Evaluation of Composition and Antimicrobial Activity of Supercritical Fluid Extract of Leaves of *Vitex negundo*

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Supercritical fluid extract of leaves of *Vitex negundo* was tested for its antimicrobial potential and was compared with that of ethanol extract, ether extract and hydrodistilled oil of leaves. The chemical constituents of extracts were studied by chromatographic techniques. Extracts were evaluated for antimicrobial potential against bacterial strains like Staphylococcus aureus, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa* and yeast *Candida albicans*. Extracts showed prominent antibacterial activity against *Bacillus subtilis* and *Staphylococcus aureus*. Supercritical fluid extract exhibited good antibacterial potential.

Key words: *Vitex negundo*, supercritical fluid extract, antimicrobial activity

Searching among medicinal plants is one way to find new compounds, either as lead compounds or as drugs *per se*. Possible role of herbal drugs for bacterial, fungal and viral diseases is being explored. In present study *in vitro* antimicrobial activity of supercritical fluid extract was investigated and compared with that of different extracts of leaves of plant *Vitex negundo* Linn.

Vitex negundo Linn., belonging to family Verbenaceae, is an aromatic shrub distributed throughout India. In the Ayurvedic system of medicine it is used as a drug of choice in the management of pain, inflammation and other related diseases. Leaves were reported to possess significant anticonvulsant and analgesic activity. Leaves contain many polyphenolic compounds, terpenoids, glycosidic iridoids and alkaloids.

Supercritical fluid extraction is an environment friendly process as it works in closed loop and it is not a new generator of carbon dioxide that is growing concern in global warming. Supercritical carbon dioxide is safe for extraction of natural products thus avoid the use of conventionally used organic solvents which are suspected to be carcinogens. Extracts prepared by supercritical fluid extraction are free of biological contamination as well as heavy metals and has longer shelf life. The environmental benefits of supercritical fluid CO_2 as extracting solvent are well known. It is a potential solvent for replacement of many undesirable organic solvents, which present a threat to the environment, health and safety in the work place[1]. In view of this background, supercritical fluid extract of leaves of *Vitex negundo* Linn was prepared.

Leaves of the plant *Vitex negundo* Linn were collected locally during August to October. Plant material was identified and authenticated by examination of the morphological characteristics at the Agharkar Institute, Pune as leaves of *Vitex negundo* Linn. family Verbenaceae (Auth08-013).

Pure cultures of *Bacillus subtilis* (ATCC6633), *Staphylococcus aureus* (ATCC6538P), *Escherichia coli (*ATCC8739), *Pseudomonas aeruginosa* (ATCC9027) and *Candida albicans* (ATCC10231) were sourced from M. K. Ranganekar Laboratories, Mumbai, where it was procured from National collection of industrial microorganisms, Pune.

The supercritical fluid extract was prepared using methodology given by Vagi *et al.*[2]. Petroleum ether extract and ethanol extract were prepared separately by macerating 10 g of powdered leaves of *Vitex negundo* Linn. with 200 ml of solvent for 3 days and evaporating the solvent under vacuum (0.33 bars) at 45° on rotary evaporator. Steam distilled oil was obtained by hydrodistillation from dried pulverized leaves. All extracts were collected and concentrated to dryness *in vaccuo*.

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Presence of flavonoids and terpenoids in extracts were confirmed by TLC. Ten microliter solution of each extract (1000 µg/ml) was applied in triplicate on a precoated aluminum (10×10 cm^2) TLC plate (silica gel 60 F_{254}, E. Merck) of uniform thickness of 0.2 mm. Plates conditioned for 20 min in a pre-saturated twin-trough chamber (Camag, Muttenz, Switzerland, 10×10 cm^2) were developed in solvent system[3] comprising of ethyl acetate:formic acid:acetic acid:water (100:11:11:27 v/v/v/v) to a distance of 8 cm. After development, plates were air dried. The visualization of flavonoids and phenolic acids was achieved by spraying the plates with NP/PEG reagent. Plates were observed under 365 nm under UV- light.

