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Isolation, Identification and Characterization of Secondary Metabolites in Methanol Extract of Stem Bark of Acacia Nilotica

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Abstract
Acacia nilotica is commonly known as babul or kikar belongs to the fabaceae family and showed diverse pharmacological actions. Significance of various parts of this plant is mentioned in the traditional medicine system of India. The methanol extract of stem bark of Acacia nilotica *was investigated for its phytochemical constituents through chromatographic and spectroscopic techniques. Lupenone, Niloticane, Acanilol-A and Acacetin secondary metabolites were successfully isolated, identified and characterized. These compounds were purified using silica gel column chromatography followed by TLC and structurally elucidated using mass spectrum, FTIR spectrum, ^{1}HNMR spectrum and ^{13}CNMR spectrum data.*
Keywords: Acacia Nilotica*, Methanol Extract, Phytochemicals, Secondary Metabolites, Lupenone, Niloticane, Acanilol-A, Acacetin.*

1. INTRODUCTION

Acacia genus belongs to the family fabaceae and Acacia genus comprises of 800 plant species. They occupy the largest area of Rajasthan, Gujarat and the Deccan [1]. Some of its species used in traditional medicine system. Acacia nilotica is commonly known as babul or kikar in Rajasthan [2]. Bark of this plant showed antimicrobial activity and play role for hair wash [3]. It bark powder is used as tooth powder [4,5]. In ayurvedic medicine bark, leaves, flowers and pods of this plant are used against bleeding piles, cold, cough, cancer, congestion, fever, diarrhea, dysentery, gall bladder, leucoderma, hemorrhoid, leprosy, tuberculosis, ophthalmia, sclerosis, small pox and menstrual problems [6,7]. Tannins, stearic acid, vitamin-C, carotene, protein, arabin, calcium, magnesium and selenium are found in different parts of this plant [8,9]. This plant contains different types of bio-active compounds such as gallic acid, ellagic acid, isoquercitin, leucocyanadin, kaempferol-7-diglucoside, glucopyranoside, rutin, and their derivatives [10-14].

Experimental

General Experimental Procedures

TLC was conducted on aluminium sheet Kieselgel 60 F_{254} (E.Merck). Silica gel (E.Merck, 60-120 mesh, 560gm) used for column (1.7m × 4.5cm) chromatography. The FTIR spectra were recorded on FTIR SHIMADZU 8400S spectrometer with nujol. The ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ at 400 MHz and 100 MHz on a Brucker NMR instrument, respectively, using TMS as internal standard. FAB mass spectra were recorded on JEOL SX 102 /DA-6000 mass spectrometer using Argon /Xenon as FAB gas.

Plant Material: 2.7 kg stem bark of Acacia nilotica collected from village Baldhan Kalan, District Rewari, Haryana India. Plant material was shade dried and it takes approx 4 weeks. Shade dried plant material (1.2 kg) was coarsely grind to powder.

Extraction: The plant material (stem bark of Acacia nilotica) was extracted with methanol for 12 hours × 6 days. Obtained extract was concentrated under reduced pressure to give crude extract. 24 gm of methanol extract of stem bark of Acacia nilotica was obtained.

Column Chromatography: Methanol extract was subjected to silica gel column chromatography (60-120 mesh, column size: 1.7 m × 4.5 cm) with gradient elution using hexane and ethyl acetate mixtures. Fractions were collected and monitored using TLC. Based on R_f values and purification profiles, four compounds were isolated.

