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Isolation and characterization of oleonolic acid and lupeol from *Vitex negundo* leaves

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Abstract
Vitex negundo is commonly known as nirgundi belonging to family Verbanaceae and is found throughout India. Ithas been traditionally reported for the treatment of depression, malaria, venereal diseases, asthma, wounds, skin diseases, anti-inflammatory, analgesic, ulcers and snake bite. The secondary metabolites in a pure form such as flavonoids, iridoids, sesquiterpene, diterpenes, lignans and plant steroids have been isolated and identified previously. In the present research work a triterpenoids i.e. oleonolic acid and lupeol isolated and characterized for the first time from *Vitex negundo* leaves.

Keywords: Triterpene, lupeol, nirgundi, Vitex

Introduction
Vitex negundo is herb which is found throughout India, locally known as nirgundi and belongs to family Verbenaceae. In Sanskrit language, the word "nirgundi" is used for plant or any substance that protects the body from the disease(s). The usefulness of the nirgundi has been written in De Materia Medica as well as in the fundamental texts of Ayurveda and Charaka Samhita [1, 2]. Ithas been reported theuse of *Vitex negundo* in traditional medicine for the treatment of depression, venereal diseases, malaria, asthma, allergy, wounds, skin diseases, anti-inflammatory, analgesic, ulcers and snake bite [3]. Various pure compounds such as flavonoids [4] iridoids [5], sesquiterpene [6], diterpenes [7], lignans [8] and plant steroids [9] have been isolated and identified.
Upon phytochemical investigations of about 30 species from the genus Vitex have revealed that the major secondary metabolites of these plants are diterpenoids, ecdysteroids, flavonoids and iridoid glycosides. Other components, such as lignans, phenylpropanoids, sesquiterpenoids and triterpenoids have been also found in some Vitex plants, but with less frequency [10].
But these studies are not enough to identify and characterize the phytochemicals present in the plant. Hence the present study initiated to isolate and characterize the constituents from *Vitex negundo* leaves.

Materials and Methods
Collection and Authentication
Leaves of *Vitex negundo* belonging to family verbanaceae were collected in the month of august from local region of Bhopal district, Madhya Pradesh (India) and were authenticated from Dr Vinayak Naik, Senior Research Scientist, Piramal Life Sciences India Ltd. Goregaon (E), Mumbai. A voucher specimen of the plant (No. NPIL/PLS/08-689) has been deposited for future reference.

Extraction and isolation
All solvents used for extraction were of technical grade, which were distilled and dried before use. Solvents used for Column Chromatography and preparative TLC were of Analytical Reagent grade. Adsorbent for column chromatography was silica gel G 60-120 (Merck).

Procedure for Extraction and Isolation
The air dried powdered leaves (1000g) were defatted with petroleum ether (60-80ºC) and remaining marc was extracted with ethanol (70%v/v) and concentrated in rotary evaporator under reduced pressure to get ethanolic extract (55g). Ethanolic extract was dissolved in ethanol and water (1:2 v/v) and partitioned with ethyl acetate and n butanol in 50mL portion for several times till complete extraction takes place. Resulting ethyl acetate fraction was concentrated under reduced pressure (22g) and was chromatographed on silica gel column (70cmX15cm, 60-120mesh, 2kg) chromatography and preparative TLC [11].

Column was first eluted with chloroform, then polarity of mobile phase was gradually increased by adding methanol in different concentrations (100:0, 95:5, 90:10, 85: 15, 80:20, 70:30, 60:40v/v).
Total 232 fractions each of 50 mL were collected and TLC was performed of each fraction individually. Fractions were pooled on the basis of their TLC profile, pooled fractions (36-40) and (55-75) were selected for the isolation of constituents. Further purification was performed by preparative TLC of isolated constituents.

Detection of Phytosterols
Salkowski test
A few crystals of compound A and B were dissolved in chloroform, to this solution few drops of concentrated sulphuric acid was added. Reddish colour produces shows the presence of phytosterols [12].

LibermannBurchard test
A few crystals of compound A and B were dissolved in chloroform, to this solution few drops of concentrated sulphuric acid followed by few drops of diluted acetic acid, 3mL of acetic anhydride. A bluish green colour formation indicates the presence of phytosterols [13].

Acetylation
The isolated pure compounds were acetylated by refluxing with acetic anhydride and previously distilled pyridine under dry conditions. The reaction mixture was allowed to stay overnight at room temperature and the mixture was concentrated to dryness under reduced pressure [14].

Analytical methods
TLC was performed on silica gel GF $_{254}$ precoated (Merck) plates. IR spectra was recorded with FTIR (Shimadzu), ^{1}H and ^{13}C spectra recorded on Bruker (300MHz and 75.4MHz) in $CDCl_3$ used TMS as internal standard. ESIMS were measured using a Q-TOF micro mass spectrometer (Waters, USA).

Results and Discussion
Ethanolic extract obtained was 5.5% w/w. ethyl acetate extract obtained after partitioned of ethanolic extract was 22g upon column chromatography of ethyl acetate fraction (36-40) yielded (12mg) and fraction (55-75) yielded (10mg) of pure compounds by preparative TLC.
Upon qualitative test performed on pure compounds indicated, it is of triterpenoidal nature.

