Bangladesh Pharmaceutical Journal 16(1): 33-37, 2013

Pharmacological and Phytochemical Screenings of Ethanol Extract of *Etlingera linguiformis* (Roxb.) R.M.Sm. Growing in Bangladesh

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Abstract

The present study was conducted to investigate the bio-activities of ethanol extract of *Etlingera linguiformis* (Roxb.) R.M.Sm. as well as to determine the chemical profiles of the extract. The antibacterial and antifungal activities of the crude extract were evaluated by the disc diffusion method against 4 Gram positive and 7 Gram negative pathogenic bacteria and 7 fungi using Ciprofloxacin and Fluconazole as standards, respectively. The minimum inhibitory concentration (MIC) was determined by the serial dilution method. The anti-atherothrombosis activity was assessed by using Streptokinase (SK) as standard. Moreover, the *in-vitro* anti-inflammatory and membrane stablization tests were performed. In the anti-bacterial and antifungal activity test, the zones of inhibition were found within the range of 10.0-15.0 and 10.0-22.0 mm, respectively. The highest zone of inhibition was obtained against *Bacillus cereus* (15.0 mm) and *Blastomyces dermatitidis* (22.0 mm). In the minimum inhibitory concentration (MIC) test the crude extract inhibited the growth of *Blastomyces dermatitidis* significantly at 31.2 µg/ml. In the anti-atherothrombosis activity test, the extract revealed moderate clot lysis by 15.15%. Moreover, the extract produced inhibition of protein denaturation and haemolysis by 34% and 38.98% in the *in vitro* anti-inflammatory and membrane stablization tests. Preliminary phytochemical screenings of the crude extractives demonstrated the presence of alkaloids, steroids, tannins, reducing sugars and gums. The extract also exhibited good biological activities. Therefore, the plant should be subjected to systematic bioactivity guided isolation in order to obtain the active molecules.

Key words: *Etlingera linguiformis,* Antimicrobial, Minimum Inhibitory Concentration, Anti-atherothrombosis, Anti-inflammatory, Membrane Stablization.

Introduction

The plants which naturally synthesize and accumulate some secondary metabolites, like alkaloids, glycosides, tannins, volatile oils and contain minerals and vitamins possess medicinal properties (De Smet, 1997). In the past, peoples of ancient civilizations depended greatly on local flora and fauna for their survival (Dossey, 2010). Ethnomedicine is a sub-field of ethnobotany or medical anthropology that deals with the study of traditional medicines: not only have those that have relevant written sources but especially those, whose knowledge and practices been orally transmitted over the centuries (Acharya *et al.,* 2008). *Etlingera linguiformis* (Roxb.) R.M.Sm. (Family: Zingiberaceae, Bengali name: Tera) is a tall, leafy, perennial herb, upto 2 m high, has 30-45 cm long, oblong, lanceolate, glabrous leaves, with stout, copiously stoloniferous aromatic rhizome. In Bangladesh the plant grows in Chittagong Hill Tracts, Moulvi Bazar and in India it is available in Darjeeling. This plant is used as a medicinal plant in Bangladesh and pieces of rhizomes are chewed with betel leaf to cure sore throat in Hathazari area of Chittagong (Website-1, 2). Young leaves and shoots are used as leafy vegetables. Rhizomes are used as medicine to cure jaundice (Ramana *et al.,* 2012). The rhizomes are strongly aromatic which contain about 0.4% essential oil with a fennel like smell, composed of about 19 components, such as methyl chevicol, methyl eugenol, β-pinene, asarone, eucalyptol and α-pinene. The fresh leaves contain 0.15% essential oils, composed of about 39 components; a few are eucalyptol, β-pinene, α-pinene, linalool, β-elemene, α-selinene, β-terpinyl acetate, α-phellandrene and juniper camphor (Website-3).

As part of our ongoing research with medicinal plant of Bangladesh (Kuddus *et al.,* 2012; Amin *et al.*,2012) the

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present study has been undertaken to evaluate the antimicrobial, anti-inflammatory, membrane stabilization and anti-atherothrombosis activities of the species to find out evidence for its folk uses and to introduce *E. linguiformis* grown in Bangladesh as a source of new drug candidate.

