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Evaluation of Phytochemical and Antimicrobial study of Extracts of *Vitex negundo* Linn

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Abstract

The persistent increase in the number of antibiotic resistant strains of microorganisms has led to the development of more potent but more expensive antibiotics. In most developing countries of the world these antibiotics are not readily affordable, thus making compliance difficult. This calls for research into alternative source of antimicrobials. Present work was carried out to assess the antimicrobial activity of *V.negundo* against some multidrug resistant pathogenic bacteria. *V.negundo* flower buds were collected, air dried and soxhlet extracted by using standard method for flavonoid extraction. These extracts were then tested for antimicrobial activity using disc diffusion method. MIC, MBC & TA was also calculated. flower buds bound flavonoid extract of *Vitex* showed highest antibacterial activity (IZ=25mm, AI=1.38±0.026) against *Bacillus subtilis* and (IZ=25mm, AI=1.04±0.010) against *Raoultella planticola*. The minimum inhibitory concentration (MIC) of *Vitex* was 0.039 mg/ml against *Bacillus subtilis, raoultella planticola and Agrobacterium tumifaciens*. The most active extract of bound flavonoid of flower buds was analyzed by GCMS study, which revealed eighty compounds in it. Two flavonoids were found in the flowers buds of *Vitex negundo*, as Kampferol-3-O-rutinoside (RT=30.342) and 5-hydroxy-3,6,7,3',4'-pentamethoxy flavones(RT=49.408), that are not has been reported earlier in flower buds of *Vitex negundo*. Flavonoids of flower buds extract of *V.negundo* Linn has the potential to be developed into an antimicrobial agent.

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Key words:
Flavonoids, Kaempferol, disc diffusion, GCMS, MIC, MBC, TA

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INTRODUCTION:

Infectious diseases are the cause of death accounting for approximately one-half of all death in tropical countries. Death from infectious diseases, ranked 5th in 1981, has become 3rd leading cause of death in 1992, with an increase of 58%.[1] The clinical efficacy of many existing antibiotics is being threatened by the emergence of multidrug resistant

pathogens.[2] There is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanism of action because there has been an alarming increase in the incidence of new and re-emerging infectious diseases. [3] Natural products of higher plants may give a new source of antimicrobial agents with possibly novel mechanism of action. Contrary to the synthetic drugs, antimicrobial of plant origin are generally not associated with side effects and have an enormous therapeutic potential to heal many infectious diseases. [4]

Vitex negundo Linn. belonging to familyVerbenaceae (which comprises 75 genera and nearly 2500 species), commonly known as 'Five leaved chaste tree (Eng)'. Although, all parts of *V. negundo* are used as medicine in the indigenous system of medicine, the leaves are the most potent for medicinal use. The decoction of leaves is used for treatment of inflammation, eye-disease, toothache, leucoderma, enlargement of the spleen, ulcers, cancers, catarrhal fever, rheumatoid arthritis, gonorrhea, sinuses, scrofulous sores, bronchitis and as tonics, vermifuge, lactagogue, emmenagogue, antibacterial, antipyretic, antihistaminic, analgesic, insecticidal,ovicidal, feeding deterrence, growth inhibition and morphogenetic agents.

The objective of this study was to evaluate the potential of plant extracts and phytochemicals on standard microorganism strains as well as multi-drug resistant bacteria. In the present work an extraction and screening for antibacterial activity of the flavonoids of flowers buds of *Vitex negundo* has been undertaken.

MATERIAL AND METHODS

Plant collection and authentication:

Different parts of ***Vitex negundo* L.**, (leaf, stem, root, fruit, and flower buds) were collected in the month of September and October 2009 from the western parts of India (Jaipur, Rajasthan). Plants were identified by senior taxonomist at department of Botany, university of Rajasthan and (voucher specimen no: RUBL20838) was submitted to the herbarium, Botany department, university of Rajasthan.

Preparation of Extracts:

Flavonoid extraction:

Selected plant parts were separately washed with sterilized water; shade dried, and finely powdered using a blender. Each sample was subjected to extraction, following the method of Subramanian and Nagarjan, 1969. [5] Hundred grams of each finely powdered sample was soxhlet extracted with 80% hot methanol (500ml) on a water bath for 24 h and filtered. Filtrate was re- extracted successively with petroleum ether (fraction I), ethyl ether (fraction II), and ethyl acetate (fraction III) using separating funnel. Petroleum ether fractions were discarded as being rich in fatty substances, where as ethyl ether and ethyl acetate fractions were analyzed for free and bound flavonoids respectively. Ethyl acetate fraction of each of the samples was hydrolyzed by refluxing with 7% H_2SO_4 for 2 h (for removal of bounded sugars) and the filtrate was extracted with ethyl acetate in separating funnel. Ethyl acetate extract thus obtained was washed with distilled water to neutrality. Ethyl ether (free flavonoids) and ethyl acetate fractions (bound flavonoids) were dried in vacuo and weighed [Table 2]. The extracts were stored at 4°c and were re-suspended in their respective solvents to get 10mg/ ml for antimicrobial assay.

