Phytochemical and Biological Investigations of *Ixora lutea* Hutch.

Shapna Sultana[1], Mohammad S. Rahman[1], Md. Aslam Hossain[1], Md. Khalid Hossain[2], and Mohammad A.Rashid[1,2]

[1]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Dhaka, Dhaka-1000, Bangladesh
[2]Centre for Biomedical Research, University of Dhaka, Dhaka-1000, Bangladesh

ABSTRACT: Two triterpenoids, lupeol (**1**) and stigmasterol were isolated from the petroleum ether soluble fraction of the methanolic extract of *Ixora lutea* Hutch. In our preliminary screening, the petroleum ether, carbon tetrachloride, chloroform soluble fractions of methanol extract of the leaves and stem were subjected to antioxidant, antimicrobial and brine shrimp lethality bioassay. All of the fractions showed moderate to potent antioxidant activity, of which the chloroform soluble fraction demonstrated the strongest antioxidant activity with the IC_{50} value of 3.0 μg/ml. In case of antimicrobial screening, all extractives showed mild growth inhibitory activity. However, in the brine shrimp lethality bioassay, the petroleum ether soluble fraction was found to be most cytotoxic among the partitionates having LC_{50} value 0.938 μg/ml.

Key words: *Ixora lutea*, rubiaceae, lupeol, stigmasterol, antioxidant activity, brine shrimp lethality bioassay, antimicrobial.

INTRODUCTION

Ixora lutea Hutch. (Bengali name Rangan; Fam-Rubiaceae) is a flowering shrub that grows all over Bangladesh.[1] The plant is used in diarrhoea, dysentery, leucorrhoea, haemoptysis and catarrhal bronchitis.[2] This genus has also been reported to exhibit immunomodulatory, anti-inflammatory and antipyretic activities.[3,4] Previous phytochemical studies led to the isolation of 5,7-dihydroxyflavone-5-O-β-D-xylopyranoside, mussaenosidic acid, 11-hydroxy-5-dodecen-2-one, 8,10,12,14-octadecatetraenoic acid[5] and cycloartenol esters.[6] We herein, report the isolation of lupeol (**1**) and stigmasterol as well as preliminary antioxidant, cytotoxic and antimicrobial activities of the extractives of *I. lutea* for the first time.

Correspondence to:
Mohammad A. Rashid Tel.: 880-2-8612069,
Fax: 880-2-8612069,
E-mail: rashidma@univdhaka.edu; rashid_phdu@yahoo.com

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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 with vanilllin-sulfuric acid followed by heating for 5 minutes at 110ºC.

Plant material. Aerial parts of the plant, *I. lutea* were collected from Dhaka in the month of September 2007. A voucher specimen (DACB 32885) for this collection has been deposited in Bangladesh National Herbarium, Mirpur, Dhaka for future reference.

Extraction and isolation. The dried powdered aerial parts (170 g) of *I. lutea* was soaked in 1.0 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 g) of the concentrated methanol extract was fractionated by the modified Kupchan partitioning method[7] into pet-ether, carbon tetrachloride, chloroform and aqueous soluble fractions. The methanol extract (5 g) was dissolved in 100 ml of 10% aqueous methanol and extracted three times with petroleum ether (100 ml X 3). The remaining aqueous phase was then increased in polarity to 20% H_2O and extracted three times with carbon tetrachloride (100 ml X 3). The remaining aqueous phase was increased further in polarity to 40% H_2O and extracted three times with dichloromethane (100 ml X 3). The subsequent evaporation of solvents afforded petroleum ether (PESF, 1.5 g), carbon tetrachloride (CTSF, 1.0 g), chloroform (CFSF, 1.5 g) and aqueous soluble (AQSF, 0.8 g) crude materials.

The petroleum ether soluble partitionate was fractionated by column chromatography (CC) over silica gel (60-120 mesh) using petroleum ether-ethyl acetate and ethyl acetate-methanol mixtures of increasing polarities to give 60 fractions (each 25 ml). Preparative thin layer chromatography of fractions eluted with 20% ethyl acetate in petroleum ether using 15 % ethyl acetate in toluene as mobile phase, afforded compound **1** (10 mg). Stigmasterol (5 mg**)** was obtained as crystals from fractions eluted with 30% ethyl acetate in petroleum ether.

