Phytochemical and Biological Investigations of *Eurya acuminata* (Theaceae)

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ABSTRACT: The methanol extract of the powdered leaf of *Eurya acuminata* was investigated for isolation of secondary metabolites and two compounds were obtained by using VLC, column chromatography and TLC. The compounds were identified as phytol (**1**) and β-sitosterol by extensive spectroscopic studies, including high field NMR analyses as well as co-TLC with authentic samples. The methanol extract of leaf of *E. acuminata* and its organic and aqueous soluble partitioning materials were evaluated for cytotoxic, thrombolytic and antimicrobial properties. In the cytotoxicity study the aqueous fraction of crude methanolic extract showed significant lethality towards brine shrimp having LC_{50} value 8.821 µg/ml as compared to standard vincristine sulfate (0.404 µg/ml). In the study for thrombolytic property, different extract of *E. acuminata* exhibited various thrombolytic activity ranging from 13.66 to 31.89 % as compared to standard streptokinase (46.51 %). No antimicrobial activity was observed from leaf extracts.

Key words: *Eurya acuminata*, phytol, β-sitosterol, brine shrimp lethality, thrombolytic, antimicrobial.

INTRODUCTION

Eurya is regarded as a genus of the family Theaceae. The genus has 326 species of which 156 have accepted names. *Euryaacuminata* (DC) (Bengali name: Lopat, Sagolerbori) is an evergreen shrub upto 3.5 m height, having narrowly oblong-elliptic, serrulate attenuate leaves approximately 5-7.6 cm long. It is found widely in Japan, China, India, Srilanka, Thiland, Vietnam, Taiwan[1] and in the hilly forests of Bangladesh.

Chemical investigations of various *Eurya* species reported various compounds like anthrocyanins, ellagic acid, caffeine, flavone, flavonols, β-D-glucopyranoside[2], euryanoside[3], chrysoeriol etc. The present study has been undertaken to isolate and identify biologically active secondary metabolites. We, herein, report the isolation of β-sitosterol and phytol, an acyclic diterpene, for the first time from this plant and the evaluation the cytotoxic, thrombolytic and antimicrobial activities.

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MATERIALS AND METHODS

General experimental procedures. Preparative TLC was conducted over glass plates coated with silica gel 60 PF_{254} (0.5 mm thickness, Merck) and compounds were detected with vanillin H_2SO_4 spray reagent. Gel permeation chromatography was performed using Sephadex LH-20. ^1H NMR spectra were recorded in $CDCl_3$ (δ values were reported in reference to $CHCl_3$ at 7.25 ppm) on a Bruker Avance 100 and 400 MHz Ultrashield NMR Spectrophotometer equipped with broadband and selective (^1H and ^{13}C) inverse probes.

Plant material. *E. acuminate* (D.C.) was collected from Moulvibazar Eco park, Sylhet, Bangladesh in the month of January, 2015 and was identified by the taxonomist of Bangladesh National

Herberium, Dhaka, Bangladesh. A voucher specimen (Accession no. DACB 40862) of the plant has been deposited in National Herbarium, Mirpur, Dhaka, Bangladesh for future reference.

Extraction and isolation. The powdered leaf (900 g) of *E. acuminata* was soaked in methanol (3L) for 15 days and filtered through a cotton plug followed by Whatman filter paper number 1. The extract was then concentrated with a Buchii rotary evaporator. An aliquot of the crude methanolic extract (32.5 g) was fractionated by vacuum liquid chromatographic (VLC) technique using silica gel 60H and petroleum ether, petroleum ether-dichloromethane, dichloromethane-ethyl acetate, ethyl acetate, ethyl acetate-methanol and methanol in increasing order of polarity. This provided 32 VLC fractions. Following TLC screening of fractions of VLC, fractions 7-11 and 16 were subjected to column chromatography over lipophilic Sephadex (LH-20) and petroleum ether-chloroform combination as mobile phase. VLC fraction 11 and column fraction 19 yielded two compounds namely phytol (**1,** R_f = 0.60 in ethyl acetate-toluene, 15:85) and β-sitosterol (R_f = 0.60 in ethyl acetate-toluene, 15:85).

Sample preparation for biological investigations. Crude extract was undertaken for solvent-solvent partitioning by using the protocol designed by Kupchan[4] and modified by VanWagenen *et al*.[5] The crude extract of leaf (5 g) was dissolved in 10 % aqueous methanol to make the mother solution which was successively partitioned by petroleum ether, dichloromethane, chloroform in order of increasing polarity by using separating funnel.

Cytotoxicity by brine shrimp lethality bioassay. In brine shrimp lethality bioassay[6] dimethyl sulfoxide (DMSO) was used as a solvent and negative control while vincristine sulfate (VS) served as the positive control. For cytotoxicity screening, DMSO solutions of the test samples were applied against *Artemia salina* in a 1-day *in vivo* assay. For the experiment, 4 mg of each of the test samples was dissolved in DMSO and solutions of varying concentrations (400, 200, 100, 50, 25, 12.50, 6.25, 3.125, 1.563 and 0.781 µg/ml) were obtained by serial dilution.