Extraction And Isolation Of The Constituents: Isolation of Lupenone: Compound-A was isolated when column was eluted with Hexane + EtOAc in 90 : 10 ratio. The solvent was removed under reduced pressure. The obtained white amorphous powder was crystallized with methanol. Melting point of this compound was determined as 216-218 °C. MS (m/z): 425 $(M+H)^+$, 424, 409, 381, 368, 355, 313, 245, 232, 218, 205, 189, 175, 161, 149, 135, 121, 109. IR (KBr, cm^{-1}): 1745 (=C=O, stretching), 1652 (>C=C< stretching), 1392 and 1367 (C–H stretching of >C-$(CH_3)_2$ group). ^{1}H NMR (δ ppm, $CDCl_3$): 4.67 (s, 1H, C-29), 4.54 (s, 1H, C-29), 2.34 (m, 2H, C-21), 1.67 (s, 3H, C-30), 0.96 (s, 3H, C-23), 0.91 (s, 3H, C-28), 0.83 (s, 3H, C-26), 0.80 (s, 3H, C-24), 0.76 (s, 3H, C-25), 0.74 (s, 3H, C-27), 1.26-1.78 (remaining 23 protons). ^{13}C NMR (δ ppm, $CDCl_3$): 39.90 (C-1), 36.20 (C-2), 214.50 (C-3), 46.84 (C-4), 55.43 (C-5), 21.10 (C-6), 34.68 (C-7), 41.38 (C-8), 49.74 (C-9), 38.33 (C-10), 20.98 (C-11), 26.30 (C-12), 37.94 (C-13), 42.72 (C-14), 29.60 (C-15), 36.20 (C-16), 43.17 (C-17), 44.10 (C-18), 48.38 (C-19), 151.14 (C-20), 30.85 (C-21), 40.11 (C-22), 16.10 (C-23), 24.02 (C-24), 18.10 (C-25), 16.96 (C-26), 15.63 (C-27), 19.59 (C-28), 110.00 (C-29), 21.34 (C-30). Molecular formula $C_{30}H_{48}O$.

Isolation of Niloticane: Compound-B was isolated when column was eluted with Hexane + EtOAc in 70 : 30 ratio. The solvent was removed under reduced pressure. Colour of obtained amorphous powder compound was pale yellow and melting point of this compound was determined as 178–180°C. MS (m/z): 319 $(M+H)^+$, 301, 283. IR (KBr, cm^{-1}): 3365 (-O-H, stretching), 2920 (-C-H, stretching, sp^3), 1712 (=C=O, stretching), 1442 (CH_2, bending), 1070 (-C-O, stretching), 890 (=C-H, bending). ^{1}H NMR (δ ppm, $CDCl_3$): 3.47 (t, 1H, C-3), 4.91 (s, 1H, -OH at C-3), 0.89 (s, 3H, C-18), 0.92 (s, 3H, C-19), 2.16 (t, 1H, C-5), 1.08 (s, 3H, C-20), 1.99 (s, 3H, C-17), 5.96 (s, 1H, -OH at C-12), 5.15 (s, 1H, C-12), 6.32 (m, 1H, C-15), 5.12 (d, 2H, C-16), 1.67- 1.91 (m, for remaining 10 protons). ^{13}C NMR (δ ppm, $CDCl_3$): 36.84 (C-1), 27.72 (C-2), 78.50 (C-3), 38.70 (C-4), 54.08 (C-5), 21.08 (C-6), 28.44 (C-7), 38.43 (C-8), 62.19 (C-9), 39.33 (C-10), 207.63 (C-11), 73.44 (C-12), 133.84 (C-13), 150.27 (C-14), 132.91 (C-15), 121.62 (C-16), 18.48 (C-17), 23.78 (C-18), 20.78 (C-19), 22.16 (C-20). Molecular formula $C_{20}H_{30}O_3$.

