

Fractionation, Characterization and Evaluation of Biocidal Potential of Active Principles of Leaves of *Vitex Negundo* Linn

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Three chemical compounds, *n*-pentatriacontane, *p*-hydroxybenzoic acid and 5-hydroxyisophthalic acid have been isolated and characterized on fractionation of hexane and methanol extracts of leaves of *Vitex negundo* L. Presence of *n*-pentatriacontane in the leaf extracts of this plant. The antimicrobial activities of the separated fractions against fungal pathogens like *Mycogone perniciosa* and *Rhizoctonia solani* and six bacteria *Bacillus megatrium*, *Escherichia coli*, *Pseudomonas fluorescense*, *Sarcina lutea*, *Staphylococcus* spp. and *Xanthomonas* spp. have been reported.

Key Words: Biocidal potential, *Vitex Negundo* Linn.

INTRODUCTION

In continuation with the anti-fungal and anti-bacterial activity observed by us¹ in different types of aqueous and methanol extracts of the leaves of *Vitex negundo* L., which made it clear that these extracts are highly potent against some of the fungal/bacterial pathogens, and keeping in view the possibility of presence of newer unreported compounds in addition to the already reported ones²⁻⁶, an attempt has been made in the present communication for the fractionation of the extracts of leaves of *V. negundo* as well as for getting them separated into various components, purifying them and subjecting to various methods of identification. The separated compounds are expected to be wide spectrum antimicrobial chemical substances having selective/specific fungicidal/bactericidal properties. The isolation, separation and identification of such compounds also becomes significant from this viewpoint also. An attempt has also been made to study the specific activity of the separated components/fractions against some fungi and bacteria for which the investigations have not been made so far.

EXPERIMENTAL

The plant of *V. negundo* commonly called as Nirgundi and belonging to

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Verbenaceae was collected from Sanjay forest, Pantnagar (latitude 29° 05' 28.5", longitude 79° 29' 32.4" and altitude 238 m). The collected material was carried in polythene bags to the laboratory and dried in shade for 22 days. Fungal and bacterial cultures were procured from the department of Plant pathology, College of Agriculture, G.B. Pant University of Agriculture and Technology, Pantnagar. They were cultured in our laboratory in petriplates containing potato dextrose agar media. Leaves of *V. negundo* were dried in shade for 22 days, ground in mixer to pass 100 mm sieve, 10 g dry powder of dry leaves was extracted four times with 5 mL hexane/methanol for 48 h at room temperature for preparation of extracts; all extracts of one type were combined and concentrated by flash evaporation at 40°C to 2 mL (*i.e.*, 20 μ L extract equivalent to 100 mg of plant material).

Mycogone pernicioso and *Rhizoctonia solani*, the two important fungal pathogens which cause the wet bubble disease of mushroom and sheath blight disease of rice respectively, were selected for studying the fungicidal potential of the separated fractions/compounds separated from the fractions. In addition to it, six bacteria *Bacillus megatrium*, *Escherichia coli*, *Pseudomonas fluorescence*, *Sarcina lutea*, *Staphylococcus* spp. and *Xanthomonas* spp. were also employed for these studies. The antimicrobial activity of hexane and methanol fractions was studied by using the paper disc method⁷, incubating the petriplates at 28°C and measuring the inhibition zones after four days of incubation. Dumb-bell shaped growth was observed in case of the extracts containing bioactive components.

The extracts were subjected to gradient elution column chromatography using the following parameters: Column length: 60 cm (for hexane extract) or 45 cm (for methanol extract); diameter: 5 cm; adsorbent: silica gel H (60–120 mesh size); eluent (see Table-1). A total of 127 fractions (of 75 mL each) and 64 fractions (of 50 mL each) were collected in case of the hexane and methanol extracts respectively.

