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Chemical and Antioxidant Studies of *Citrus macroptera*

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Abstract

Lupeol **(1)** and Stigmasterol **(2)** were isolated from the crude extracts of the stem bark of *Citrus macroptera* (Family: Rutaceae) for the first time. The n-hexane, dichloromethane and methanol extracts were screened *in vitro* for antioxidant activity using the 1, 1-diphenyl-2-picrylhydrazyl-hydrate (DPPH) free radical scavenging assay. The hot methanol extract showed potential antioxidant activity (IC_{50}: 178.96 µg/ml) whereas the cold methanol and dichloromethane extracts showed moderate activity (IC_{50}: 242.78 µg/ml and 255.78 µg/ml respectively). Mild antioxidant activity was observed with the n-hexane extract of the stem bark of *Citrus macroptera* (IC_{50}: 422.94 µg/ml).

Key words : *Citrus macroptera*, Rutaceae, Lupeol, Stigmasterol, Antioxidant.

Introduction

Citrus macroptera (Bengali name- Shatkara; English name - Wild orange; Family- Rutaceae) is a tree which grows in Indochina, Myanmar, Thailand, Indonesia, Malayasia and Papua New Guinea (Carpenter and Reece, 1969) In Bangladesh, *Citrus macroptera* is widely distributed in the district of Sylhet. Previous phytochemical investigations resulted in the isolation of alkaloids like (+) ribalinine and isoplaty desmine, (Gaillard *et al,* 1995) aromatic compounds like cinnamic acid, syvingaldehyde, vanilline and methyl vanillate, (Gaillard *et al,* 1995) coumarins like bergamottin, psoralen, marmin, severine and geiparvarin (Dreyer and Huey, 1973) So far no details biological studies have been carried out on this plant. In this paper, the isolation and structure elucidation of the Lupeol **(1)** and Stigmasterol **(2)** by using spectroscopic techniques and the preliminary antioxidant activities of the organic extractives are being reported.

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Materials and Methods

General experimental procedure

The ^{1}H NMR sprectra was recorded using a Bruker DPX-400 (400 MHz) instrument. For NMR studies deuterated chloroform was used and the δ values for ^{1}H spectra were referred to the residual nondeuterated solvent signals.

Plant material

Stem bark of *C. macroptera* was collected from Asam, India and identified by a taxonomist.

Extraction and Isolation

The powdered stem bark (260 g) of *C. macroptera* was extracted in a Soxhlet apparatus for 7 days with n-hexane, 10 days with dichloromethane and 10 days with methanol. Separately 24 g powdered stem bark was soaked in 250 ml methanol for 7 days for cold extraction. All the extracts were filtered through a cotton plug followed by Whatman filter paper number 1 and then concentrated by using a rotary vacuum evaporator to provide n-hexane (1.28 g), dichloromethane (3.43 g), hot methanol (0.523 g) and cold methanol (2.77 g) extractives.

The hot methanol extract (0.523 g) was fractionated by column chromatography over silica gel (Kieselgel 60, mesh 70-230). The column was eluted with petroleum ether, dichloromethane and methanol mixture of increasing polarities to provide 31 fractions. Lupeo (Aratanechemuge, *et al*, 2004; Haque, 2006) (1) (3.6 mg) was isolated from fractions (11+12) eluted with 72.5-75% dichloromethane in petroleum ether. The R_f value of the compound was determined as 0.53 in chloroform-methanol (97:3) on silica gel PF_{254} plate and the yield value was 0.0014%.

The cold methanol extract (2.77 g) was fractionated by column chromatography over silica gel (Kieselgel 60, mesh 70-230). The column was eluted with n-hexane, ethyl acetate and methanol mixture of increasing polarities to provide 22 fractions. Stigmasterol (Khan, 1991) (**2**) (2.0 mg) was isolated from fraction-2 eluted with 12.5% ethyl acetate in n-hexane. The R_f value of the compound was determined as 0.33 in toluene-ethyl acetate (95:5) on Silica gel PF_{254} plate and the yield value was 0.0083%.

Lupeol (1)

White crystals; ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.68 (1H, m, Hb-29), 4.56 (1H, m, Ha-29), 3.18 (1H, dd, *J* = 11.4, 4.6 Hz, Hα- 3), 2.37 (1H, m, H-19), 1.67 (3H, s, CH_3-30), 1.02 (3H, s, CH_3-27), 0.96 (3H, s, CH_3-26), 0.94 (3H, s, CH_3-25), 0.82 (3H, s, CH_3-24), 0.78 (3H, s, CH_3-23), 0.75 (3H, s, CH_3-28).

