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SCREENING FOR ANTIMICROBIAL PROPERTIES OF *VITEX NEGUNDO. L* FROM RURAL AREAS OF WARANGAL DIST/A.P. INDIA

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ABSTRACT

Antimicrobial properties of different parts of *Vitex negundo* were evaluated on bacterial strains viz, *Bacillus cereus* ATCC 11778, *Bacillus subtilis* ATCC 6633, *Staphylococcus epidermidis* ATCC 12228, *Enterobacter aerogenes* ATCC 13046, *Escherichia coli* ATCC 25922, *Klebsiella Pneumoniae* NCIM 2719, *Proteus vulgaris* NCTC 8313, *Pseudomonas aeruginosa* ATCC 27853,*Psuedomonas putida* ATCC 12842, *Salmonella typhimurium* ATCC 23564. The solvents used for extraction of plant parts were hexane, chloroform and methanol. In vitro antibacterial activity was tested by agar diffusion method. The most susceptible gram positive bacteria was *Bacillus cereus*, while the most susceptible gram negative bacteria were *Klebsiella pneumonia, Pseudomonas aeruginosa, Pseudomonas putida.* The extracts of root and bark have shown moderate antimicrobial activity when compared with flower and leaves. Among these bacteria *Proteus vulgaris* is resistant against all extracts of leaves and flowers. Intermediate activity was observed with bark and root extracts. The significance of antibacterial activity of active extracts was compared with standard antimicrobics; streptomycin 2mg/cup.The results obtained in the present study suggest that *Vitex negundo* can be used in treating diseases caused by test organisms.

KEY WORDS

Antimicrobial activity, *Vitex negundo*, Phytochemicals, Solvent extraction.

INTRODUCTION

Infectious diseases are the main cause of human death world wide. Antibiotic resistance of infectious agents has become a global concern[1]. The clinical efficacy of many existing antibiotics is being threatened by the emergence of multi drug pathogens[2]. Many infectious diseases are known to be treated with herbal remedies. Natural products either as pure compounds or as standardized plant extracts offer unlimited opportunities for new drugs because of unmatched availability of chemical diversity. There is a an urgent need to explore and discover new antimicrobial compounds with diverse chemical structures and novel mechanism of action for new and reemerging infectious diseases[3]. Therefore, researchers are increasingly turning their attention to folk medicine, looking for new leads for developing better drugs against microbial infections[4]. The increasing failure of chemotherapeutics coupled with antibiotic resistance exbited by pathogenic microbial infectious agents has led to the screening of several medicinal plants for their potential antimicrobial activity[5].

Plant based microbials have enormous therapeutics as they can serve the purpose with out any side effects that are often associated with synthetic compounds. The potential for developing antimicrobials from higher plant is rewarding as it will lead to development of phytomedicine to act against microbes.

In recent years, secondary plant metabolites (phytochemical) previously with unknown pharmacological activity have been extensively investigated as a source of medicinal agents[6]. Thus, it is anticipated that phytochemicals with adequate antibacterial efficacy will be used for the treatment of bacterial infections[7].

In the context of discussion, a study was undertaken to evaluate the activity of *Vitex negundo* against several gram positive and gram negative bacterial strains *in vitro*. *Vitex negundo* a wild and small tree grows extensively in this region. It is considered as a valued medicinal plant in folklore, rural medicine and also ayurvedha. Leaves of *Vitex negundo* have been investigated for anti-inflammatory activity in past including its mechanism and action[8-12]. First noticed non steroidal anti-inflammatory drug (NSAID) like activity of *Vitex negundo.* Similarly, fresh leaves of *Vitex negundo* have been suggested to possess anti-inflammatory and pain suppressing activities possibly mediated via prostaglandin (PG) synthesis inhibition, antihistaminic, membrane stabilizing and antioxidants activities.

Even though some works have been reported on antimicrobial properties on *Vitex negundo* by other Indian investugators[13-17], these works give precise information on antimicrobial property of this plant. Hence in this present investigation was carried to evaluate and compare the antibacterial activity of all parts of *Vitex negundo* in increasing polarity solvents (hexane, chloroform and methanol) extracts against eleven prominent Gram positive and Gram negative bacteria. Here in present study, extraction is based on increasing polarity of solvents and activity was measured in increased concentration *i.e.* 100mg/ml where this gives the any precise activity of extracts. Moreover most of authors read the zone of inhibition including the cup or disc, but in this work it is excluding the cup or well diameter and read against an broad spectrum standard antibiotic as positive control. The antimicrobial activity of plant chemicals greatly depends upon region, age and climatic conditions[18, 19].

