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BIOLOGICAL AND PHYTOCHEMICAL INVESTIGATIONS ON *Ajania fruticulosa*

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Ajania fruticulosa (Ledeb.) Poljakov (Synonym: *Tanacetum gracile*) is a medicinal herb belonging to the family Asteraceae. It is found in the Sarabi forest, Quetta, Pakistan. The genus *Tanacetum* comprises several species that are widespread in many countries of Europe, Asia, and North America. An extensive literature survey showed that phytochemical investigations on the species of *Tanacetum* have resulted in the isolation of a number of interesting natural products [1–6]. The plants of the genus *Tanacetum* have been used in popular medicine as expectorants, vermifuges, antiseptics, and spasmolytics [7]. In Bulgaria, the dry leaves and flowers of *T. vulgare* are used as antiseptic and spasmolytic and for protecting hair against dandruff [8]. The leaves of *T. parthenium* ("feverfew") are used as a popular British traditional herbal remedy for the prophylaxis of migraine [9, 10].

The methanolic extract of *Ajania fruticulosa* showed antibacterial activity against *Bacillus cereus*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Salmonella typhi*, *Shigella boydii*, and *Staphylococcus aureus* at concentrations of 200 μg/100 μL, and antifungal activity against *Trichophyton schoenleinii*, *Pseudallescheria boydii*, *Aspergillus niger*, *Microsporum canis*, *Trichophyton simii*, and *Fusarium oxysporum* at concentrations of 400 μg/mL. Amoxicillin, ampicillin, and cephalexin were used as standard antibacterial antibiotics, whereas ketoconazole and miconazole were used as standard antifungal antibiotics to compare the extent of activity of samples [11]. The methanolic extract of *Ajania fruticulosa* showed 40.3% inhibition of acid phosphatase enzyme at 1 mM concentration. The methanolic extract of *Ajania fruticulosa* showed 35.5% inhibition of acetylcholinesterase enzyme at 1 mM concentration. Based on the extent of inhibition, it was concluded that the crude methanolic extract of *Ajania fruticulosa* showed a moderate inhibition of these enzymes.

Phytochemical investigations on *Ajania fruticulosa* have resulted in the identification of six known compounds, β-amyrin (**1**), 14α-taraxeran-3-one (**2**), 14α-taraxeran-3α-ol (**3**), octacosyl-*p*-coumarate (**4**), octacosanyl ferulate (**5**), and 3-hydroxy-4-methoxybenzaldehyde (**6**), which were isolated for the first time from this plant. The structures of these compounds were elucidated by comparing data of different spectroscopic techniques such as UV, IR, HR-MS, and NMR with the reported data [12–16]. Only the methanolic extract of *Ajania fruticulosa* was tested for antimicrobial and enzyme inhibition bioassays, which were of interest for the isolation of compounds from this plant, but unfortunately the quantity of these compounds was not sufficient for the bioassay screening.

Plant Material. The whole plant of *Ajania fruticulosa* (4.5 kg) was collected from Sarabi Forest, Quetta, Pakistan. The plant was identified by Prof. Dr. Rasool Baksh Tareen, and a voucher specimen (Herbarium No. RBT-TG-01-1998-BUH) was deposited in the Herbarium of the Department of Botany, University of Balochistan, Quetta, Pakistan.

Extraction and Isolation. The air-dried whole plant of *Ajania fruticulosa* (4.5 kg) was extracted with methanol (20 L). The removal of the solvent yielded 206 g of the crude methanolic extract. The methanolic extract was fractionated into different fractions on the basis of solvent-solvent extractions. The chloroform and ethyl acetate fractions were subjected to column chromatography and preparative TLC to afford the pure compounds **1–5** and **6**, respectively.

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14α-Taraxeran-3-one (2). After elution of compound **1** from fraction F-1, the polarity of the solvent system was gradually increased by increasing the quantity of chloroform in *n*-hexane. When 1.5 L of *n*-hexane–chloroform (8:2) was passed through the column, compound **2** was eluted along with a minor impurity of compound **3**. The impurity was removed by TLC on precoated silica gel plates using *n*-hexane–chloroform (8:2) as the solvent system to afford the pure crystalline compound **2** (12.3 mg), mp 229–230°C; $[\alpha]_D^{25}$ –340° (*c* 0.52 in $CHCl_3$). IR ($CHCl_3$, ν_{max}, cm^{-1}): 1722 (C=O stretching vibrations), 3051 (C-H stretching vibrations); El-MS, *m/z:* (rel. int. %): 426 (24), 411 (86), 341 (5), 302 (18), 273 (32), 205 (37), 109 (76), 69 (100). HR-EI-MS, *m/z*: 426.3842 ($C_{30}H_{50}O$, calcd 426.3834), 411.3622 ($C_{29}H_{47}O$, calcd 411.3626, $M^+ - CH_3$), 341.3180 ($C_{25}H_{41}$, calcd 341.3208, $M^+ - C_5H_9O$), 302.2608 ($C_{21}H_{34}O$, calcd 302.2618, $M^+ - C_9H_{16}$), 273.2227 ($C_{19}H_{29}O$, calcd 273.2218, $M^+ - C_{11}H_{21}$), 205.1882 ($C_{15}H_{25}$, calcd 205.1906, $M^+ - C_{15}H_{25}O$), 109.1010 (C_8H_{13}, calcd 109.1017, $M^+ - C_{22}H_{37}O$), 69.0683 (C_5H_9, calcd 69.0704, $M^+ - C_{25}H_{41}O$). ^{1}H NMR ($CDC1_3$, 500 MHz, δ, ppm): 0.71 (3H, s, CH_3-28), 0.85 (3H, s, CH_3-24), 0.93 (3H, s, CH_3-27), 0.98 (3H, s, CH_3-29), 0.99 (3H, s, CH_3-30), 1.03 (3H, s, CH_3-25), 1.15 (3H, s, CH_3-26), 1.16 (3H, s, CH_3-23), 2.38 (2H, m, H_2-2).

