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Isoflavones from the Bark of *Ormosia robusta* (Fabaceae) baker.

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ABSTRACT

Bark of *Ormosia robusta* (Fabaceae) Baker has been investigated for isolation of secondary metabolites and evaluation of bioactivities. Two prenylated isoflavones, Warangalone (1) and Erysenegalensein M (2), along with two triterpenoids, Betulinic acid (3) and Lupeol (4), have been isolated from the methanolic extract of the bark by using chromatographic analysis. Structures of these compounds were elucidated by extensive spectroscopic analysis and by comparing the data with the published one. This is the first report of isolation of these compounds from this plant. Subsequently the methanolic extract was fractionated with four organic solvents and all the fractions were studied to evaluate their *in vitro* bioactivities. Significant variance is observed for polyphenol content as well as free radical scavenging activity (4.17 - 270.42 mg of gallic acid equivalent/gm of extract and DPPH IC_{50} value: 1.21-6.80 µg/ml) depending on the nature of the solvent partitioned with. The results of the brine shrimp lethality bioassay (LC_{50} value: 1.49 -33.15 µg/ml) indicate that the plant possesses cytotoxic principal and have considerable toxic potencies.

Keywords: *Ormosia robusta*, Fabaceae, Isoflavones, Triterpenoids

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INTRODUCTION

Ormosia robusta belongs to the Fabaceae family and is found mainly in Arunachal Pradesh, Sibsagar and the Cachar District of Assam, Sylhet and Chittagong in Bangladesh and Myanmar [1-3]. It is mainly used as a timber and no phytochemical or biological study has been carried out on this plant till to date. The aim of the present investigation was to isolate the secondary metabolites as well as to evaluate the antioxidant potential and brine shrimp lethality bioassay of extracts of bark of *O. robusta.*

MATERIALS AND METHODS

Experimental

For vacuum liquid chromatography (VLC) and gel permeation chromatography columns were packed by kieselgel 60H and Sephadex (LH-20) respectively. Analytical thin layer chromatography (TLC) was performed on precoated (TLC Silica gel 60 F_{254}-Merxk KGaA) plates using UV light, vanillin/H_2SO_4 reagents to visualize the spots. RV10 Basic (IKA, Germany) was used for rotary evaporation. UV spectra were taken by a UV-visible spectrophotometer (SPECTRAmax-PLUS384 UV–vis). Nuclear magnetic resonance (NMR) spectra in duterated chloroform ($CDCL_3$) were recorded on a Bruker Avance 400 MHz NMR spectrometer.

Plant Material

Bark of *O. robusta* was collected in July 2008 from the Cox's Bazar, Bangladesh. The plant was identified by a taxonomist and a voucher specimen was submitted in the Bangladesh National Herbarium (accession no. 34372) for future reference.

Preparation of the extract

Collected plant materials were chopped after washing, sun dried and powdered followed by extraction with methanol in room temperature for one week. The crude methanolic extract (MEOR) was then filtered through a cotton plug followed by the filtration through Whatman no. 1 filter papers and was concentrated to dryness with rotary evaporator at low temperature and pressure. MEOR was investigated to isolate and characterize the secondary metabolites and bioactivities were tested on MEOR as well as the fractionated parts, obtained by successive partition of the crude extract with *n*-hexane (HEOR), carbon tetra chloride (CTCOR), chloroform (CLOR) and ethyl acetate (EAOR) by using modified Kupchan Partitioning method [4].

Isolation and identification of isoflavones and triterpenoids

25 gm of the dried MEOR was mixed well with silica gel (kieselgel 60H) and then subjected to

VLC (packed with kieselgel 60H). The sample was then subjected to gradient elution in the column using ethyl acetate (EA) in petroleum ether (PE) (0% to 100%) solvent system. Total 24 fractions were collected and marked as 1 to 24, each of which was analyzed on precoated TLC plate.

After initial TLC screening fraction 4 (8% EA in PE) and 7 (15 % EA in PE) were subjected to gel permeation chromatography (packed with Sephadex LH-20). Compound **1** & **2** were isolated and purified from the sub-fractions (46-48) of VLC fraction 7 and compound **3** was isolated from the sub-fractions (18-20) of VLC fraction 4. Compound **4** was crystallized in the VLC fraction 9 (25 % EA in PE) and then collected and purified by washing with EA.

