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Original Article

Pharmacological and phytochemical screenings of ethanol extract of *Leea macrophylla* Roxb.

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Abstract

The antimicrobial, anti-inflammatory, membrane stabilizing and anti-atherothrombosis activities of crude ethanol extract of leaves of *Leea macrophylla* Roxb. have been investigated. In antimicrobial assay by disc diffusion method, the extract showed mild to moderate antimicrobial activity with zone of inhibition ranging from 9-12 mm and 16-31 mm for test bacteria and fungi, respectively where the growth of *Aspergillus niger*, *Blastomyces dermatidis, Candida albicans, Pityrosporum ovale*, *Trichophyton* sp. *Microsporum* sp. and *Cryptococcus neoformans* were strongly inhibited. The extract produced inhibition of protein denaturation and haemolysis by 47.4% and 57.63% in the in vitro anti-inflammatory and membrane stabilization tests, respectively. On the other hand, the crude extract exhibited 20.61% clot lysis compared to the standard streptokinase (SK) (81.53%) in the anti-atherothrombosis activity studies. Preliminary phytochemical screenings with the crude extract revealed the presence of alkaloids, glycosides, tannins, flavonoids, reducing sugars and gums.

Keywords: *Leea macrophylla*, antimicrobial, MIC, anti-inflammatory, membrane stabilization, anti-atherothrombosis.

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1. Introduction

Bangladesh has a rich and prestigious heritage of herbal medicines amongst the South Asian countries. More than 500 species of medicinal plants are estimated to grow in Bangladesh and about 250 species of them are used for the preparation of traditional medicines. However, majority of these plants have not yet undergone chemical, pharmacological and toxicological studies to investigate their bioactive compounds [1]. In

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continuation of the global efforts [2-4] for isolation of new and potent bioactive compounds from plants, the present study has focused on the biological investigations of the medicinal plant, *Leea macrophylla* Roxb.

Leea macrophylla Roxb. (Bengali name –Dholsamudra, Fam: Leeaceae) is a low shrub, about 1 m high, which is rarely distributed in some parts of Chittagong Hill tracts in Bangladesh and also found throughout the warmer regions of India, Nepal, Cambodia, Laos, Myanmar and Thailand. Roots are tuberous, perennial and leaves are simple, large up to 60 cm long. The plant has various ethno-pharmacological uses and almost all parts of the plants possess potential curative properties. The crude extract of this plant is reported to have anti-urolithiatic effect of cystone in rat [5]. Powder of leaves mixed with honey is given to cancer patients [6-7]. Leaf juice is recognized as anti-inflammatory agent and used in boils, arthritis, gout and rheumatism [8]. The dried powder of root with clarified butter is prescribed in morning as age sustainer. Leaves of the plant are used as vegetable by tribal people [9]. It is also applied externally to allay pain and to stop the effusion of blood [10]. It has also ethno-botanical uses in goiter, gastric tumor, lipoma and tetanus [11]. Phytochemical studies with the seed extracts revealed the presence of phenolic, saponin, glycoside, carbohydrate and protein types of compounds [12]. We, here in, report the antimicrobial, anti-inflammatory, membrane stabilization, anti-atherothrombosis activities and preliminary phytochemical screening of leaf extracts of *L. macrophylla*.

2. Materials and Methods

Collection of plant materials:

The leaves of *L. macrophylla* were collected from the hilly areas of Forest Research Institute, Chittagong in the November, 2011. The plant was identified by the experts of Bangladesh Forest Research Institute Herbarium, Chittagong where a voucher specimen has been deposited. After collection, the samples were sun dried for 7 days followed by oven drying for 24 h at 50 ^{0}C to facilitate proper grinding. Then about 150 g of powdered leaf was extracted with ethanol (99.8%) in a Soxhlet apparatus (Quickfit, England) for 10 h and the extract thus obtained was concentrated with a rotary evaporator (Heidolph, 560-91110-00-0, Germany) at reduced temperature and pressure to yield 15.45 gm (10.30%) of extractive for *L. macrophylla*.

Preliminary phytochemical screening:

For preliminary phytochemical screenings, the crude extract was subjected to various tests (Table-1) for determination of chemical nature (secondary metabolites) of the extract [13].

Antimicrobial screening:

The antibacterial and antifungal activities of the crude extract was evaluated by the disc diffusion method [14] against 4 Gram positive and 7 Gram negative pathogenic bacteria and 7 fungi (Table-2) using ciprofloxacin and fluconazole as standards. The organisms were obtained as pure culture from the Microbiology Lab., Department of Pharmacy, BGC Trust University, Chittagong, Bangladesh. The antimicrobial activity of the test agents was determined by measuring the diameter of zone of inhibition expressed in mm. The experiments were carried out in triplicate and the results have been shown as mean ± SEM (Standard error of mean).

Minimum inhibitory concentration (MIC):

The minimum inhibitory concentration (MIC) of the crude extract was determined by the serial tube dilution technique [15] in broth medium, containing graded concentration of the plant extract inoculated with the test organisms (Table-2).

