition of prostaglandin synthesis may account for the analgesic activity of the leaf extract as it contributes in large part to the analgesic activity of the NSAIDs. Castor oil is made up of 90% ricinoleate (Mekeon, Lin and Stafford 1999), which when metabolised is responsible for the observed effects of the oil. The active metabolite ricinoleic acid is responsible for its diarrhoea inducing property, which diminishes Na+ and C1- permeability in the intestine (Gaginella and Phillips 1975); it is also associated with endogenous stimulation of prostaglandins release (Zavala *et al.* 1998). Ethanol extract of the leaf of *P. paludosa* was found to inhibit the severity of diarrhoea induced by castor oil. It is possible that the extract was able to inhibit electrolyte permeability in the intestine due to castor oil and or through the inhibition of prostaglandins release (Adzu *et al.* 2003). We suggest that saponins and/or flavonoids present in the leaf extract might be responsible for its antidiarrhoeal activity. The mortality rate of brine shrimp was found to increase with the increase in concentration of the sample and the extract of leaves of *P. paludosa* showed significant (p=0.001) toxicity to the brine shrimp nauplii. These results tend to suggest possible antitumor, antibacterial or pesticidal activities. The free radical, DPPH is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors, and to evaluate antioxidant activity. It has also been used to quantify antioxidants in complex biological systems in recent years. The DPPH method can be used for solid or liquid samples and is not specific to any particular antioxidant component, but applies to the overall antioxidant capacity of the sample. A measure of total antioxidant

capacity will help us understand the functional properties of the plant extract. In the quantitative antioxidant assay using DPPH, the ethanolic extract of *P. paludosa* leaves showed strong radical scavenging effect in comparison to the effect of the tocopherol though it showed weak scavenging effect in comparison to the effect of standard ascorbic acid. In a previous study on antioxidative activity of *Phoenix dactylifera,* antioxidant state (plasma vitamin C, E and A and b-carotene) increased significantly on administration of different extracts (Mohamed and Al-Okbi 2004). It has been recognised for some time that several classes of flavonoids play significant roles in many physiological processes and show antioxidant and fungicidal activity (Larson 1988) and are natural antihistamines. Flavonoid and flavonol-lignan derivatives inhibit lipid peroxidation and are potent quenchers of triplet oxygen.

CONCLUSION

Pharmacological evaluation of *P. paludosa* leaf extract reveals some interesting activities like cytotoxicity, antidiarrhoeal, analgesic as well as the antioxidant activities of this plant. From these we can assume that different active secondary metabolites are present in its extracts and perhaps some of these compounds may function in a synergistic manner. However, further studies are necessary to elucidate the mechanism lying with this effect. This report may serve as a stepping stone for future research on the biological and pharmacological activities of *P. paludosa* leaf extract.

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