Chemical and Biological Investigations of *Justicia gendarussa* (Burm. f)

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ABSTRACT: In this study, three compounds, stigmasterol (**1**), lupeol (**2**), 16-hydroxylupeol (**3**) were isolated from the petroleum ether soluble fraction of a methanol extract of whole plant of *Justica gendarussa* (Burm. f). The crude methanol extract as well as its petroleum ether, carbon tetrachloride and chloroform soluble Kupchan fractions were studied for antioxidant, antimicrobial and cytotoxic activities. Among the different fractions tested for antioxidant activity, the chloroform soluble partitionate was the most potent with IC_{50} value of 18.80 µg/ml as compared to *tert*-butyl-1-hydroxytoluene (IC_{50}=17.69 µg/ml). Antimicrobial screening of the different extractives was conducted by the disc diffusion method and all the fractions except chloroform soluble partitionate exhibited mild antimicrobial activity with zone of inhibition ranging from 7-10 mm. In brine shrimp lethality bioassay, the petroleum ether soluble materials demonstrated the highest toxicity with LC_{50} of 1.27µg /ml. This is the first report of isolation of compounds from *J. gendarussa*, and its antioxidant, antimicrobial and cytotoxic properties.

Key words: *Justica gendarussa,* antioxidant, antimicrobial, cytotoxicity, stigmasterol, lupeol, 16-hydroxylupeol.

INTRODUCTION

Justica gendarussa (Burm. f) **(**Family-Acanthaceae, common name Black adusa) is a shade loving, quick growing, evergreen shrub found throughout India and also in all Asian countries like Malaysia, Indonesia, Sri Lanka and Bangladesh.[1,2] The plant is used in traditional medicinal practice for chronic rheumatism, inflammations, bronchitis, vaginal discharges, dyspepsia, eye diseases and fever.[1,3] *Justicia* has been found to contain lignans, naturally occurring phenolic dimers[3] and triterpenoids.[4]

For the first time, we herein, report the isolation of stigmasterol (**1**), lupeol (**2**) and 16-hydroxylupeol (**3**) from a methanol extract of *J. gendarussa* as well as its antioxidant, antimicrobial and cytotoxic properties of the various extractives.

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MATERIALS AND METHODS

Instruments. ^{1}H NMR spectra were recorded using a Bruker AMX-400 (400 MHz) instrument and the spectra were referenced to the residual non-deuterated solvent ($CDCl_3$) signal. Gel permeation chromatography was conducted over Sephadex (LH-20). Preparative TLC was carried out by using normal phase Si-gel 60 F_{254} (Merck) on glass plates (20×20 cm) of 0.5-mm thickness. Spots on TLC and preparative TLC plates were visualized after spraying the developed plates with 1% vanillin in sulfuric acid, followed by heating at 110 °C for few minutes.

Plant materials. Samples of *Justicia gendarussa* were collected from Kishoregonj, Bangladesh in November, 2009, identified by an expert taxonomist and a voucher specimen (accession no. 34728) for this collection has been deposited in Bangladesh National Herbarium, Dhaka, Bangladesh.

Preparation of extracts. The air-dried and powdered whole plant (0.5 kg) of *J. gendarussa* was soaked in 2.5 L of methanol for 10 days at room temperature and then filtered through a cotton plug, followed by Whatman filter paper number 1. The extract was concentrated with a rotary evaporator. An

aliquot (5.0 g) of the concentrated methanolic extract was fractionated by the modified Kupchan partitioning method[5] into petroleum ether (PEF, 1.8 g), carbon tetrachloride (CTF, 0.9 g), chloroform (CLF, 0.7g) and aqueous (AQF, 1.1 g) soluble fractions.

Isolation of the compounds. The petroleum ether soluble fraction was chromatographed over Sephadex (LH-20) and the column was eluted with *n*-hexane-dichloromethane-methanol mixture (2:5:1) followed by mixtures of dichloromethane and methanol in order of increasing polarities (9:1; 1:1) and finally with 100% methanol to yield 50 fractions of 2 mL each.

