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Research Article

A TRITERPENOID ANTIOXIDANT AGENTS FOUND IN *HOLOPTELEA INTEGRIFOLIA (ROXB) PLANCH.*

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ABSTRACT

An ornamental and medicinal plant *Holoptelea integrifolia (Roxb) Planch* (Ulmaceae) has been collected from Pakistan. From plant bark, two medicinal pentacyclic triterpenodis, betulinic acid (3β-Hydroxy- lup-20(29)-en-28-oic acid) and betulin (Lup-20(29)-ene-3β, 28-diol) were isolated for the first time from the bark of the plant. The structure of compounds 1 and 2 were ascertained via advanced spectroscopic technique, as these structures were also compared with authenticated structures of betulin and betulinic acid and come across with similar data.

Keywords: *Holoptelea integrifolia (Roxb) Planch,* bark, Ulmaceae, Betulinic acid and Betulin.

INTRODUCTION

Holoptelea integrifolia (Roxb) Planch (Ulmaceae) is a medicinal and ornamental plant which is widely distributed all over tropical and temperate regions of Northern Hemisphere including Pakistan, India, Nepal, Srilanka, Cambodia, Laos, Myanmar, Vietnam e.t.c. It is also abundantly found in sub Himalayan hills of Assam[1,2].

In Indo Pak subcontinent it is a widely used herb in traditional medical system against different aliments, as stem bark is highly recommended by local healers to relieve inflammation in edema and in Nepal; bark is used externally to relieve rheumatic swellings[3]. It is also employed in dyspepsia, piles, hemorrhoids and leprosy. Plant is considered as an excellent antidiabetic, laxative and carminative, whereas leaves, seeds and stem bark are topically used in the form of paste in ring worm, scabies and other skin diseases. Although very little phytochemical work have been done on the bark of plant but according to previous literature survey it contains 2α. 3α-dihydroxyolean–12-en-28-acid, hederagenin, friedelan-3β-ol, friedelin[4,5], epifriedelinol, 2-aminonaphthaquinone, β-sitosterol, β -D-glucose[6] and two triterpenoid fatty acid esters as Holoptelin A and B[7].

EXPERIMENTAL

Plant material

Bark of *H. integrifolia (Roxb) Planch* was collected in January 2009, from Karachi region and identified by Professor of Pharmacognosy Department, University of Karachi, Pakistan. Bark has been deposited in the herbarium of Department of Pharmacognosy, Faculty of Pharmacy, University of Karachi, Pakistan having voucher specimen number 0045.

Extraction

Fresh bark of *H. integrifolia (Roxb.) Planch* was cleaned, shade dried and weighed (10kg)

properly, chopped into small pieces. Bark had been percolated in absolute methanol (Merck, Germany) at room temperature for 15 days then filtered off and filtrate was followed by evaporation under reduced pressure and controlled temperature on the rotary evaporator. The reddish brown extract was lyophilized to a powdered form (500g), out of this 200g extract was used for biological activities while 300g for chemical analysis.

General methods

Rotary evaporator and lyophilizer by Eyela Japan, Column chromatography (CC) was performed on silica gel particle size 0.063 – 0.200 mm (70-230 mesh) ASTM (Merck, Germany) was used. EI-MS [ion source energy (70eV), ion source temperature 250 ºC] was recorded on Finnigan MAT-312. Hitachi U-3200 spectrometer has measured UV data, Shimadzu FT-IR 8400S spectrometer for IR spectra and Bruker Vector-22 spectrophotometer recorded IR data. ^{1}H and ^{13}C NMR data were acquired with a Bruker AV-600 spectrometer using $CDCL_3$ as solvent.

Fractionation and Isolation

The crude methanolic extract was suspended in distilled water (450 mL) and sequentially partitioned with *n*-hexane (3 x 450mL), ethyl acetate (3 x 450mL) (EtOAc) and butanol (3 x 450mL) (BuOH) respectively to obtain each extract separately in dried form after evaporation. The dried EtOAC extract 40g was divided in to two part A and B. Part A 20g was utilized for the pharmacological screening while Part B 20g was acidified with 0.5N HCl and extracted with chloroform. The crude $CHCl_3$ extract (19g) were subjected to column chromatography eluted with n-Hexane-Chloroform gradient as a result of this two fractions (A and D) were obtained with Hexane: Chloroform; 9:1(A) and 1:9 (D) respectively. The active fraction of friedelin was eluted with Hexane-$CHCL_3$, 9:1 ratio while betulinic acid and betulin were eluted by $CHCL_3$-Hexane 1:9 ratio in a crude form. These compounds were further purified by flash silica gel (230-400 mesh size) and through TLC monitoring three compounds were detected in a pure state by cerium ammonium sulfate reagent in fractions A and D respectively. Thereafter structure determination were made by their spectral data.

