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Screening of Preliminary Phytochemical Analysis and *In-Vitro* Antimicrobial Activity of Stem Bark Extracts of *Vitex negundo* L.

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ABSTRACT

Antimicrobial activity of stem bark of *Vitex negundo* was studied against nine pathogenic bacteria and three fungal strains by agar well diffusion method. Antimicrobial activity was recorded for hexane, chloroform and methanol extracts. Methanol extract exhibited higher degree of antimicrobial activity compared to chloroform and hexane extracts. *Escherichia coli* was turned out be the most susceptible bacterium to the crude stem bark using the standard Ciprofloxacin (5 µg/disc) and nystatin. Minimum inhibition concentration values of hexane, chloroform and methanol extracts were determined by the agar dilution method ranged between 31.2 and 1000 µg. The study suggested that the stem bark extracts possess bioactive compounds with antimicrobial activity against the tested bacteria and fungi revealing a significant scope to develop a novel broad spectrum of antimicrobial drug formulation from *V. negundo*.

Keywords: *Vitex negundo,* solvent extracts, pytochemicals, antimicrobial activity.

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INTRODUCTION

Vitex negundo (L.) belongs to family Verbenaceae, commonly known as Nirgundi and distributed throughout India. Bark occurs in channelled pieces, 0.3- 0.5 cm thick; outer surface yellowish grey, rough, lenticelular, longitudinally channeled and transversely cracked; inner surface darker than outer, blackish and smooth; fracture short and splintery; taste slightly bitter.

It is widely used in traditional systems of medicine including Ayurveda, Unani and Siddha for management of headache, inflammation, leucoderma, enlargement of the spleen, rheumatoid arthritis, gonerrhoea, bronchitis, fever, cold and cough, lactagogue and emmenagogue as juice, decoction and also as vapor [1-3]. It contains fragrant, volatile oil and resins with nishindaside, negundoside (irridoid glycoside) and artemetin [4,5]. Besides, several alkaloids, glycosides, flavonoids, reducing sugars, sterols, resin and tannins have also been reported [6]. Every part of this plant is valuable in medicine and various preparations of plant have been mentioned in indigenous system of medicines for various skin diseases [7], anti-inflammatory [8], rheumatism [9], antibacterial [10,11]. The availability of few reports on antimicrobial activity, the present study led to the screening of preliminary phytochemical analysis and antimicrobial activity of different solvent extracts of stem bark of *V. negundo*.

MATERIALS AND METHODS

Chemicals, Media and Antibiotics

The organic solvents i.e., hexane, chloroform, methanol and dimethyl sulphoxide (DMSO) were obtained from Rankem company, India. Nutrient broth, Nutrient agar and Saboraud dextrose agar were obtained from Hi-media, Mumbai, India. The antibacterial agents Ciprofloxacin (5 µg/disc) and nystatin were obtained from Axiom Laboratories Ltd., India.

Stem bark collection

The stem bark of *Vitex negundo* was collected from Kambalakonda, Visakhapatnam, Andhra Pradesh. The specimen was authenticated by Prof. M. Venkaiah, Department of Botany, Andhra University, Visakhapatnam and voucher specimen was deposited in the Herbarium, Botany department (BDH), Andhra University, Visakhapatnam.

Stem bark extract

The stem bark was dried in shade (25 - 28 ^{0}C) for a month. The dried stem bark was ground using a mechanical grinder. Sequential extraction of it was done using hexane, chloroform, followed by methanol. The filtrates were concentrated by removing the solvents under reduced pressure at 40 ^{0}C using a rotary evaporator. The concentrated crude extracts were labeled and stored at 4 ^{0}C [12].

At the time of testing known quantity of crude (100, 200, 300 mg/ml) were dissolved in DMSO.

Microbial strains and Growth conditions

Bacteria were selected for the antibacterial activity that include Gram-negative *Escherichia coli* (MTCC B1560), *Klebsiella pneumoniae (*MTCC B4030), *Pseudomonas aeruginosa* (MTCC B2297), *Proteus vulgaris* (MTCC B7299) and Gram positive *Bacillus subtilis* (MTCC B2274), *Enterococcus faecalis (*MTCC B3159), *Micrococcus luteus (*MTCC B1538), *Staphylococcus aureus* (MTCC B3160), *Streptococcus pneumoniae* (MTCC B2672) and three fungal strains *Aspergillus niger* (MTCC 4325), *Candida albicans* (MTCC 4748) and *Saccharomyces cerevisiae* (MTCC 4742) were procured from IMTECH, Chandigarh, India. Broth and agar were prepared according to the manufacturer's instructions.

Before testing, the bacterial suspension was transferred to nutrient broth and cultured at 37 ^{0}C. Inoculates were prepared by adjusting the turbidity of the medium to match the 0.5 MC farland standard. The fungal cultures were maintained on Saboraud dextrose agar, incubated at 25 ^{0}C for 4 days. The fungal growth

was harvested and washed with sterile normal saline and finally suspended in 100 ml of sterile normal saline and then suspension was stored in refrigerator till used.