MATERIALS AND METHODS

Fresh plant parts of *Vitex negundo* were collected randomly from rural parts of Warangal district, Andhra Pradesh, India. The taxonomic identity of the plant was confirmed by Department of Botany, Kakatiya University, Warangal.

PREPARATION OF PLANT EXTRACT

The plant was collected in different seasons for preparation of extracts from different parts. The preparation of various extracts were made from the shade dried and powdered parts of *Vitex negundo L*. Ten grams of each of the dried and powdered materials were macerated separately with 100 ml of n-hexane, chloroform and methanol for 48 hours. Extracts were concentrated under reduced pressure. The condensed products were weighed and kept at 4^0C prior to test.

HPTLC Finger printing

Chromatography was performed on 3 x10cm HPTLC (Merk, Germany). The extracts were prewashed with methanol and activated at 110^0 C for five minutes. The methanol extracted sample was applied as 4mm bandwidth using a camag equipped with 100µl syringe. A constant application rate of 5µl/sec was used. Mobile phase was hexane: ethyl acetate (4:6) and chromatogram were scanned at 254nm[20].

Phytochemical Analysis

Preliminary phytochemical analysis was made for presence of alkaloid, flavanoides, carbohydrates, glycosides, proteins and amino acids, steroids, vitamin C, fat and fixed oil[21]

Antimicrobial assay

Antimicrobial activity of the different parts was determined using a slightly modified cup

plate method Muller Hinton Agar was used. Each organism at exponential growth was separately suspended in normal saline solution and a transmittance (T) of 75 – 77% at 530nm was made which is equal to 10^6 CFU/ml. Plant extracts were prepared in DMSO at concentration of 100mg/ml. Each plate was inoculated with 20µl of microbial suspension. 100µl of each extract was added to each cup. The plates containing bacteria were incubated at 37^o C for 24hrs. The positive antimicrobial activity was read based on growth inhibition zone and compared with the solvent as negative control and streptomycin 20mg / ml *i.e.* 2mg/cup was used as standard control.

Test microorganisms

The microbial strains used in the present investigations were obtained from National Chemical laboratory (NCL) Pune, India. They were maintained at 4^0C on nutrient agar slants. *Bacillus cereus ATCC 11778, Bacillus subtilis ATCC 6633, Staphylococcus epidermidis ATCC 12228, Enterobacter aerogenes ATCC 13046, Escherichia coli ATCC 25922, Klebsiella Pneumoniae NCIM 2719, Proteus vulgaris NCTC 8313, Pseudomonas aeruginosa ATCC 27853, Pseudomonas putida ATCC 12842 and Salmonella typhimurium ATCC 23564.*

RESULTS

In the HPTLC finger printing of methanol extract gave eight spots at the Rf values: 0.09, 0.11, 0.20, 0.44, 0.56, 0.72, 0.86. Purity of sample extract was confirmed by comparing the absorption spectra at start, middle and end position of band. HPTLC is an invaluable quality assessment tool for the evaluation of botanical materials. It allows for the analysis of a broad range of compounds both efficient and cost effective. The corresponding HPTLC chromatograms are presented in fig 4