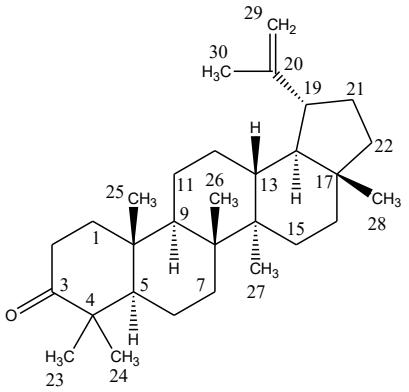
Isolation of Acanilol-A: Compound-C was isolated when column was eluted with Hexane + EtOAc in 50 : 50 ratio. Melting point of this white amorphous powder was 207-209 °C. MS (m/z): 356 $(M)^+$, 341, 313. IR (KBr, cm^{-1}): 3248 (-O-H, stretching), 2920 (-C-H, stretching, sp^3), 1655 (>C=O, stretching), 1627 (C=C, stretching), 1197 (-C-O-, stretching). ^{1}H NMR (δ ppm, $CDCl_3$): 13.42 (s, 1H, -OH at C-8), 7.39 (s, 1H, C-1), 6.78 (s, 1H, C-4), 6.59 (s, 1H, C-11), 6.39 (s, 1H, C-9), 5.18 (s, 2H, C-5), 4.05 (s, 3H, -OCH_3 at C-2), 3.98 (s, 3H, -OCH_3 at C-3), 3.92 (s, 3H, -OCH_3 at C-10). ^{13}C NMR (δ ppm, $CDCl_3$): 109.71 (C-1), 151.82 (C-2), 152.54 (C-3), 111.33 (C-4), 70.38 (C-5), 175.38 (C-7), 161.30 (C-8), 97.62 (C-9), 164.40 (C-10), 92.63 (C-11), 158.75 (C-12), 145.44 (C-13), 121.73 (C-14), 130.64 (C-15), 140.50 (C-16), 105.33 (C-17), 56.79 (-OCH_3 at C-2), 58.20 (-OCH_3 at C-3), 56.04 (-OCH_3 at C-10). Molecular formula $C_{19}H_{16}O_7$.

Isolation of Acacetin: Compound-D was isolated when column was eluted with Hexane + EtOAc in 30 : 70 ratio. Pale yellow amorphous powder was obtained. Melting point of this compound was 262-264 °C. MS (m/z): 285 $(M+H)^+$, 270, 257, 240, 153, 133, 119. IR (KBr, cm^{-1}): 3515 (-O-H, stretching), 3022 (Ar =C-H, stretching), 1697 (-C=O, stretching), 1575, 1570 (Ar C-C, stretching), 1535 (-C=C-, stretching), 1215 (-C-O, stretching), 1032 (-C-O-C, stretching), 815, 765 (-C-H, bending). ^{1}H NMR (δppm, $CDCl_3$): 12.89 (s, 1H, -OH at C-5), 9.12 (s, 1H, -OH at C-7), 7.42 (d, 2H, C-3' and C-5'), 6.91 (d, 2H, C-2' and C-6'), 6.72 (s, 1H, C-3), 6.24 (s, 1H, C-8), 6.15 (s, 1H, C-6), 3.96 (s, 3H, -OCH_3 at C-4'). ^{13}C NMR (δppm, $CDCl_3$): 163.74 (C-2), 105.69 (C-3), 182.28 (C-4), 160.52 (C-5), 99.24 (C-6), 164.48 (C-7), 94.74 (C-8), 159.11 (C-9), 104.33 (C-10), 124.05 (C-1'), 127.90 (C-2'), 113.52 (C-3'), 162.28 (C-4'), 113.85 (C-5'), 127.10 (C-6') 59.23 (-OCH_3 at C-4'). Molecular formula $C_{16}H_{12}O_5$.

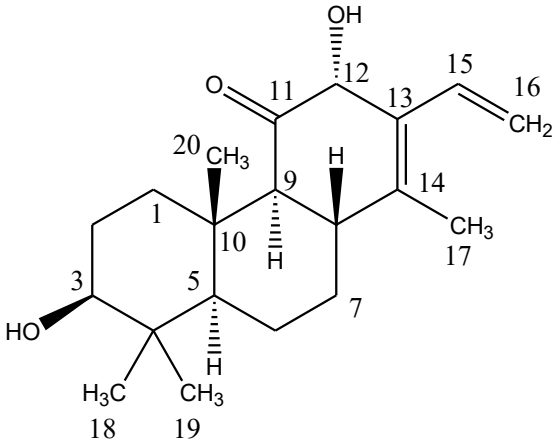
2. RESULTS AND DISCUSSION

Characterization of Lupenone: The molecular ion peak in the mass spectrum appeared at m/z 425 $[M+H]^+$, and other significant fragmentation peaks appeared at m/z 424, 409, 381, 368 and 355 indicating a molecular formula of $C_{30}H_{48}O$ and support a triterpenoid skeleton. The IR spectrum (KBr, cm^{-1}) showed a strong absorption band at 1745 cm^{-1}, characteristic of a ketonic carbonyl (C=O) group. A band at 1652 cm^{-1} suggests the presence of an olefinic (>C=C<) group. Absorptions at 1392 and 1367

