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Antibacterial potential of flavonoids and alkaloids of *Vitex negundo* Linn., and *Andrographis paniculata* Nees

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ABSTRACT

The *in vitro* antibacterial activity of flavonoid and alkaloid extracts of various plant parts *of V. negundo* and *A. paniculata* was investigated using Disc Diffusion Assay against four Gram negative (E. *aerogens, R. planticola, A. tumefaciens and K. pneumoniae*) and one Gram positive bacteria (*B. subtilis*). Minimum inhibitory concentration of the extracts was evaluated by micro broth dilution method, while minimum bactericidal concentrations were determined by sub culturing the relevant samples. The extracts exhibited antimicrobial activities with zones of inhibition ranging from 9 to 23.5 mm. *A. tumefaciens* was found to be most susceptible organism in the investigation against which all the extracts showed positive response. Stem free flavonoid extract of *V. negundo* and stem bound flavonoid extract of *A. paniculata* was recorded as most active extract as it showed significant zone of inhibition against all the tested pathogens. The range of MIC and MBC was recorded 1.25-0.039mg/ml. Results of the present study reveal that extracts of both the selected plants are showing great antimicrobial potential against tested pathogen, and may be exploited for future antibacterial drugs.

Introduction

Plant-based antimicrobials represent a vast untapped source for medicines and further exploration of plant antimicrobials is needed as antimicrobials of plant origin have enormous therapeutic potential. They are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects of synthetic antimicrobials [1]. They may act as lead compounds for the pharmaceutical industry or as the base for the development of new antimicrobials [2, 3].

Although pharmacological industries have produced a number of new antibiotics in the last three decades, resistance to these drugs in microorganisms has increased. In general, bacteria have the genetic ability to transmit and acquire resistance to drugs, which are utilized as therapeutic agents [4]. The problem of microbial resistance is growing and the outlook for the use of antimicrobial drugs in the future is still uncertain. Therefore, actions must be taken to reduce this problem and one of the ways to overcome this problem is to encourage research to develop new drugs, which might be synthetic or natural. Since the synthetic drugs are mostly associated with side effects, hence more emphasis should be given to develop safe, natural plant based drugs. According to World Health Organization [5] medicinal plants would be the best source to obtain a variety of drugs. About 80% of individuals from developed countries use traditional medicine, which has compounds derived from medicinal plants. Therefore, such plants should be investigated to better understand their properties, safety and efficiency [6] .Many plants have been used because of their antimicrobial traits, which are due to compounds synthesized in the secondary metabolism of plants. Hence, more studies pertaining to the use of plants as therapeutic agents should be emphasized, especially those related to the control of antibiotic resistant microbes. The objective of this study was to evaluate the potential of plant extracts and phytochemicals on standard microorganism strains. Further the synergistic effects of extracts for antimicrobial activity in association with antibiotics against drugs resistant bacteria, was also evaluated. Two plants namely *Vitex negundo* Linn, and *Andrographis paniculata* Nees, were selected in the present study, for assessment of their antibacterial activity.

In the present work flavonoids and alkaloids of the two selected plants were tested for their antibacterial activity.

Material and methods

Different parts of *V. negundo* (leaf, stem and root) and *A. paniculata* (leaf, stem and root) were collected in the month of February to April 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 & RUBL20873) 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 [7]. 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

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dried in vaccuo and weighed. The extracts were stored at 4°c and were re-suspended in their respective solvents to get 10mg/ ml for antimicrobial assay.

Extraction of Alkaloids: Alkaloids were extracted from different parts of the selected plants by well established methods [8] after preliminary detection of alkaloids. Finely powered sample (100g) of plant parts were extracted with 10% acetic acid in ethanol for 4 h. Extracts were concentrated and were made alkaline by NH_4OH. Precipitate thus obtained was collected by centrifugation, washed with 1% NH_4OH, filtered, dried in vaccuo and weighed. Extracts thus obtained were stored at 4°C in air tight glass vials for further use.

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 [9] 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 [10] 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 for 24 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. 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 [11].

Result

The data pertaining to antimicrobial potential of the selected plants are tabulated in Table 1 to 6 respectively.