Hexane fraction number '2', methanol fractions number '18–20' and methanol fractions number '31–35' only, could be collected in sufficient quantity. Fraction '2' of hexane extracts (being named as compound 'A' henceforth) was found to be TLC single and subjected to various analytical techniques like spectral studies etc. for the purpose of identification. Fraction '18–20' of the methanol extract were mixed and subjected to TLC. Four spots were obtained. The spot with second highest R_f value (being named as component 'B' henceforth) could be separated in good quantity using preparative TLC (adsorbent = silica gel G, eluent = CHCl_3 + CH_3OH (8 : 2 by volume, visualizer = I_2 , plate thickness = 0.5 mm). It was subjected to identification studies. Fractions '31–35' of methanol extract were also mixed together and treated similarly to separate four components out of which the major colourless component 'C' with second highest R_f was collected in good amount using preparative TLC as described above and could be used further for identification studies.

TABLE-1
ELUENTS USED FOR FRACTIONATION OF EXTRACTS OF LEAVES OF
V. NEGUNDO

Hexane extract		Methanol extract	
Eluent	Fraction number*	Eluent	Fraction number**
Hexane	1-7	Methanol	1-10
Hexane + CHCl ₃ (75 : 25)	8-14	CHCl ₃ + Methanol (90 : 10)	11-15
Hexane + CHCl ₃ (50 : 50)	15-21	CHCl ₃ + Methanol (80 : 20)	16-20
Hexane + CHCl ₃ (25 : 75)	22-28	CHCl ₃ + Methanol (70 : 30)	21-25
CHCl ₃	29-35	CHCl ₃ + Methanol (60 : 40)	26-30
CHCl ₃ + EtOAc (75 : 25)	36-42	CHCl ₃ + Methanol (50 : 50)	31-35
CHCl ₃ + EtOAc (50 : 50)	43-49	CHCl ₃ + Methanol (40 : 60)	36-40
CHCl ₃ + EtOAc (25 : 75)	50-56	CHCl ₃ + Methanol (30 : 70)	41-45
EtOAc	57-64	CHCl ₃ + Methanol (20 : 80)	46-50
EtOAc + Acetone (75 : 25)	65-71	CHCl ₃ + Methanol (10 : 90)	51-55
EtOAc + Acetone (50 : 50)	72-78	Methanol	56-64
EtOAc + Acetone (50 : 50)	79-87		
Acetone	88-98		
Acetone + Methanol (75 : 25)	99-105		
Acetone + Methanol (75 : 25)	106-117		
Acetone + Methanol (75 : 25)	118-122		
Methanol	123-127		

*Fractions of 75 mL each.

†Fractions of 50 mL each.

RESULTS AND DISCUSSION

Compound 'A' from hexane extract: This component was recrystallized in petroleum ether (60-80°C) forming colourless needles and characterized as a saturated long chain wax like alkane, pentatriacontane with the molecular formula CH₃·(CH₂)₃₃·CH₃ and molecular weight 492, melting point [73.8°C (lit. 75°C)¹⁵], UV-Vis spectrum [λ_{\max} at 213 nm signifying the $\sigma \rightarrow \sigma^*$ transitions], IR spectrum in KBr [2920 cm⁻¹ (m) and 2850 m (due to C—H stretching); 1370 m, 1465 s, and 1460 s (due to C—CH₃ bending); 730 s and 720 s (cm⁻¹) which may be due to —(CH₂)_n— in plane bending vibrations with the possibility of a large value of 'n' due to highly intense bands^{10, 12, 13}; ¹H NMR spectrum in CDCl₃ [δ = 0.88, T, (6-H) (triplet of —CH₃ group split by adjacent —CH₂ group) and δ = 1.254, S, (66 H) (broadening of split —CH₂ peaks and their merging caused by the virtual coupling of protons of —CH₃ and —CH₂ characterized by a value of coupling constants of protons of —CH₂ groups greater than the difference in their δ values¹⁰ and the protons of many —CH₂ groups behaving as a unit¹³ with integral peak area values suggested the ratio of protons to be 1 : 11. ¹³C NMR spectrum in MeOH [observed shifts at 31.93, 29.70, 29.37, 28.87 and 22.70 ppm

(calculated δ being 21.3, 25.8, 32.6, 32.5, and 32.7 ppm)¹⁰] and mass spectrum [base peak at $m/z = 57$ and M-2 peak at $m/z = 490$; low intensity molecular ion peak and fragmentation characterized by clusters of peaks besides the feature that the corresponding peaks of each cluster are 14 mass units apart, C₃ to C₅ being the most abundant fragments, the fragment abundances decreasing in a smooth curve down to M-C₂H₅ and the M-CH₃ peak being very weak—all characteristics of straight chain wax type saturated hydrocarbon¹⁰].

