Phytochemical and Biological Investigations of *Ixora arborea* Roxb.

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ABSTRACT: A total of four compounds namely betulin (**1**), erythrodiol (**2**), lupeol (**3**), and stigmasterol were isolated from the leaf extract of *Ixora arborea* for the first time. The structures of the isolated compounds were established by extensive spectroscopic studies. In our preliminary screening, the petroleum ether, carbon tetrachloride, dichloromethane and aqueous soluble fractions of the methanol extract were subjected to antioxidant, antimicrobial and brine shrimp lethality bioassays. All of the fractions showed significant antioxidant activity along with the total phenolic content, of which the methanolic extract of leaves (MEL) showed the highest free radical scavenging activity with IC_{50} value 33.0 µg/ml. In the brine shrimp lethality bioassay, among all the extractives the petroleum ether soluble fraction (PESF) demonstrated significant cytotoxicity with LC_{50} value of 0.622 µg/ml. However, in case of antimicrobial screening, only the methanolic crude extract showed mild growth inhibitory activity against the tested microorganisms.

Key words: *Ixora arborea*, betulin, erythrodiol, lupeol, stigmasterol, antioxidant, cytotoxicity, antimicrobial

INTRODUCTION

Ixora arborea Roxb. (Bengali name- Shet rangan; Eng.name- Torchwood tree; Fam-Rubiaceae) is distributed in the tropics and grows all over Bangladesh. The plant is an evergreen tree with white flowers and commonly grown in gardens as a decorative plant. Its flowers are used in pulmonary troubles and whooping cough.[1,2] Torchwood tree is a folk remedy for urinary diseases.[3] The decoction of bark is used for anaemia and general debility; fruits and roots are given to females when urine is highly coloured.[4] Previous phytochemical studies with *I. arborea* revealed the occurance of kaempferol-rutinoside, ixoral, β-sitosterol, chrysin-O-β-dioxylopyranoside, and dimethoxycoumarin.[4] We, herein, report betulin (**1**), erythrodiol (**2**), lupeol (**3**) and stigmasterol from a methanolic extract of *I. arborea* as well as antioxidant, cytotoxic and antimicrobial activities of the extractives for the first time.

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Dhaka Univ. J. Pharm. Sci. **8**(2): 161-166, 2009 (December)

MATERIALS AND METHODS

General experimental procedure. The ^1H NMR spectra were recorded using a Bruker AMX-400 (400 MHz) instrument and the spectra were referenced to the residual nondeuterated solvent signal. PTLC (20 X 20 cm) and TLC (20 X 5 cm) were carried out using Merck Si gel 60 PF_{254} on glass plates at a thickness of 0.5 mm. Spots on TLC and

PTLC plates were visualised by spraying the developed plates with vanillin-sulfuric acid followed by heating for 5 minutes at 110 ºC. All solvents used in this study were of reagent grade.

Plant material. The leaves of *I. arborea* were collected from Dhaka in the month of November 2008. A voucher specimen (DACB 32925) for this collection has been deposited in the Bangladesh national herbarium for future reference.

Extraction and isolation. The powdered leaves (210 g) of *I. arborea* was soaked in 1.5 L methanol for 7 days and filtered through a cotton plug followed by Whatman filter paper number 1. The extract was then concentrated by using a rotary evaporator. A portion (5 gm) of the concentrated methanol extract was fractionated by the modified Kupchan partitioning method[5] into petroleum ether, carbon tetrachloride, dichloromethane and aqueous soluble fractions. Subsequent evaporation of solvents afforded extracts of petroleum ether (PESF, 1.5 g), carbon tetrachloride (CTSF, 1.2 g), dichloromethane (DCMF, 0.8 g) and aqueous soluble (AQSF, 0.8 g) materials.

The petroleum ether soluble fraction was fractionated by column chromatography (CC) over silica gel (60-120 mesh) using petroleum ether-ethyl acetate mixtures of increasing polarities to give 112 fractions (each 25 ml). Preparative TLC of fractions eluted with 5% ethyl acetate in petroleum ether was run using 5 % ethyl acetate in toluene as the mobile phase which afforded compounds **1** (10.0 mg) and **2** (9.0 mg). Similar purification of fraction eluted with 10% and 12 % ethyl acetate in petroleum ether using 10% ethyl acetate in toluene yielded compounds **3** (5.0 mg) and stigmasterol (5.0 mg), respectively.

