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Isolation of Phenolic Compounds from *Acacia nilotica* with Topical Antiinflammatory Activity

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A quath of *Acacia nilotica* was prepared according to Ayurvedic method from powder of shade dried aerial parts of the plant. Chromatographic separation by repeated column chromatography followed by purification using mixed solvent crystallizations of the quath yielded the phenolic compounds 3,5-dihydroxy-4-methoxy benzoic acid (**1**) and 3,4-dimethoxy-4-hydroxy benzoic acid (syringic acid, **2**). Both these compounds are known compounds and have been isolated for the first time from this plant. Antiinflammatory activity was also studied for compound **1**.

Key Words: *Acacia nilotica,* **Phenols, Antiinflammatory Activity.**

INTRODUCTION

In Ayurvedic system of medicines, plants are used in different forms for various formulations like churna (powder of specific part of the plant), bhasma (processed ash), quath (water concentrate as per prescribed procedure) etc. In Ayurveda, quath is one of the potent formulations which is used for various ailments, prepared as mentioned in Ayurveda by boiling powdered plant material with water to get water concentrate.

In the present study, quath of shade dried aerial parts of *Acacia nilotica* was prepared by using powdered material and water in the ratio 1 : 8 (w/v). The water extract (quath) was concentrated to dryness to get fine powder which was subjected to separation and further purification. Two compounds were isolated. 3,5-dihydroxy-4-methoxy-benzoic acid, (**1**) and 3,4-dimethoxy-4-hydroxy benzoic acid (syringic acid), (**2**).

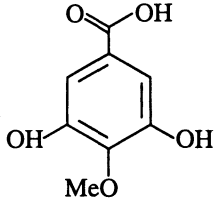

Fig. 1. 3,5-Dihydroxy-4-methoxy benzoic acid (**1**)

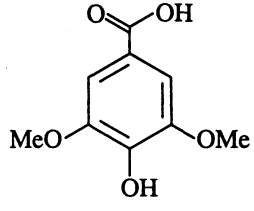

Fig. 2. 3,5-Dimethoxy-4-hydroxy benzoic acid (**2**)

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EXPERIMENTAL

IR spectrum was recorded on FTIR-8300 Shimadzu spectrometer, NMR spectra on Bruker DRX-500, operating at 500 MHz for ^{1}H and at 125 MHz for ^{13}C NMR spectrum and Varian Mercury Plus instrument, operating at 300 MHz for ^{1}H and at 75 MHz for ^{13}C NMR spectrum at 24°C using residual signal of non-deuterated solvents as internal reference. Mass spectrum, EIMS was recorded on Finnigan-Mat 1020C mass spectrometer using ionization energy of 70 eV and LCMS on LCMS-MS Perkin-Elmer Applied Biosystems SCIEX-2000. Thickness of the mice ears was measured using Digitrix mark II micrometer, Japan. Indomethacin was purchased from Fluka, Germany and 12-O-tetradecanoyl phorbol-13-acetate (TPA) was purchased from Sigma-Aldrich, USA.

The aerial parts of *Acacia nilotica* (L.) Willd (Mimosaceae) were collected from forests near Pune. The plant specimen was authenticated by matching with the voucher specimen AHMA: 17000, available with the Agharkar Herbarium of Maharashtra Association, Pune, India.

The animal experiments were conducted as per guidelines suggested by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) under Ministry of Statistics and Programme Implementation, Government of India.

The experiments were carried out using Swiss albino mice which were originally obtained from the National Institute of Virology, Pune, India. They had been inbred at Agharkar Research Institute, Pune, India for several generations. These animals were housed in polypropylene cages at 25 ± 2°C and 10 : 14 h light and dark cycle. They were maintained on commercially available Amrut brand animal feed and water *ad libitum*. After pilot studies, pharmacological studies were carried out using six animals for each treatment.

The air shade dried powder of *Acacia nilotica* (10.0 g) was boiled with distilled water (80.0 mL) and concentrated to get the quath (20 mL). The quath was filtered and the residue was washed with water. The water solution was concentrated to dryness under reduced pressure to afford fine powdered mass (2.724 g). Methanol-soluble part of this solid (extract **F**, 2.108) was separated and subjected to further separation.

Chromatographic separation of constituents of extract F

The extract **F** (2.1 g) was fractioned over silica gel coloumn chromatography (2.5 × 60 cm) (1 : 20 g) starting with toluene (200 mL) followed by toluene/ethyl acetate 3 : 1 (1200 mL), toluene/ethyl acetate 7 : 3 (400 mL), toluene/ethyl acetate 13 : 7 (1000 mL), toluene/ethyl acetate 3 : 2 (200 mL), toluene/ethyl acetate 1 : 1 (200 mL), ethyl acetate (200 mL) and finally MeOH (200 mL). The fractions of 200 mL volume were collected. The progress of the column chromatographic separation was monitored by thin layer chromatography. Fractions showing similar compositions were combined together to obtain twelve major fractions.