All the compounds were characterized by extensive spectroscopic analysis. By comparing the ^{1}H NMR data of the compounds with reported data [5-8], the compounds were identified.

Determination of Total Phenolic Content

The total phenolic contents of the extracts (MEOR, HEOR, DMOR, CLOR and EAOR) were determined with the Folin-Ciocalteau reagent using the method of Spanos and Wrolstad [9]. To 0.50 ml of each sample (three replicates), 2.5 ml of Folin-Ciocalteau (10%) reagent and 2 ml of Na_2CO_3 (7.5%, w/v) were added and the mixtures were incubated at 45 °C for 15 min.. The absorbance of all samples was measured at 765 nm using a UV-visible spectrophotometer. Results were expressed as milligrams of gallic acid equivalent per gram of extract (mg GAE/gm of extract).

Determination of free radical scavenging activity

Free radical scavenging activity of the different fractions was determined by decrease in the absorbance of methanol solution of DPPH (2, 2-Diphenyl-1-picrylhydrazyl) [10]. 2 ml of the different concentrations (500 µg/ml to 0.977 µg/ml) of the test samples were mixed with 3 ml of DPPH solution in methanol (20µg/ml). After 30 minutes of reaction period at room temperature in dark place the absorbance was measured at 517 nm against methanol as blank by UV spectrophotometer. The percentage inhibition of DPPH radical was calculated by comparing the results of the test with those of the control (not treated with extract) using the following equation

Percentage inhibition = (1– absorbance of test/absorbance of control) × 100

IC_{50} values (concentration of samples required to scavenge 50% of the free radicals) were calculated from the regression equation, prepared from the concentration of the samples and percentage inhibition of free radical formation. Synthetic antioxidant reagent, butylated hydroxytoluene (BHT), was used as positive controls.

Brine Shrimp lethality bioassay[11]

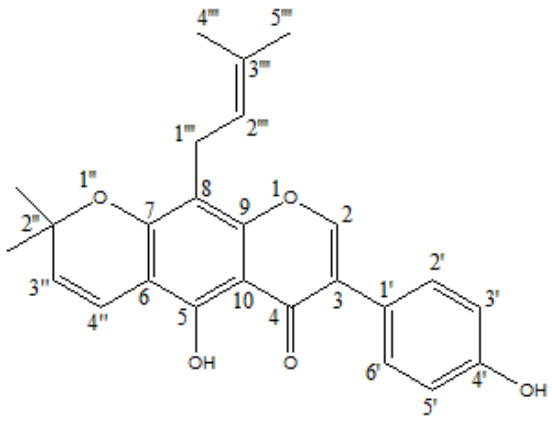
For testing the cytotoxic potential of the extracts brine shrimp lethality bioassay was performed. Brine shrimp eggs (*Artemia salina* Leach) were hatched in simulated sea water at a temperature of 37^0C and pH at 8.4 with continuous oxygen supply to get nauplii. Test samples of different concentrations (400 µg/ml to 0.781 µg/ml) were prepared by dissolving extracts in dimethylsulfoxide (DMSO). The nauplii were counted by visual inspection and were taken in vials containing 5 ml of simulated sea water. Test samples and positive controls using vinblastine sulphate were added to the pre-marked vials through micropipette. After an incubation period of 24 hours, the survivors were counted. The percent (%) mortality was calculated for each dilution. The concentration vs mortality data were analysed statistically by using probit analysis and linear regression. The effectiveness or the concentration-mortality relationship of plant product was calculated as a median lethal concentration (LC_{50}) value.

RESULTS AND DISCUSSION

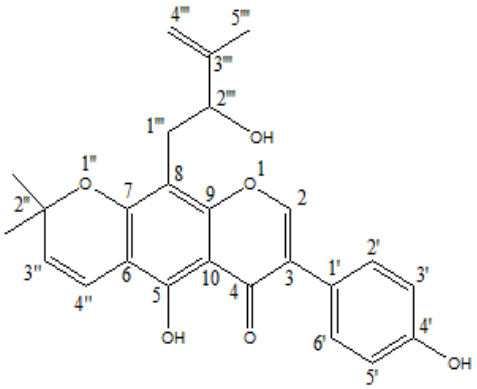
Repeated chromatographic separation and purification of the VLC fractions of the methanolic extract of the stem bark of O. robusta afforded two prenylated isoflavones (1 and 2) and two pentacyclic triterpenoids (3 and 4).