Materials and Methods

Collection and identification: The aerial parts of *E. linguiformis* (Roxb.) was collected from Chittagong Botanical Garden, Shitakando, Chittagong, Bangladesh in the November, 2011. The plant was identified by the experts of Bangladesh Forest Research Institute Herbarium, Chittagong where voucher specimen has been deposited.

Drying and grinding: After collection, the plant was separated from other plant parts and dust and then washed with running tap water. The plant was then subjected for shade dry at temperature not exceeding 50 ºC. The dry materials were grounded into a coarse powder with the help of a grinder and stored in airtight container and in a cool and dark until extraction commenced.

Hot extraction by Soxhlet extractor: Exactly 130 gm of powder was extracted with 750 ml of ethanol (99.98%) in a Soxhlet apparatus (Quickfit, England) (Bhal and Bhal 1992). The extract was concentrated with a rotary evaporator (Heidolph, Germany) under reduced temperature and pressure to provide a gummy residue (15.49 gm, yield 11.92%).

Preliminary phytochemical investigation: For preliminary phytochemical investigation the crude extract was subjected to various tests (Table 1) to determine the chemical nature of the extractive (Ali, 1993).

Screening for antimicrobial activity: The antibacterial and antifungal activities of the crude extract was evaluated by the disc diffusion method against 4 Gram positive and 7 Gram negative pathogenic bacteria and 7 fungi (Table 2) using Ciprofloxacin and Fluconazole as standards, respectively (Bauer *et al.,* 1951). The organisms were collected from the Microbiology Lab., Department of Pharmacy, BGC Trust University, Chittagong, Bangladesh. The test organisms were maintained on nutrient agar slopes and were sub-cultured at regular intervals. These microorganisms served as test pathogens for antibacterial sensitivity test. The antimicrobial activity of test agent 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.

Minimum inhibitory concentration (MIC): The minimum inhibitory concentration (MIC) of ethanol extract of *E. linguiformis* was determined (Table 2) by the serial tube dilution technique (Turnidge *et al.,* 2003) in broth medium, containing graded concentration of the plant extract inoculated with the organisms.

Anti-inflammatory activity: To determine the anti-inflammation activity (Tables 3) of the ethanol extract of *E. linguiformis*, 12 clean centrifuge tubes (three for positive control, acetyl salicylic acid, three for negative control, 99.8% ethanol and six for crude extract) were used. 1.0 ml of 5% egg albumin solution was kept into all test tubes. Then 1 ml of acetyl salicylic acid (0.1 mg), 1 ml of ethanol and 1ml of ethanol extract (1000 mg/kg) added to the positive and negative and test groups marked test tubes, respectively. The pH (5.6±0.2) of all the reaction mixtures was adjusted by 1N HCl. These were heated, cooled and after filtration, the absorbance was measured spectrophotometrically at 660 nm (Olajide *et al.,* 2004).

Membrane stabilization activity: For this study, three clean centrifuge tubes were taken for positive control (acetyl salicylic acid), three for negative control (99.8% ethanol) and six for crude ethanol extract and 1.0 ml of 10% RBC suspension was added to each tube. Then 1.0 ml ethanol and 1.0 ml acetyl salicylic acid were added to the negative control and positive control tubes respectively. On the other hand, for the test group, 1.0 ml of ethanol extract (1000 mg/kg) was mixed. The pH (7.4±0.2) of the reaction mixtures was adjusted by phosphate buffer. The tubes were then incubated in water bath and after cooling these were centrifuged at 2500 rpm for 5 minutes. After filtration the absorbance of the supernatants were taken at 556 nm. The total inhibition of haemolysis was then calculated (Table 4) by determining the % inhibition of haemolysis (Shinde *et al*., 1999).