Determination of Antimicrobial activity

Antibacterial and antifungal activity of stem bark extracts of *V. negundo* were determined using agar well method [13]. For susceptibility test, 100 µl of inoculums, equivalent to 10 CFU was mixed with 6 ml of nutrient agar (to ensure even distribution of bacteria) and poured immediately into the sterile petriplates. The petriplates were left to solidify for 10 minutes. A sterilized 6 mm borer was used to make wells in the centre of the divided areas. About 50 µl each extract was then pipette into the wells. Petriplates with bacteria and test extracts was incubated at 37 ^{0}C for 16-18 hr after which the inhibition zone (IZ) was measured using an antibiotic zone reader scale (HiAntibiotic ZoneScale-c).

For the antifungal activity, the same method as for bacteria was adopted of nutrient agar, Saboraud dextrose agar was used. The inoculated medium was incubated at 25 ^{0}C for two days for the *C. albicans*, *S. cerevisiae* and three days for *A. niger*. About 500 µg of nystatin was dissolved in 1 ml of sterile de ionized water. About 10 µl of 0.5 mg/ml nystatin (equivalent to 5 µg dose) and 10 µl of DMSO was pipette into wells. For bacteria Ciprofloxacin (5 Dg/disc) was used (Axiom Laboratories Ltd., India). The experiments were conducted in triplicates each and diameter of the IZ surrounding each well was recorded.

The extracts that exhibited IZ were subjected to minimum inhibition concentration (MIC) assay by using two-fold serial dilution method [14]**.** A quantity of 0.6 g of each extract was dissolved in 300 ml sterile nutrient broth which yields initial concentration of 2000 µg/ml. Subsequently, two-fold serial dilution was made from the stock to obtain 1000, 500, 250, 125, 62.5, 31.2 µg/ml concentrations. One ml of standardized inoculums of each test organism was introduced into each extract nutrient broth mixture and then incubated at 37 ^{0}C. The lowest concentration inhibiting growth was regarded as the MIC of the extracts. For the fungi, the inoculated medium was incubated at 25 ^{0}C for two (*C. albicans*, *S. cerevisiae*) to three (*A. niger*) days.

Statistical analysis

Each experimental data from triplicates was subjected to one way ANOVA using Minitab version 15. A significant level of 0.05 was used for all statistical analyses.

RESULTS AND DISCUSSION

The antimicrobial activity of the three different solvent extracts of *V. negundo* revealed that the methanol extract had significant activity against all the tested microorganisms, while the chloroform and hexane extracts possessed moderate activity and least activity, respectively (Table 1). The results of the present study are significant at level of $p>0.05$.

Methanol extract exhibited the highest IZ against *P. vulgaris* followed by *P. aeruginosa*, while chloroform was active against *P. vulgaris* and *P. aeruginosa*, and hexane extract active against *K. pneumoniae, E. feacalis* and *C. albicans*. *E. coli* and *E. feacalis* were sensitive to hexane and chloroform extracts at high concentration whereas *A. niger* was resistance to hexane and chloroform extracts. *K. pneumoniae, P. vulgaris* and *C. albicans* were sensitive to three solvent extracts and exhibited broad spectrum of antimicrobial activity. Methanol extract of *P. aeruginosa* also showed high zone value than the standard antibiotic ciprofloxacin.

From the MIC values (Table 2), it was observed that *P. vulgaris* and *C. albicans* showed the least MIC value for methanol extract. The chloroform extract showed the least MIC value against *P. vulgaris* whereas hexane extract against *P. vulgaris* and *C. albicans*. The preliminary phytochemical analysis revealed the presence of steroids, flavonoids, proteins, lipids, coumarins, phenols, tannins, alkaloids, saponins and triterpenoids in the three solvent extracts of *V.negundo* stem bark (Table 3).

The medicinal plants are rich in secondary metabolites which include alkaloids, glycosides, flavonoids, steroids, related active metabolites, which are of great medicinal value and have been extensively used in the drug and pharmaceutical industry. Recently, a number of studies have been reported on the phytochemistry of medicinal plants, particularly on the stem bark [15-18]. Interestingly, in the present investigation, stem bark of

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V. negundo has been screened for the presence of steroids, flavonoids, carbohydrates, proteins, lipids, iridoids, coumarins, phenols, tannins, alkaloids, saponins and triterpenoids. The results also revealed that all the solvent extracts possessed antimicrobial activity. Of which methanol extract showed the higher degree of antimicrobial activity on selected microorganisms. This is accordance with the previous study reported that methanol was the most effective solvent for plant extraction than chloroform and hexane [19]. This may be as a result of the presence of a few compounds extracted into the solvent and they may not have been enough inhibitory activity on pathogens.