14α-Taraxeran-3α-ol (3). After the elution of compounds **1** and **2** from the column, the polarity of the solvent system was gradually increased by increasing the ratio of chloroform in *n*-hexane. The slow moving compound **3** was eluted with *n*-hexane–chloroform (7:3). TLC examination on precoated silica gel plate using *n*-hexane–chloroform (7:3) showed the presence of minor impurities. Therefore, it was further subjected to purification by preparative TLC on silica gel plates using the same solvent system, which afforded pure crystalline compound **3** (10.6 mg), mp 237–238°C; $[\alpha]_D^{25}$ –4.02° (*c* 1.5 in $CHCl_3$). IR ($CHCl_3$, ν_{max}, cm^{-1}): 3448 (O-H stretching vibrations) and 3049 (C-H stretching vibrations). EI-MS, *m/z* (rel. int. %): 428 (39), 413 (31), 275 (42), 165 (87), 109 (82), 69 (98); HR-EI-MS, *m/z*: 428.3967 ($C_{30}H_{52}O$, calcd 428.4018), 413.3731 ($C_{29}H_{49}O$, calcd 413.3783, M^+-CH_3), 275.2352 ($C_{19}H_{31}O$, calcd 275.2374, M^+-$C_{11}H_{21}$), 165.1256 ($C_{11}H_{17}O$, calcd 165.1279, $M^+ - C_{19}H_{35}$), 109.1007 (C_8H_{13}, calcd 109.1017, $M^+ - C_{22}H_{39}O$), 69.0671 (C_5H_9, calcd 69.0704, base peak, $M^+ - C_{25}H_{43}O$). ^{1}H NMR (400 MHz, $CDC1_3$, δ, ppm, J/Hz): 0.84 (3H, s, CH_3-28), 0.92 (3H, s, CH_3-24), 0.93 (3H, s, CH_3-26), 0.95 (3H, s, CH_3-27), 0.98 (3H, s, CH_3-29), 0.99 (3H, s, CH_3-30), 1.01 (3H, s, CH_3-25), 1.16 (3H, s, CH_3-23), 3.72 (1H, br.dd, $J_{3\beta,2\alpha} = 5.1$, $J_{3\beta,2\beta} = 2.6$, H-3β).

Antibacterial Activity. The antibacterial activity was determined with the agar well diffusion method [17]. A loopful of 10^4–10^6 suspension of 24 h old broth of each bacterium was streaked on the surface of Muller-Hinton agar (BBL-USA) plates. Wells were dug in the agar with the help of a sterile cork borer. Stock solutions of test compounds or crude extracts containing 2 mg/ml were prepared in sterile dimethyl sulfoxide (DMSO). Dilutions of the stock solution containing 50, 100, 150, and 200 µg were prepared in DMSO. Then 100 µL of each dilution was added in the respective wells. The plates were then incubated at 37°C for 24 h and zones of inhibition were measured in millimeters. Ampicillin, amoxicillin, cephalexin, and tobramycin were used as standard antibacterial antibiotics to compare the extent of activity of the test samples [17].

Antifungal Activity. For the determination of antifungal activity, Sabouraud Dextrose Agar (SDA) slants were used [17]. Then 1 mL of test compound containing 100 mg/mL was added to each test tube. For the control, 1 mL of DMSO was added instead of the test compounds. After solidification of the medium, the slants were streaked with different fungi. These slants were incubated at 30°C for 7–10 days. The growth inhibition of each fungus against the test compound was recorded. Ketoconazole, miconazole, nystatin, and griseofulvin were used as standard antifungal antibiotics in order to compare the activity of the test compounds [17].

Acid Phosphatase Inhibition. *p*-Nitrophenylphosphate (PNP) was purchased from Boehringer, whereas potato acid phosphatase (EC 3.1.3.2) was acquired from Sigma. Buffer and other chemicals were of extra pure analytical grade. Acid phosphatase inhibition was determined spectrophotometerically on a 96-well microtiter plate reader (Molecular Devices, USA) using PNP as substrate by the modified method of Takahisa [18]. The reaction was carried out in 9 mM citrate buffer (pH 4.8) at 37°C. In a typical assay, 20 µL buffer, 90 µL enzyme, and 5 µL test compound solution were mixed and incubated for 60 min. The reaction was started by adding 90 µL of PNP. Hydrolysis of PNP was determined by monitoring the change in color from transparent to yellow due to the release of inorganic phosphate from PNP at a wavelength of 405 nm [18].

Acetylcholinesterase Inhibition. Electric eel acetylcholinesterase, acetylthiocholine, 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), and eserine were purchased from Sigma (St. Louis, Missousi, USA). Acetylcholinesterase inhibition was also determined spectrophotometrically using acetylthiocholine as substrate by the modified method of Ellman [19]. The reaction was carried out in 100 mM sodium phosphate buffer (pH 8.0) at 25°C. In a typical assay, 165 µL buffer, 10 µL enzyme, and 5 µL test compound solution were mixed and incubated for 30 min. DTNB (10 µL) was then added, and the reaction was then initiated by adding 10 µL of acetylthiocholine. Hydrolysis of acetylthiocholine was determined by monitoring the formation of the yellow 5-thio-2-nitrobenzoate anion as a result of the reaction of 5,5′-dithiobis-2-nitrobenzoic acid with thiocholine

released by the enzymatic hydrolysis of acetylcholine at a wavelength of 412 nm. The concentration of the compound that inhibited the enzyme activity by 50% (IC_{50}) was determined by interpolation using increasing levels of the test substances [19].

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