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Artigo

Bioactivity of *Excoecaria agallocha*

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RESUMO: "Bioatividade de *Excoecaria agallocha*". Os resultados dos estudos neurofarmacológicos, microbiológicos e toxicológicos do extrato etanólico da casca de *Excoecaria agallocha* são reportados. O extrato (dosagens de 100 e 200 mg/kg) produziu uma diminuição profunda na atividade exploratória de maneira dose-dependente. Ele também mostrou um efeito sedativo marcante conforme evidenciado por uma redução significativa no comportamento total e potencialização do tempo de sono induzido por tiopental sódico. A totalidade destes efeitos mostrou que o extrato possui ação depressora sobre o sistema nervoso central (SNC). O extrato de *E. agallocha* exibiu significante atividade antibacteriana *in vitro* contra *Staphylococcus aureus*, *Shigella dysenteriae, Shigella sonnei* e *Enterococci* com as zonas de inibição medindo entre 11 a 15 mm. Enquanto o extrato mostrou considerável toxicidade em *Artemia salina* (DL_{50} = 20 mg/mL), ele exibiu apenas baixo nível de toxicidade em camundongos.

Unitermos: *Excoecaria agallocha,* Euphorbiaceae, tiopental sódico, teste de campo aberto, teste do orifício cruzado.

ABSTRACT: The results of neuropharmacological, microbiological and toxicological studies on the ethanol extract of the bark of *Excoecaria agallocha* are reported. The extract (100 and 200 mg/kg dosages) was found to produce a profound decrease in exploratory activity in a dose-dependent manner. It also showed a marked sedative effect as evidenced by a significant reduction in gross behaviour and potentiation of sodium thiopental-induced sleeping time. The totality of these effects showed that the extract possesses depressant action on the central nervous system (CNS). The extract of *E. agallocha* exhibited significant *in vitro* antibacterial activity against *Staphylococcus aureus*, *Shigella dysenteriae, Shigella sonnei* and *Enterococci* with the zones of inhibition ranging from 11 to 15 mm. While the extract showed considerable brine shrimp toxicity (LD_{50} = 20 mg/mL), it displayed only low level of toxicity in mice.

Keywords: *Excoecaria agallocha,* Euphorbiaceae, sodium thiopental, open field test, hole cross test.

INTRODUCTION

Excoecaria agallocha L. (Euphorbiaceae) is a small mangrove tree that grows widely in the tidal forests and swamps of the Sundarbans and other coastal areas of Bangladesh (Ghani, 2003; GRIN database, 2008). This plant is also found in the countries of temperate and tropical Asia, Australasia and South-western Pacific (GRIN database, 2008). This plant has traditionally been used to treat sores and stings from marine creatures, and ulcers, as a purgative and an emetic, and the smoke from the bark to treat leprosy (Ghani, 2003). The bark oil has been reported to be effective against rheumatism, leprosy and paralysis. However, the milky sap of this tree can cause temporary blindness if it enters the eyes. The sap can also cause skin blisters and irritation. Clinical trials carried out on this plant showed its potential as anti-HIV, anticancer, antibacterial and antiviral agent (Peter et al., 1999). Previous phytochemical studies on *E. agallocha* revealed the presence of diterpenoids (ISI database, 2008; Ji-Dong et al., 2007; Li et al., 2007; Wang et al., 2006, 2005; Wang and Guo, 2005; Kang et al., 2005), triterpenoids (Zou et al., 2006), flavonoid (Konishi et al., 2003) and phorbole esters (Ericson et al., 1995). As part of our continuing phytochemical and bioactivity studies on Bangladeshi medicinal plants and also in other parts of the world (Alam et al., 2008a,b; Ali et al., 2008; Datta et al., 2007, 2004; Saha et al., 2007; Uddin et al., 2007a-c, 2006a,b, 2005, 2004; Rouf et al., 2006; Haque et al., 2004; Rahman et al., 2004; Delazar et al., 2006; Shoeb et al., 2007; Auzi et al., 2007; Zamani et al., 2007; Razavi et al., 2008), we now report on the neuropharmacological and antibacterial activities, and acute toxicty of the ethanol extract of the

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bark of *E. agallocha* in laboratory rodents as well as brine shrimp toxicity.

MATERIAL AND METHODS

Plant material

The bark of *Excoecaria agallocha* L. was collected during October 2003 from the Sundarbans of Karomjol, Dacope region. The plant was identified at Bangladesh National Herbarium where a voucher specimen was deposited (Accession No. -30209).