Betulin (1): White crystalline mass; ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.75, 4.67 (each 1H, br.s, H_2-29), 3.78 (1H, d, J=11.2, H_b-28), 3.34 (1H, d, J=10.8, H_a-28), 3.18 (1H, dd, J = 11.2, 5.2 Hz, H-3), 2.37 (1H, m, H-19), 1.67 (3H, s, Me-30), 1.01 (3H, s, Me-23), 0.97 (3H, s, Me-27), 0.96 (3H, s, Me-26), 0.82 (3H, s, Me-25), 0.76 (3H, s, Me-24); ^{13}C NMR (100 MHz, $CDCl_3$): 38.6 (C-1), 27.2 (C-2), 79.0 (C-3), 38.8 (C-4), 55.3 (C-5), 18.3 (C-6), 34.0 (C-7), 41.0 (C-8), 50.5 (C-9), 37.2 (C-10), 20.9 (C-11), 25.2 (C-12), 37.3 (C-13), 42.8 (C-14), 27.0 (C-15), 29.2 (C-16), 47.8 (C-17), 48.8 (C-18), 47.8 (C-19), 150.5 (C-20), 29.7 (C-21), 34.2 (C-22), 28.0 (C-23), 16.0 (C-24), 16.1 (C-25), 15.6 (C-26), 14.8 (C-27), 60.6 (C-28), 109.7 (C-29), 19.1 (C-30).

Erythrodiol (2): White amorphous; ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.18 (1H, t, J=3.0 Hz, H-12), 3.54 (1H, d, J= 10.8 Hz, H_b-28), 3.21 (1H, d, J= 10.8 Hz, H_a-28), 3.18 (1H, dd, J= 11.2, 5.2 Hz, H-3), 1.15 (3H, s, Me-30), 0.99 (3H, s, Me-27), 0.93 (3H, s, H-29), 0.92 (3H, s, Me-23), 0.88 (3H, s, Me-26), 0.87 (3H, s, Me-25), 0.78 (3H, s, Me-24); ^{13}C NMR (100 MHz, $CDCl_3$): 38.6 (C-1), 27.2 (C-2), 79.0 (C-3), 38.8 (C-4), 55.3 (C-5), 18.4 (C-6), 32.6 (C-7), 39.8 (C-8), 46.7 (C-9), 36.9 (C-10), 23.6 (C-11), 122.4 (C-12), 144.1 (C-13), 41.8 (C-14), 25.6 (C-15), 22.0 (C-16), 39.9 (C-17), 42.4 (C-18), 46.5 (C-19), 31.1 (C-20), 34.1(C-21), 31.0 (C-22), 28.1 (C-23), 15.5 (C-24), 15.6 (C-25), 16.7 (C-26), 25.9 (C-27), 69.9 (C-28), 33.2 (C-29), 23.6 (C-30).

lupeol (3): Colorless gum; ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.68 (1H, br. s, H_a-29), 4.57 (1H_b, br. s, H_b-29), 3.18 (1H, dd, *J* = 11.2, 5.2 Hz, H-3), 2.36 (1H, m, H-19), 1.67 (3H, s , Me-30), 1.02 (3H, s, Me-27), 0.96 (3H, s, Me-26), 0.94 (3H, s, Me-25), 0.82 (3H, s, Me-24), 0.78 (3H, s, Me-23), 0.75 (3H, s, Me-28).

Stigmasterol: Amorphous; ^{1}H NMR (400 MHz, $CDCl_3$): identical to that acquired for authentic sample and published values[15].