Table 1: Antimicrobial activity of solvent extracts of *V. negundo* stem bark.

Zone of inhibition (mm)											
	Hexane extract			Chloroform extract			Methanol extract			Controls	
OR	100	200	300	100	200	300	100	200	300	C/N	D
EC	–	–	10±0.3	–	–	12±0.3	12±0.4	14±0.1	21±0.4	18[C]	–
KP	13±0.1	14±0.7	16±0.1	12±0.6	14±0.5	18±0.0	13±0.0	17±0.8	23±0.0	22[C]	–
PA	–	–	12±0.1	12±0.4	16±0.4	20±0.0	18±0.4	20±0.4	24±0.3	24[C]	–
PV	11±0.4	13±0.3	15±0.2	16±0.2	18±0.4	20±0.3	20±0.5	24±0.8	26±0.4	25[C]	–
BS	–	10±0.2	12±0.7	12±0.7	14±0.3	16±0.3	12±0.3	14±0.7	16±0.9	22[C]	–
EF	–	-	10±0.2	–	–	14±0.1	12±0.7	15±0.4	18±0.0	24[C]	–
ML	–	–	–	11±0.1	13±0.4	19±0.0	13±0.4	19±0.4	23±0.8	26[C]	–
SA	–	10±0.2	12±0.7	12±0.7	14±0.3	16±0.3	12±0.3	15±0.7	16±0.9	22[C]	
SP	–	10±0.2	12±0.7	12±0.7	14±0.3	16±0.3	16±0.3	18±0.3	20±0.9	22[C]	
AN	–	–	–	–	–	–	10±0.0	12±0.2	13±0.4	18[N]	–
CA	10±0.7	12±0.3	16±0.2	12±0.9	16±0.3	19±0.1	20±0.3	22±0.2	24±0.1	23[N]	–
SC	–	10±0.2	12±0.5	–	10±0.6	12±0.2	12±0.9	14±0.7	17±0.9	20[N]	–

All the values of inhibitory activity for the extracts tested are significant at 0.05 levels.
OR: Organisms; C: Ciprofloxicin; N: Nystatin; D: DMSO
EC: *Escherichia coli*; KP: *Klebsiella pneumoniae*; PA: *Pseudomonas aeruginosa*; PV: *Proteus vulgaris*; BS: *Bacillus subtilis*; EF: *Enterococcus faecalis*; ML: *Micrococcua leutes*; SA: *Staphyloccocus aureus*; SP: *Streptomyces pneumoniae*; AN: *Aspergillus niger*; CA: *Candida albicans*; SC: *Saccharomyces cerevisiae.*

Table 2: MIC of the solvent extracts of *V. negundo* stem bark.

Organisms	Hexane extract	Chloroform extract	Methanol extract
E. coli	>1000	>1000	500
K. pneumoniae	1000	1000	250
P. aeruginosa	1000	250	62.5
P. vulgaris	500	62.5	31.2
B. subtilis	>1000	1000	500
E. feacalis	1000	1000	250
M. leuteus	>1000	500	62.5
S. aureus	>1000	500	250
S. pneumoniae	1000	500	62.5
A. niger	>1000	>1000	1000
C. albicans	500	250	31.2
S. cerevisiae	>1000	500	500

Table 3: Preliminary phytochemical constituents of *V. negundo* stem bark.

Phytochemicals	Hexane extract	Chloroform extract	Methanol extract
Steroids	+	+	+
Flavonoids	+	+	+
Carbohydrates	-	-	-
Proteins	-	+	+
Lipids	-	+	+
Iridoids	-	-	-
Coumarins	-	+	+
Phenols	-	-	+
Tannins	-	+	+
Alkaloids	+	+	+
Saponins	-	-	+
Triterpenoids	+	+	+

+: positive
-: negative

It was reported that the ethanol extract of *V. negundo* leaves showed the spectrum of inhibition on S. *paratyphi, K. pneumoniae, V. cholera, S. mutans* and *E. Coli* [20] whereas leaves, stem, root, flower and fruit extracts exhibited antimicrobial activity against E. *coli, S. aureus, P. mirabilis and P. aeruginosa* [21] and the present study showed antimicrobial activity against *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *P. vulgaris*, *B. subtilis*, *E. faecalis*, *M. leutes*, *S. aureus*, *S. pneumoniae*, *A. niger*, *C. albicans* and *S. cerevisiae.*

V. negundo is already considered as medicinal plant. The plant is said to be a source of many bioactive principles acting against some human ailments and in the present study stem bark extract exhibited the high degree of antimicrobial activity against all tested bacterial and fungal strains. The present study also suggests that stem bark posses bioactive compounds responsible for exerting antimicrobial action against infectious diseases caused by bacteria and fungi. Therefore, it is concluded that alcohol extracts of stem bark of *V.negundo* brings to light the scope to develop a novel broad spectrum of antimicrobial drug formulation.

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