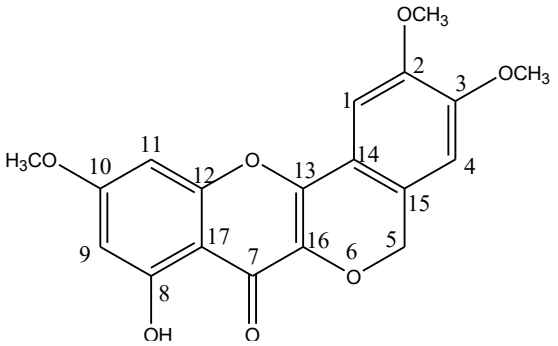
cm^{-1} are due to C–H stretching vibrations associated with gem-dimethyl groups (>C–$(CH_3)_2$), which are commonly found in triterpenes. In the ^{1}HNMR spectrum (δppm, $CDCl_3$) two singlet peaks was observed at δ 4.67 and δ 4.54 for the protons attached at C-29 position while another singlet peak observed at δ 1.67 for the proton attached at C-30 position. Six singlet peaks also observed at different δ values i.e. δ 0.96 (s, 3H, C-23), 0.80 (s, 3H, C-24), 0.76 (s, 3H, C-25), 0.83 (s, 3H, C-26), 0.74 (s, 3H, C-27) and 0.91 (s, 3H, C-28), for the methyl groups at C-23, C-24, C-25, C-26, C-27 and C-28 respectively. A multiplet also observed at δ 2.34 (m, 2H, C-21) for the 2 protons attached at position C-21. The broad region between δ 1.26-1.78 accounts for the overlapping methylene and methine protons in the aliphatic backbone. In the ^{13}CNMR spectrum (δppm, $CDCl_3$), absorptions observed at 16.10 (C-23), 24.02 (C-24), 18.10 (C-25), 16.96 (C-26), 15.63 (C-27) and 19.59 (C-28) confirmed the presence of six methyl groups. The signals observed at 110.00 and 151.14 were assigned for C-29 and C-20 carbon atoms respectively. The absorption for methyl group at C–30, which is attached to olifinic carbon atom, was appeared at 21.34. The absorption at 214.50 showed the presence of carbonyl group attached at C-3 position. The ^{13}CNMR values of other carbon atoms in compound-A were recognized as 39.90 (C-1), 36.20 (C-2), 46.84 (C-4), 55.43 (C-5), 21.10 (C-6), 34.68 (C-7), 41.38 (C-8), 49.74 (C-9), 38.33 (C-10), 20.98 (C-11), 26.30 (C-12), 37.94 (C-13), 42.72 (C-14), 29.60 (C-15), 36.20 (C-16), 43.17 (C-17), 44.10 (C-18), 48.38 (C-19), 151.14 (C-20), 30.85 (C-21) and 40.11 (C-22). On the basis of above spectral data analysis, compound-A was identified as Lupenone [15].