Anti-atherothrombosis activity: An aliquot of 4.0 ml venous blood from each of the 10 healthy volunteers was collected and distributed in three different pre-weighed sterile microcentrifuge tubes (1 for crude extract, 1 for streptokinase as standard and 1 for ethanol as negative control). After clot formation the clot weight was

determined by removing the serum without disturbing the clot. Then 100 µl (5µg/µl) of ethanol extract, 100 µl of streptokinase and 100 µl of ethanol (99.8%) were added to the microcentrifuge tubes marked as test, positive control and negative control respectively. After incubation at 37 ºC for 90 minute, the percentage of clot lysis (Table 5) was calculated (Prasad *et al.,* 2006).

Results and Discussion

Preliminary phytochemical screening: The crude extractive when tested with various chemical reagents demonstrated the presence of alkaloids, steroids, tannins, reducing sugars and gums as shown in Table 1.

Pharmacological studies: In the disc diffusion anti-bacterial activity test, the zones of inhibition was found within the range of 10.0-15.0 mm. The highest zone of inhibition (15.0 mm) was obtained against *B. cereus*, followed by 13.0, 12.0, 11.0, 11.0, 11.0 and 10.0 mm against *S. aureus, P. aeruginosa, B. subtilis, B. megateriuum, S.* Paratyphi and *Shigella sonnei*, respectively. The crude extract strongly inhibited the growth of *B. cereus* and *P. aeruginosa* when compared with standard ciprofloxacin. But the tested extract produced no inhibition to the *E. coli*, *S. dysenteriae*, *S.* Typhi and *V. cholera* (Table 2).

During the minimum inhibitory concentration (MIC) determination, the crude extract inhibited the growth of *Blastomyces dermatitidis* significantly at the dose of 31.2 µg/ml followed by *Bacillus cereus* and *Micrococcus* (62.50 µg/ml), *S. aureus, A. niger*, and *C. albicans* by 125.0 µg/ml and *Bacillus subtilis*, *B. megateriuum*, *P. aeruginosa*, *S.* Paratyphi and *P. ovale* by 250.0 µg/ml (Table 2).

Table 1. Chemical groups present in the extract of *Etlingera linguiformis.*

Test for	Test performed	EtOH Extract of *E. linguiformis*
Alkaloids	Meyer'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 sugars	Fehling's test	+
	Benedict's test	+
Gums	Molisch's test	+

(+) = present; (-) = absent

In the present study for *in-vi*tro anti-inflammatory test, the crude ethanol extract of *E. linguiformis* (1000 mg/kg) showed mean inhibition of protein denaturation 34±0.002 whereas, for ASA it was found to be 52±0.0007 (Table 3). The ability of ethanol extract of *E. linguiformis* to inhibit thermal and hypotonic solution-induced protein denaturation was found to be mild.

The test extract (1000 mg/kg) inhibited the heat induced haemolysis of RBCs by 38.98±0.00736 whereas, the standard aspirin showed 89.83±0.002041 (Table 4). The stabilization activity for the crude extract of *E. linguiformis* was found to be reasonable. Although the precise mechanism of this membrane stabilization is yet to be elucidated, it is thought that the ethanol extract of the plant may possibly inhibit the release of lysosomal

Table 2. Antibacterial activity of *Etlingera linguiformis* at 500 µg/disc and standard.

Test organisms	Diameter of zone of inhibition (mm)		MICs (µg/ml)
	EtOH Extract (500 µg/disc)	Standard (30 µg/disc)	
Gram positive bacteria		**Ciprofloxacin**	
Bacillus cereus	15.0 ± 2.00[c]	13.0 ± 1.26	62.5
B. megateriuum	11.0 ± 1.00[c]	12.0 ± 0.58	250.0
B. subtilis	11.0 ± 2.00[c]	15.0 ± 1.04	250.0
Staphylococcus aureus	13.0 ± 0.00[c]	14.0 ± 0.76	125.0
Gram negative bacteria			
Escherichia coli	nd	15.0 ± 0.50	nd
Pseudomonas aeruginosa	12.0 ± 1.00[d]	11.0 ± 1.04	250.0
Salmonella Typhi	nd	12.0 ± 0.50	nd
Salmonella Paratyphi	11.0 ± 1.00[c]	12.0 ± 1.50	250.0
Shigella dysenteriae	nd	15.0 ± 0.58	nd
Sh. sonnei	10.0 ± 1.00[a]	14.0 ± 0.29	nd
Vibrio cholerae	nd	14.0 ± 0.29	nd
Fungi		**Fluconazole**	
Aspergillus niger	14.0 ± 2.65[d]	14.0 ± 0.76	125.0
Blastomyces dermatitidis	22.0 ± 3.00[a]	12.0 ± 0.76	31.5
Candida albicans	15.0 ± 2.65[c]	13.0 ± 0.50	125.0
Cryptococcus neoformans	10.0 ± 1.00[c]	12.0 ± 1.50	nd
Microsporum sp.	20.0 ± 2.65[b]	11.0 ± 1.32	62.5
Pityrosporum ovale	11.0 ± 1.00[c]	13.0 ± 1.26	250.0
Trichophyton sp.	14.0 ± 1.00[d]	14.0 ± 0.50	125.0

