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STEROIDS AND TRITERPENOIDS FROM GREY MANGROVE *AVICENNIA MARINA*

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Abstract

Phytochemical investigation on the pneumatophores (aerial roots) of *Avicennia marina* (Forssk.) Vierh., resulted in the isolation of two sterols, Stigmasterol-3-*O*-β-D galactopyranoside (**1**) and Stigmasterol (**2**) and three triterpenoids, Lupeol (**3**), Taraxerol (**4**) and Betulinic acid (**5**). Compound (**1**) is reported for the first time from this plant, whereas, the remaining four other compounds have already been reported but not from the mangroves of Pakistan. Compound (**1**) was also found to possess anti-glycation activity which is reported here also for the first time.

Introduction

Avicennia marina (Forssk.) Vierh., (Avicenniaceae) is the most dominant and widespread species of mangrove in Pakistan (Saifullah, 1997). The plant grows in sheltered coastal habitats of tropical region of the world, especially in estuaries and deltas. *A. marina* is an important mangrove species. Its bark, leaves and fruit have been used in traditional medicine for the treatment of skin diseases, rheumatism, small pox, ulcers and fodder for livestock. (Fauvel *et al.,* 1993; Bandaranayake, 2002). It also possesses antimalarial and cytotoxic activity (Miles *et al.,* 1998). It is also a source of alcohol, amino acid, carbohydrate, fatty acid, hydrocarbons, inorganic salts, minerals, phytoalexins, carboxylic acid, steroids, tannins, triterpenes, vitamins, iridoid glucosides and fatty acids (Bandaranayake, 2002; Hogg & Gillan, 1984; Kanig & Rimper, 1985). The present study deals with isolation and structure elucidation of steroids and triterpenoids from its aerial roots.

Materials and Methods

Pneumatophores (aerial roots) of grey mangrove *A. marina* were collected from the backwater of Karachi Harbour known as Sandspit. The 6 kg shaded-dried roots were extracted with methanol at room temperature and later were evaporated to yield the residue (152g). The whole residue was then extracted with ethyl acetate.

For column chromatography (CC), silica gel (70-230 mesh) and for flash chromatography (FC), silica gel (230-400 mesh) was used. TLC was performed on pre-coated silica gel G-25-UV 254 plates. Detection was carried out at 254nm, and by Ceric sulphate reagent purity was checked on TLC with different solvent system using Hexane, acetone, ethyle acetate chloroform and methanol giving single spot. H-NMR, ^{13}CNMR, Cosy, HMQC and HMBC Spectra were run on Bruker Spectrometers operating at 500, 400 and 300MHz. The chemical shifts are given in ppm and coupling constants in Hz. EI-MS spectra were recorded on a JMS-HX-110 spectrometer, with a data system.

The extract was subjected to cover a silica gel column using hexane with gradient of Ethyl acetate up to 100% and followed by methanol different fractions were collected and loaded on silica gel (flash silica 230-400 mesh). Stigmasterol-3-*O*-β-D galactopyranoside (Compound **1)** was eluted with methanol and chloroform (1: 9) and Stigmasterol (Compound **2**), Lupeol (Compound **3**), Teraxerol (Compound **4**), and Betulinic acid (Compound **5**) from Ethyl acetate: hexane (3: 7) to purify.

Antiglycation assay: It is a nucleophilic reaction in which protein amino group or lipid molecule is covalently linked with the carbonyl group of reducing sugar such as glucose and fructose to form glycated product called Glycation, Glycosylation, Non-enzymatic reaction or Advance glycated ends product (AGEs). It is a posttranslational modification of protein which occur by hyperglycemia and long term complication such as Cataract, Neuropathy, Nephropathy, Wound healing, Alzheimer's disease (AD) etc. functioning of biomolecules.

Chemicals: Bovine serum albumin (BSA) was purchased from Research Organics Cleveland, while others chemicals {glucose anhydrous, trichloroacetic acid (TCA) sodium azide (NaN_3), dimethyl sulfoxide (DMSO), Sodium dihydrogen phosphate (NaH_2PO_4), Sodium chloride (NaCl), disodium hydrogen phosphate (Na_2HPO_4), potassium chloride (KCl), potassium dihydrogen phosphate (KH_2PO_4), and sodium hydroxide (NaOH)} were purchased from Sigma Aldrich.