Selected Test Microorganisms:

Pathogenic microorganisms selected for study include five bacteria, viz., *E. aerogens* (MTCC 2822), *B. subtilis* (MTCC 121), *K. pneumoniae* (MTCC 4030), *R. planticola* (MTCC 2271) and *A. tumefaciens* (MTCC 431). Selected microorganisms were procured from IMTECH, Chandigarh, India.

Bacterial strains were grown and maintained on "Muller- Hinton Agar Medium".

Antimicrobial assay:

Disc diffusion assay [6] was performed for screening. MH agar and SD agar base plates were seeded with the bacterial and fungal inoculums respectively (inoculum size 1×10^8 CFU/ml for bacteria and 1×10^7 cell/ml for fungi.) Sterile filter paper discs of Whatmann no.1 (6mm in diameter) were impregnated with 100µl of each of the extract of concentration (10mg/ml) to give a final concentration of 1 mg/disc. Discs were left to dry in vaccuo so as to remove residual solvent, which might interfere with the determination. Discs with extract were then placed on the corresponding seeded agar plates. Each extract was tested in triplicate along with streptomycin (1mg/disc) and terbinafine (1mg/disc) as standard for bacteria and fungi respectively. The plates were kept at 4°C for diffusion of extract, thereafter were incubated at 37°C for bacteria (24h) and 27°C for fungi (48h). Activity index for each extracts was calculated [Table 1] by the standard formula viz

Activity index = IZ produced by extract/ IZ produced by standard

Where, IZ = inhibition zone.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal (MBC)/ fungicidal (MBF) concentration:

Minimum inhibitory concentration (MIC) was determined for each plant extract showing antimicrobial activity against test pathogens. Broth micro dilution method [7] was followed for determination of MIC values. Plant extracts were resuspended in acetone (which has no activity against test microorganisms) to make 10 mg/ml final concentration. Two fold serially diluted extracts were added to broth media of 96-wells of micro titer plates. Thereafter 100µl inoculum(for bacteria 1×10^8 CFU/ ml and 1×10^7 cell/ml for fungi) was added to each well. Bacterial and fungal suspensions were used as negative control, while broth containing standard drug was used as positive control. Micro titer plates were then incubated at 37°C for24 h for bacteria and 28°C for 48 h for fungi. Each extract was assayed in duplicate and each time two sets of micro plates were prepared, one was kept for incubation while another was kept at 4°C for comparing the turbidity in the wells of micro plate. The MIC values were taken as the lowest concentration of the extracts in the well of the micro titer plate that showed no turbidity after incubation. The turbidity of the wells in the micro titer plate was interpreted as visible growth of microorganisms. The minimum bactericidal/ fungicidal concentration (MBC/MFC) was determined by sub culturing 50 µl from each well showing no apparent growth [Table 1]. Least concentration of extract showing no visible growth on sub culturing was taken as MBC/MFC.

Total activity (TA) determination:

Total activity is the volume up to which test extract can be diluted without losing the ability to kill microorganisms. It is calculated by dividing the amount of extract from 1 g plant material by the MIC of the same extract or compound isolated and is expressed in ml/g[8] [Table 2].

Gas Chromatography and Mass Spectroscopy Analysis:

The qualitative and quantitative composition of bound flavonoid extract of *Vitex negundo* was studied by GCMS on a GCM Spectrophotometer (Shimadzu) consisting of GC-2010 gas chromatograph and GC-MS QP 2010 plus GC mass spectrophotometer in Jawaharlal Nehru University, New Delhi [Table 3].

RESULTS:

The data pertaining to the antimicrobial potential of the plant extract are tabulated in table 1, 2 & 3 respectively.