Structural elucidation of compound A
It was white amorphous powder. m.p.:172-173°C. Mass spectrum showed molecular ion peak m/z at 515(M+1) which corresponds to molecular formula $C_{30}H_{48}O_3$

IR spectrum: An intensely broad band at 3443 cm-1 showed presence of OH streching, 2923, 1730 (carbonyl stretch), 1230 (C-O stretch, ether).

^{1}H NMR (300 MHz, $CDCl_3$, δ, TMS=0): 0.67(3H, S), 0.76(3H, S), 0.80 (3H, S), 0.83(3H, S), 0.85(3H, S), 0.91(3H, S), 1.09(3H, S), 2.04(9H, S), 2.82(2H, d, J=10.8Hz), 3.69(1H, d, J=11.7Hz), 3.87(1H, d, J=11.7Hz), 4.79(1H, m), 5.34(1H, d, J=15.3Hz)

^{13}C NMR (75.4Hz, $CDCl_3$, δ, TMS=0): 143.62, 80.94, 77.43, 76.59, 74.54, 46.52, 45.85, 41.05, 40.52, 39.30, 38.11, 37.70, 36.79, 33.05, 32.29, 31.92, 31.23, 30.67, 30.20, 29.69, 28.04, 27.62, 25.88, 23.57, 22.46, and 22.69
The Compound A gave a positive Liebermann-Burckhardt test. It showed a molecular ion at m/z 515 corresponding to $C_{30}H_{48}O_3$. The ^{1}HNMR spectrum of compound A showed seven tertiary methyl groups at δ 0.67, 0.76, 0.80, 0.83, 0.85, 0.91 and 1.09 on an oleanane skeleton. A doublet of one proton at δ 3.69 and a doublet of one vinyl proton at δ 5.34 were assigned to H-18 and H-12, respectively, suggesting an olea12-ene skeleton. In ^{13}C-NMR spectrum, the signal corresponding to the carboxyl C-28 appeared at δ 143.62. The spectral data were similar to the ones reported for oleanolic acid.

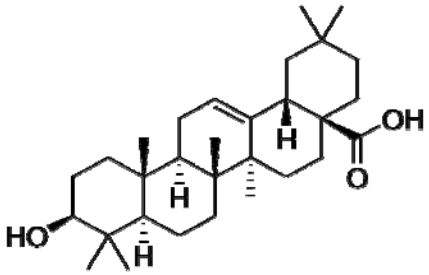

Fig 1: Chemical structure of Oleonolic acid

Characterization of compound B
It was yellowish amorphous powder. m.p: 217-219°C. Mass spectrum of isolated compound showed molecular ion m/z 271.3[M+1] corresponding to the molecular formula $C_{30}H_{50}O$.

IR Spectrum: Intensely broad band at 3454 cm -1, moderate intense band at 1194 cm -1 observed for the OH bond vibration of hydroxyl group. Carbonyl stretch was observed at 1696 cm -1.The corresponding C=C vibrations was shown at 1453 cm -1 was weakly intense band.

^{1}H NMR (300 MHz, $CDCl_3$, δ, TMS=0): 0.83(3H, S), 0.88(3H, S), 0.93(3H, S), 0.94(3H, S),1.00(3H, S),1.06(3H, S),1.18(3H, S), 2.21(1H, S), 3.18(1H, m), 5.05(2H, S), 5.11(2H, S).

^{13}C NMR (75.4Hz, $CDCl_3$, δ, TMS=0): 79.05, 77.43, 64.39, 59.07, 55.19, 47.72, 47.24, 46.83, 42.08, 41.72, 40.02, 39.80, 38.78, 37.15, 36.90, 34.74, 33.75, 32.94, 31.93, 29.70, 28.75, 27.28, 26.94, 26.62, 25.99, 23.69, 23.53, 23.37, 23.27 and 22.69,
^{1}H NMR spectrum, revealed the presence of seven tertiary methyl protons at δ 0.83, δ 0.88, δ 0.93, δ 0.94, δ 1.00, δ 1.06, and δ 1.18. A sextet of one proton at δ 2.21 corresponds to 19 β –H is characteristic of lupeol. H-3 proton appeared as multiplet at δ 3.18 while two broad singlets at δ 5.05 and δ 5.11 due to two exomethylene protons attached at C29.
^{13}C NMR spectrum,showed seven methyl groups at δ 28.75 (C- 23), δ 27.28 (C-28), δ 26.62 (C-25), δ 25.99 (C-26), δ 23.69(C-24), δ23.37(C-27), and δ 22.69 (C-30). The deshielded signals at δ 79.05 was due to presence of hydroxyl group at C-3. The comparison with the spectral data with reported led us to propose the structure as lupeol.

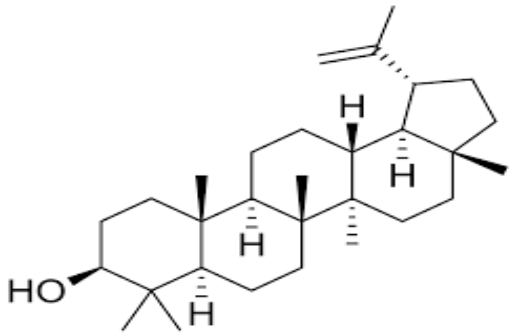

Fig 2: Chemical structure of Lupeol

Conclusion

The physical, chemical and spectral evidence of compound A and compound B confirms that the given constituents are oleonolic acid and lupeol respectively.

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