Test for anti-inflammatory activity:

The anti-inflammatory activity of crude extract was determined by using inhibition of albumin denaturation technique [16]. To conduct the experiment, 3 clean centrifuge tubes were taken for standard

(positive control), negative control and crude ethanol extract. 1.0 ml of 5% egg albumin solution was added to the tubes. Then 1.0 ml of ethanol was added to the control tubes. 1.0 ml acetyl salicylic acid (0.1%) was added as positive control group. On the other hand, for test group 1 ml ethanol extract (500 µg/ml) was mixed to the "test" marked tube. The pH (5.6 ± 0.2) of the all reaction mixtures was adjusted with 1N HCl and heated at 57 °C for 20 min. After cooling and filtering through Whatman no. 1 filter paper, the absorbance was measured spectrophotometrically at 660 nm. The test was repeated for three times and the percentage inhibition of protein denaturation was calculated as follows:

I% inhibition = $(A_{control} – A_{sample}) \times 100/A_{control}$

Test for membrane stabilization activity:

The membrane stabilizing activity was assessed by using hypotonic solution induced haemolysis of human erythrocyte [17]. For this study, 3 clean centrifuge tubes were taken for standard, positive control and crude extract and marked accordingly. About 1.0 ml of 10% RBCs suspension was added to all tubes and 1.0 ml ethanol and 1.0 ml acetyl salicylic acid were added to the negative control and positive control marked tube, respectively. On the other hand, 1.0 ml crude extract (500 µg/ml)was mixed to the test group. Then all the tubes were treated with 1.0 ml of hypotonic solution. The pH (7.4 ± 0.2) of the reaction mixtures was adjusted by phosphate buffer. All centrifuge tubes containing reaction mixtures were incubated at 56 °C for 30 min in a water bath. The tubes were cooled under running tap water and then centrifuged at 2500 rpm for 5 min. The absorbance of the supernatants was measured at 556 nm with a visible spectrophotometer. The test was repeated for three times. The percentage inhibition of protein denaturation was calculated as follows:

I% inhibition = $(A_{control} – A_{sample}) \times 100/A_{control}$

Test for anti-atherothrombosis activity:

The thrombolytic activity of the crude extract was evaluated by previously described method [18] using streptokinase as standard. For this study, 4 ml venous blood was drawn from healthy volunteers and distributed in three (for extract, reference standard and for negative control) pre-weighed sterile micro-centrifuge tubes (0.5 ml/tube) and incubated at 37 °C for 45 min. After clot formation, serum was completely removed without disturbing the clot and each tube was weighed again to determine the weight of clot (clot weight = weight of clot containing tube – weight of tube alone). Then, 100 µl of ethanol extract at a dose of 5 µg/µl, 100 µl of streptokinase and 100 µl of ethanol were separately added to the pre-marked tubes containing the clot. The tubes were then incubated at 37 °C for 90 min and observed for clot lysis. Afterwards, the fluid released was removed and tubes were again weighed to observe the difference in weight after clot disruption. Difference obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis. The experiment was repeated for three times in different days with fresh blood samples collected from 10 healthy volunteers (male and female) having no history of contraceptives and anticoagulants.

Statistical analysis:

The primary data obtained from the experiments were manipulated as the source of responses. As indicated before, seven samples were prepared for each of the bioassays and data were expressed as mean ± SEM (standard error of mean). Statistical analysis was performed by student's t-test (n=7). Differences were considered statistically significant when $p <0.5$.

3. Results and Discussion

Preliminary phytochemical screening:

In preliminary phytochemical screenings, the crude extract demonstrated the presence of alkaloids, steroids, tannins, reducing sugars as shown in Table 1.

Pharmacological studies:

In the antimicrobial sensitivity test, the crude extract displayed mild to moderate antimicrobial activity against the test organisms (Table 2). The test sample demonstrated strong antifungal activity against *Pityrosporum ovale* (31±1), Trichophyton sp. (28±1), *Candida albicans* (26±1), *Cryptococcus neoformans* (26±1), Microsporum sp. (26±1) while the growth of all tested bacteria were moderately inhibited. During the MIC determination, the ethanol extract inhibited the growth of test organisms at 31.25-250 µg/ml (Table 2). The low MIC value (31.25 µg/ml) of the extract was against *Candida albicans*, *Pityrosporum ovale*, Trichophyton sp., Microsporum sp. and *Cryptococcus neoformans* suggested the presence of compounds having potent antifungal activity in the extractive.

In the *in vitro* anti-inflammatory screening, the ethanol extract (500 µg/ml) showed mean inhibition of protein denaturation 47.4±0.001 as compared to that of 52.35±0.0007 produced by standard acetyl salicylic acid (Table-3). So, the ability of ethanol extract of the plant to inhibit thermal and hypotonic solution induced protein denaturation was found to be significant.