In the present investigation free and bound flavonoid extracts of flower buds were tested against five different pathogenic bacteria, both of them showed antibacterial activities against one of the selected pathogens [Figure 1]. Among all, most susceptible organism in the investigation was *Agrobacterium tumefacians*, against which both the extracts showed inhibition zone. Bound flavonoids of flower buds showed maximum IZ against all the selected pathogens [Figure 2]. The maximum inhibition zone was observed against *Bacillus subtilis* (IZ=25mm, AI=1.38±0.026) & *Roultella planticola* (IZ=25mm, AI=1.04±0.010) by the bound flavonoid extract of flower buds of *Vitex negundo*. The range of MIC & MBC of extracts recorded was 0.039-0.312mg/ml &0.039-0.625mg/ml respectively. In the case of bound flavonoid bactericidal effect was found against *R.planticola* & whereas in case of fee flavonoid bactericidal effect was observed for *K.pneumoniae*.

Total activity as a measure of potency was also determined. Most potent extract was of bound flavonoid of flower buds of *Vitex negudo* which showed high value of total activity (410.25ml/g) against *B.subtilis* & *A.tumefacians*.

Phytochemical Analysis:

According to the results obtained in antimicrobial assay, the most active extract i.e. bound flavonoid of flower buds was chosen for GC-MS analysis. Analysis of the chemical composition of the extract by GCMS facilitated the identification of components in extract, which revealed total 80 compounds in it. Interpretation on mass spectrum GCMS was conducted using the database of National Institute Standard and Technology (NIST) having more than 6200 patterns. The spectrum of unknown component was compared with the spectrum of the known components stored in the NIST library. The Retention time, Name, Molecular weight and Structure of the components of the test extract were ascertained [Table 3, Figure 3].

The major compounds identified in bound flavonoid extract of flower buds of *Vitex* were Phenol (26.83%), Naphthalene (4.95%), 2,3-dihydrobenzofuran (6.79%), Phenol,2,3-Bis (1,1-dimethyl) (4.49%), Flavones 4'-OH,5-OH, 7-di-O-glucoside or Kampferol-3-O-rutinoside(0.25%) [Figure 5], 5-hydroxy-3,6,7,3',4'-pentamethoxy flavones (0,80%) [Figure 4] and many more.

DISCUSSION:

Ethyl acetate, ethanol and essential oil extracts of *V.negundo* Linn. has already been tested for antibacterial activity.[9] Crude ethanol extract of fruit (seed) has also been examined for in vitro antifungal activity.[10] Screening of the plant under investigation (*V.negundo*) so far however has not been worked out for flavonoids. Mostly the crude extracts have been screened, that too without MIC, MBC/MFC and TA determination. Such studies could only indicate their antimicrobial potential but are not helpful in establishing them as an antibiotic, hence cannot replace the existing antibiotics.

Similarly 5-hydroxy-3,6,7,3',4'-pentamethoxy flavones has already been isolated from leaves of *Vitex negundo* & its antimicrobial activity was tested against *B.subtilis, S.aureus, M.pyogen, P.aeruginosa and E.coli.*[11] , and the same flavonoid was also isolated from bark of *Vitex negundo*[12], and from leaves of *Vitex negundo* [13]. But till date the isolated two flavonoid named as Kampferol-3-o-rutinoside and 5-hydroxy-3,6,7,3',4'-pentamethoxy flavones have not been reported in flower buds of *Vitex negundo*. It is worth mentioning that IZ of bound flavonoid of flowers buds against most of the tested pathogens, found to be more as compared to standard drugs. In the light of the fact this study advocates the use of selected plant by the pharmaceutical industries for preparing flavonoids based antimicrobial drugs for resistant pathogens.

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Table 1: Antimicrobial assessment of free and bound flavonoid extract of flower buds extract of *Vitex negudo* against different pathogenic bacteria.

Test Pathogens	Extract	IZ	AI	MIC	MBC
Bacillus subtilis	E1	-	-	-	-
	E2	25	1.38± 0.026	0.039	0.078
Enterobector aerogens	E1	-	-	-	-
	E2	21.5	0.97± 0.010	0.156	0.312
Routella planticola	E1	15	0.62± 0.012	0.312	0.625
	E2	25	1.04± 0.010	0.039	0.039
Agrobacterium tumifacians	E1	22.5	1.02± 0.015	0.078	0.156
	E2	23.5	1.07± 0.080	0.039	0.078
Klebesilla pneumonia	E1	18	0.86± 0.011	0.156	0.156
	E2	18.5	0.88± 0.010	0.156	0.312