Thrombolytic property. The thrombolytic property was assessed by standard Streptokinase (100 µl) as positive control and water as negative control.[7] Aliquots (5 ml) of venous blood were drawn from healthy volunteers who were distributed in ten different pre-weighed sterile eppendorf tubes (0.5 ml/tube) and incubated at 37º C for 45 minutes. After clot formation, the serum was completely removed without disturbing the clot and each eppendorf tube having clot was again weighed to determine the clot weight (clot weight = weight of clot containing tube - weight of tube alone). To each eppendorf tube containing pre-weighted clot, 100 µl aqueous solution of different extract along with the crude extract, positive and negative control were added separately to the eppendorf tubes. All the eppendorf tubes were then incubated at 37º C for 90 mins and observed for clot lysis. Then the released fluid was removed and tubes were again weighed to observe the difference in weight after clot disruption. Clot lysis was expressed as percentage:

% Clot lysis = (Weight of the lysis clot / Weight of clot before lysis) × 100 %

***In vitro* antimicrobial activity.** The samples were tested for antimicrobial activity by the disc diffusion method.[8] The screening was done against 13 strains of bacteria. The results thus obtained were compared with standard antibiotic, vancomycin (30 µg/disc).

RESULTS AND DISCUSSION

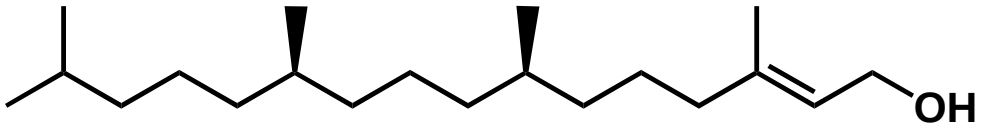
The chemical study of leaf of *E. acuminata* led to the isolation and identification of two bioactive compounds **1** (phytol), an acyclic diterperne and β-sitosterol.

The ^{1}H NMR spectral data (400 MHz, $CDCl_3$) of compound **1** demonstrated a triplet with coupling constant J = 6.8 Hz at δ 5.40 indicating the presence of a monosubstituted olefinic group. Doublet at δ 4.14 (J = 6.8 Hz) stands for oxymethylene proton. Four singlet at δ 0.83, 0.84, 0.85 and 0.86 revealed the presence of four angular methyl groups. Another singlet at δ 1.66 indicated the presence of methyl group substituted on olefinic carbon. Also, there are 9

peaks for 9 methylene groups. The ^{13}C NMR showed the presence of 20 carbon atoms along with 2 olefinic carbons at δ_c 123.1 and 140.4. Oxymethylene carbon was evident from the chemical shift at δ_c 59.46.

HMBC spectrum shows significance proton carbon correlation. Prominent peaks were observed for H-1/C-2, H-20/C-3, H-19/C-7, H-18/C-11 and H-17/C-15. Considering the above data as well as by comparison with published ^{13}C NMR data revealed the compound to be phytol.[9] This is the first report of isolation of phytol from this plant.

β-sitosterol was identified by co-TLC with the authentic sample in different solvent systems.



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Cytotoxicity by brine shrimp lethality bioassay. In the cytotoxicity screening the LC_{50} values obtained from the best fit line slope were found to be significant (in comparison with VS, 0.404 µg/ml) for AQF (8.821 ± 0.31 µg/ml), DCMF (9.1097 ± 0.58 µg/ml), CFF(11.806 ± 0.85 µg/ml) (Table 1).

Table 1. Cytotoxic activity of Leaf extracts of *E. acuminata*

Sample	Cytotoxic activity (LC_{50} in µg/ml)
AQF	8.821±.31
DCMF	9.109±0.58
CFF	11.806±0.85
PEF	60.402± 10.79
MEF	130.231±8.853
Standard (vincristine sulphate)	0.404

AQF = Aqueous fraction, DCMF = Dichloromethane soluble fraction, CFF = Chloroform soluble fraction, PEF = Pet ether soluble fraction, MEF = Methanol soluble fraction.

Thrombolytic activity. The extractives of leaf of *E. acuminata* were assayed for thrombolytic activity to determine the ability of clot lysis. Standard streptokinase at 37 °C showed 46.51 % lysis of the clot as compared to distilled water showing a negligible lysis of clot (13.67 %). In this study, pet ether fraction of leaf extract showed the highest thrombolytic property (31.89 %) (Table 2).

Table 2. Thrombolytic activity of leaf extract of *E. acuminata*

Sample	% of clot lysis leaf part
AQF	13.66
DCMF	14.38
CFF	17.01
PEF	31.89
MEF	25.52
Blank	13.67
Standard (streptokinase)	46.51

***In vitro* antimicrobial activity.** *In vitro* antibacterial activity of the test samples was investigated against six gram positive and seven gram negative bacteria. All the test samples were found to be inactive (data not shown).

CONCLUSION

Two compounds were isolated by successive chromatographic separation and purification of a crude methanolic extract of *E. acuminata*. The brine shrimp lethality bioassay indicated the presence of moderate cytotoxic principles in the tested extractives. In thrombolytic study, the crude methanolic extract and its partitioning fractions (petroleum ether, dichloromethane, chloroform and aqueous) of the leaf of *E. acuminata* demonstrate clot lysis by 25.52 %, 31.89 %, 14.38 %, 17.01 % and 13.66 %, respectively, whereas streptokinase showed 46.51 % clot lysis. Therefore, considering the potential bioactivity, the plant material can further be

studied extensively to find out their unexplored efficacy and rationalize their uses as traditional medicine.

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