RESULT AND DISCUSSION

The whole bark of *H. integrifolia* was subjected for extraction and fractionation, in which the partitioned EtOAC fraction (20gm) was undergone for isolation and detection followed by chromatographic procedures i.e. thin layer chromatography and column chromatography. In chromatographic operation of $CHCL_3$ extract gave several compounds from bulk of fractions and out of them fraction of solvent system of Hx: $CHCL_3$, as 1 (Hx): 9($CHCL_3$) were purified to obtained triterpenoids by two sub fractions **a** & **b**. These sub fractions were rechromatographed to yield betulin (381mg) from sub fractions **a** and from **b** betulinic acid (645mg) had been isolated from different polarities. Their structure determination was carried out by advance spectroscopic techniques. As a result of comparative spectral data (obtained and reported), since compound *betulin* is first time ever isolated from the bark of *H. integrifolia*, while betulinic acid was previously reported in seeds of the same resource.

3β-Hydroxy- lup-20(29)-en-28-oic acid as betulinic acid (M.P 282-285 ^{o}C) from sub fraction **b** end absorption at 199 nm in UV spectrum was in the favor of absence of conjugated unsaturated system in the molecule. IR spectrum showed sharp absorption band at the 1687cm^{-1} indicating the presence of carboxyl group in the molecule, another peak appeared at 1638cm^{-1} due to the olefinic double bond while appearance of broad absorption in the region between 3434-3072cm^{-1} indicates the hydroxyl group in the molecule. Compound showed a molecule ion peak at m/z 456 that appeared at an exact mass of m/z 456.2 on high resolution mass corresponding to molecular formula $C_{30}H_{48}O_3$. In the mass spectrum the other fragments appeared at m/z 438 (M+- H_2O), 411 (M+- COOH), 248

(31), 220(22), 207(48), 203(28) and 189(100). In the olefinic region of ^{1}H- NMR of the compound two doublets integrating for one proton each resonated at δ 4.95 and δ 4.78 (J=2.0Hz). Both doublets were correlated to the methylene at δ 109.7 in HMQC and were assigned to the terminal olefinic methylene in the structure and that was in complete agreement with the olefinic absorption observed at 1687cm^{-1} in the IR spectrum of the compound. Since sharp singlets of three proton integration each exhibited at δ 1.80, 1.23, 1.08, 1.07, 1.02 and 0.82. In ^{1}H- NMR were ascribed to the proton of six tertiary methyl groups i.e. H-30, H-23, H-27, H-25, H-26 and H-24 respectively.

Lup-20(29)-ene-3β, 28-diol as betulin (M.P 254-255 ^{o}C) with end absorption in UV region at 203 λ max, while the IR absorption of the compound was observed at 3382,1725,1638, 1592, 1275 and 882 cm^{-1} which revealed the OH group and alcoholic characters. The mass spectrum of betulinic acid as analogous with betulin having molecular ion peak at 442 m/z (M^+ $C_{30}H_{50}O_2$) while according to fragmentation pattern other major peaks were observed at m/z 411, 234, 216, 207,203 and 189 confirms the presence of lupine type structure of compound. The intensity of 411 peak in mass spectra (M-CH_2OH) recalls that the structure of betulinic acid analogous betulin with 2- OH group distinguishable to compound 4. In ^{1}H-NMR spectrum of compound correspondingly showed many methyl signals at up field and the absence of aromatic protons suggested a terpenoidal skeleton. The spectrum and the mass data of all these components were found identical to authentic and their reported values[8].

The distinguishable position in ^{13}C-NMR of betulin were found at C-17= 47.8 ppm (authenticated and reported) and C-28= 60.8 & 60.6 (authenticated and reported) whereas in the betulinic acid the value of the same carbon signals depicted in the spectrum were at 56.3 & 56.26 (authenticated and reported) and 180.5 & 179.62 (authenticated and reported) due to the attachment of **CH_2OH** and **COOH** respectively, as shown in Table 1 and figure 1 & 2.

Consequently these compounds were identified as Betulinic acid and Betulin respectively on the basis of detailed spectroscopic analysis and their comparisons with the data reported in the chemical literature for these components.

The occurrence of these lupane series compounds are in diversified natural resource, according to chemo taxonomical point of view *betulinic acid, betulin* and their derivatives are distributed among wide range of families in the plant kingdom. Plant species having these compounds and their derivatives are *Betula alba, Vitex negundo, Nerium oleander, Betula utilis* and *Saussurea lappa*.