Lupeol (1). Colorless gum; ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.74 (1H, br. s, H_a-29), 4.61 (1H, br. s, H_b-29), 3.19 (1H, dd, *J* = 11.2, 5.2 Hz, H-3), 2.36 (1H, m, H-19), 1.69 (3H, s , CH_3-30), 1.03 (3H, s , CH_3-26), 0.98 (3H, s, CH_3 -23), 0.97 (3H, s, CH_3 -27), 0.94 (3H, s, CH_3 -25), 0.82 (3H, s, CH_3 -28), 0.76 (3H, s, CH_3 -24).

Stigmasterol. Colorless needles; m.p.160-164 ºC; ^{1}H NMR spectrum was superimposable to of an authentic sample.

Antimicrobial screening. The disc diffusion method[9,10] was used to test antimicrobial activity of the extractives against thirteen 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 cerevacae)* collected as pure cultures from the Institute of Nutrition and Food Science (INFS), University of Dhaka, Bangladesh. Solutions of known concentration (µg/ml) of the test samples were made by dissolving measured amount of the samples in calculated volume of solvents. Dried and sterilized filter paper discs (6 mm diameter) were then impregnated with known amounts of the test substances using micropipette and the residual solvents were completely evaporated. Discs containing the test materials were placed on to nutrient agar medium uniformly seeded with the test microorganisms. Standard disc of kanamycin (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 (4^{o}C) for 24 hours to allow maximum diffusion of the test materials and kanamycin. The plates were then incubated at 37^{o}C for 24 hours to allow maximum growth of the organisms. The test material having antimicrobial activity inhibited the growth of the microorganisms and a clear, distinct zone of inhibition was visualized surrounding the discs. 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 value was taken.

Brine shrimp lethality bioassay. Brine shrimp lethality bioassay[11-13] 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 *in vivo* assay. For the experiment, 4 mg of each of the petroleum ether, carbontetrachloride and chloroform 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 using DMSO. Vincristine sulphate was used as positive control.

Antioxidant activity. The antioxidant activity (free radical scavenging activity) of the extracts on the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) was determined by the method of Brand-Williams *et al*.[14] In the experiment, 2.0 mg of each of the extract was dissolved in methanol. 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. 2 ml of a methanol solution of the extract of each concentration 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. Then the absorbance was determined at 517 nm and from these values the corresponding percentage of inhibitions were calculated by using the following equation:

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

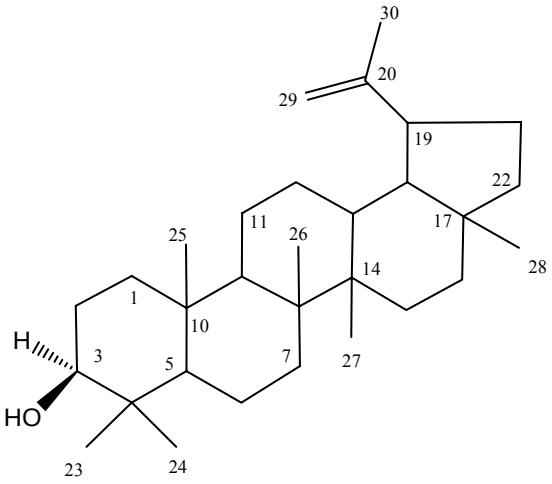
Then % inhibitions were plotted against respective concentrations used and from the graph IC_{50} was calculated by using *tert*-butyl-1-hydroxytoluene (BHT), a potential antioxidant, was used as positive control.

Statistical análisis. For each of the extracts, three samples were prepared for each of the bioassays. 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 aerial parts of *I. lutea* provided two compounds, the structuresof which were determined by NMR spectral analysis as well as by comparison with previously reported values.

The ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of compound **1** showed one double doudlet of one proton intensity at δ 3.19 (*J*=11.2, 5.2 Hz) typical for H_α-3 of a triterpene type-carbon skeleton. The chemical shift and the splitting pattern suggested that the proton was attached to a carbon which also carried a hydroxyl substituent with a beta (β) orientation. The spectrum displayed two singlets at δ 4.74 and δ 4.61 (1H each) assignable to protons at C-29 characteristic of a lupine type triterpenoid. A multiplet of one proton intensity at δ 2.36 was ascribed to H-19. The spectrum also displayed seven singlets at δ 0.76, 0.82, 0.94, 0.97, 0.98, 1.03 and 1.69 (3H each) assignable to protons of methyl groups at C-4 (H_3-24), C-17 (H_3-28), C-10 (H_3-25), C-14 (H_3-27), C-4 (H_3-23), C-8 (H_3-26) and C-20 (H_3-30), respectively. By comparing the ^{1}H NMR data of compound **1** with published values[15] as well as by Co-TLC with an authentic sample established its identity as lupeol.



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Stigmasterol melted at 160-164ºC, which was in agreement to that reported for stigmasterol. Again, the ^{1}H NMR spectral data was identical to that reported for stigmasterol.[8] These features along with Co-TLC with authentic sample confirmed the identity of stigmasterol.

Petroleum ether (PFSF), carbon tetrachloride (CTSF), chloroform (CFSF) and aqueous (AQSF) soluble fractions (500 µg/disc) of the methanol extract were screened against 13 test bacteria and 3

fungi. Most were found to be insensitive to the test organisms. However, the CTSF showed weak antimicrobial activity against *S. lutea, A. niger* and *S. cerevacae* with the zone of inhibition 10 mm each.

In case of brine shrimp lethality bioassay, the lethality of the petroleum ether (PESF), carbon tetrachloride (CTSF), chloroform (CFSF) and aqueous (AQSF) soluble fractions of the methanolic extract was evaluated against *A. salina*. Table 1 shows the results of the brine shrimp lethality testing after 24 hours of exposure to the samples and the positive control, vincristine sulphate (VS). The LC_{50} were found to be 0.938, 11.12, 9.56, 15.27 and 0.50 µg/ml for PE, CT, CF, AQ and VS, respectively. In comparison with vincristine sulphate, the cytotoxicity exhibited by the petroleum ether and carbon tetrachloride soluble fractions of the methanolic extract of *I. lutea* were significant. This clearly indicates the presence of potent bioactive priciples in these extractives, which might be very useful as antiproliferative, antitumor, pesticidal and other bioactive agents.[11-13]

Table 1. LC_{50} data of test samples of *I. lutea*

Samples	LC_{50} (µg/ml)
VS	0.501 ± 0.31
PE	0.938 ± 1.21
CT	11.12 ± 1.33
CF	09.56 ± 0.98
AQ	15.27 ± 1.45

The values of LC_{50} are expressed as mean ± SD (n=3); VS: vincristine sulphate (Std.); PE: pet-ether soluble fraction of the methanolic extract; CT: carbon tetrachloride soluble fraction of the methanolic extract; CF: chloroform soluble fraction of the methanolic extract; AQ: aqueous fraction of the methanol extract.

Table 2. IC_{50} values of *I. lutea* extractives and ascorbic acid

Samples	IC_{50} (µg/ml)
BHT	20.0 ± 0.34
PE	220.0 ± 0.98
CT	25.0 ± 1.25
CF	3.0 ± 1.33
AQ	236.0 ± 1.29

The values of IC_{50} are expressed as mean ± SD (n=3); BHT: *tert*-butyl-1-hydroxytoluene (Std.); PE: Pet ether soluble fraction of the methanolic extract; CT: carbon tetrachloride soluble fraction of the methanolic extract; CF: chloroform soluble fraction of the methanolic extract; AQ: aqueous fraction of the methanolic extract.

In case of antioxidant screening (Table 2), the chloroform soluble fraction showed the highest antioxidant activity with IC_{50} value of 3.0 µg/ml. At the same time, the carbon tetrachloride soluble fraction of the methanolic extract also exhibited moderate antioxidant activity (IC_{50}=25.0 µg/ml), where the aqueous and pet ether soluble fractions showed weak free radical scavenging with the IC_{50} values 236.0 and 220.0 µg/ml, respectively. These results denote the presence of antioxidant principles in the extractives.

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