Solution of 2 mg/ml in chloroform for each extract was prepared by sonicating mixture for 1 min in bath sonicator. Five microliters of each test solution was applied in triplicate on a precoated aluminum TLC plate as mentioned above and developed in solvent system comprising of toluene:ethyl acetate, 93:7 v/v[4] to a distance of 8 cm. After development, plates were air dried. The visualization of constituents on plate was achieved by spraying plate with Anisaldehyde/ sulphuric acid reagent and heating plate at 100º for 10 min then evaluated under visible light.

Paper disc method was used for identification of microorganisms sensitive to *Vitex negundo* extracts and well-diffusion method was used to confirm the antibacterial activity on sensitive microorganisms. Amoxicillin hydrochloride for bacteria (200 µg/ml) and miconazole for yeast (200 µg/ml) were used as positive control. The extracts were mixed with aqueous solution of 10% DMSO (dimethyl sulphoxide) using Tween 80 (0.001%) as surfactant to make uniform dispersion of the strength 25 mg/ml, 6 mg/ml and 3 mg/ml. All samples were sonicated for 5 min on bath sonicator. Aqueous solution of DMSO (10%) and Tween 80 (0.001%) is used as vehicle control.

Paper disc method was used for preliminary screening of extracts for antimicrobial activity. Two milligrams of each extract was loaded on sterile discs of diameter 5 mm made up of Whatman filter paper No. 1 and positive control discs were charged with amoxicillin hydrochloride (20 µg) and miconazole (20 µg). All discs were dried aseptically and were placed on pre-incubated swabbed plates such that each plate contains not more than 4 discs. Well-diffusion method was used for further screening of antimicrobial activity of extracts on sensitive microorganisms. 100 µl of each solution of was added to respective wells. The plates containing bacterial cultures were incubated at 37º for 24 h and those containing yeast were incubated at 25º for 72 h. Diameter of zones of inhibition was measured in millimeter. The inhibition zones were compared with those obtained for antibiotics.

Percent yields of various extracts of leaves of *Vitex negundo* Linn. are given in Table 1. TLC studies showed that the ethanol extract contained flavonoids as the major constituents along with a few terpenoids (fig 1a). Essential oils (terpenoids) were found to be the major constituents of supercritical fluid extract (fig 1b) and steam distilled oil (fig. 1c). Petroleum ether extract contained terpenoids and chlorophyll derivatives.

The zone of inhibition for miconazole by paper disc method was 14 mm. However none of the extracts showed activity against *Candia albicans*. Vehicle control did not show any zone of inhibition in both the assays. Zone of inhibition of amoxicillin hydrochloride (20 µg) was found to be 12 mm in paper disc method and 28 mm in well-diffusion

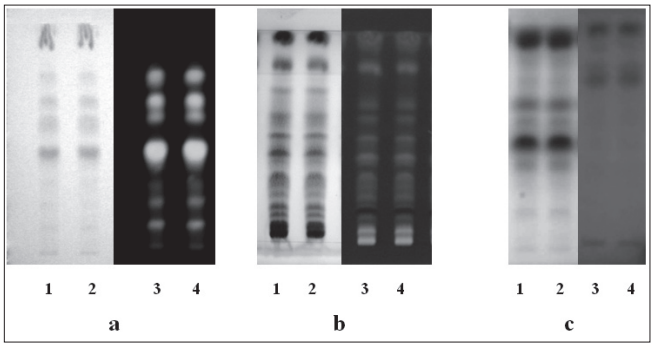

Fig. 1: TLC pattern of various extracts of leaves of *Vitex negundo* (a) flavonoids in ethanol extract of leaves of *Vitex negundo*, track 1 and 2 indicate TLC pattern exposed to visible light and track 3 and 4 at 366nm after derivatisation with NP/PEG reagent (b) TLC pattern of supercritical fluid extract of leaves of *Vitex negundo* observed at 366nm (track 3 and 4) and in visible light after derivatisation with Anisaldehyde sulphuric acid reagent (track 1 and 2). (c) TLC pattern of hydrodistilled oil of leaves of *Vitex negundo* observed at 254nm (track 3 and 4) and in visible light after derivatisation with Anisaldehyde sulphuric acid reagent (track 1 and 2).

TABLE 1: % W/W YIELD OF THE LEAF EXTRACTS OF *VITEX NEGUNDO* LINN.