Sodium phosphate buffer (pH 7.4), was prepared by mixing Na_2HPO_4 and NaH_2PO_4 (67 mM) containing sodium azide (3 mM), phosphate buffer saline (PBS) pH 10 was prepared by mixing NaCl (137 mM) + Na_2HPO_4 (8.1 mM) + KCl (2.68 mM) + KH_2PO_4 (1.47 mM) + pH 10 was adjusted with NaOH (0.25 mM), while BSA (10 mg / mL) and anhydrous glucoses (50 mg / mL) solutions were prepared in Sodium phosphate buffer.

Sample Preparation: Sample was prepared in DMSO for pure compound (2 mM /mL).

***In vitro* glycation**: In 96-well plate assays, each well contains 60 µL reaction mixtures (20 µL BSA (10 mg / mL + 20 µL of glucose anhydrous (50 mg / mL) + 20 µL test sample) (Nakagawa *et al.*, 2002) Glycated control contains 20 µL BSA + 20 µL glucose + 20 µL Sodium phosphate buffer, while blank control contains 20 µL BSA an Fumiod 40 µL Sodium phosphate buffer. Reaction mixture was incubated at 37°C for 7-days (Yamaguchi *et al.*, 2000). After incubation, 6 µL (100%) of TCA was added in each well and centrifuged (15,000 rpm) for 4 minutes at 4°C. After centrifugation, the pellets were rewashed with 60 µL (10%) of TCA (Mastuura *et al*., 2002). The supernatant containing glucose, inhibitor and interfering substance was removed and pellet containing AGE-BSA were dissolved in 60.0 µL PBS (Mastuura *et al*., 2002). Assessment of fluorescence spectrum (ex. 370 nm), and change in fluorescence intensity (ex. 370 nm to em. 440 nm) based on AGEs were monitored by using a spectrofluorimeter (RF-1500, Shimadzu, Japan). (Hye & Kyong, 2003). The % Inhibition was calculated through the following formula:

% Inhibition = [1 - (Fluorescence of sample / Fluorescence of glycated) X 100]

Results and Discussion

From the methanol soluble fraction of *Avicennia marina*, compound **1** (Fig. 1) was isolated for the first time from this species. The compound was isolated by column chromatography of methanol soluble fraction. The FAB MS showed the $[M-H]^+$ at *m/z* corresponded to the molecular formula $C_{35}H_{58}O_6$ (calcd.574.4239). The ^{1}H-NMR spectrum ($CDCl_3$, 500MHz) of compound **1** showed a downfield broad singlet at δ 5.22 which was assigned to the C-6 olefinic proton the C-3proton appeared as a double doublet at δ 3.68 (J = 12Hz, J = 4.4Hz). The 3H singlets at δ 1.5,0.95, 0.91, 0.83, 0.81 and 0.76 were due to C-21, C-19, C-29, C-26, C-27 and C-18 methyls protons, respectively. The C-1 anomeric proton appeared as a doublet at δ 4.3 as a doublet ($J_{1,2}$ = 7.7Hz) indicating the presence of a β sugar. The compound was identified as stigmasterol-3-*O*-β-D galactopyranoside (Ahmed *et al*., 1992) in comparison with the reported data.

The compound showed moderate anti-glycation activity.

Name of compound	**% Inhibition**
Stigmasterol-3-O-β-D galactopyranoside	58.4
Rutin (Standard)	85.5

The compound **2** (Fig. 2) was isolated as white crystal from hexane fraction of this plant. Its H-NMR completely corresponded to the data for Stigmasterol-3-*O*-β-D galactopyranoside except addition of galactose group. The ^{13}C-NMR spectrum was also similar except additional peaks for sugar moiety. It was identified as Stigmasterol (Rubinstein *et al*., 1950) in comparison with the reported data.

Compound 3 (Fig. 3) was isolated as a white crystal from the n- hexane extract of this plant. The molecular formula was established by HR-EIMS (m/z 426) as $C_{30}H_{50}O$. Its data is similar to that identified by Monaco & Pervitata (1984) as Lupeol.

Compound **4** (Fig. 4) was isolated as white crystal from the methanolic extract of this plant. Its molecular formula was established by HR-EIMS (m/z 426) as $C_{30}H_{50}O$. It was already identified as Taraxerol by Ogihara *et al*., (1987).

Compound 5 (Fig. 5) was isolated as white crystal from methanolic extract. Molecular formula was established by HR-EIMS (*m/z* 456) as $C_{30}H_{48}O_3$. It was identified earlier as Betulinic acid by Siddique *et al*., (1988).