Antioxidant activity. The antioxidant (free radical scavenging) activity of the extracts on the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) was determined by the method developed by Brand-Williams *et al*., 1995.[6] Here, 2.0 mg of each of the test sample was dissolved in methanol and solution of varying concentrations such as 500 µg/ml, 250 µg/ml, 125 µg/ml, 62.50 µg/ml, 31.25 µg/ml, 15.62 µg/ml, 7.8125 µg/ml, 3.91 µg/ml, 1.95 µg/ml and 0.98 µg/ml were obtained by serial dilution technique. Then 2 ml of each of the test sample was mixed with 3 ml of a DPPH-methanol solution (20 µg/ml) and was allowed to stand for 20 minutes for

the reaction to occur. The absorbance was determined at 517 nm and from these values the corresponding percentage of inhibitions were calculated by using the following equation:

$$\% \text{ inhibition} = [1- (ABS_{sample} / ABS_{control})] \times 100$$

Then % inhibitions were plotted against respective concentrations used and from the graph IC_{50} was calculated using ascorbic acid, a potential antioxidant, as the positive control.

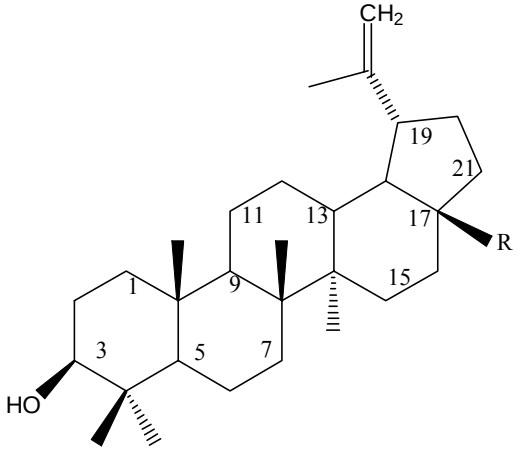
Brine shrimp lethality bioassay. Brine shrimp lethality bioassay[7-9] technique was applied for determination of general toxic property of the plant extractives. DMSO solutions of the samples were applied against *Artemia salina* in a 1-day ex-vivo assay. For the experiment, 4 mg of each of the methanolic crude extract and its petroleum ether, carbon tetrachloride and dichloromethane and aqueous soluble fractions were dissolved in DMSO and solutions of varying concentrations (400, 200, 100, 50, 25, 12.50, 6.25, 3.125, 1.563, 0.781 µg/ml) were obtained by serial dilution technique for each extract. Vincristine sulphate was used as positive control.

Antimicrobial Screening. The disc diffusion method[10,11] was used to test antimicrobial activity of the extractives against 13 bacteria (*Bacillus cereus, B. megaterium, B. subtilis, Staphylococcus aureus, Sarcina lutea, Escherichia coli, Pseudomonas aeruginosa, Salmonella paratyphi, S. typhi*, *Shigella boydii, S. dysenteriae, Vibrio mimicus & V. parahemolyticus*) and 3 fungi (*Candida albicans, Aspergillus niger & Sacharomyces cerevisiae)* collected as pure cultures from the Institute of Nutrition and Food Science (INFS), University of Dhaka, Bangladesh. The antimicrobial activity of the test agents was determined by measuring the diameter of zone of inhibition expressed in mm. The experiment was carried out in triplicate and the mean values were taken.

Statistical analysis. For each of the extracts, three samples were prepared for each of the bioassay. The zone of inhibition, LC_{50} and IC_{50} were calculated as mean ± SD (n=3) for the antimicrobial screening, brine shrimp lethality bioassay and antioxidant activity, respectively.

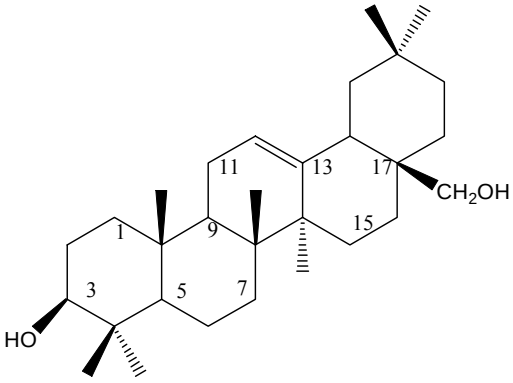
RESULTS AND DISCUSSION

Repeated chromatographic separation and purification of the petroleum ether soluble partitionate of a methanolic extract of the leaves of *I. arborea* provided four compounds, the structures of which were determined by extensive NMR spectral analysis of NMR data as well as by comparison with previously reported values.