IZ= Inhibition zone in mm (mean value; include 6 mm diameter of disc),
AI= Activity Index (IZ developed by extract/ IZ developed by standard),
SEM, (-) = No activity, Extracts assayed in triplicate
IZ of standard drug streptomycin against) *E. aerogens*(22mm), *B. subtilis* (18mm), *K. pneumoniae (17mm), R. planticola* (30mm), *A. tumefaciens (28mm).*
MIC = Minimum Inhibitory Concentration (mg/ml)
MBC = Minimum Bactericidal (mg/ml)
E1=Free flavonoids
E2= Bound flavonoids

Table 2: Total activity of the extracts of *Vitex negundo:*

Test pathogens	Extract	Quantity of extract mg/g dried plant part	Total activity (ml/g)
B. subtilis	E1	5.4	-
	E2	16	410.25
E. aerogens	E1	5.4	-
	E2	16	102.56
R. Planticola	E1	5.4	173.07
	E2	16	177.77
A. tumifacians	E1	5.4	887.57
	E2	16	410.25
K. Pnumoniae	E1	5.4	34.16
	E2	16	51.28

*Total activity= Extract per gram dried plant part; MIC

Table 3: Important compounds identified in the GC-MS analysis of bound flavonoid extract of flower – buds extract of *Vitex negundo.*

Peak	R time	Area %	Chemical formula	Compound name	Molecular weight
1	6.176	26.83	C6H6O	Phenol	94
2	8.983	4.95	C10H8	Naphthalene	128
3	9.558	6.79	C8H8O	2,3-dihydro benzofuran	120
4	12.175	1.89	C14H30	Tetradecane	198
5	13.958	3.33	C8H8O3	4-hydroxybenzoicacidmethylester	152
6	14.392	4.49	C14H22O	Phenol,2,4-Bis(1,1-dimethyl)	206
7	14.850	1.75	C11H20O4	Azelacicacid, dimethylester	216
8	20,625	4.49	C17H34O2	Palmiticacid methylester	270
9	24.925	0.13	C12H20O	5,5,8a-trimethyl-3,5,6,7,8,8a-hexahydro-2H-chromene	180
10	24.925	0.13	C12H20O	5,5,8a-trimethyl-3,5,6,7,8,8a-hexahydro-2H-chromene	180
11	30.342	0.25	C27H30O15	Flavones 4'-OH,5-OH,7-di-O-glucoside.OR Kaempferol-3-o-rutinoside	594
12	30.650	1.21	C16H22O4	1,2-benzenedicarboxilic acid,mono(2-ethylhexaylester)	278
13	34.975	0.34	C15H24O3	Ovidin A	252
14	49.408	0.80	C20H20O8	5-hydroxy-3,6,7,3',4'-pentamethoxy flavones	388
15	55.783	4.30	C30H50O7Si	3,5,7-tris(trimethylsiloxy)-2[3,4-di(trimethylsiloxyphenyl]4H-a-bezopyran-4-one	662

Figure 1: Graph showing antimicrobial activity of flavonoids of flowers of Vitex negundo Linn

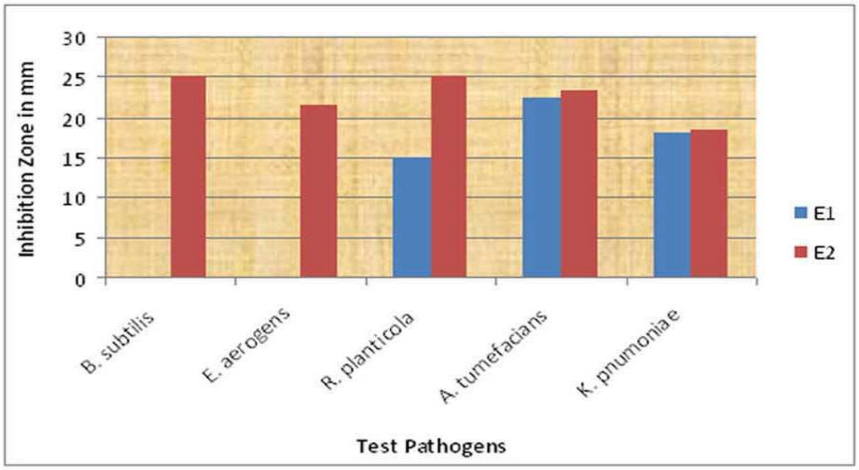
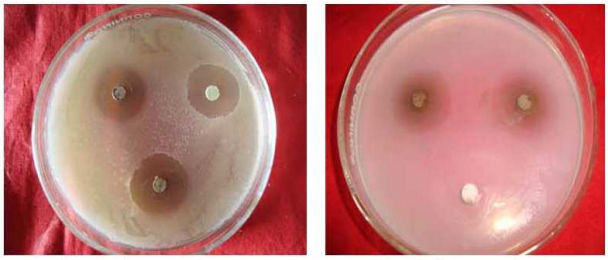