Both of these compounds have great medicinal significance as reported in literature. Betulinic acid has valuable biological potential such as, inhibitors of HIV - 1 entry, HIV- protease or of reverse transcriptase (RT), anti bacterial (against gram positive), anti malarial, anti inflammatory, anthelmintic (*Caenorhabditis elegans*), antioxidant and also acquire in tree species worthy for timber purposes (up to 2.5%)[9,10,11].

This compound differs from *doxorubicin* "Classical" anticancer drug but it induces apoptosis by amendment of mitochondrial function and has capability of extremely discriminatory growth inhibitor of human melanoma, neuroectodermal and malignant tumor cells. It is substantiate to be a novel anticancer drug and currently going through preclinical development for the treatment of malignant melanomas.

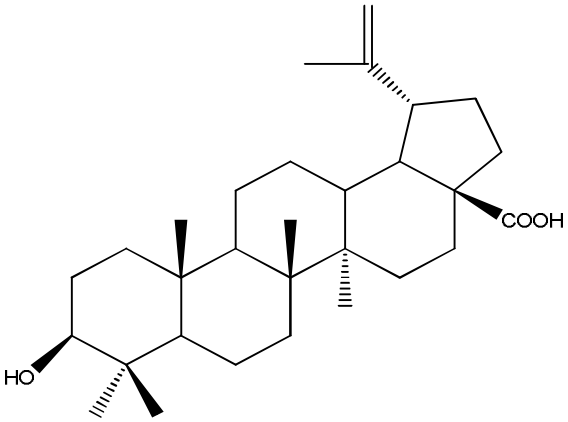
Betulin had significant anticancer effect on cervix carcinoma, hepatoma, lung adenocarcinoma, and breast cancer. While exhibits moderate activity on other human cancer cells as prostate carcinoma and minor cell inhibition in human erythroleukemia. Both compounds b. acid and betulin also have antiviral properties against herpes simplex type I, influenza FPV/Rostock and ECHO 6 viruses also having promising lead to a

potential new generation of target oriented insect controls.

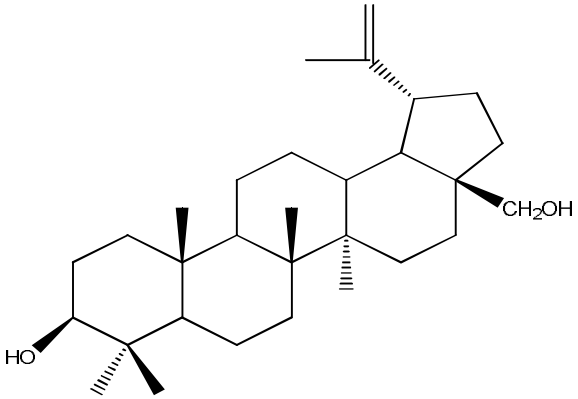
These potential pharmacologically significant pentacyclic triterpenoids of lupane series are not only important as chemically but also has great important biologically for derivatization and further development of new compounds as precursor [12, 13].

Table 1: ^{13}C-NMR data of compounds 1 and 2

^{13}C	Betulinic Acid		Betulin	
	(Authentic)	Comp. 1	(Authentic)	Comp. 2
1	38.7	38.72	38.8	38.74
2	27.4	27.41	27.4	27.43
3	78.9	79.0	79.0	79.0
4	38.8	38.87	38.3	38.73
5	55.3	55.35	55.4	55.34
6	18.3	18.29	18.3	18.3
7	34.3	34.33	34.3	34.28
8	40.7	40.7	41.0	40.96
9	50.5	50.52	50.6	50.45
10	37.2	37.21	37.4	37.36
11	20.8	20.85	20.9	20.86
12	25.5	25.50	25.6	25.26
13	38.4	38.38	37.0	37.19
14	42.4	42.44	42.8	42.75
15	30.5	30.54	27.1	27.09
16	32.1	32.15	29.3	29.22
17	56.3	56.26	47.8	47.82
18	46.8	46.87	47.8	47.82
19	49.2	49.27	48.8	48.81
20	150.3	150.39	150.3	150.47
21	29.7	29.70	29.8	29.8
22	37.0	37.02	34.0	33.9
23	27.9	27.99	28.0	28.0
24	15.3	15.34	15.3	15.35
25	16.0[a]	16.02[a]	16.1[b]	16.0[b]
26	16.1[a]	16.12[a]	16.1[b]	16.11[b]
27	14.7	14.69	14.7	14.78
28	180.5	179.62	60.8	60.6
29	109.6	109.70	109.6	109.68
30	19.4	19.37	19.4	20.86



BETULINIC ACID



BETULIN

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