Characterization of Niloticane: Molecular formula of the compound was determined to be $C_{20}H_{30}O_3$ based on the mass spectrum, which showed a molecular ion peak at m/z 319 $[M+H]^+$, along with fragment peaks at m/z 301 and 283. The IR spectrum (KBr, cm^{-1}) showed a broad absorption at 3365 cm^{-1} due to –O-H stretching, a strong peak at 1712 cm^{-1} corresponding to a ketonic carbonyl group (>C=O) and absorption at 2920 and 1442 cm^{-1} representing aliphatic –C–H stretching and CH_2 bending, respectively. Additional peaks at 1070 cm^{-1} and 890 cm^{-1} indicated C–O stretching and olefinic C–H bending vibrations. In the ^{1}HNMR spectrum (δppm, $CDCl_3$) of this compound observed a triplet at δ 3.47 ppm (1H, C-3) indicates a proton on a carbon bearing a hydroxyl group, supported by a singlet at δ 4.91 ppm (1H) corresponding to the -OH proton at C-3. The spectrum shows methyl singlets at δ 0.89 (3H, C-18), δ 0.92 (3H, C-19), δ 1.08 (3H, C-20) and δ 1.99 (3H, C-17) which are typical of tertiary methyl groups. The presence of signals at δ 5.15 (s, 1H, C-12), δ 5.96 (s, 1H, –OH at C-12) indicates the presence of hydroxyl group at C-12 position while signals observed at δ 6.32 (m, 1H, C-15) and 5.12 (d, 2H, C-16) suggesting olefinic protons. A triplet signal observed at δ 2.16 ppm (1H, C-5) for the one proton at C-5 position. Remaining protons observed as multiplet at δ 1.67-1.91 ppm. Peak observed in the ^{13}CNMR spectrum (δppm, $CDCl_3$) at δ 207.63 ppm is indicate the presence of carbonyl group (C=O) at C-11 position. Signals observed at δ 78.50 ppm (C-3) and δ 73.44 ppm (C-12) suggest hydroxylated methine carbons, supporting the presence of -OH groups at C-3 and C-12 positions. The olefinic nature of the molecule is evident from the signals at δ 133.84 (C-13), 150.27 (C-14), 132.91 (C-15) and 121.62 (C-16), which correspond to sp^2 hybridized carbons forming a double bond system. Remaining signals obtained at 36.84 (C-1), 27.72 (C-2), 78.50 (C-3), 38.70 (C-4), 54.08 (C-5), 21.08 (C-6), 28.44 (C-7), 38.43 (C-8), 62.19 (C-9), 39.33 (C-10), 18.48 (C-17), 23.78 (C-18), 20.78 (C-19), 22.16 (C-20). On the basis of above spectral data analysis, compound-B was identified as Niloticane [16-17].

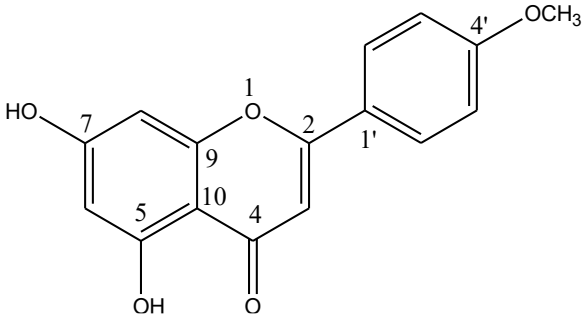


Characterization of Acanilol-A: The molecular ion peak observed at m/z 356 $[M]^+$ and key ion peaks at 341 and 313 in the mass spectrum corresponds to a molecular formula of $C_{19}H_{16}O_7$. In IR spectrum (KBr, cm^{-1}) a broad peak observed at 3248 cm^{-1} indicate the –O-H group, while a strong absorption at 1655 cm^{-1} is due to a carbonyl (>C=O) group. Bands at 1627 cm^{-1} correspond to aromatic C=C stretching and the peak at 1197 cm^{-1} is assigned to C–O stretching vibrations of methoxy groups. The ^{1}HNMR spectrum (δppm, $CDCl_3$) exhibits characteristic signals indicative of a highly substituted aromatic compound. A downfield singlet at δ 13.42 ppm (s, 1H, -OH at C-8) corresponds to a hydroxyl proton at C-8, suggesting strong intramolecular hydrogen bonding typically observed in phenolic structures. The spectrum shows four aromatic singlets at δ 7.39 (C-1), 6.78 (C-4), 6.59 (C-11), and 6.39 ppm (C-9), each integrating for one proton, indicating a tetrasubstituted aromatic ring system with isolated protons. A singlet at δ 5.18 ppm integrating for two protons is attributed to a methylene group at C-5, likely positioned adjacent to an aromatic ring or within a side chain. Additionally, three sharp singlets at δ 4.05 (s, 3H, -OCH_3 at C-2), 3.98 (s, 3H, -OCH_3 at C-3), and 3.92 ppm (s, 3H, -OCH_3 at C-10), each integrating for three protons, correspond to methoxy groups attached at C-2, C-3, and C-10, respectively. The ^{13}CNMR spectrum (δppm, $CDCl_3$) of this compound displays signal at δ 175.38 (C-7) indicates a carboxylic group and three methoxy carbon signals are observed at δ 56.79 (–OCH_3 at C-2), 58.20 (–OCH_3 at C-3) and 56.04 (–OCH_3 at C-10), confirming the presence of methoxy substitutions on the aromatic ring. A peak observed at δ 161.30 (C-8) indicates a carbon attached with hydroxyl group. Other Signals observed at δ 109.71 (C-1), 151.82 (C-2), 152.54 (C-3), 111.33 (C-4), 70.38 (C-5), 97.62 (C-9), 164.40 (C-10), 92.63 (C-11), 158.75 (C-12), 145.44 (C-13), 121.73 (C-14), 130.64 (C-15), 140.50 (C-16) and 105.33 (C-17). On the basis of above spectral data analysis, compound-C was identified as Acanilol-A [18].