In the present study total 18 extracts (12 flavonoid extract and 6 alkaloid extracts) were tested against five different bacterial strains. All the tested extracts showed antibacterial potential against one or the other tested pathogen. The most susceptible organism in the investigation was *A. tumefaciens* followed by *K. pneumoniae, R. planticola, E. aerogens* and *B. subtilis*. The bound flavonoid extract of leaf and stem of *V. negundo* showed maximum zone of inhibition against A. *tumefaciens* (IZ=17.5mm, AI=0.80±0.023) with the same values of MIC (0.156mg/ml) and MBC (0.312mg/ml) respectively. Whereas free flavonoid extract of stem of *A. paniculata* showed highest inhibition zone (IZ=20mm, AI= 0.30±0.023mm) with very low and similar values of MIC and MBC (0.039mg/ml) respectively. Same values of MIC and MBC showed its bactericidal nature. The least susceptible organism in the complete study was *B. subtilis* against which free flavonoid extract of stem of *V. negundo* showed moderate inhibition zone (IZ=9.5mm, AI= 0.527±0.01), whereas bound flavonoid extract from leaf of *A. paniculata* also showed significant zone of inhibition (IZ=15.5mm,AI= 0.541±0.021) against the same tested pathogen.

Alkaloid extracts of both the selected plants also showed positive response to one or the other tested pathogens. Stem alkaloid extract of *V. negundo* was recorded as most active extract as compared to the other parts, as it had shown remarkable zone of inhibition against all the tested bacteria which is at par to the inhibition zone produced by the standard drug streptomycin. The extract showed maximum zone of inhibition (IZ=23.5mm, AI=1.12±0.023) against *K. pneumoniae* with significantly low values of MIC and MBC (MIC=0.078, MBC=0.156). Root alkaloid extract of *A. paniculata* was found more active than leaf and stem which showed maximum activity against *A. tumefaciens* (IZ=15mm).

The extracts exhibited antimicrobial activities with zones of inhibition ranging from 9 to 23.5mm, whereas range of MIC and MBC was recorded 1.25-0.039mg/ml. similar values of MIC and MBC for a particular extract showed its bactericidal nature whereas different values declares its bacteriostatic nature. In case of V. negundo bactericidal effect was recorded against *A. tumefaciens, K. pneumoniae and E. aerogens* whereas in case of *A. paniculata* bactericidal effect was observed against all the tested pathogens by one or the other selected extract.

Total activity (TA) as a measure of potency was also determined. Most potent extract under study was stem free flavonoid of *V. negundo* which showed high value of TA (393.58ml/g) against *E. aerogens* whereas root free flavonoid of *A. paniculata* showed higher value of total activity (476.92ml/g) against *R. planticola*.

Discussion

Plant synthesizes variety of phytochemicals as part of their normal metabolic activities. Chemical profile of a single plant may vary over a time, as it reacts to changing conditions.

Table 1: Antimicrobial activity of flavonoid and alkaloid extract of different parts of *V.negundo*.

Plant part	Extract	*B. subtilis*		*E. aerogens*		*R. planticola*		*A. tumefaciens*		*K. pneumoniae*	
		IZ	AI	IZ	AI	IZ	AI	IZ	AI	IZ	AI
Leaf	E1	-	-	7	0.318±0.55	12.5	0.52± 0.010	13	0.59± 0.010	15.5	0.74± 0.01
	E2	-	-	15	0.68± 0.01	19.5	0.81± 0.015	17.5	0.80± 0.023	16	0.76± 0.002
	A1	-	-	7	0.31± 0.001	13.5	0.56± 0.31	12	0.55± 0.031	15.5	0.74± 0.03
Stem	E1	9.5	0.527± 0.010	18.5	0.84± 0.015	13	0.54± 0.01	12.5	0.57± 0.081	12	0.57± 0.021
	E2	-	-	14.5	0.65± 0.021	17	0.71± 0.01	17.5	0.80± 0.01	15	0.71± 0.010
	A2	19	1.05± 0.015	18	0.81± 0.015	20	0.83± 0.015	14	0.64± 0.020	23.5	1.12± 0.023
Root	E1	-	-	-	-	-	-	12.5	0.57± 0.023	11.5	0.55± 0.032
	E2	-	-	14	0.63± 0.015	20	0.83± 0.023	14.5	0.66± 0.05	-	-
	A3	10	0.55± 0.010	-	-	16	0.67± 0.081	12	0.55± 0.081	16	0.76± 0.001

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* (24mm), *A. tumefaciens* (28mm).
E1- free flavonoid, E2- bound flavonoid, A- alkaloid

Table 2: Antimicrobial activity of flavonoid and alkaloid extract of *A. paniculata*