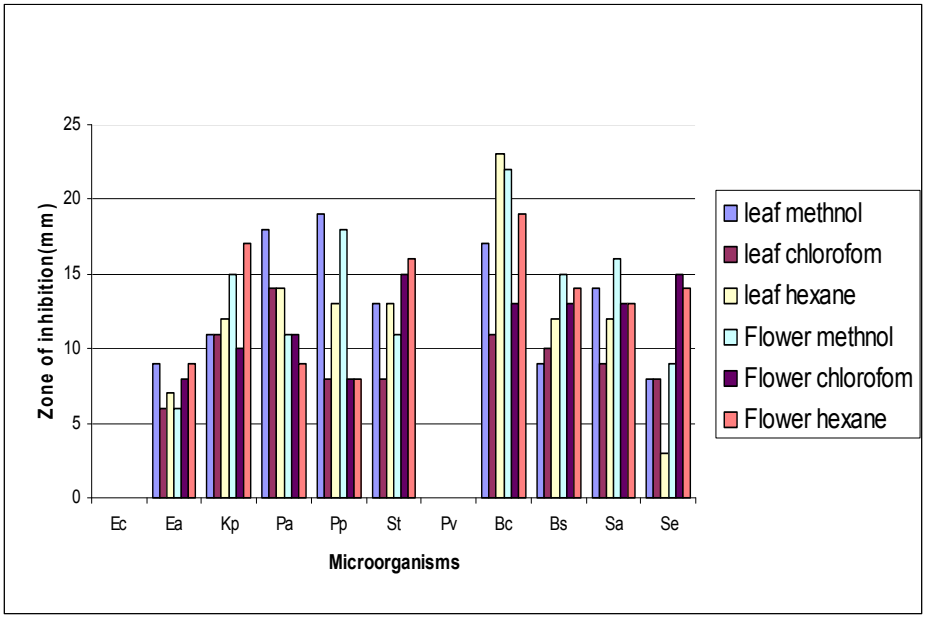

Figure 1
Antimicrobial activity of Vitex negundo leaf and flower extract

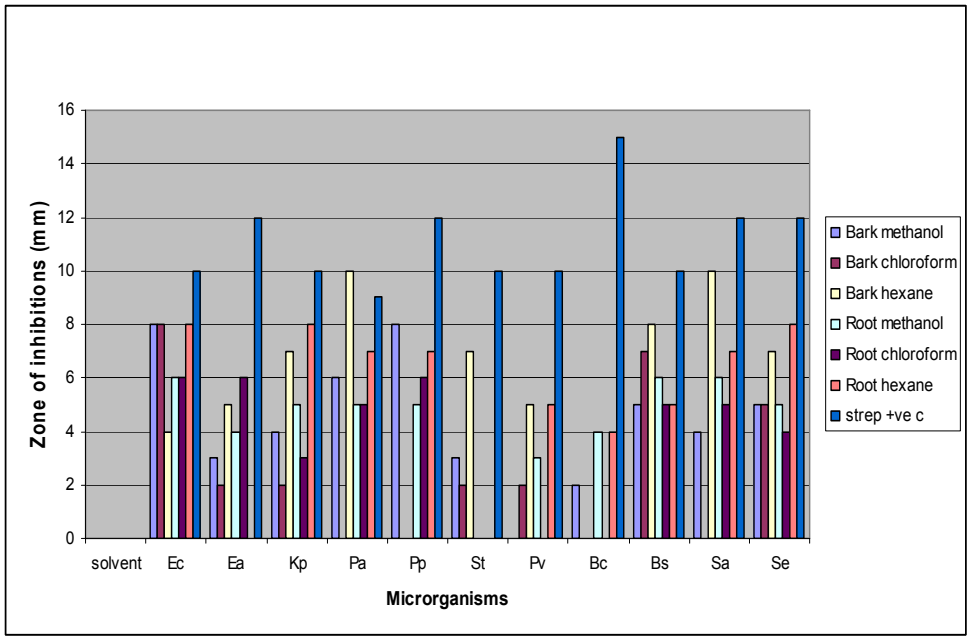

Figure 2
Antimicrobial activity of Vitex negundo bark and root extracts Vitex negundo L.