Stigmasterol (2)

Colorless needles; ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.35 (1H, m, H-6), 5.13 (1H, dd, *J* = 14.4, 8.4 Hz, H- 22), 5.03 (1H, dd, *J* = 14.4, 8.4 Hz, H- 23), 3.51 (1H, m, H-3), 1.0

(3H, s, CH_3-10), 0.91 (3H, d, *J* = 6.4 Hz, CH_3-20), 0.85 (3H, d, *J* = 7.4 Hz, CH_3-27), 0.81 (3H, d, *J* = 7.4 Hz, CH_3-26), 0.67 (3H, s, CH_3-13).

Screening for antioxidant activity

Antioxidant activities of the extracts were determined on the basis of their scavenging potential of the stable DPPH free radical in both qualitative and quantitative assay.

i) Qualitative assay

A suitably diluted stock solutions were spotted on pre-coated silica gel TLC plates and the plates were developed in solvent systems of different polarities (polar, medium polar and non-polar) to resolve polar and non-polar components of the extracts. The plates were dried at room temperature and were sprayed with 0.02% DPPH in ethanol. Bleaching of DPPH by the resolved band was observed for 10 minutes and the color changes (yellow on purple background) were noted (Sadhu *et al*, 2003).

ii) Quantitative assay

Quantitative assay was performed on the basis of the modified method (Gupat *et al*, 2003) Stock solution (10 mg/ml) of the plant extracts were prepared in ethanol from which serial dilutions were carried out to obtain concentrations of 1, 5, 10, 50, 100, 500 µg/ml. Diluted solutions (2 ml) were added to 2 ml of a 0.004% ethanol solutions of DPPH (Aldrich, USA), mixed and allowed to stand at 25 ^{O}C for 30 min for reaction to occur. The absorbance was determined as 517 nm and from these values corresponding percentage of inhibitions were calculated. Then % inhibitions were plotted against log concentration and from the graph IC_{50} was calculated. The experiment was performed in triplicate and average absorption was noted for each concentration. Ascorbic acid (Loba, India) was used as positive control.

Results and Discussion

Repeated chromatographic separation and purification of the hot and cold methanol extracts of the stem bark of *C. macroptera* provided a total of two compounds **(1 and 2),** the structure of which were determined by extensive NMR spectral analysis as well as by comparison of their spectral data with previously reported values.

The ^{1}H NMR spectrum of compound **1** revealed signal for one double doublet of one proton intensity at δ 3.18 typical for H-3 of the pentacyclic triterpenoid Lupeol. The spectrum displayed two multiplets at δ 4.68 and δ 4.56 (one-proton each) assignable to protons at C-29. The spectrum also showed seven singlets at δ 0.75, 0.78, 0.82, 0.94, 0.96, 1.02 and 1.67 (three-proton each) assignable to protons of methyl groups at C-17 (H_3-28), C-4 (H_3-23, H_3-24), C-10 (H_3-25), C-8 (H_3-26), C-14 (H_3-27) and C-20 (H_3-30) respectively. By comparing the ^{1}H NMR spectra of compound **1** with that of

previously published data, (Aratane-chemuge, *et al*, 2004; Haque, 2006) it was confirmed as Lupeol.

The ^{1}H NMR spectra of compound **2** revealed a one-proton multiplet at δ 3.51, the position and multiplicity of which was indicative of H-3 of the steroidal nucleus. The typical signal for the olefinic H-6 of the steroidal skeleton was evident from a multiplet at δ 5.33 integrating for one-proton. The olefinic protons (H-22 and H-23) appeared as characteristics downfield signals at δ 5.13 and δ 5.03 respectively in the ^{1}H NMR spectrum. Each of the signal was observed as double doublets (J = 14.4, 8.4 Hz) which indicated coupling with the neighboring olefinic and methine protons. The spectrum further revealed signals at δ 0.67 and δ 1.00 (three-proton 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 0.81 (J = 7.4 Hz) and 0.85 (J = 7.4 Hz) which could be attributed to the two methyl groups at C-25. The doublet at δ 0.91 (J = 6.4 Hz) was demonstrative of a methyl group at C-20. These spectral features are in close agreement to those observed for Stigmasterol (Khan, 1991). On this basis, the identity of compound **2** was confirmed as Stigmasterol.