Component 'B' of methanol extract: This compound was crystallized as colourless prisms in EtOH, responded positively for acid and phenolic groups¹¹ and characterized as *p*-hydroxy benzoic acid, C₇H₆O₃ with molecular weight = 138, on the basis of melting point [213°C (Lit. 213–214°C)¹⁵] UV-Vis spectrum in EtOH [the principal π - π^* band at 255 nm which matches the reported value for *p*-hydroxy-benzoic acid¹⁶], IR spectrum in KBr [3385 (intermolecular H-bonded O—H stretching), 2822, 2663 and 2546 (overtone and combinations of O—H in-plane stretching vibrations), 1926 (overtone of C—H out of plane bending mode and combination bands in aromatic systems), 1674 (characteristic band of *p*-hydroxybenzoic acid¹⁰), 1594, 1608 and 1509 (C—C ring stretch of phenyl nucleus), 1447 and 1422 (ring stretch bands for the coupled O—H in-plane bending and C—O stretching with lower frequency band being stronger) 1316 and 1291 (O—H in-plane bending vibrations in carboxylic acids and phenols both), 1243 (sharp band due to C—H in-plane bending in phenyl ring), 1128, 1101 and 1012 cm⁻¹ (C—O stretching)]; ¹H NMR [$\delta = 12.70$ (S) single peak due to rapid carboxylic proton exchange with the protons of OH of hydroxy acids^{10, 12}, $\delta = 7.8$ – 7.9 , (D), (2H) and at $\delta = 6.81$ – 6.84 , (D), (2H) with the same values of coupling constant 'J' = 0.0289 (due to the 4-H of the ring which matches perfectly with the structure of *p*-hydroxybenzoic acid), ¹³C NMR [$\delta = 122.94$, 133.0, 116.14, 163.29, and 170.07 which match with the values reported¹⁴ for *p*-hydroxy-benzoic acid]; Mass spectrum [base line peak at $m/z = 121$ (M-17), molecular ion peak at $m/z = 138$ (which is also the molecular weight of *p*-hydroxybenzoic acid), at $m/z = 93$ (M-45 fragment)].

Component 'C' of methanol extract: This compound was crystallized as light brown crystals in EtOH and characterized as 5-hydroxy-1,3-benzene dicarboxylic acid (5-hydroxyisophthalic acid), with molecular weight 182, on the basis of melting point [287°C (sub) (Lit.15, 288°C)], positive test for acid and phenolic groups, UV-Vis spectrum in MeOH solvent [π - π^* band at 310 nm, K band for —COOH substituted ring at 232 nm], IR spectrum (in KBr) [3075 (intramolecular H-bonded O—H stretching), 1941, 1861, 1704, 1594, 1489, 1457 and 1424, 1385, 1344, 1290, 1263, 1211, 1180 cm⁻¹ and the bands due to 1,3,5-trisubstitution in the benzene ring], ¹H NMR spectrum [$\delta = 12.70$ (S) protons of —COOH and phenolic group shifting the absorption downfield due to intermolecular H-bonding, $\delta = 8.129$ – 8.138 , (T), (1H) and $\delta = 7.628$ – 7.633 , (D), (2H) (peak area and coupling constants supporting the structure of 5-hydroxyisophthalic acid)], ¹³C NMR spectrum [assigning number '1', and '3' to the benzene ring carbon attached to —COOH groups, '5' to that attached to —OH group and C-7 and C-8 to the carbon of the two —COOH groups, the observed δ are 133.65, 123.15, 121.72, 158.92 and 169.07 ppm for C-1 and C-3, C-2, C-4 and C-6, C-5, and C-7 and

C-8 respectively, which are in good agreement with the calculated values¹⁰, i.e., 134.3, 123.8, 121.4, 155.9 and 168.0 ppm respectively]. Mass spectrum [base line peak at $m/z = 182$ (representing the molecular ion peak as well) which is the molecular weight of 5-hydroxyisophthalic acid. The M-17, M-45, M-90 and M-107 peaks at $m/z = 165, 137, 92$ and 75 respectively, further supported the expected fragmentation pattern of 5-hydroxyisophthalic acid].