Fig: 3
Aerial *parts of vitex negundoL*

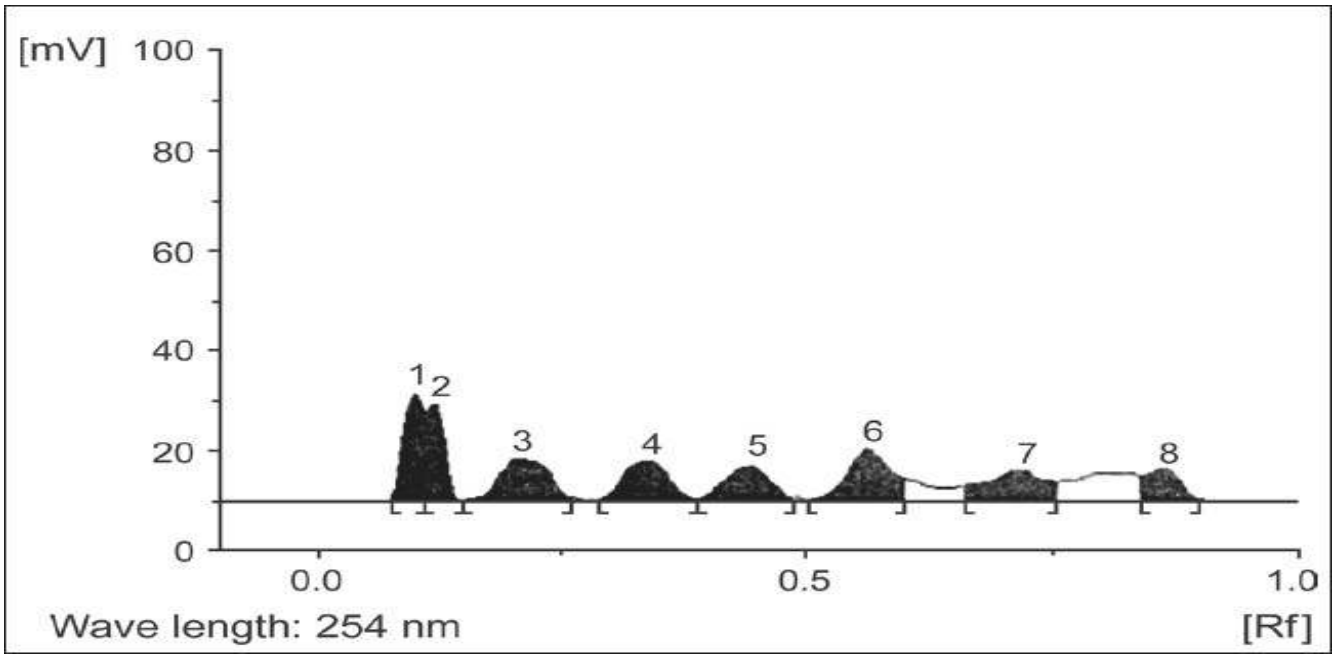

Figure 4
HPTLC fingerprint profile of methanolic extract of V. negundol . L (leaf)

The extracts of different parts of *Vitex negundo* with hexane, chloroform and methanol and their phytochemical constituents &percentage are summarized in Table -1&3. The data presented in Table-2 reveals the antimicrobial activity of the methanol, chloroform and hexane extracts of *Vitex negundo* plant parts. The results indicate that the extracts from the *Vitex negundo L.* studied showed inhibition of growth of some of the tested microorganisms to various degrees. The methanol and chloroform was found to be the most effective antimicrobial agent compared to the hexane. The methanolic extract was active against more than 70 percent of microorganisms investigated while chloroform and hexane. *Bacillus cereus* was most susceptible gram positive bacteria followed by *S.epidermidis* and *S.aureus* while *B.subtilis* was least susceptible gram positive bacteria. *Proteus vulgaris* was the complete resistant gram negative bacterial strain followed by *S.aureus, S.typhimurium E.coli* against all extracts. *K.pneumoniae* was most susceptible gram negative bacteria. The inhibitory activities of all extracts shown in Table 2 are comparable with standard antimicrobics streptomycin 2mg/cup.There was no inhibition of growth with the vehicle control (DMSO).

Table – 1

Percent of yield of extracts obtained though different solvents from leaf, flower, bark and root of Vitex negundo L.

Plant part	Solvent	Yield(in Gms)	Yield (in %)
Leaf	Methanol	2.3778	23.77
Leaf	Chloroform	0.3	3
Leaf	Hexane	0.257	2.5
Flower	Methanol	2.0922	20.9
Flower	Chloroform	0.4701	4.7
Flower	Hexane	0.57	5.7
Bark	Methanol	1.2	12
Bark	Chloroform	0.06	0.6
Bark	Hexane	0.01	0 .1
Root	Methanol	1.256	12.56
Root	Chloroform	0.046	0.46
Root	Hexane	0.01	0.1

Salmonella typhimurium (leaf&Flower)

Klebsiella pneumoniae (leaf&Flower)

Klebsiella peumoniae(bark)

Salmonella typhimurium(bark)

*(*Klebsiella *pneumoniae (root)*

Staphylococcus aureus (root)

Antibacterial activity of different extracts of leaf, flower, bark and root of *Vitex negundo* against test organisms.