1. R = CH_2OH

3. R = CH_3



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The ^{13}C NMR spectrum (100 MHz, $CDCl_3$) of compound **1** displayed 30 carbon resonances indicative of a triterpenoid-type carbon skeleton. The DEPT experiments indicated that 24 out of the 30 carbon atoms in **1** had attached protons. Thus, it exhibited signals for 6 methyl (28.0, 19.0, 16.1, 16.0, 15.6, 14.8), 12 methylene (109.7, 60.6, 38.0,

34.2, 34.0, 29.7, 29.2, 27.2, 27.0, 25.2, 20.9, 18.3), 6 methine (79.0, 55.3, 50.5, 48.8, 47.8, 37.3) and 6 quaternary (150.5, 47.8, 42.8, 41.0, 38.8, 37.2) carbons.

The ^{1}H NMR spectrum of compound **1** showed a double doublet (J = 11.2, 5.2 Hz) at δ 3.18 which could be assigned for H-3 in the triterpene nucleus. Two broad singlets at δ 4.75 and δ 4.67 revealed the presence of vinylic protons at H_2-29. On the other hand, the spectrum also showed six three proton singlets at δ 1.67, 1.01, 0.97, 0.96, 0.82 and 0.76 assignable to methyl protons at C-20 (Me-30), C-4 (Me-23), C-14 (Me-27), C-8 (Me-26), C-10 (Me-25), and C-4 (Me-24), respectively. The multiplet of one proton intensity at δ 2.37 was assigned to H-19. The ^{1}H-^{1}H COSY spectrum of **1** revealed two mutually coupled doublet (J= 11.0 Hz) at δ 3.34 and 3.78 which could be ascribed to the oxymethylene protons at C-28.

These ^{1}H and ^{13}C NMR spectral data of compound **1** were almost identical to those published for betulin.[12] On this basis, the identity of compound **1** was established as betulin, the identity of which was further substantiated by co-TLC with authentic sample. Although it is a known natural product, this is the first report of isolation of betulin (**1**) from *I. arborea*.

The ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of compound **2** displayed a characteristic double doublet at δ 3.18 (J = 11.2, 5.2 Hz) indicative of H-3 in a triterpene nucleus. The downfield signal at δ 5.18 was assigned to the olefinic proton at H-12. The ^{1}H-^{1}H COSY spectrum exhibited coupling between two doublets (J= 10.8 Hz) at δ 3.21 and 3.54 assignable to the hydroxymethyl protons at C-28. On the other hand, the ^{1}H NMR spectra also showed seven three proton singlets at δ 1.15 (Me-30), 0.99 (Me-27), 0.93 (Me-29), 0.92 (Me-23), 0.88 (Me-26), 0.87 (Me-25) and 0.78 (Me-24) demonstrative of the protons of seven methyl groups in **2**. The ^{13}C NMR spectrum of compound **2** displayed 30 carbon resonances, while the DEPT experiments indicated that 23 out of the 30 carbon atoms in **2** were protonated. Thus, it exhibited signals for 7 methyl (33.2, 28.1, 25.9, 23.6, 16.7, 15.6, 15.5), 11 methylene (69.9, 46.5, 38.6, 34.1, 32.6, 31.0, 27.2, 25.6, 23.6, 22.0, 18.4), 5 methine (122.4, 79.0, 55.3, 47.6, 42.4) and 7 quaternary (144.1, 41.8, 39.9, 39.8, 38.8, 36.9, 31.1) carbons. On this basis, compound **2** was characterized as erythrodiol, the identity of which was confirmed by comparison of the spectral data with previously reported values.[13]

The ^{1}H (400 MHz, $CDCl_3$) and ^{13}C (100 MHz, $CDCl_3$) $CDCl_3$ spectra of compound **3** were almost identical to those recorded for betulin (**1**). However, the oxymethylene proton and carbon resonances (δ_H 3.34, 3.78, each 1H, J= 11.0 Hz); (δc 60.6) in **1** were replaced by a methyl group signal at δ 0.78. Thus, both ^{1}H and ^{13}C NMR spectra of compound **3** showed seven methyl group resonances, instead of six observed in case of betulin. On this basis as well as by comparison of these data with previously reported values allowed to characterize compound **3** as lupeol.[14] Again the identity of **3** as lupeol was further confirmed by co-TLC with an authentic sample.