Compound 1 was isolated as a yellow needle like crystals. Comparison of the ^{1}H NMR (400 MHz, CDCl3) data of compound **1** with those reported by Talla et al[5], proved the compound as Warangalone (8(3,3-dimethylallyl)-4'-hydroxy-2''',2''' dimethylpyran [6,7- b]isoflavone).

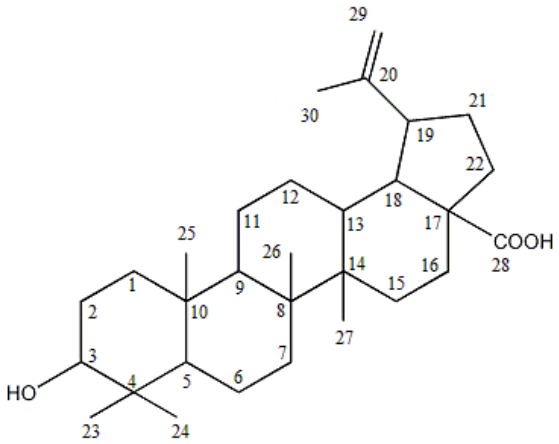
Warangalone (1); ^{1}H NMR ($CDCl_3$, ppm): 7.88 (1H, s, H-2), 7.39 (2H, d, J= 7.4 Hz, H-2'/6'), 6.88 (2H, d, J= 7.4 Hz, H-3'/5'), 5.61(1H, d, J= 10.0 Hz, H-3''), 6.73(1H, d, J= 10.0 Hz, H-4''), 1.40 (6H, s, H- 5''/6''), 3.39 (2H, d, J= 7.6 Hz, H-1'''), 5.18 (1H, t, J= 7.2 Hz, H-2'''), 1.61 (3H, s, H-4'''), 1.74 (3H, s, H-5''') and 13.05 (1H, s, 5-OH)

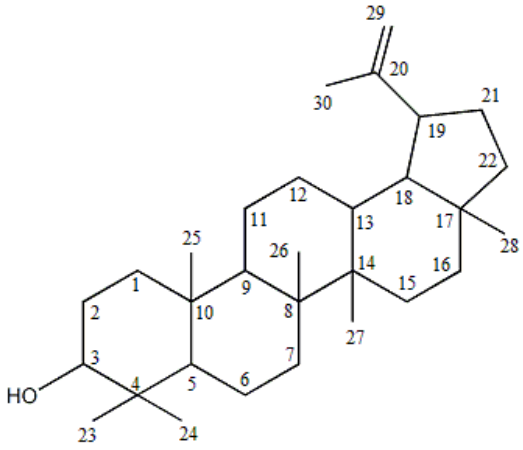


Compound 1



Compound 2





Compound 3 **Compound 4**

Compound 2 was also isolated as yellow oil. Comparison of the ^{1}H NMR (400 MHz, $CDCl_3$) data of compound 2 with those reported by Wandji et al [6] identified the compound as Erysenegalensein M (5,4'-dihydroxy-8-(2''-hydroxy-3'''-methylbut-3'''-enyl)-2'',2''-dimethylpyrano [5'',6'':6,7] isoflavone).

Erysenegalensein M; ^{1}H NMR ($CDCl_3$, ppm): 7.88 (1H, s, H-2), 7.40 (2H, br s, H-2'/6'), 6.89 (2H, s, H-3'/5'), 5.62 (1H, d, J= 10.0 Hz, H-3''), 6.75(1H, d, J= 10.0 Hz, H-4''), 1.50 (3H, s, H-5'), 1.49 (3H, s, H-6'), 2.96 (1H, dd, J= 14.0, 5.6 Hz, H-1'''), 2.98 (1H, dd, J= 14.0, 4.8 Hz, H-1'''), 4.33 (1H, dd, J= 8.0, 4.8 Hz, H-2'''), 4.83 (1H, s, H-4'''), 4.95 (1H, s, H-4'''), 1.88 (3H, s, H-5''') and 13.16 (1H, s, 5-OH)

Compound 3 and 4 was identified as betulinic acid and lupeol respectively by comparing the spectroscopic data with the published one reported by Parvin et al [7] and Aratanechemuge et al [8] respectively.