Screening for antioxidant activity:

DPPH is one of the free radicals widely used for testing preliminary radical scavenging activity of a compound or a plant extract.

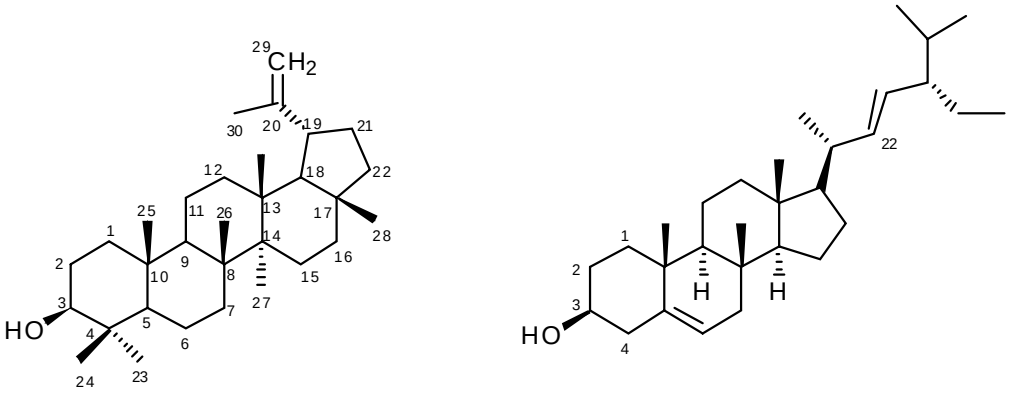
i) Qualitative assay: The color changes (yellow on purple background) on the TLC plates were observed due to the bleaching of DPPH by the resolved bands.

ii) Quantitative assay: The hot methanol extract of the stem bark of *Citrus macroptera* showed potential antioxidant activity with the IC_{50} value of 178.96 µg/ml whereas the cold methanol and the dichloromethane extracts showed moderate activity with the IC_{50} of 242.78 µg/ml and 255.78 µg/ml respectively. The n-hexane extract showed mild activity (IC_{50}: 422.94 µg/ml) against DPPH free radical (Table I). It is evident that all possess antioxidant activity.

Most of the tannins, flavanoids and phenolic compounds may be responsible for antioxidants properties of many plants (Larson, 1988). In these experiments, antiradical activity may be due to the presence of antioxidant principles in the extracts. The free radical scavenging property may be one of the mechanisms by which this plant is effective in its ethno pharmacological uses against different ailments. Further studies comprising of phytochemical investigations of the used plant and evaluation for antioxidant activity using other methods (e.g. various biochemical assays both *in vivo* and *in vitro*) are essential to characterize them as biological antioxidants.

Table I. Evaluation of antioxidant activity of the plant extracts

Sample	Concentration (µg/ml)	Inhibition (%) (mean ± SD)	IC_{50} (µg/ml)
Methanol hot extract	1	10.89 ± 0.006	178.96
	5	30.64 ± 0.008	
	10	42.82 ± 0.007	
	50	52.69 ± 0.005	
	100	71.67 ± 0.01	
	500	77.18 ± 0.011	
Methanol cold extract	1	17.95 ± 0.011	242.78
	5	20.89 ± 0.012	
	10	30.64 ± 0.011	
	50	38.84 ± 0.012	
	100	52.3 ± 0.021	
	500	73.97 ± 0.033	
Dichloromethane extract	1	2.31 ± 0.006	255.78
	5	9.36 ± 0.004	
	10	30.0 ± 0.103	
	50	31.67 ± 0.008	
	100	47.82 ± 0.008	
	500	77.94 ± 0.005	
n-Hexane extract	1	1.15 ± 0.002	422.94
	5	11.41 ± 0.002	
	10	12.18 ± 0.006	
	50	24.23 ± 0.006	
	100	48.59 ± 0.002	
	500	50.77 ± 0.005	
Ascorbic acid	1	44.91 ± 0.92	131.29
	5	47.95 ± 0.98	
	10	53.86 ± 1.78	
	50	56.295 ± 1.32	
	100	63.49 ± 1.51	
	500	68.03 ± 0.81	



1 **2**

Fig. 1: Secondary metabolites identified as the constituents of *citrus macroptera*

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