Characterization of Acacetin: The molecular ion peak observed in the mass spectrum at m/z 285 $[M+H]^+$ and other fragmentation peaks at m/z 270, 257 and 240 suggest its molecular formula is $C_{16}H_{12}O_5$. A broad band at 3515 cm^{-1} in the IR spectrum (KBr, cm^{-1}) indicated the presence of –O-H groups while another strong band at 1697 cm^{-1} was attributed to the carbonyl (>C=O) group. Other significant bands observed at 1575 and 1570 cm^{-1} confirmed aromatic C=C stretches and peaks at 1215 and 1032 cm^{-1} represented C–O and C–O–C stretching vibrations, respectively. Bands at 815 and 765 cm^{-1} were associated with aromatic C–H bending, consistent with a substituted benzene ring. In the ^{1}H NMR

spectrum (δppm, $CDCl_3$) observed singlet at δ 12.89 ppm (1H) corresponds to the hydroxyl proton at C-5 position, indicative of strong intramolecular hydrogen bonding with the adjacent carbonyl group. Another hydroxyl proton appears at δ 9.12 ppm (s, 1H), attributed to the free -OH group at C-7 position. The aromatic region shows two doublets at δ 7.42 ppm (2H, d) and δ 6.91 ppm (2H, d), corresponding to the protons at C-3', C-5' and C-2', C-6' respectively. A singlet at δ 6.72 ppm (1H) corresponds to the proton at C-3 and two another singlets at δ 6.24 ppm (1H, C-8) and δ 6.15 ppm (1H, C-6) corresponds to the proton at C-8 and C-6 position respectively. A methoxy singlet at δ 3.96 ppm (3H) corresponds to the $–OCH_3$ group at C-4′. In the ^{13}C NMR spectrum signal observed at δ 182.28 ppm corresponds to the carbonyl carbon at C-4 position. The peaks observed at downfield shifts at δ 160.52 (C-5), 164.48 (C-7) and 159.11 (C-9) suggest the presence of hydroxyl groups at position C-5, C-7 and C-9 respectively. Peak observed at 59.23 ($-OCH_3$ at C-4') and162.28 (C-4') confirm the presence of $-OCH_3$ group at C-4' position. Other signals are observed at 163.74 (C-2), 105.69 (C-3), 99.24 (C-6), 94.74 (C-8), 104.33 (C-10), 124.05 (C-1'), 127.90 (C-2'), 113.52 (C-3'), 113.85 (C-5'), 127.10 (C-6'). On the basis of above spectral data analysis, compound-D was identified as Acacetin (5,7-dihydroxy-4′-methoxyflavone) [19-21].



3. CONCLUSION

Secondary metabolites i.e. Lupenone, Niloticane, Acanilol-A and Acacetin have been isolated from the methanol extract of stem bark of Acacia nilotica by using various chromatographic techniques. Identification and characterization of these phytochemicals was done on the basis of mass spectrum, FTIR spectrum, ^{1}HNMR spectrum and ^{13}CNMR spectrum data. These isolated molecules can be tested and used in various diseases because of their bioactive nature. Presence of these secondary metabolites in the stem bark of Acacia nilotica proves the significance of this plant.

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Conflict of Interest

Authors declare no conflict of interest.

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