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PISTAGREMIC ACID, A NOVEL ANTIMICROBIAL AND ANTIOXIDANT ISOLATED FROM *Pistacia integerrima*

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In the present research, a new triterpenic compound was isolated from galls of Pistacia integerrima. *The structure of this novel compound was identified using modern sophisticated spectroscopic techniques such as HR-EI-MS, FAB, 1D, and 2D NMR spectral data and single crystal X-ray diffraction methods. The compound was confirmed to be 2-methyl-6-(4,4,10,13,14-pentamethyl-3-*O*-2,3,4,5,6,7,10,11,12,13,14,15,16,-17-tetradecahydro-1*H*-cyclopenta[a]-phenanthren-17-yl)hept-2-enoic acid and named pistagremic acid, which has antimicrobial and antioxidant properties.*

Keywords: *Pistacia integerrima*, pistagremic acid, antimicrobial, antioxidant.

Pistacia integerrima J.L. Stewart belongs to the family Anacardiacea. It is commonly known as kakarsinghi. It is distributed in the eastern Himalayan Range from Indus to Kumaon [1] at a height of 12000 to 8000 ft. *P. integerrima* is a medium-sized deciduous tree that can achieve a height of 40 ft. *P. integerrima* is a significant medicinal plant and is used as anti-inflammatory, antidiabetic, blood cleanser, tonic for gastrointestinal disorders, and cough expectorant [2]. In Pakistan, galls of *P. integerrima* are used for treatment of hepatitis and other liver disorder [3]. It has also been reported to possess CNS depressant activity [3–5]. In the current investigation, we have discovered a potential bioactive chemical constituent that may be responsible for its folk use in treating microbial infections. Moreover, we have also discussed the structure elucidation of the bioactive compound on the basis of NMR data, which has already been established by single X-ray crystallography [6]. The isolated compound was scrutinized for its antimicrobial and antioxidant profile.

Pistagremic acid (**1**) was isolated in white crystalline form. The IR spectrum of the title compound displayed characteristic absorption bands for the carbonyl group (1712 cm^{-1}), carboxylic group (3360 cm^{-1}), chelate carboxylic group (1690, 1672 cm^{-1}), and unsaturation (1612 cm^{-1}). Its molecular formula was assigned as $C_{30}H_{46}O_3$ based on HR-EI-MS (*m/z* 454.34456; calcd for $C_{30}H_{46}O_3$, 454.3447). The ^{13}C NMR spectrum (Table 1) showed the presence of a ketone and a carboxylic acid at δ 218.1 and 173.6 ppm, respectively. In the ^1H NMR spectrum (Table 1), a total of seven methyl signals was observed, including a secondary methyl at δ 0.91 (J = 6.0 Hz, H-21) and six tertiary methyls at δ 0.79, 0.87, 0.98, 1.08, 1.09, and 1.90 (each 3H, s). Also, a trisubstituted olefinic proton resonated at δ 6.06 (1H, m, H-24) in the proton spectrum of compound **1**. The structure of **1** was validated through advanced 2D NMR techniques such as COSY, HSQC, and HMBC. The HMBC spectrum (Fig. 1) showed cross peaks for H-18 and H-19 to C-13, C-17, C-14 and C-10, C-9, C-5, respectively. Similarly, H-30 of the methyl group exhibited HMBC correlations to C-3 and C-4. Also, the proton signals of H-1, H-2, H-28, and H-29 possessed HMBC cross peaks for the ketonic carbon assigned to C-3. The olefinic proton (H-24) was correlated to C-23, C-25, and C-26. Based on the above arguments and single crystal X-ray crystallographic studies, the structure of **1** was assigned as 2-methyl-6-(4,4,10,13,14-pentamethyl-3-*O*-2,3,4,5,6,7,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[a]-phenanthren-17-yl)hept-2-enoic acid, as shown in Fig. 1 [6].

Antibacterial Effect. The isolated compound was tested against five various gram-positive and gram-negative bacteria. Pistagremic acid (**1**) exhibited promising antibacterial action against *K. pneumoniae, Straptodirimu* and *B. stearothermophus* with inhibition zones 15, 20, and 14 mm, respectively. Streptomycin was used as standard antibacterial drug. PA was less antibacterial than streptomycin.

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TABLE 1. ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) Chemical Shifts of **1** ($CDCl_3$, δ, ppm, J/Hz)

C atom	δ_H	δ_C	DEPT	C atom	δ_H	δ_C	DEPT
1	1.61 m, 1.71 m	33.6	CH_2	16	1.92 m, 1.23 m	28.2	CH_2
2	2.50 m	34.9	CH_2	17	1.48 m	50.1	CH
3	–	218.1	C	18	0.79 s	22.0	CH_3
4	–	47.3	C	19	0.98 s	21.6	CH_3
5	1.69 m	52.4	CH	20	1.49 m	36.0	CH
6	1.72 m, 1.45 m	21.4	CH_2	21	0.91 (d, J = 6.0)	18.2	CH_3
7	1.92 m, 2.03 m	26.8	CH_2	22	1.60 m, 1.15 m	35.9	CH_2
8	–	134.7	C	23	1.20 m, 1.59 m	26.9	CH_2
9	–	146.0	C	24	6.06 m	147.2	CH
10	–	35.6	C	25	–	125.8	C
11	1.65 m	20.3	CH_2	26	–	173.6	C
12	1.81 m, 1.62 m	34.6	CH_2	27	1.90 s	20.5	CH_3
13	–	43.5	C	28	1.08 s	26.7	CH_3
14	–	51.2	C	29	1.09 s	21.1	CH_3
15	1.47 m, 1.50 m	34.0	CH_2	30	0.87 s	24.2	CH_3

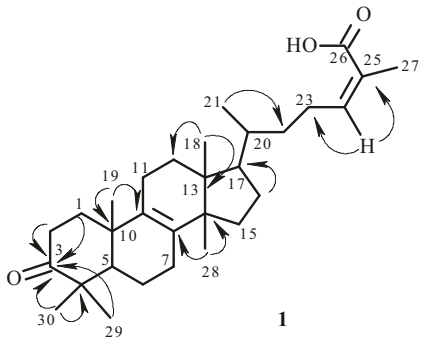

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Fig. 1. Selected HMBC correlations of pistagremic acid (**1**).