Rechromatography of fraction 3 + 4

Fraction 3 + 4 (1.473 g), containing compound **1** was rechromatographed by column chromatography on silica gel (1 : 70 g) using toluene/ethyl acetate 17 : 3

(300 mL) as eluent followed by toluene/ethyl acetate 4 : 1 (200 mL), toluene/ethyl acetate 3 : 1 (200 mL), toluene/ethyl acetate 7 : 3 (400 mL), toluene/ethyl acetate 13 : 7 (100 mL), toluene/ethyl acetate 1 : 1 (100 mL), ethyl acetate (100 mL) and finally MeOH (100 mL). The column chromatography afforded impure compound **1** (0.91 g). It was purified by repeated crystallization using methanol to yield crystals of compound **1** (0.385 g).

Compound **1** was obtained as white crystals and it was analyzed for $C_8H_8O_5$ ([M]+ 184): m.p. 191.0°C; UV spectrum: λ_{max} 217.8, 274.2 nm; IR (nujol) ν_{max}: 3519, 3359, 1691, 1618, 1535, 866, 721 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): 6.95 δ (s, 2H, H-2 and H-6), 8.0 δ (s, 2H, C-3 and C-5 —O<u>H</u>), 7.75 δ (s, 1H, —COO<u>H</u>), 3.7 δ (*s*, 3H, C-4-O-C<u>H</u>$_3$); ^{13}C NMR (125 MHz, DMSO + $CDCl_3$): 119.923 δ (s, C-1), 109.015 δ (d, C-2 and C-6), 145.255 δ (s, C-3 and C-5), 138.208 δ (s, C-4), 166.794 δ (s, <u>C</u>OOH), 51.451 δ (q, C-4-O<u>C</u>H$_3$); EIMS m/z: (M+) 184 (60%), 169 (1), 153 (100), 139 (3), 125 (27), 111 (9), 97 (16), 91 (6), 85 (18), 69 (29), 57 (53).

Rechromatography of fraction 6

Fraction 6 (0.147 g), containing compound **2** was rechromatographed by column chromatography on silica gel (1 : 70 g) using toluene/ethyl acetate 17 : 3 (100 mL) as eluent followed by toluene/ethyl acetate 4 : 1 (100 mL), toluene/ethyl acetate 3 : 1 (100 mL), toluene/ethyl acetate 7 : 3 (500 mL), toluene/ethyl acetate 13 : 7 (300 mL), toluene/ethyl acetate 1 : 1 (200 mL), ethyl acetate (100 mL) and finally MeOH (100 mL). The column chromatography afforded impure compound **2** (0.048 g). It was purified by repeated crystallization using methanol to provide crystals of compound **2** (0.015 g).

Compound **2** was obtained as white crystals and it was analyzed for $C_9H_{10}O_5$ ($[M]^+$ 198): m.p. 205.0°C; UV spectrum: λ_{max} 216.0, 270.0 nm; IR (nujol) ν_{max}: 3519, 3359, 1695, 1620, 1542, 1448, 1028, 866, 769 cm^{-1} ; ^1H NMR (300 MHz, Pyridine–d_5): 7.931 δ (s, 2H, H-2 and H-6), 3.507 δ (s, 6H, C-3 and C-5-O-C<u>H</u>$_3$); ^{13}C NMR (75 MHz, pyridine-d_5): 121.448 δ (s, C-1), 109.198 (d, C-2 and C-6), 146.138 δ (s, C-3 and C-5), 139.080 (s, C-4), 168.636 δ (s, -<u>C</u>OOH), 54.271 δ (q, C-4-O<u>C</u>H$_3$); LCMS *m/z*: 198 (M+), 196, 181, 179.1, 157.3.

Topical antiinflammatory activity study of Compound 1

Ear edema was induced[1] on the right ear of mouse by topical application of 2.5 g of TPA in 20 L of acetone. The left ear (control) received the vehicle (acetone). The compound **1** paste in acetone 0.5 and 1 mg/ear and indomethacin 0.5 mg/ear was applied to right ear simultaneously with TPA. The thickness of the ears was measured before and at 4th hour after induction of inflammation. The edema was measured as an increase in the ear thickness due to TPA application. The results were calculated as mean ± SEM and were analyzed by students t-test for calculation of significance of results (Table-1).