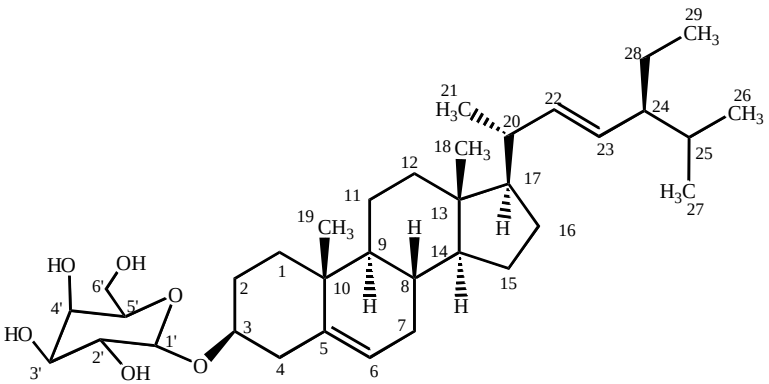

Fig. 1. Stigmasterol-3-*O*-β-D galactopyranoside.

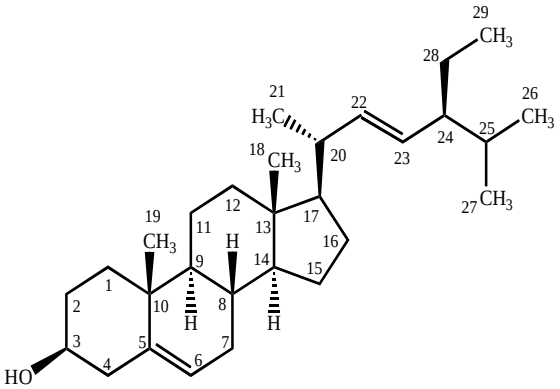

Fig. 2. Structure of Stigmasterol.

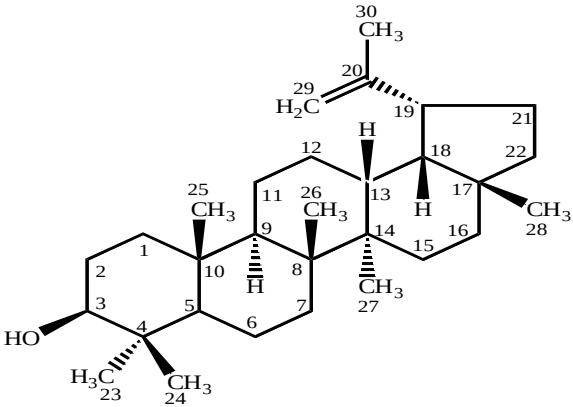

Fig. 3. Structure of Lupeol.

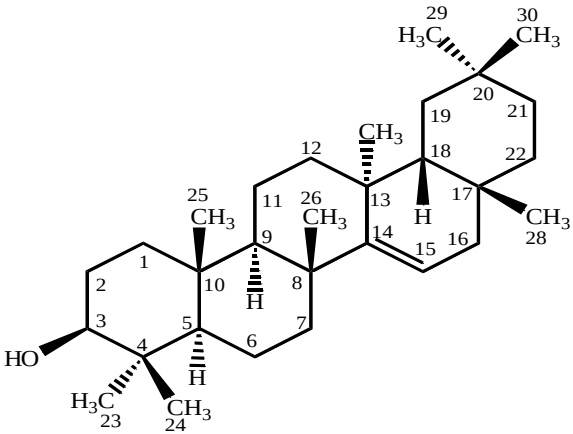

Fig. 4. Structure of Taraxerol.

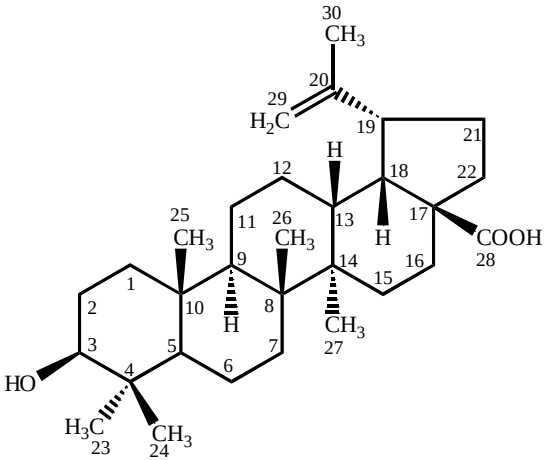

Fig. 5. Structure of Betulinic acid.

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