The ethanol extract (500 µg/ml) also inhibited the heat induced total haemolysis of human RBCs by 57.63±0.002, whereas the standard aspirin showed 89.83±0.002 (Table 4). The result provides evidence for moderate membrane stabilization as an additional mechanism to support its anti-inflammatory effect.

Addition of 100 µl streptokinase, a positive control (30,000 IU), to the clot along with 90 min of incubation at 37 ^{0}C showed 81.53% clot lysis. On the other hand, after treatment of clots with 100 µl of test sample, 20.61% clot lysis was obtained (Table 5). So, the plant provides weak activity against clot formation.

Table 1: Chemical analysis for phytoconstituents in the crude extract of *L. macrophylla*

Examination	Test performed	Result
Alkaloids	Mayer's test	+
	Dragendorff's test	+
	Wagner's test	+
	Hager's test	-
	Tannic acid test	-
Glycosides	Salkowski test	-
	Libermann-burchared test	-
Steroids	Salkowski test	+
	Libermann-burchared test	+
Tannins	Ferric chlorides test	+
	Potassium dichromate test	+
Flavonoids	Conc. HCl and alcoholic test	-
Saponins	Shake test (aq. solution)	-
Reducing sugar	Fehling's test	+
	Benedict's test	+
Gums	Molisch's test	+

(+) = present; (-) absent

Conclusion

The present study was conducted to evaluate the biological activities of ethanol extract of *L. macrophylla* as well as to determine the phytochemical profiles of the extract. Preliminary phytochemical screenings with the crude extractives demonstrated the presence of alkaloids, steroids, tannins, reducing sugars and gums. This plant showed moderate antibacterial and strong antifungal activities. The ability of the ethanol extract of this plant to inhibit thermal and hypotonic solution induced protein denaturation was found to be significant and provides evidence for mild membrane stabilization as an additional mechanism of their anti-inflammatory effect.

Table 2: Antimicrobial activity of *L. macrophylla* at 500 µg/disc and standard 30 µg/disc.

Test organisms	Diameter of zone of inhibition (mm)		Minimum inhibitory concentrations (µg/ml)
	Crude extract	Standard	
Gram positive bacteria		**Ciprofloxacin**	
Bacillus cereus	11±1[d]	12.8±1.26	250
B. megateriuum	nd	14.2±0.76	nd
B. subtilis	10±1[b]	14.8±1.04	250
Staphylococcus aureus	nd	12.3±0.58	nd
Gram negative bacteria			
Escherichia coli	12±1[d]	14.7±0.58	250
Pseudomonas aeruginosa	11±1[d]	11.3±1.04	250
Salmonella Paratyphi	12±2[d]	15.5±0.50	nd
S. Typhi	nd	12.5±0.50	nd
Shigella dysenteriae	9±1[c]	12.5±1.50	250
S. sonnei	9±1[b]	13.8±0.29	250
Vibrio cholerae	nd	13.8±0.29	nd
Fungi		**Fluconazole**	
Aspergillus niger	16±2.65[d]	13.7±0.76	125
Blastomyces dermatidis	25±1[a]	11.7±0.76	62.5
Candida albicans	26±1[a]	11.5±1.50	31.25
Cryptococcus neoformans	26±1[a]	14.5±0.50	31.25
Microsporum sp.	26±1[a]	11.5±1.32	31.25
Pityrosporum ovale	31±1[a]	13.0±0.50	31.25
Trichophyton sp.	28±1[a]	12.7±1.26	31.25

[a]p<0.001, [b]p<0.02, [c]p<0.05, [d]p<0.10; The diameter of zone of inhibition are expressed as mean ± SEM (n=7); SEM: standard error of mean; Zone of inhibitions under 8 mm were considered as less active and were discarded. nd: Not detected.

Table 3: *In vitro* anti-inflammatory activity of test sample and controls.

Test groups	**Total inhibition of protein denaturation**
Control	00.00±0.0004
Positive control (ASA 0.1%)	52.35±0.0007[b]
EELM (500 µg/ml)	47.4±0.001[a]

[a]p <0.02, [b]p <0.001; Total inhibition of protein denaturation = % MIPD ±SEM; ASA = Acetyl salicylic acid, EELM = Ethanol Extract of *L. macrophylla*

Table 4: *In-vitro* membrane stabilization test for sample and controls.

Test groups	Total inhibition of haemolysis
Control	00.00±0.00736
Positive control (ASA 0.1%)	89.83±0.002041[a]
EELM (500 µg/ml)	57.63±0.002041[b]

Total inhibition of haemolysis = %IMHLs ± SEM, [a]p<0.01, [b]p<0.02.

Table 5: Anti-atherothrombosis activity of test sample and controls.

Controls/extract	Clot lysis (%)
Ethanol (Negative control)	2.49±0.39
Streptokinase (Positive control)	81.53±3.7049
EELM	20.61±1.762

Values are expressed as mean ± SEM (standard error of mean)

So, the results obtained from this study indicate that this plant species could be useful in the search for new natural bioactive compounds.

Conflict of Interest: None

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