Phytochemical and Biological investigations of *Phoenix paludosa* Roxb.

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ABSTRACT: Lupeol (**1**), epilupeol (**2**) and β-sitosterol (**3**) were isolated from the *n*-hexane and the carbon tetrachloride soluble fraction of a methanol extract of the leaves of *Phoenix paludosa* Roxb. The *n*-hexane, carbon tetrachloride and chloroform soluble materials from the concentrated methanol extract were subjected to antimicrobial screening and brine shrimp lethality bioassay. All of the partitionates showed insensitivity to microbial growth, while the *n*-hexane, chloroform and methanol soluble fractions showed significant cytotoxicity having LC_{50} 2.17 μg/ml, 2.77 μg/ml and 2.46 μg/ml, respectively. This is the first report of isolation of the compounds **1**-**3** and bioactivities of *P. paludosa.*

Key words: *Phoenix paludosa*, Aracaceae, Lupeol, Epi-Lupeol, β-sitosterol, Brine shrimp lethality bioassay, Antimicrobial.

INTRODUCTION

Phoenix paludosa Roxb. (Bengali name- Hantal, Hital; Family- Aracaceae) is thorny palm with slender stems of 6 to 7 meter in height which grows all over the Sundarban, Bangladesh. The leaves and fruits look like those of date palm.[1,2] The genus *Phoenix* is reported to have diuretic, analgesic,[3] ameliorative,[4] antioxidant and antimutagenic activities.[5,6] Previous phytochemical investigations on *P. paludosa* led to the isolation of 3-*O*-caffeoylshikimic acid, 4-*O*-caffeoylshikimic acid, cosmosiin, ergostane-3,6-diol, ergost-4-ene-3,6-dione, ergost-4-en-3-one, estrone, isoquercitrin, mannose, rhipocephalin, stigmasta-4,22-diene-3,6-dione and vitexin.[7] Here, the preliminary antimicrobial and cytotoxic activities of the extractives as well as isolation of lupeol (**1**), epilupeol (**2**) and β-sitosterol (**3**) from this plant are reported for the first time.

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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 in NMR laboratory of BCSIR, Dhaka. PTLC was carried out using Merck Si gel 60 PF_{254} on glass plates (20 cm X 20 cm) at a thickness of 0.5 mm. TLC was conducted on normal-phase Merck Si gel 60 PF_{254} as glass plates. Spots on TLC and PTLC plates were visualised by spraying with vanilllin-sulfuric acid followed by heating for 5 minutes at 110ºC.

Plant Material. Leaves of *P. paludosa* were collected from Sundarban, Khulna in the month of August 2004 and dried. A voucher specimen for this collection has been deposited in the herbarium of the Department of Botany, University of Dhaka.

Extraction and Isolation. The powdered leaves (100 g) of *P. paludosa* were soaked in 0.5 L methanol for 15 days and filtered through a cotton plug followed by Whatman filter paper number 1. The extract was then concentrated with a rotary evaporator. A portion (5 g) of the concentrated methanol extract was fractionated by the modified Kupchan partitioning method[8] into *n*-hexane, carbon tetrachloride, chloroform and aqueous soluble fractions. Evaporation of solvents afforded *n*-hexane (1.50 g), carbon tetrachloride (0.70 g), chloroform (1.30 g) and aqueous soluble (1.2 g) materials.

The *n*-hexane soluble fraction was fractionated by Vacuum Liquid Chromatography (VLC) packed with fine silica gel (kiesel gel 60H). The column was then eluted with *n*-hexane followed by mixtures of *n*-hexane and ethyl acetate and then ethyl acetate and methanol and finally with methanol. The polarity was gradually increased by adding increasing proportions of ethyl acetate and methanol. A total of 21 fractions were collected each in 100 ml beakers. Re-chromatography of the fraction eluted with 12% ethyl acetate in *n*-hexane (stationary phase- silica gel PF_{254}, mobile phase: hexane-ethyl acetate, 95:5, thickness of plates - 0.5 mm) afforded compound **1** (8 mg). Again, the fraction eluted with 15% ethyl acetate in *n*-hexane afforded compound **2** (6 mg) when subjected to preparative thin layer chromatography over silica gel using 5% ethyl acetate in toluene.