Betulinic acid; ^{1}H NMR ($CDCl_3$, ppm): 3.16 (1H, dd, J= 11.2, 4.8 Hz, H-3), 2.99 (1H, m, H-19), 0.96 (3H, s, H-23), 0.74 (3H, s, H-24), 0.82 (3H, s, H-25), 0.97 (3H, s, H-26), 0.93 (3H, s, H-27), 4.73 (1H, br.s, H-29), 4.60 (1H, br.s, H-29) and 1.68 (3H, s, H-30)

Lupeol; ^{1}H NMR ($CDCl_3$, ppm): 3.18 (1H, dddd, J= 5.6, 5.6, 5.6, 5.6 Hz, H-3), 2.36 (1H, dt, Jt= 11.2 Hz, Jd= 6.0 Hz, H-19), 0.96 (3H, s, H-23), 0.75 (3H, s, H-24), 0.82 (3H, s, H-25), 1.02 (3H, s, H-26), 0.94 (3H, s, H-27), 0.78 (3H, s, H-28), 4.56 (1H, br.s, H-29), 4.67 (1H, br.s, H-29) and 1.68 (3H, s, H-30)

The results of total phenolic content and DPPH scavenging activity are presented in (Table: 1). Total phenolic content in the analyzed fractions were found in between 4.17 to 270.42 mg of GAE/gm of extract with the highest amount in EAOR (270.42 $\pm$ 5.39 mg of GAE/gm of extract).

High level of total phenolic exhibits high antioxidant capacity since this relationship was also reported in previous studies on other plants [12, 13]. In the present study, we observed a similar relations (Table: 1) since EAOR has the lowest IC_{50} value (1.21µg/ml) and hence is the best DPPH free radical scavenger among all fractions, an indication of high antioxidant activity. Lowest phenolic content was found in HEOR (4.17 ± 1.23 mg of GAE/gm of extract) and similarly lowest scavenging activity with a high IC_{50} value (6.80 µg/ml).

Different mortality rate of the nauplii in all samples and unchanged nauplii (no lethality/mortality) of the control group suggest that all the fractions have toxic activities. It is required to be mentioned that previous studies by Genest et al[14] reported the acute toxicity of *Ormosia* seeds in animals. The obtained LC_{50} values were found in the range of 1.49 to 33.15 µg/ml where the lowest LC_{50} value (1.49 µg/ml) was obtained from the HEOR fraction and the highest one (33.15 µg/ml) from the EAOR, whereas the used standard Vincristine sulphate showd the LC_{50} value of 0.451 µg/ml. Presence of Betulinic Acid and Lupeol, two compounds with inhibitory activity against few cancer cells[15-18], justifies the plant's cytotoxic potential. The outcome of the experiment necessitates the further research on this species to find out the other active principles responsible for the toxic effect.

Table 1: *In vitro* bioactivities of different fractions of *Ormosia robusta* bark.

Sample	TPH[a]	DPPH[b]	Brine Shrimp Lethality bioassay[c]
MEOR	128.55 ± 4.01	1.73 ± 0.69	19.37 ± 1.47 (17.07 – 21.03)
HEOR	4.17 ± 1.23	6.80 ± 0.16	1.49 ± 0.23 (1.22 - 1.75)
CTCOR	101.18 ± 6.74	1.81 ± 0.91	2.26 ± 0.61 (1.56– 2.96)
CROR	42.13 ± 1.41	3.19 ± 1.19	9.42 ± 1.30 (7.95 – 10.90)
EAOR	270.42 ± 5.39	1.21± 0.76	33.15 ± 2.17 (30.69 – 35.61)
BHT	-	21.45± 1.74	-
Vinblastine sulphate	-	-	0.54 (0.645- 0.443)

Values are the mean of three determinations ± standard deviation (SD). See text for the identification of extracts and fractions.

[a]Total Phenolic content. Values are expressed as mg of Gallic acid equivalent (GAE)/gm of extract.

[b] IC_{50} value for DPPH scavenging (µg/ml)

[c] Values are expressed as LC_{50}(µg /ml) for brine shrimp lethality. Lower and upper limit for 95% confidence interval are displayed in the parenthesis.

CONCLUSION

The research work done by a phytochemical study identified the presence of four chemical constituents as warangalone, erysenegalensein M, lupeol and betulinic acid from the bark of *O. robusta* for the first time. Besides different solvent extracts showed significant bioactivities and suggests the possibility of potent substance to be present. Further investigation will be directed to identify and find out the pharmacological effects of the isolated compounds and isolation of more potent secondary metabolites having useful activity.

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