Antifungal Effect. Compound **1** was tested against *T. longifusus*, *C. albicans*, *A. flavus*, *M. canis*, *F. salani,* and *C. glaberata.* Among the tested fungi, the growth of *C. albicans*, *M. canis*, *F. salani,* and *C. glaberata* was inhibited with PA, with inhibition zones 12, 18, 12, and 14 mm, respectively. The standard antifungal drug used was miconazole.

Antioxidant Effect. The compound showed concentration-dependent free radical scavenging potential. The percent DPPH free radical effect of the tested compound was 2.44, 3.33, 6.22, 9.99, 14.55, 18.92, and 30.55 at the tested concentrations of 5, 10, 20, 40, 60, 80, and 100 ppm, respectively. The antioxidant effect of the reference drug BTH was better than our tested sample.

EXPERIMENTAL

General Procedures. Melting point was determined on a BICOT apparatus (Bibby Scientific Ltd.). IR spectrum was recorded on an FT-IR Nicolet 380 spectrometer (Thermo Scientific). ^{1}H (400 MHz, A), ^{13}C (400 MHz, A), HMBC (600 MHz, A), and HSQC (400 MHz) spectra were recorded on a Bruker spectrospin NMR instrument in $CDCL_3$. HR-EI-MS were scanned using a Jeol-JMS-HX-110 mass spectrometer; an EI source 2500, 70 eV chrystallography machine and a Bruker Kappa APEXII CCD diffractometer were used to confirm structure. Column chromatography was performed on Merck silica gel 60 (0.063–0.200 mm). TLC was carried out using Merck aluminum plates pre-coated with silica gel 60 F254.

Plant Material. *P. integerrima* galls were collected from Toormang, Razagram area of district Dir, Khyber Pukhtun Khawa Province of Pakistan in February, 2010. The plant material was identified by Prof. Dr. Abdur Rashid of the Department of Botany, University of Peshawar, Pakistan. A voucher specimen (No. RF-895) was deposited in the Herbarium of the department.

Extraction and Isolation. The shade-dried and crushed bark of *Pistacia integerrima* (14 kg) was subjected to cold extraction with MeOH. MeOH extract (600 g) was suspended in water and successively partitioned with hexane, $CHCl_3$, EtOAc, and BuOH according to the standard protocol [7, 8]. The EtOAc fraction (30 g) was subjected to column chromatography on

Merck silica gel 60 (0.063–0.200 mm), 5 × 60 cm. The column was first eluted with hexane–acetone (100:0→0:100) as the solvent system. A total of 33 fractions, RF-1–33, was obtained based on TLC profiles. Fraction RF-20 obtained using a hexane–acetone 100:0→15:100 gradient contained colorless crystals of various sizes and was separated from the solution by decantation. The crystals were washed with *n*-hexane several times. To obtain pure and larger crystals, these crystals were regrown from a mixture of hexane–acetone–chloroform (70:20:10), resulting in a compound named pistagremic acid (50 mg).

Antibacterial Bioassay. The antibacterial activity was assayed using a modified agar well diffusion method [9]. Muller-Hinton agar was used as medium. The cultures were taken in triplicate at an incubation temperature of 37°C for 24 to 72 h. The broth culture (0.6 mL) of the test organism was placed in a sterile Petri dish to which 20 mL of sterile molten MHA was added. Holes were bored in to the medium using 0.2 mL of the pistagremic acid (**1**). Streptomycin was used as standard antimicrobial agent at a concentration of 2 mg/mL. Inoculation was done for 1 h to make possible the diffusion of the antimicrobial agent into the medium. Incubation was done at 37°C for 24 h, and the diameters of the zone of inhibition of microbial growth were measured in the plate in millimeters (mm).

Antifungal Bioassay. The antimicrobial effect of the title compound against various fungal strains was assaged. The antifungal activity was determined by the tube dilution method [10]. In the described method, miconazole was used as the standard drug. The compound was dissolved in DMSO-d_6 (2 mg/5 mL). Sterile Sabouraud′s dextrose agar medium (5 mL) was placed in a test tube and inoculated with the sample solution (400 μg/mL) kept in slanting position at room temperature overnight. The fungal culture was then inoculated on the slant. The samples were incubated for 7 days at 29°C, and growth inhibition was observed.

DPPH Free Radical Scavenging Assay. The antioxidant activity was determined spectrometrically using the DPPH radical scavenging assay [11], with slight modification. Various concentrations were taken and the compound dissolved in distilled methanol and diluted to 50 mL. From this stock solution, different microgram solutions of 20, 40, 50, 60, 80, and 100 ppm were prepared by dilution. Then 5 mL of each solution was taken in a test tube and 1 mL of 0.001 M of DPPH solution was added to it. All these solutions were kept in the dark for 30 min. Also, 5 mL methanol was taken and 1 mL of DPPH solution was added to serve as the control solution. At the end of the incubation period, the mixtures were examined for antioxidant activity using an Optima UV-visible spectrophotometer at a wavelength of 517 nm. The experiments were performed in triplicate. The % DPPH was determined using the formula:

$$\%\text{DPPH} = \text{Control abs} - \text{Extract abs} \times 100/\text{Control}.$$

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