Plant part	Extract	*B. subtilis*		*E. aerogens*		*R. planticola*		*A. tumefaciens*		*K. pneumoniae*	
		IZ	AI	IZ	AI	IZ	AI	IZ	AI	IZ	AI
Leaf	E1	11.5	0.80± 0.113	-	-	7.5	0.32± 0.010	13	0.50± 0.023	14	0.372±0.120
	E2	15.5	0.541±0.021	-	-	16.5	0.50± 0.010	13.5	0.623±0.008	16.5	0.50± 0.010
	A1	9	0.12± 0.07	7	0.30±0.001	-	-	12.5	0.62± 0.003	-	-
Stem	E1	13	0.742±0.021	16.5	0.360±0.031	-	-	20	0.30± 0.023	10.5	0.201±0.002
	E2	12.5	0.73± 0.060	9.5	0.321±0.20	10.5	0.425±0.023	15.5	0.510±0.010	12.5	0.221±0.010
	A2	-	-	-	-	12.5	0.83±0.017	14.5	0.90± 0.001	14	0.37± 0.005
Root	E1	12.5	0.89± 0.080	7.5	0.50± 0.010	18.5	0.23± 0.062	14.5	0.452±0.01	9.5	0.50± 0.023
	E2	13.5	0.650.008	15	0.330±0.076	13.5	0.210±0.010	14.5	0.50± 0.001	13	0.526±0.021
	A3	11	0.37± 0.023	10.5	0.57± 0.100	9.5	0.61±0.021	15	0.56± 0.023	-	-

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* (24mm), *A. tumefaciens* (28mm).
E1- free flavonoid, E2- bound flavonoid, A- alkaloid

Table 3: MIC and MBC/MFC values of flavonoids and alkaloids of *V. negundo against test pathogens*

Plant part	Extract	*B. subtilis*		*E. aerogens*		*R. planticola*		*A. tumefaciens*		*K. pneumoniae*	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Leaf	E1	-	-	0.625	1.25	0.312	0.625	0.312	0.625	0.156	0.312
	E2	-	-	0.625	1.25	0.156	0.312	0.156	0.312	0.156	0.156
	A1	-	-	1.25	2.5	0.312	0.625	0.312	0.312	0.156	0.312
Stem	E1	0.625	1.25	.078	0.312	0.625	1.25	0.312	0.625	0.312	0.625
	E2	-	-	0.312	0.312	0.312	0.625	0.156	0.312	0.156	0.312
	A2	0.312	0.625	0.156	0.312	0.156	0.312	0.312	0.312	0.078	0.156
Root	E1	-	-	-	-	-	-	0.312	0.625	0.312	0.625
	E2	-	-	0.312	0.625	0.078	0.156	0.156	0.312	-	-
	A3	0.625	1.25	-	-	0.312	0.625	0.312	0.625	0.156	0.312

MIC = Minimum Inhibitory Concentration (mg/ml)
MBC = Minimum Bactericidal (mg/ml)
E1- free flavonoid, E2- bound flavonoid, A- alkaloid

Table 4: MIC and MBC/MFC values of flavonoids and alkaloids of *A. paniculata against test pathogens*

Plant part	Extract	*B. subtilis*		*E. aerogens*		*R. planticola*		*A. tumefaciens*		*K. pneumoniae*	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Leaf	E1	0.312	0.625	-	-	1.25	2.5	0.625	0.625	0.312	0.625
	E2	0.156	0.312	-	-	0.078	0.156	0.312	0.625	0.078	0.156
	A1	0.625	1.25	1.25	2.5	-	-	0.625	1.25	-	-
Stem	E1	0.312	0.625	0.156	0.156	-	-	0.039	0.039	0.625	1.25
	E2	0.312	0.312	0.312	0.625	0.625	1.25	0.039	0.078	0.312	0.312
	A2	-	-	-	-	0.312	0.625	0.156	0.312	0.156	0.312
Root	E1	0.312	0.625	1.25	1.25	0.039	0.078	0.156	0.156	0.625	1.25
	E2	0.156	0.312	0.078	0.156	0.156	0.312	0.156	0.312	0.312	0.625
	A3	0.312	0.625	0.625	1.25	0.625	0.625	0.078	0.156	-	-

MIC = Minimum Inhibitory Concentration (mg/ml)
MBC = Minimum Bactericidal (mg/ml)
E1- free flavonoid, E2- bound flavonoid, A- alkaloid