Extraction

The bark of *E. agallocha* (200 g) was dried at room temperature, ground, and Soxhlet-extracted with 80% aqueous ethanol. The solvent was completely removed by rotary evaporator to obtain dark reddish gummy exudates (yield 7.5%).

Animals

Swiss albino mice (20-30 g) and rat (120-140 g) of either sex were obtained from the Animal house of the International Centre for Diarrhoeal Disease and Research, Bangladesh (ICDDR, B). The animals were housed under standard laboratory conditions (relative humidity 55-65 %, r.t. 23.0 ± 2.0 ^{o}C and 12 h light: dark cycle). The animals were fed with standard diet and water *ad libitum*. The University Animal Research Ethical Committee approved the experimental protocol.

Neuropharmacological activity

Sodium thiopental-induced sleeping time

A sub-hypnotic dose of sodium thiopental (35 mg/kg) was i.p. injected to mice 20 min after a similar injection of vehicle or the drug. Sleeping time was determined as the interval between the loss and the recovery of the righting reflex (Ferrini et al., 1974). Groups of male mice (n=5) were injected with sodium thiopental (35 mg/kg i.p) 15 minutes after administration of either normal saline or *E. agallocha* extract (100 and 200 mg/kg), and the time interval between losing and regaining of righting reflex was measured as sleeping time.

Open field test

This experiment was carried out as described by (Gupta et al., 1971). The animals were divided into control and test groups containing five mice each. The test group received *E. agallocha* extract at the doses of 100 and 200 mg/kg body weight orally whereas the control group received vehicle (1% Tween 80 in water).The floor of an open field of half square meter was divided into a series of squares each alternatively coloured black and white. The apparatus had a wall of 40 cm height. The number of squares visited by the animals was counted for 3 min at 0, 30, 60, 120 and 240 min after oral administration of the test drugs.

Hole cross test

The method was adopted as described by Takagi et al. (1971). A steel partition was fixed in the middle of a cage having a size of 30 × 20 × 14 cm. A hole of 3 cm diameter was made at a height of 7.5 cm in the centre of the cage. The number of passage of a mouse through the hole from one chamber to other was counted for a period of 3 min at 0, 30, 60, 120, 180 and 240 min after oral administration of the extract.

Hole-board test

The method described by File and Wardill (1975) was used. The test was carried out 30 min after oral treatment of extract at the doses of 100 and 200 mg/kg. Chlorpromazine hydrochloride (4 mg/kg, i.p.) was used as a reference, while control animals were treated with 1% Tween 80. The number of head-dips by each mouse was recorded for 240 min.

Antimicrobial activity

The antimicrobial activity of *E. agallocha* extract was studied against *Staphylococcus aureus*, *Staphylococcus epidermidis, Shigella dysenteriae, Shigella sonnei, Shigella flexneri, Enterococci,* clinical isolates. All bacterial strains were kindly provided by IMTECH, Chandigarh (India). Cultures of these bacteria were grown in a nutrient broth at 37 °C and maintained on nutrient agar (Himedia, India) slants at 40 °C. The antibacterial property was studied by the disc diffusion method (Chattopadhyay et al., 2002) using extract 200 mg/disc. Control disks contained solvents only (50% aqueous ethanol). Gentamycin was used as positive controls. Minimum inhibitory concentration (MIC) was evaluated by the micro dilution method using 5 mL of liquid broth with different concentrations of extract.

Acute toxicity studies

Ethanol extract of *E. agallocha* suspended in 5% Tween 80 was administered to the groups of mice in a single oral dose by gavages using a feeding needle (at least three doses). The control group received an equal volume of the 5% Tween 80 vehicle. Seven animals of both sexes were used for each dosage level. They were deprived of food, but not water 16-18 h prior to the administration of the test suspension. Observations of toxic symptoms were made and recorded systematically at one, two, four and six hours after administration.

Finally, the number of survivors was noted after 24 h and these animals were then maintained for a further 13 days with observations made daily. At the conclusion of the experiment, all surviving animals were sacrificed with an injection of pentobarbital and their organ liver were excised and weighed. The pathological observations of the liver tissues were performed on gross and microscopic bases. Histological examinations were performed on the preserved tissues with particular emphasis on those which showed gross pathological changes. The toxicological effect was assessed on the basis of mortality, which was expressed as LD_{50}. (Muller and Kley, 1982; Lorke, 1983) If a test at one dose level of at least 5 g/kg body weight produced no compound-related mortality, then a full study using three dose levels might not be necessary.