Figure 2: Inhibition zone of bound flavonoids of flower of V.negundo

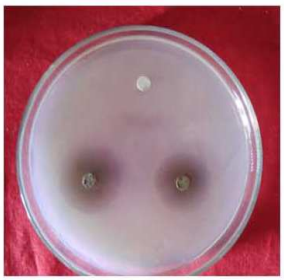


Activity against *A. tumefacians*

Activity against *B. subtilis*

Activity against *E. aerogens*

Activity against *K. pneumonia*



Activity against *R. planticola*

Figure 3: GC –MS analysis of bound flavonoids extract of flower buds of Vitex

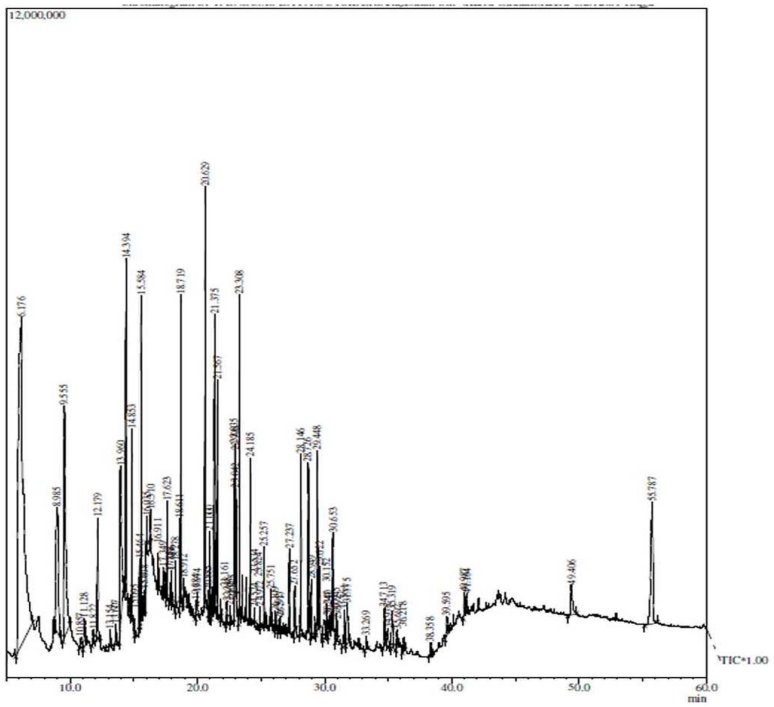


Figure 4: Mass spectrum of 5-hydroxy-3, 3', 4', 6, 7 – pentamethoxy flavones.

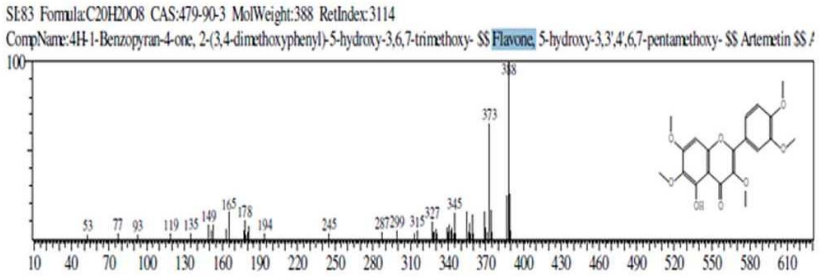
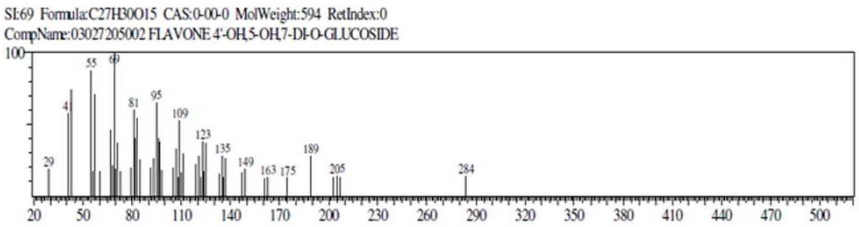
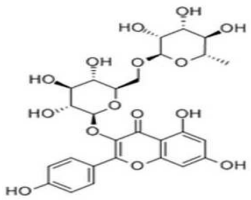


Figure 5: Mass spectrum & structure of Kampferol-3-O-runtinoside.





Kampferol-3-O-rutinoside

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