RESULTS AND DISCUSSION

Compound **1**, a white crystalline solid, $C_8H_8O_5$, was purified by repeated mixed solvent crystallization; m.p. 191.0°C; EIMS m/z ($[M]^+$) 184. IR spectrum of

compound **1** shows characteristic bands at 3529 cm^{-1} (hydroxy), broad peak at 3359 cm^{-1} (carboxyl hydroxy), 1691 cm^{-1} (acid carbonyl attached to benzene ring) and 1618 and 1535 cm^{-1} (aromatic C—H stretching). ^1H NMR spectrum shows singlet at 8.0 δ (2H) for hydroxy protons, singlet at 7.75 δ for carboxylic acid proton, singlet at 6.95 δ (2H) for aromatic protons and singlet at 3.7 δ for methyl group. The ^{13}C NMR spectrum of compound **1** shows 6-carbon atoms. The signals appear at 166.79 δ (s) for carboxyl carbonyl group, 145.26 δ (d) for C-3 and C-5 carbons of aromatic ring bearing hydroxyl group, 138.21 δ (*s*) for C-4 aromatic carbon attached to methoxy group, 119.92 δ (*s*) for C-1 attached to carboxyl group, 109.02 δ (*d*) for C-2 and C-6 carbons of aromatic ring and 51.45 δ (*q*) for methyl of methoxy group. This data also confirms the adjacent positions of hydroxy groups with respect to methoxy group. In mass spectrum, the molecular ion peak is observed as $[M]^+$ at m/z 184. The base peak at m/z 153 can be obtained by $[M]^+$-OMe. Similarly, the peak m/z 125 ($[M\text{-}Me\text{-}OH]^+$-CO) also confirms the structure for compound **1** as 3,5-dihydroxy-4-methoxy benzoic acid. This is the known compound and has been already isolated from plants[2-4]. Though this is a known compound, this has been isolated from *Acacia nilotica* for the first time and the antiinflammatory activity was reported for the first time for this compound. Effect of Compound **1** on TPA induced local inflammation was found to be significant though it was less than indomethacin (Table-1).

TABLE-1
EFFECT OF COMPOUND 1 ON TPA INDUCED LOCAL INFLAMMATION

Sr. No.	Treatment		Difference in % inhibition of	
			ear thickness (mm^{-3} ± SEM)	inflammation
1.	Control		21.55 ± 0.83	—
2.	Compound 1	0.5 mg/ear	13.33 ± 0.66[a]	38.15
3.	Compound 1	1.0 mg/ear	8.66 ± 0.66[a]	59.81
4.	Indomethacin	0.5 mg/ear	10.83 ± 1.11[a]	49.74

[a] Significant as compared to control $p < 0.001$.

Compound **2**, a white crystalline solid, $C_9H_{10}O_5$, was purified by repeated mixed solvent crystallization; m.p. 205.0°C; EIMS m/z ($[M]^+$) 198. IR spectrum shows characteristic bands at 3519 cm^{-1} (hydroxyl), broad peak at 3359 cm^{-1} (carboxyl hydroxy), 1695 cm^{-1} (acid carbonyl attached to benzene ring) and 1620 cm^{-1} and 1542 cm^{-1} (aromatic C—H stretching). ^1H NMR spectrum shows singlet at 7.931 δ (2H) for aromatic protons and singlet at 3.507 δ for methoxy methyl groups. The APT spectrum of compound **2** shows 6-carbon atoms. The signals are appeared at 168.836 δ (s) for carboxyl carbonyl group, 146.138 δ (d) for C-3 and C-5 carbons of aromatic ring bearing hydroxyl group, 139.080 δ (s) for C-4 aromatic carbon attached to methoxy group, 121.448 δ (s) for C-1 attached to carboxyl group, 109.198 δ (d) for C-2 and C-6 carbons of aromatic ring and 54.271 δ (q) for methyl of methoxy groups. This data also confirms the adjacent positions of methoxy groups with respect to hydroxy group. In mass spectrum,

the molecular ion peak is observed as $[M]^+$ at m/z 198. The peaks are observed at m/z 196 $[M\text{-}H_2]^+$, m/z 181 $[M\text{-}H_2\text{-}Me]^+$. The base peak observed at m/z 179.1 $[M\text{-}H_2\text{-}OH]^+$ is due to loss of hydrogen molecule followed by hydroxy radical. This data also reveals the structure for compound **2** as 3,4-dimethoxy-4-hydroxy benzoic acid. This is a known compound[3, 5-14] but has been isolated from *Acacia nilotica* for the first time. From literature survey, this compound is recognized as syringic acid.

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