Table-2
Presence *of different phytochemicals in different parts of Vitex negundo L.*

Solvent	Plant part	Colour of extract	Alkaloids	Carbohydrate	Tannins & phenols	Glycosides	Flavanoids	Saponins	Steroids	Gums & resins
Methanol	Leaf	Dark green	+ + +	+	+ + +	++	+ +	+	+	+
	Flower	light green	+	++	++	++	+++	++	+	-
	Bark	green	+++	++	+++	+	++	+	+	-
	Root	light yellow	-	+	-	-	-	+	-	-
Chloroform	Leaf	greenish	-	+	-	-	+++	+	+	-
	Flower	light green	+	+	+	-	++	++	+	+
	Bark	black green	+	-	-	+	++	+	++	-
	Root	brownish	-	+	-	-	+	-	-	-
n-Hexane	Leaf	green	+	+	+	+	-	++	-	+
	Flower	light green	+++	-	-	+	+	++	+	+
	Bark	yellow green	+	++	+	-	-	++	+	-
	Root	yellow	-	+	-	+	-	-	+	-

High amount (+++), Relatively high (++), Trace amount (+), Absent (-)

Tabe-3

Antimicrobial *activity of the methanol, chloroform and hexane extracts of different parts Vitex negundo L*

zone of inhibition in (mm)

Plant Parts	Solvent	Extract conc.	Ec	Ea	Kp	Pa	PP	St	Pv	Bc	Bs	Sa	Se
Leaf	methanol	10mg/cup	12	9	11	18	19	13	--	17	9	14	8
Leaf	chloroform	10mg/cup	11	6	11	14	8	8	--	11	10	9	8
Leaf	hexane		08	7	12	14	13	13	--	23	12	12	3
Flower	methanol	10mg/cup	ND	6	15	11	18	11	--	22	15	16	9
Flower	chloroform	10mg/cup	ND	8	10	11	8	15	--	13	13	13	15
Flower	hexane	10mg/cup	ND	9	17	9	8	16	--	19	14	13	14
Bark	methanol	10mg/cup	8	3	4	6	8	3	--	2	5	4	5
Bark	chloroform	10mg/cup	8	2	2	--	--	2	2	--	7	--	5
Bark	hexane	10mg/cup	4	5	7	10	--	7	5	--	8	10	7
Root	methanol	10mg/cup	6	4	5	5	5	--	3	4	6	6	5
Root	chloroform	10mg/cup	6	6	3	5	6	--	--	--	5	5	4
Root	hexane	10mg/cup	8	--	8	7	7	--	5	4	5	7	8
+ve control streptomycin		2mg/ml	10	12	10	9	12	10	10	15	10	12	12
-ve control DMSO													

Microorganisms: *Ec-Escherishia coli, EA-Enterobacter aerogenes; kp-Klebseilla pneumoniae; Pa-Pseudomonas aeruginosa; Pp-Pseudomonas putida;St-Salmonella typhimurium;Pv-Proteus vulgaris;Bc-Bacillus cereus Bs-Bacillus subtilis;Sa-Staphylococcus aureus;Se-Staphylococcus epidermidis.* Positive control streptomycin 2mg/ml. Negative control – DMSO; Values are mean of duplicates; -- no inhibition zone

DISCUSSION

Several factors are known to influence the active principle present in the plant. Polarity of the extracting solvent greatly influences the antimicrobial property. The activity of plant extracts against both gram positive and gram negative bacteria may be an indicative of the presence of broad spectrum antibiotic compounds or simply general metabolic toxins in the plant. Generally gram negative bacteria are resistant than gram positive bacteria[22, 23]. Traditional practitioners make use of water preliminary as solvent, but our studies showed that the methanol, hexane and chloroform of this plant parts were certainly much better and powerful. This may due to the better solubility of their active components in organic solvents[24]. These observations can be rationalized in terms of polarity of compounds being extracted by each solvent and, in addition to their ability to dissolve or diffuse in different media used in assay. The growth media also seem to play an important role in the determination of antibacterial activity[25]. Lin reported that Muller- Hinton agar appears to be the best medium to explicate the antibacterial activity and same was used in present study.