[a]p<0.02, [b]p<0.05, [c]p<0.10, [d]p<0.50; MICs: Minimum inhibitory concentrations nd: Not detected; The diameter of zone of inhibition is expressed as mean±SEM (n = 3); SEM: standard error of mean; Zone of inhibition under 8 mm was considered as less active and was discarded.

content of neutrophils at the site of inflammation. That provides evidence for poor membrane stabilization as an additional mechanism of their anti-inflammatory effect.

Table 3. *In-vitro* anti-inflammatory activity of ethanol extract of *E. linguiformis.*

Test groups	SD	SEM	Inhibition of protein denaturation
Control (Ethanol)	0.0005	0.0004	0.00 ± 0.00108
Positive control (ASA 0.1 mg)	0.001	0.0007	52 ± 0.0007[b]
EEEL (1000 mg/kg)	0.0005	0.0004	34 ± 0.002[a]

*SEM = Standard error of mean, Total inhibition of protein denaturation = %MIPD ±SEM, [a]p<0.02, [b]p<0.001; EEEL = Ethanol Extract of *E. linguiformis;* ASA = Acetyl salicylic acid

In the study for anti-atherothrombosis activity test, addition of 100 µl SK, a positive control (30,000 I.U.) to the clots along with 90 min of incubation at 37 °C, showed 81.53% clot lysis. On the other hand, clots when treated with 100 µl ethanol (negative control) showed only negligible clot lysis (2.49%). After treatment of clots with 100 µl of *E. lingueformis* clot lysis 15.15% was obtained. Statistical representation of the effective clot lysis percentage, by the crude extract, positive thrombolytic control (streptokinase) and negative control (ethanol) is tabulated in Table 5.

Table 4. Tabulation for *in-vitro* membrane stabilization test of ethanol extract of *E. linguiformis.*

Test groups	SD	SEM	Total inhibition of haemolysis
Control (Ethanol)	0.010408	0.00736	00.00 ± 0.00736
Positive control (ASA 0.1 mg)	0.002887	0.002041	89.83 ± 0.002041[a]
EEEL (1000mg/kg)	0.010408	0.00736	38.98 ± 0.00736[b]

*Total inhibition of haemolysis = %IMHLs ± SEM, [a]p < 0.01, [b]p < 0.05

Table 5. Result of anti- atherothrombosis activity of ethanol extract of *E. linguiformis.*

Controls/extract	Clot lysis (%)
Ethanol (negative control)	2.49 ± 0.39
Streptokinase (positive control)	81.53 ± 3.7049
EEEL	15.15 ± 2.432

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

Conclusion

The present research was conducted to investigate the preliminary biological activities of ethanol extract of *E. linguiformis* (Roxb.) R.M.Sm. 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 antimicrobial activity with low MIC against several micro-organisms. The ability of ethanol extract of this plant to inhibit thermal- and hypotonic solution- induced protein denaturation was found to be mildly significant and provides evidence for poor membrane stabilization as an additional mechanism of their anti-inflammatory effect. So, the results obtained from this study indicate that this plant species could be useful in the search for new natural bioactive compounds and thus warrant for further studies.

Acknowledgements

The authors wish to thank the authority of the department of Pharmacy, BGC Trust University, Chittagong, Bangladesh for supplying test organisms to perform these investigations.

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