The fourth compound was identified as stigmasterol by direct comparison of its ^{1}H NMR spectrum[15] with that acquired for stigmasterol as well as by Co- TLC.

In case of screening for antioxidant activity (Table 1), the crude methanolic extract of leaves (MEL) showed the highest free radical scavenging activity with IC_{50} value 33.0 µg/ml. At the same time the PESF also exhibited strong antioxidant potential having IC_{50} value 40.0 µg/ml. For *I. arborea* highest amount of phenolic content was found in methanolic extract of leaves having TPC value of 28.30 mg of GAE/ gm of extractive and this fraction showed significant antioxidant activity having an IC_{50} value of 33.0 µg/ml. At the same time the total phenolic content of the PESF was 19.23 mg of GAE/ gm of extractive, which also demonstrated significant free radical scavenging activity (IC_{50}= 40.0 µgm/ml). Therefore a positive correlation was seen between the total phenolic content and antioxidant activity. This is in accordance with the fact that phenolic compounds have potent free radical scavenging activity.[16,17]

Table 1 shows the results of the brine shrimp lethality testing of various extractives of *I. arborea* after 24 hours of exposure to the samples and the positive control, vincristine sulphate (VS). The LC_{50} were found to be 2.825, 0.622, 2.427, 5.345, 2.289, 0.451 µg/ml for MEL, PESF, CTSF, DCMF, AQSF and VS, respectively. In comparison with vincristine sulphate, the cytotoxicity exhibited by all of the extractives were significant whereas the petroleum ether soluble fractions of the methanolic extract of *I. arborea* demonstrated the highest cytotoxicity.

In case of antimicrobial screening, the crude extract of *I. arborea* showed mild growth inhibitory activity against the tested microorganisms. The zone of inhibition produced by the methanol extract was 10.0 ± 0.23, 10.0 ± 0.17, 10.0 ± 0.32, 9.0 ± 0.21, 9.0 ± 0.43, 10.0 ± 0.57, 10.0 ± 0.54, 10.0 ± 0.56, 10.0 ± 0.76, 9.0 ± 0.24, 10.0 ± 0.43, 10.0 ± 0.47, 10.0 ± 0.67, 9.0 ± 0.53, 9.0 ± 0.86 and 10.0 ± 0.85 mm against *Bacillus cereus, B. megaterium, B. subtilis, Staphylococcus aureus, Sarcina lutea, Escherichia coli, Pseudomonas aeruginosa, Salmonella paratyphi, S. typhi*, *Shigella boydii, S. dysenteriae, Vibrio mimicus*, *V. parahemolyticus, Candida albicans, Aspergillus niger* & *Sacharomyces cerevisiae*, respectively. The remaining extractives did not show any noticeable inhibitory growth of the organism at 400 µg/disc.

Table 1. LC_{50} and IC_{50} data of test samples of *I. arborea.*

Samples	LC_{50} (µg/ml)	TPC (mg of GAE/ gm of extractives)	IC_{50} (µg/ml)
VS	0.451 ± 0.31	-	-
AC	-	-	5.25 ± 0.21
MEL	2.825 ± 1.13	28.30 ± 0.21	33.0 ± 1.34
PESF	0.622 ± 1.21	19.23 ± 0.29	40.0 ± 2.07
CTSF	2.427 ± 1.33	11.76 ± 0.37	115.0 ± 1.24
DCMF	5.345 ± 0.98	8.98 ± 0.04	160.0 ± 3.35
AQSF	2.289 ± 1.45	6.23 ± 1.08	250.0 ± 1.47

The values of $LC_{50,}$ TPC and IC_{50} are expressed as mean ± SD (n=3); VS: vincristine sulphate (Std.); AC: Ascorbic acid (Std.); MEL: methanolic extract of leaves; PESF: pet-ether soluble fraction of the methanolic extract; CTSF: carbon tetrachloride soluble fraction of the methanolic extract; DCMF: dichloromethane soluble fraction of the methanolic extract; AQSF: aqueous fraction of the methanol extract.

CONCLUSION

From the study, it is evident that, the extractives of *I. arborea* showed significant antioxidant and cytotoxic activities. Further investigation is required to isolate the bioactive principles. The bioactivities shown by the extractives of *I. arborea* support the traditional uses of this plant against various diseases.

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