The carbon tetrachloride soluble fraction was fractionated by column chromatography (CC) over Sephadex (LH-20) using *n*-hexane-dichloromethane-methanol (2:5:1) to provide 30 fractions, each 25 ml. Preparative thin layer chromatography [stationary phase- silica gel F_{254}, mobile phase-toluene-ethyl acetate (95 : 5); thickness of plates-0.5 mm] afforded compound **3** (5 mg).

Lupeol (**1**): colorless crystalline mass; ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.67 & 4.55 (each 1H, br. s, H_2-29), 3.20 (1H, dd, *J*=11.5, 5.03 Hz, H_α-3), 2.36 (1H, m, H-19), 1.67 (3H, s, H_3-30), 1.02 (3H, s, H_3-26), 0.95 (3H, s, H_3-23), 0.93 (3H, s, H_3-27), 0.84 (3H, s, H_3-25), 0.82 (3H, s, H_3-28), 0.78 (3H, s, H_3-24).

Epilupeol (**2**): white powder; ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.67 & 4.55 (each 1H, br.s, H_2-29), 3.37 (1H, t, *J*=3.0 Hz, H_β -3), 2.36 (1H, m, H-19), 1.67 (3H, s, H_3-30), 1.02 (3H, s, H_3-26), 0.95 (3H, s, H_3-23), 0.93 (3H, s, H_3-27), 0.84 (3H, s, H_3-25), 0.82 (3H, s, H_3-28), 0.78 (3H, s, H_3-24).

β-sitosterol (**3**): amorphous powder ; ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.34 (1H, d, *J* = 6.0 Hz, H-6), 3.50 (1H, m, H-3), 1.00 (3H, s, H_3-19), 0.91 (3H, d, *J* = 6.4 Hz, H_3-21), 0.85 (3H, d, *J* = 6.0 Hz, H_3-29), 0.83 (3H, d, *J* = 6.0 Hz, H_3-26), 0.81 (3H, d, *J* = 6.0 Hz, H_3-27), 0.67 (3H, s, H_3-18).

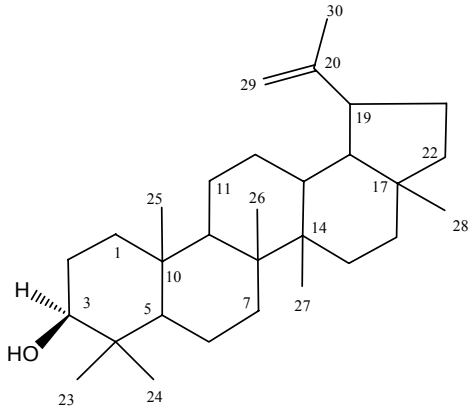
Bioassays. The antimicrobial activity of the Kupchan fractions was determined by the disc diffusion method[9] against thirteen bacteria (*Bacillus cereus, B. megaterium, B. subtilis, Staphylococcus aureus, Sarcina lutea, Escherichia coli, Pseudomonas aeruginosa, Salmonella paratyph, S. typhi*, *Shigella 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. The samples were dissolved separately in chloroform and applied to sterile discs at a concentration of 400 µg/ disc and carefully dried to evaporate the residual solvent. Standard kanamycin disc (30 µg/disc) was used as the positive control in the experiment.

For cytotoxicity screening, DMSO solutions of the plant extractives were applied against *Artemia salina* in a 1-day *in vivo* assay, the experimental details of which could be found elsewhere.[10-12] For the experiment, 4 mg of each of the Kupchan fraction was 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.

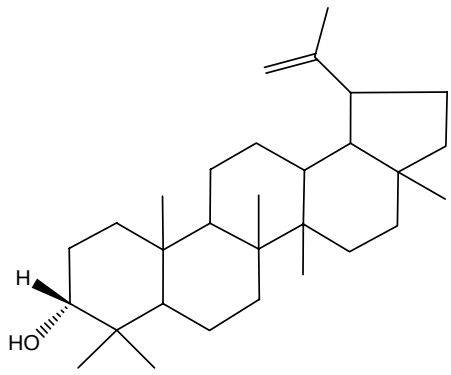
Statistical analysis. For each of the extractives, three samples were prepared for each of the bioassay. The zone of inhibition and LC_{50} were calculated as mean ± SD (n=3) for the antimicrobial screening and brine shrimp lethality bioassay, respectively.