Brine shrimp lethality assay

Brine shrimp lethality assay (Meyer et al., 1982) was carried out to investigate the general toxicity of the extract of *E. agallocha*. Brine shrimps (*Artemia*

Table 1. Effect of the ethanol extract of *E. agallocha* on sodium thiopental (i.p.) induced sleeping in mice.

Group	Onset of action (min)	Duration of sleeping (min)
1% aq. Ttween 80	3.55±0.15	56.08±0.13
Diazepam (i.p.)	2.7±0.12*	90.80±2.56*
E. agallocha (100 mg/kg) (p.o)	6.94±0.22*	75.68±2.51*
E. agallocha (200 mg/kg) (p.o.)	5.26±0.10*	66.70±1.03*

Data was collected from the time of drug administration. Values are represented as mean ± standard error of mean. Differences in means were estimated by means of ANOVA followed by Bonferroni and Dunnet's post hoc test (N=5) Statistical significance was considered as $p< 0.05$ in all cases vs. control.

Table 2. Effect of the ethanol extract of *E. agallocha* on mice in the open field test.

Group	Number of movement				
	0 min	30 min	60 min	120 min	240 min
1% Tween 80 in water	120.60±3.50	116.60±3.44	117.40±4.45	109.80±5.76	105.00±4.89
E. agallocha (100 mg/kg, p.o)	120.60±3.97	117.80±5.40	115.20±4.96	109.60±5.77	102.80±4.81
E. agallocha (200 mg/kg, p.o)	113.60±2.07*	108.40±4.93*	98.00±8.83*	85.60±8.56*	57.80±12.75*

Data was collected after 30 min of extract administration. Values are represented as mean ±Standard deviation (N=5). General linear model followed by repetitive measures and Bonferroni and Dunnet's post hoc test. Results were considered significant at $P < 0.05$.

Table 3. Effect of the ethanol extract of *E. agallocha* on mice in the hole cross test.

Group	Number of movement					
	0 min	30 min	60 min	120 min	180 min	240 min
1% Tween 80 in water	10.20±2.39	10.00±3.61	11.60±3.21	8.40±4.39	9.80±4.65	11.60±3.05
E. agallocha (100 mg/kg, p.o)	6.80±1.79*	5.80±1.09*	4.80±0.84*	4.20±1.64*	5.40±1.82*	3.20±0.84*
E. agallocha (200 mg/kg, p.o)	2.80±0.84*	3.60±0.55*	3.40±1.14*	3.00±1.00*	2.80±1.48*	1.80±1.09*

Data was collected after 30 min of drug administration. Values are represented as mean ± standard deviation (N=5). General linear model followed by repetitive measures and Bonferroni and Dunnet's post hoc test. Results were considered significant at $P < 0.05$.

Table 4. Effect of the ethanol extract of *E. agallocha* on mice in the hole board test.

Group	Observations				
	0 min	30 min	60 min	120 min	240 min
1% Tween 80 in water	12.80±1.30	13.20±8.4	15.20±0.48	16.20±0.45	17.40±0.55
E. agallocha (100 mg/kg, p.o)	15.80±0.84*	14.00±1.41*	12.20±1.79*	11.20±2.95*	9.60±1.52*
E. agallocha (200 mg/kg, p.o)	10.80±1.92*	10.00±1.87*	8.40±1.52*	7.60±1.52*	6.40±1.34*

Data was collected after 30 min of drug administration. Values are represented as mean ±Standard deviation (N=5). General linear model followed by repetitive measures and Bonferroni and Dunnet's post hoc test. Results were considered significant at $P < 0.05$.