Compound **1** was isolated as pure needlelike crystalline mass from the column fractions eluted with *n*-hexane-dichloromethane-methanol (2:5:1). Preparative TLC (toluene-ethyl acetate, 9: 1) of the column fractions eluted with *n*-hexane-dichloromethane-methanol (2:5:1) afforded compound **2** as colorless solid. Compound **3** was purified by preparative TLC of column fractions (eluted with methanol-dichloromethane, 1:9) with 20% ethyl acetate in toluene.

DPPH free radical scavenging activity. The free radical scavenging activity of the extractives were determined by the method developed by Brand-Williams *et al.*[6] based on the scavenging activity of the stable 1,1 diphenyl-2-picrylhydrazyl (DPPH) free radical. In short, 2.0 ml of a methanol solution of the extract at different concentrations were mixed with 3.0 ml of a DPPH methanol solution (20 µg/ml) and the mixture was kept in dark for 20 minutes for reaction to occur. The absorbance of the resultant solution was determined at 517 nm and the percent inhibition was calculated from $[(A_o–A_1)/A_o] \times 100$, where A_o is the absorbance of the control and A_1 is the absorbance of the test sample.

Antimicrobial screening. The disc diffusion method[7] was used to evaluate the antimicrobial activity of the extractives against 13 bacteria and 3 fungi (Table-2), collected as pure cultures, from the Institute of Nutrition and Food Sciences (INFS), University of Dhaka, Bangladesh. Solutions of known concentration (400 µg/ml) of the test samples were made by dissolving measured amount of the samples in calculated volume of solvents, $CHCl_3$ or CH_3OH. Dried and sterilized filter paper discs (6 mm diameter) were then impregnated with known amount of the test substance using micropipette and the residual solvents were completely evaporated. Standard disc of ciprofloxacin (30 µg/disc) and blank discs (impregnated with solvents followed by evaporation) were used as positive and negative control, respectively. These plates were then kept at low temperature (4º C) for 24 hours to allow maximum diffusion of the test materials and ciprofloxacin. The plates were finally incubated at 37º C for 24 hours to allow maximum growth or inhibition of growth of the organisms. 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 mean values were taken.

Evaluation of cytotoxicity. Brine shrimp lethality bioassay[8,9] technique was applied for the determination of general toxic property of the plant extractives. DMSO solutions of the samples were applied against *Artemia salina* in a 1-day assay. For the experiment, 4 mg of each of the extractive 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 using DMSO. Vincristine sulphate (10, 5, 2.5, 1.25, 0.625, 0.3125, 0.15625, 0.078125 and 0.0390 µg/ml) was used as positive control.

Compound **1**: ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.28 (1H, br. s, H-6), 4.74 (1H, dd, *J*=15.0, 6.5 Hz, H-22), 4.68 1H, dd, *J*=15.0, 6.5 Hz, H-23), 3.50 (1H, m, H-3), 1.01 (3H, s, Me-10), 0.95 (3H, d, *J*= 6.4 Hz, Me-20), 0.89 (3H, d, *J*=6.0 Hz, Me-25), O.83 (3H, d, *J*=6.0 Hz, Me-25), 0.74 (3H, t, *J*=7.4, Me-28), 0.67 (3H, s, Me-13).

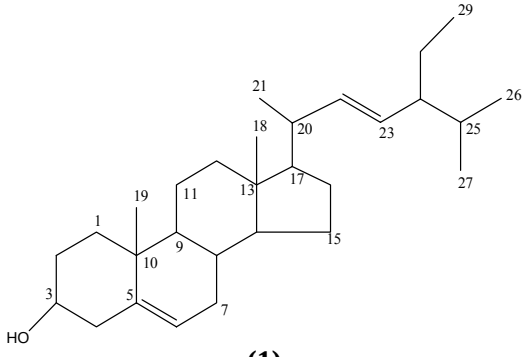
Compound **2**: ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.67 (1H, br. s, H_a-29), 4.55 (1H_b, br. s, H-29), 3.20 (1H, dd, *J*=11.5, 5.0 Hz, H-3), 2.28 (1H, m, H-19), 1.67 (3H, s, Me-20), 1.02 (3H, s, Me-8), 0.95 (3H, s, Me-4), 0.93 (3H, s, Me-14), 0.84 (3H, s, Me-10), 0.82 (3H, s, Me-17), 0.78 (3H, s, Me-4).