RESULTS AND DISCUSSION

Repeated chromatographic separation and purification of the *n*-hexane and carbon tetrachloride soluble fractions of a methanolic extract of the leaves of *P. paludosa* yielded three compounds (**1-3**). The structures of which were solved by extensive analysis NMR data as well as by comparing with published datas.

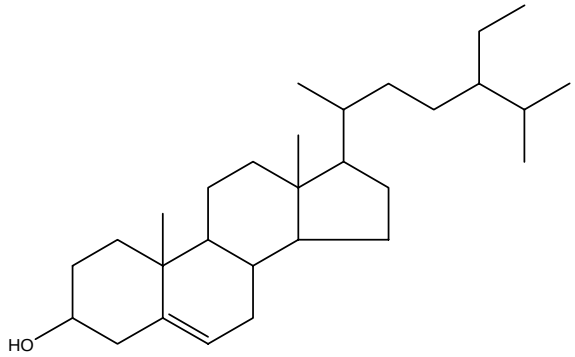


1



2

The ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of compound **1** showed a double doublet (J = 11.5, 5.03 Hz) of one proton intensity at δ 3.20 typical for an oxymethine proton at C-3 of a triterpene. The splitting pattern of this proton confirmed the β (beta) orientation of the C-3 oxygenated substituent. The spectrum displayed two broad singlets at δ 4.67 and δ 4.55 (1H each) assignable to the vinylic protons at C-29. It also showed seven singlets at δ 0.95, 0.78, 0.84, 1.02, 0.93, 0.82 and 1.67 (3H each) assignable to 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 the ^{1}H NMR data of compound **1** with previously published data,[13] it was identified as lupeol. The identity of **1** was further substantiated by Co-TLC with an authentic sample of lupeol, previously isolated in our laboratory.



3

The ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of compound **2** exhibited a triplet (J=3.0 Hz) of one proton intensity at δ 3.37 characteristic for H-3 of a terpene type carbon skeleton. The absence of a double doublet and the appearance of a triplet suggested that the hydroxy group was at the α (alpha)-position, thus confirming the β orientation of the C-3 proton (14). The spectrum displayed two broad singlets at δ 4.67 and δ 4.55 (1H each) assignable to the vinylic protons at C-29. A multiplet at δ 2.28 could be ascribed to proton at C-19. The spectrum also displayed seven singlets at δ 0.95, 0.78, 0.84, 1.02, 0.93, 0.82 and 1.67 (3H each) for methyl 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. On this basis and by comparing these ^{1}H NMR data with previously published values,[14] compound **2** was identified as epilupeol. Again, the identity of **2** as epilupeol was further substantiated by Co-TLC with an authentic sample.

The ^{1}H NMR spectrum of compound **3** readily demonstrated the steroidal nature of these compounds. The spectral data of compound **3** was superimposable to the ^{1}H NMR spectrum recorded for β-sitosterol.[15] Additionally, thin layer chromatographic analysis of **3** with authentic samples of β-sitosterol further confirmed its identification.

In the antimicrobial screening, the extractives of the *P. paludosa* were found insensitive to microbial growth (data not shown). However, in case of brine shrimp lethality bioassay, the lethality of the *n*-hexane (HF), chloroform (CF) and aqueous soluble fractions (AQF) of the methanolic extract to brine shrimp was determined on *A. salina*. Table 1 shows the results of the brine shrimp lethality testing after

Table 1. LC_{50} data of *P. paludosa* extractives and vincristine sulfate

Samples	LC_{50} (µg/ml)
VS	0.28 ± 1.25
HF	2.17 ± 1.30
CF	2.77 ± 0.95
AQF	2.46 ± 1.33

The values of LC_{50} are expressed as mean ± SD (n=3). VS: vincristine sulphate (Std.); VS: Vincristine sulphate (Std.), HF: *n*-hexane soluble fractions of the methanolic extract, CF: Chloroform soluble fractions of the methanolic extract, AQF: aqueous fraction of methanolic extract.