In general, the plant antibiotic substances appear to be more inhibiting to gram positive organisms than gram negative type. It may be remembered that penicillin and some of the other prominent antibiotics of fungal origin are also selective in their inhibitory action; most of them being inhibited to Gram positive bacteria. Unlike Gram positive bacteria, the lipopolysaccharide layer along with proteins and phospholipids are the major fungal origin are also rather selective in their inhibitory action, most of them being inhibited to Gram positive bacteria unlike gram positive bacteria, lipopolysaccharide layer along with proteins and phospholipids are the major component in the outer surface in gram negative bacteria[26]. Access of most components to the peptidoglycon layer of cell wall is hindered by the outer lipopolysaccharide layer. This explains the resistance of the Gram negative bacteria to the lytic action of most extracts exhibiting the activity. Antibacterial extracts from tested plant can be useful in warding of infectious diseases and therefore a compelling reason to suppose that, as anti-infective agents from tested plants are active against human pathogens, it can be assumed that these plants could be useful in warding infectious diseases[27,28] (Kirtkar and Basu, 1968; Nadkarni, 1997).

Presence of the phytochemical constituents such as alkaloids, flavanoides, tannin, and phenolic compounds have been reported to be important compounds in many other medicinal plants [29, 30]. The results of the present investigations, methanol, hexane and chloroform of leaves, flowers and barks posses these compounds which might shows antibacterial activity. Previously some reports concerning the antibacterial activity of *V.negundo* are present but our findings support the efficacy.

Kumar *et al.* studied the antibacterial of dichloromethane: methanol (1:1 v/v) extracts of *Vitex negundo* against different bacterial strains. Their finding conclude that none of the micro organisms including the bacterial strains like *B.subtilis, S.aureus, S.epidermidis, E.coli, and P.aeruginosa* were inhibited by dichloromethane: methanol extracts.

Ahmad *et al*. (1998) studied the antibacterial activity of the *V.negundo* while plant of hexane, alcoholic and aqueous extracts against *B.subtilis, E.coli, Proteus vulgaris, S.typhimurium, P.aeruginosa* and *S.aureus* had no activity. Valasraj *et al.*(1997) studied the antibacterial activity of ethanol extracts of *V.negundo* leaf using agar dilution method against four bacteria *B.subtilis, S.epidermidis, E.coli* and *P.aeruginosa.*

Panda et *al.* (2009) studied the antibacterial activity of *V. negundo* on bark and leaf of petroleum ether, chloroform, methanol and aqueous extracts against *B.subtilis, S.aureus, S.epidermidis, S. typhimurium, P.aeruginosa, V.cholerae,* and *V.alginolyteus* had little activity but inhibition was measured including disc and cup that measures 6mm indicates low activity moreover less concentration of extract was taken which dose not give accuracy of results.

They concluded that antibacterial activities against Gram positive bacteria were more pronounced than against Gram negative, their finding showed that at concentration 6.25mg/ml inhibition was found against B.subtilis where as other organism's viz. *S.epidermidis, E.coli* and *P.aeruginosa* were inhibited at a concentration of 25.0mg/ml. So far the antibacterial activity on *V. negundo* was tested by Kumar *et al.* (2006) and Ahmad et al (1998) resulted in negative. On the other hand, Valasraj *et al.* (1997) reported response four strains only. The antibacterial activity of *V.negundo* performed by Panda *et al*. (1999) on two parts viz. (leaf and bark) that too at low concentrations *i.e.* 50mg/ml and moreover inhibition zones have been measured including cup or disc diameter this might give improper results.

However, our results obtained have better inhibitory effect when compared with above mentioned authors. Comparison of the data obtained in this study with previously published result is problematic. Our work of antimicrobial activity of whole plant parts was fractionation based on the polarity of solvent and for their antimicrobial activity, at the double concentration.

It is necessary to point out that the chemical compounds of any plant greatly depend on geographical region, age of plant, local climatic seasonal and experimental conditions. Genetic differences are also responsible for changes of chemical compounds [31] thereby altering the biological activities studied[32].

CONCLUSION

From this study, it can be concluded that crude extract of hexane, chloroform and methanol of leaf and flower exhibited potential bactericidal properties. Present investigations together with previous studies provide support to the antibacterial properties of *Vitex negundo* parts. Therefore it can be used as antibacterial supplement in the developing countries towards the development of new therapeutic agents. Further pharmacological and clinical studies are required to understand the mechanism and the actual efficacy of these plant extracts in treating various infectious diseases.

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