Table 5. Antibacterial properties of the ethanol extract of *E. Agallocha.*

Bacterial strains	Diameter of Zone of inhibition in mm	
	Gentamycin (30 µg/disc)	Ethanolic extract of *E. agallocha* (200 µg/disc)
Gram positive		
Staphylococcus aureus	41	15
Staphylococcus epidermis	35	0
Gram negative		
Shigella sonnei	34	11
Shigella flexneri	34	0
Shigella dysenteriae	35	14
Enterococci	43	11

salina) were hatched using brine shrimp eggs in a conical shaped vessel (1 L), filled with sterile artificial seawater (prepared using sea salt 38 g/L and adjusted to pH 8.5 using 1N NaOH) under constant aeration for 48 h. After hatching, active nauplii free from egg shells were collected from brighter portion of the hatching chamber and used for the assay. Ten nauplii were drawn through a glass capillary and placed in each vial containing 4.5 mL of brine solution. In each experiment, 0.5 mL. of the plant extract was added to 4.5 mL of brine solution and maintained at room temperature for 24 h under the light and surviving larvae were counted. Experiments were conducted along with control (vehicle treated), different concentrations (1-5000 microgram/mL) of the test substances in a set of three tubes per dose.

The percentage lethality was determined by comparing the mean surviving larvae of the test and control tubes. LC_{50} values were obtained from the best-fit line plotted concentration verses percentage lethality. Vincristine sulphate was used as a positive control in the bioassay.

Statistical analysis

Statistical analysis was performed using SPSS-11.5 statistical Software for Windows. Differences in means were estimated by means of ANOVA followed by Bonferroni and Dunnet's post hoc test and General linear model followed by repetitive measures and Bonferroni and Dunnet's post hoc test. Results were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

The extract of the bark of *E. agallocha* produced significant alterations in general behaviour pattern, reduction in spontaneous mobility, and potentiation of sodium thiopental-induced sleeping time in a dose-dependent manner. The extract at the doses of 100 and 200 mg/kg significantly prolonged the duration of the sodium thiopental-induced sleeping time (Table 1). This finding is similar to that observed by Fujimori (1995) who proposed that the enhancement of barbital hypnosis is a useful index of central nervous system (CNS) depressant activity. Anxiety and sedation are principally mediated in the CNS by the $GABA_A$ receptor complex, which is also involved in other physiological functions related to behaviour, as well as in various psychological and neurological disorders such as epilepsy, depression, Parkinson syndrome and Alzheimer's disease. The $GABA_A$ receptor complex comprises a Cl- channel and binding sites for several compounds, such as benzodiazepines, barbiturates, neuroactive steroids, and a variety of other drugs like loreclezole and propofol (Korpi et al., 2002).

The validation of the anxiety was carried out by measuring external signs, through the hole cross, the hole-board and the open field tests (Tables 2-4). The exploration capacity might be considered to be an index of anxiety although it is difficult to separate it from motor activity. The results showed a considerable decrease in exploratory conduct in the mice caused by the extracts. In the open field test (Table 2), the extract at the doses of 100 and 200 mg/kg showed significant decrease in movement from its initial value of zero to 240 min. Initially, it was observed that the number of movements of the control group overall increased from 30 to 240 min.

In the hole cross test (Table 3), the extract *E. agallocha* at the doses of 100 and 200 mg/kg showed significant decrease in movement from its initial value of 0 to 240 min. Similar increased movements were also observed with the extract doses of 100 and 200 mg/kg in the hole-board test (Table 4).

The extract of *E. agallocha* exhibited significant *in vitro* antibacterial activity (Table 5) against *Staphylococcus aureus*, *Shigella dysenteriae, Shigella sonnei* and*, Enterococci* with the zones of inhibition ranging from 11 to 15 mm. However, no activity was observed against *Shigella flexneri* and *Staphylococcus epidermis* at test concentrations.

Mice treated with the dose of 400 mg/kg body weight in acute toxicity test showed some behavioural changes, e.g. response to external stimuli, reduction of

mobility and aggression, slight excitability sketching and sluggishness, after 2 h of oral administration. However, all these changes disappeared after 24 h. Death of mice was noted at 600 mg/kg. Weight gain was observed in both sexes 7 and 14 days after oral administration of *E. agallocha*. The LD_{50} value was 8000 mg/kg body weight. According to Schorderet (1992), substances with LD_{50} values greater than 5000 g/kg of body weight are considered to have low toxicity.

The brine shrimp assay is a simple and useful tool for the isolation of potentially cytotoxic compounds from plant extracts (Meyer et al., 1982). It has been established that the cytotoxic compounds usually show good activity in the BSL assay, and this assay can be recommended as a guide for the detection of antitumour and pesticidal compounds because of its simplicity and cost-effectiveness. The extract of *E. agallocha* showed considerable brine shrimp toxicity with an LC_{50} value less than 20 µg/mL.

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