Compound **3**: ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.67 (1H, br s, H-29), 4.55 (1H, br. s, H-29), 3.20 (1H, dd, *J*=11.5, 5.0 Hz, H-3), 2.98 (1H, m, H-16), 2.28 (1H, m, H-19), 1.67 (3H, s, Me-20), 1.02 (3H, s, Me-8), 0.95 (3H, s, Me-4), 0.93 (3H, s, Me-14), 0.84 (3H, s, Me-10), 0.82 (3H, s, Me-17), 0.78 (3H, s, Me-4).

RESULTS AND DISCUSSION

Solvent-solvent partitioning, followed by repeated chromatographic separation and purification of the methanol extract of *J. gendarussa* provided a total of three compounds (**1-3**). The structures of isolated compounds were solved by extensive NMR data analysis, comparison with published values as well as co-TLC with authentic samples.

The ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of compound **1** revealed a one proton multiplet at δ 3.50, the position and multiplicity of which was indicative of H-3 of the steroidal nucleus. The typical signal for the olefinic H–6 was evident from a broad singlet at δ 5.28, that integrated for one proton. The olefinic protons (H–22 and H–23) displayed the characteristic downfield signals at δ 4.74 and 4.68 respectively in the ^{1}H NMR spectrum. Each of the signal was observed as double-doublets (J = 15.0 Hz, 6.5 Hz) which indicated couplings with the neighbouring olefinic and methine protons. The spectrum further revealed signals at δ 0.67 and δ 1.01 (3H each) assignable to two tertiary methyl groups at C–13 and C–10, respectively. The ^{1}H NMR spectrum also showed two doublets centered at δ 0.83 (J = 6.0 Hz) and 0.89 (J = 6.0 Hz) which could be attributed to the methyl groups at C–25. The other doublet at δ 0.95 (J = 6.4 Hz) was demonstrative of a methyl group at C–20. On the other hand, the triplet (J = 6.5 Hz) of three-proton intensity at δ 0.74 could be assigned to the primary methyl group attached to C–28. The above spectral features are in close agreement to those observed for stigmasterol.[10] On this basis, compound **1** was characterized as stigmasterol, the identity of which was confirmed by co-TLC with an authentic sample previously isolated in our laboratory.



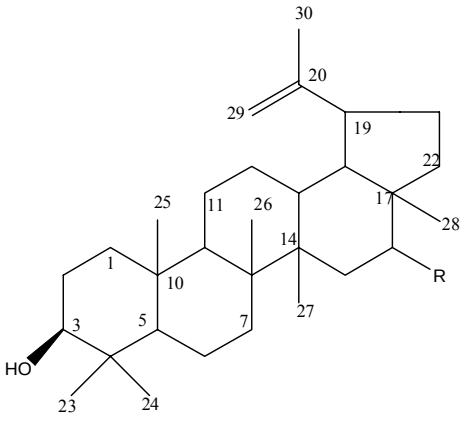
(1)

The ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of compound **2** showed a double doublet (J = 11.5, 5.0 Hz) of one proton intensity at δ 3.20 ppm, typical for an oxymethine proton at C-3 of a triterpenoid type carbon skeleton. The splitting pattern of this proton confirmed the β orientation of the C-3 oxygenated substituent. The spectrum also displayed two broad singlets at δ 4.67 and 4.55 ppm (1H each) assignable to the vinylic protons at C-29. The ^{1}H NMR spectrum further showed seven singlets at δ 0.95, 0.78, 0.84, 1.02, 0.93, 0.82 and 1.67 (3H each) which could be attributed to the methyl group protons at C-4 (H_3-23, H_3-24), C-10 (H_3-25), C-8 (H_3-26), C-14 (H_3-27), C-17 (H_3-28) and C-20 (H_3-30), respectively. By comparing these ^{1}H NMR data with previously published values,[11] compound **2** was identified as lupeol. The identity of **2** was further substantiated by co-TLC with an authentic sample of lupeol.