24 hours of exposure to the samples and the positive control, vincristine sulphate (VS). The LC_{50} obtained from the best-fit line slope were found to be 2.17, 2.77, 2.46 and 0.28 µg/ml for *n*-hexane, chloroform, aqueous soluble fractions of the methanolic extract and vincristine sulfate, respectively. In comparison with the positive control (vincristine sulphate), the cytotoxicity exhibited by the all fractions of methanolic extract was significant.

REFERENCES

1. Bangladesh National Herbarium, Mirpur, Dhaka, Bangladesh.
2. Hassan, D.Z. 2000. Some plants of Sundarbans, 1st ed., Tectona computing, Chittagong, Bangladesh, p. 80.
3. Howlader, M.A., Bachar, S.C., Begum, F., Rouf, A.S. 2006. Diuretic and analgesic effects of the methanol extract of *Phoenix sylvestris* root. *Pak. J. Pharm. Sci.* **19**, 330-332.
4. Al-Qarawi, A.A., Abdel-Rahman, H., Ali, B.H., Mousa, H.M., El-Mougy, S.A. 2005. The ameliorative effect of dates (*Phoenix dactylifera* L.) on ethanol-induced gastric ulcer in rats. *J. Ethnopharmacol.* **98**, 313-317.
5. Panwar, M., Kumar, M., Samarth, R., Kumar, A. 2005. Evaluation of chemopreventive action and antimutagenic effect of the standardized *Panax ginseng* extract, EFLA400, in Swiss albino mice. *Phytother. Res.* **19**, 65-71.
6. Vayalil, P.K. 2002. Antioxidant and antimutagenic properties of aqueous extract of date fruit (*Phoenix dactylifera* L. Arecaceae). *J. Agric. Food Chem.* **50**, 610-617.
7. Dictionary of Natural Products on CD-ROM, version 9.2. Chapman & Hall, 2001.
8. Van Wagenen, B.C., Larsen, R., Cardellina, J.H., Ran dazzo, D., Lidert, Z.C. and Swithenbank, C. 1993. Ulosantoin, a potent insecticide from the sponge Ulosa ruetzleri. *J. Org. Chem.* **58**, 335-337.
9. Bauer A.W, Kirby W.M.M, Sherris J.C. and Turck M. 1966. Antibiotic susceptibility testing by a standardized single disc method. *Am. J. Clin. Pathol.* **45**, 493-496.
10. Meyer B.N., Ferringni, N.R., Puam, J.E., Lacobsen, L.B., Nichols, D.E. and McLaughlin, J.L. 1982. Brine shrimp: a convenient general bioassay for active constituents. *Planta Med.* **45**, 31-32.
11. Persoone G. 1988. Proceedings of the international symposium on brine shrimp, *Artemia salina*. University Press, Wittern, Belgium, p. 1-3.
12. McLaughlin JL. 1982. Brine shrimp: a convenient general bioassay for active constituents. *Planta Med.* **45**, 31-32.
13. Aratanechemuge, Y., Hibasami, H., Sanpin, K., Katsuzaki, H., Kunio, I.K., Komiya, T. 2004. Induction of apoptosis by lupeol isolated from mokumen *Gossampinus malabarica* L. Merr in human promyelotic leukemia HL-60 cells. *Oncol. Rep.* **11**, 289-292.
14. Rahman, M.Z., Sultana,S.J., Faruquee, C.F., Ferdous, F., Rahman, M.S., Islam, M.S. and Rashid, M.A. 2007. Phytochemical and Biological investigation of *Erythrina variegate*. *Saudi Pharm. J.* **15**, 140-145.
15. Morales, G., Sierra, P., Mancilla, A., Paredes, A.,Loyola, L.A.,Gallardo, O. and Borquez, J. 2003. Secondary metabolites from four medicinal plants from northern Chile: antimicrobial activity and biotoxicity against *Artemia salina*. *J. Chil. Chem. Soc.* **48**, 13-18.