TABLE-2
BIOCIDAL POTENTIAL OF FRACTIONS OF HEXANE AND METHANOL EXTRACTS

S. No.	Fraction number	Inhibition zone size against the pathogen number‡			
		< 2 mm	2-5 mm	6-10 mm	> 10 mm
1.	2†	1, 2, 5, 6, 7, 8	3	4	—
2.	18*	3, 4, 5, 6	1, 2, 7, 8	—	—
3.	19*	3, 4, 5	1, 2, 7, 8	—	—
4.	20*	3, 6, 7, 8	1, 2, 4, 5	—	—
5.	31*	5, 6, 7, 8	1, 2	4	3
6.	32*	6, 7, 8	1, 5	2, 4	3
7.	33*	6, 7, 8	1	2, 3, 4	5
8.	34*	6, 7, 8	1, 2, 3, 4, 5	—	—
9.	35*	6, 7, 8	1, 2, 3, 4, 5	—	—

*Fraction from methanol extract.

†Fraction from hexane extract.

‡Pathogen number: 1. *M. pernicioso*, 2. *R. solani*, 3. *B. megatrium*, 4. *E. coli*, 5. *P. fluorescence*, 6. *S. lutea*, 7. *Staphylococcus* spp., 8. *Xanthomonas* spp.

The results of the biocidal action of fraction-2 of hexane extract, fractions 18–20 of methanol extract, and fractions 31–35 of methanol extract against the fungal and bacterial pathogens used are presented in Table-2. Fraction '2' of hexane extract containing *n*-pentatriacontane, showed no significant fungicidal potential against any of the fungal pathogens used. However, it has shown good activity against *B. megatrium* forming the inhibition zone of 5 mm. Further, its bactericidal action against *E. coli* is very good. However, it is less potent in comparison to the fractions 31–35 of methanol extract as far as the activity against *B. megatrium* and *E. coli* is concerned.

Fractions 18, 19 and 20 of methanol extract, found to be containing *p*-hydroxybenzoic acid as major component, have shown good activity (zone size 2–5 mm) against both fungal pathogens. Its fungicidal potential is next to, only, the fractions 31, 32 and 33 of the same extract in which 5-hydroxyisophthalic acid has been found to be the major constituent as described above. Fractions 18, 19 and 20 of MeOH extract exhibited good potential against *P. fluorescence*, *Staphylococcus* spp., *E. coli* and *Xanthomonas* spp. It is important to mention that a little work has been reported² on the biocidal potential of crude extracts of some parts of the plant of *V. negundo* against *R. solani* and *E. coli* and no earlier reports are available on the activity of the fractions reported in the present communication against any of the fungi or bacteria used by us.

A perusal of the data in Table-2 reveals that fraction 31–35 is most active out

of the four fractions subjected to identification studies. It possesses maximum potency against both of the fungi used (forming inhibition zones in the range 5–6 mm) as well as against the bacteria *B. megatrium*, *E. coli* and *P. fluorescence* (causing inhibition of 3–17 mm, 3–10 mm and 2–12 mm respectively). Such high activity has not been observed in any other fraction out of the 127 and 64 fractions of hexane and methanol extract respectively which have been tried by us for the evaluation of biocidal potential of the leaves of *V. negundo*.

p-Hydroxybenzoic acid and 5-hydroxyisophthalic acid have also been reported from the alcoholic extracts of leaves of *V. negundo*^{2,8}. Further, a colourless mixture of several *n*-alkanes (C_{27–37}) separated from the petroleum ether extract of seeds of *V. negundo* showed the presence of *n*-tritriacontane, *n*-hentriacontane, *n*-pentatriacontane and *n*-nonacosane on being subjected to GLC⁴. Such compounds are reportedly expected to be present in the plants with the chances that the number of —CH₂ units being 23–33 and the odd numbered members predominating over the even numbered members of the series⁹. However, *n*-pentatriacontane (compound 'A') has not earlier been reported in the leaves of *V. negundo* and we are reporting it first time in the present work.

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