Table 1. IC_{50} values of the standard and partitionate of *Justicia gendarussa* in DPPH assay

Test samples	IC_{50} value (μg/ml)
BHT	17.69
MEF	292.16
PEF	37.64
CTF	26.70
CLF	18.80

BHT = *Tert*- butyl-1-hydroxytoluene



2: R=H
3: R=OH

The ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of compound **3** showed almost similar signals to those observed for compound **2.** Additionally the spectrum revealed a multiplet of one proton intensity at δ 2.98 depicting the presence of another oxymethine proton at C_{16}. The large width half of this signal suggested that the hydroxyl group was at β position. Thus compound **3** was identified as 16-hydroxylupeol.[12] The identity of 3 as 16-hydroxylupeol was further confirmed by co-TLC with an authentic sample.

Among the different fractions tested for antioxidant activity (Table 1), the chloroform soluble partitionate demonstrated maximum free radical scavenging activity with IC_{50} value of 18.80 µg/ml followed by the two other fractions carbon tetrachloride (CTF, IC_{50} = 26.00 µg/ml) and petroleum ether (PEF, IC_{50} = 37.64 µg/ml) soluble fractions exhibiting significant antioxidant activity as well.

In the antimicrobial screening, the methanolic crude extract as well as its petroleum ether and carbon tetrachloride soluble fractions revealed mild inhibitory activity with average zone of inhibition of 7-10 mm each as compared to standard (43-45 mm) (Table 2) exhibited by ciprofloxacin. However, the chloroform soluble fraction and the negative control disc showed no inhibition of microbial growth (data not shown in the table).

Table 3 shows the results of brine shrimp lethality testing after 24 hours of exposure to the samples and the positive control, vincristine sulphate. The LC_{50} values were found to be 1.27 µg/ml for petroleum ether soluble fractions of the methanol extract revealed its toxicity to a significant degree.

Table 2. Antimicrobial activity of *Justicia gendarussa* extractives and ciprofloxacin

Test microorganisms	Diameter of zone of inhibition (mm)			
	MEF	PEF	CTF	Ciprofloxacin
Gram positive bacteria				
Bacillus cereus	9	9	10	44
B. megaterium	7	10	8	44
B. subtilis	7	7	7	44
Staphylococcus aureus	8	7	8	44
Sarcina lutea	7	8	7	44
Gram negative bacteria				
Escherichia coli	10	9	10	44
Pseudomonas aeruginosa	8	7	8	45
Salmonella paratyphi	8	8	7	44
S. typhi	9	8	7	44
Shigella boydii	9	7	8	44
Sh. dysenteriae	9	8	9	45
Vibrio mimicus	8	7	8	44
V. parahemolyticus	7	8	8	44
Fungi				
Candida albicans	7	7	8	43
Aspergillus niger	8	8	8	45
Sacharomyces cerevacae	7	7	9	43

Table 3. LC_{50} values of the standard and partitionate of *Justicia gendarussa* in brine shrimp leothalics assay.

Test Samples	LC_{50} values (µg/ml)
VS	0.451
MEF	20.62
PEF	1.27
CTC	9.36
CLF	6.89

VS = Vincristine sulphate, MEF = Methanolic extract of the whole plant, PEF = Petroleum ether soluble fraction of the methanolic extract of the whole plant, CTF = Carbon tetrachloride soluble fraction of the methanolic extract of the whole plant, CLF = Chloroform soluble fraction of the methanolic extract of the whole plant

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