Table 5: Total activity of flavonoids and alkaloids of *V. negundo*

Plant parts	Extract	Quantity of extract mg/g dried plant part	*B. subtilis*	*E. aerogens*	*R. planticola*	*A. tumefaciens*	*K. pneumoniae*
Leaf	E1	9	-	14.4	28.84	28.84	57.69
	E2	5	-	8	32.05	32.05	32.05
	A1	3.5	-	2.8	11.21	11.21	22.4
Stem	E1	30.7	49.12	393.58	49.12	49.12	98.39
	E2	6	-	19.23	19.23	38.46	38.46
	A2	6.5	20.83	41.66	41.66	20.83	83.33
Root	E1	5.5		-	-	17.62	17.62
	E2	6.5		20.83	83.33	41.66	-
	A3	9	14.4	28.84	28.84	20.83	57.69

Total activity= Extract per gram dried plant part / MIC

Table 6: Total activity of flavonoids and alkaloids of *A. paniculata*

Plant parts	Extract	Quantity of extract mg/g dried plant part	*B. subtilis*	*E. aerogens*	*R. planticola*	*A. tumefaciens*	*K. pneumoniae*
Leaf	E1	27	86.53	-	21.60	43.2	86.53
	E2	17.5	112.17	-	224.35	56.08	112.17
	A1	1.01	1.616	14.8	-	1.616	-
Stem	E1	10.67	34.19	68.39	17.07	273.58	34.19
	E2	6.67	21.37	21.37	21.37	171.02	21.37
	A2	18.5	-	-	118.58	118.58	118.58
Root	E1	18.67	59.83	14.93	476.92	119.67	59.83
	E2	2	12.82	25.64	12.82	12.82	12.82
	A3	10.2	32.69	16.32	-	130.76	-

Total activity= Extract per gram dried plant part / MIC

Plant scientists and natural products chemists are combing the flora for the phytochemicals and lead compounds, which could be developed for treatment of various diseases. During the last 10 years pace of development of new antimicrobial drugs has slowed down, while prevalence of resistance has increased multifold [12]. The problem of microbial resistance of growing and outlook for the use of antimicrobial drugs in future is still uncertain therefore, action must be taken to reduce this problem, such as controlling the use of antibiotics and carrying out research for better understanding of genetic mechanism of resistance. This prompted to evaluate plants as source of potential chemotherapeutic and antimicrobial agent along with their ethno medicinal use [13].

Earlier attempts on antimicrobial activity of the two selected plants have shown promising results against variety of microbial flora. In the current investigation, *V. negundo* showed its antimicrobial potential against test pathogens which are involved in number of human diseases. *Vitex negundo* has previously been studied for antibacterial and antifungal activities, but still the literature available is meager. Ethyl acetate, ethanol and essential oil extracts of *V. negundo* Linn. has already been tested for antibacterial activity [14]. Crude ethanol extract of fruit (seed) has also been examined for *in vitro* antifungal activity [15] .Petroleum ether, carbon tetrachloride and crude methanol extract of *V. negundo* has already tested for antimicrobial activity [16] ·Pet ether, chloroform, water and water: ethanol extract of *Vitex* leaves have also been screened for antibacterial and antifungal activity [17]. Water extract of whole aerial part of *A.paniculata* had already been screened against different pathogenic bacteria [18]. Chloroform, acetone, ethanol & water extract of *A.paniculata* leaf were also tested against *S.aureus, P.aeruginosa, B.subtilis, A.niger & P.chrysogenum* [19]. Aqueous, andrographolide and arabinogalactan protein from *A.paniculata* were evaluated for antimicrobial activity [20].

In the present investigation extracts showed more effective antibacterial potential against the Gram negative bacterial strains than the Gram positive. This is quite contradictory to the findings that plant extracts are usually more active against Gram positive bacteria than Gram negative[21]. In general, the Gram negative bacteria have shown less sensitivity to plant extracts, possibly as a result of their extra lipo-polysaccharide and protein cell wall that provides permeability barrier to the antibacterial agents [22]. This contradiction shows broad spectrum affectivity of the tested extracts against the bacterial strains.

Screening of the plant under investigation (*V.negundo* & *A. paniculata*) so far has not been worked out for flavonoids. Mostly the crude extracts have been screened, that too without MIC, MBC 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. In the present study IZ, AI, MIC, MBC and TA have been evaluated for each extract. For most of the extracts, MIC values recorded were very low, indicating strong bioefficacy of the plant.

Since the selected plants have shown good antimicrobial potency, hence can be exploited in future for preparation of an alternative drug for multidrug resistant bacteria at commercial scale.

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