Extracts	Yield in % w/w
Ethanol extract	15.73
Petroleum ether extract	1.92
Steam distillated oil extract	0.33
Supercritical fluid extract	0.882

method for both *Bacillus subtilis* and *Staphylococcus aureus*. It was observed that *Staphylococcus aureus* was moderately sensitive to ethanol extract (Tables 2 and 3). Supercritical fluid extract, petroleum ether extract and steam distillated oil extract exhibited significant antibacterial activity against the strains *Bacillus subtilis* and *Staphylococcus aureus*. However in the present study, among the five microorganisms tested *B. subtilis* was the most sensitive organism to all the extracts. The ethanol extract showed less lethal activity at the lower concentration as compared to supercritical fluid extract, petroleum ether extract and steam distillated oil extract. In present study *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans* were found resistant to all extracts tested for antimicrobial potential at stated concentration. However, Khokra *et al.* have reported activity of ethanol extract against *Pseudomonas aeruginosa* and *Escherichia coli*[5]

Volatile oil of *Vitex negundo* is reported to contain β-carryophyllene, sabinene, linalool, terpinen-4-ol, α-guaiene and globulol as major constituents along with sesquiterpenes, monoterpenes, terpenoids and sterols. A wide variety of essential oils are known to posses the antimicrobial properties and in many cases this activity is due to the presence of monoterpene constituents which exerts membrane damaging effects and stimulate leakage of cellular potassium ions which provides evidence of lethal action related to cytoplasmic membrane damage. Presence of terpenoids in supercritical fluid extract as evident by TLC pattern explains its stronger antibacterial potential.

In conclusion, our observations confirm that the supercritical fluid extract, steam distilled oil and petroleum ether extract have superior antibacterial activity to that of the ethanolic extract of leaves of *Vitex negundo* Linn. Antimicrobial activity of *Vitex negundo* against gram positive microorganisms suggests that it can be developed as indigenous antimicrobial agent.

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TABLE 2: ANTIBACTERIAL ACTIVITY OF EXTRACTS OF LEAVES OF *VITEX NEGUNDO* LINN. BY PAPER-DISC METHOD

Extracts Concentration (2mg)	Bacteria				Fungi
	S.a.	E.c.	B.s.	P.a.	C. a.
ethanol extract	0.25	-	0.21	-	-
petroleum ether extract	0.21	-	0.21	-	-
steam distillated oil extract	0.21	-	0.25	-	-
supercritical fluid extract	0.25	-	0.25	-	-

Values are activity index = inhibition zone of the test sample divided by inhibition zone of standard i. e. Amoxicillin 20 µg for bacteria (12 mm for S. a. and B. s.) and miconazole for fungi (14 mm for C.a.), zone of inhibition includes the disc diameter of 5 mm, and S. a.- *Staphylococcus aureus*, E. c.- *Escherichia coli* ,B. s- *Bacillus subtilis* P. a.- *Pseudomonas aeruginosa* C. a.- *Candida albicans*

TABLE 3: ANTIBACTERIAL ACTIVITY OF THE LEAF EXTRACTS OF *VITEX NEGUNDO* LINN. BY WELL-DIFFUSION ASSAY

Extracts	Concentration (mg/ml)	Bacteria	
		S.a.*	B.s.*
ethanolic extract	3	-	-
	6	-	0.35±0.07
	25	0.34±0.06	0.42±0.13
petroleum ether extract	3	0.42±0.08	0.42±0.08
	6	0.46±0.07	0.45±0.06
	25	0.53±0.07	0.53±0.09
steam distillated oil extract	3	0.46±0.08	-
	6	0.53±0.09	0.42±0.15
	25	0.60±0.14	0.46±0.06
supercritical fluid extract	3	0.35±0.09	0.39±0.09
	6	0.39±0.06	0.42±0.09
	25	0.53±0.09	0.5±0.08
+ve CONTROL	0.2	1	1

*Values are activity index, which is inhibition zone of the test sample divided by the inhibition zone of standard (amoxicillin 200 µg/ml), zone of inhibition in mm includes the borer diameter 8 mm and S. a.- *Staphylococcus aureus*, B. s.- *Bacillus subtilis*.

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