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Research Article

EXTRACTION AND PHARMACOLOGICAL EVALUATION OF SOME EXTRACTS OF *VITEX NEGUNDO* LINN.

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ABSTRACT

Antimicrobial efficacy of flavonoids (free and bound) of *Vitex negundo* Linn. was determined using Disc Diffusion Assay against two gram positive bacteria (*Staphylococcus aureus*. *Pseudomonas aruginosa*), and two gram negative bacteria (*Escherichia coli, Proteus mirabilis*) and four fungi (*Aspergillus flavus*, *Aspergillus niger*, *Candida albicans*, *Trycophyton mentegrophyte*).Minimum inhibitory concentration of the extracts was evaluated by micro broth dilution method, while minimum bactericidal/ fungicidal concentrations was determined by sub culturing the relevant samples. Most susceptible organism in the present study was *Candida albicans* followed by *P.aruginosa, P.mirabilis, E.coli*, and *S.aureus*. Bound flavonoids of flowers of *Vitex negundo* were found to be the most potent. Out of 10 extracts tested, 8 were found to be active, while 2 extracts showed no activity at tested concentrations. Total activity was also calculated for the extracts. Results of the present study indicate that *V.negundo* can be exploited for future antimicrobial drugs.

Keywords: *Vitex negundo*, Flavonoid, Antimicrobial, Bactericidal, Fungicidal.

INTRODUCTION

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural resources. Traditional medicines are important source of potentially useful compounds for the development of chemotherapeutic agents [1]. Emergence of pathogenic microorganisms that are resistant / multiresistant to major class of antibiotics has increased in recent years due to indiscriminate use of synthetic antimicrobial drugs [2]. In addition, high cost and adverse effects are commonly associated with popular synthetic antibiotics (such as hypersensitivity, allergic reactions, immunosuppression etc.) and are a major burning global issue in treating infectious diseases [3]. In the present scenario, there is an urgent and continuous need of exploration and development of cheaper and effective new plant based drugs with better bioactive potentials and least side effects. Hence, recent attentions have been paid to biologically active extracts and compounds from plant species used in herbal medicines [4].

Selected plant *Vitex negundo* Linn, belongs to family verbenaceae, which is a large family of herb, shrubs and trees comprising of about 75 genera and nearly 2500 species. *V. negundo* Linn, [5] is distributed in East Asia, South west china, throughout India and cultivated in Pakistan. 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 [6], antibacterial [7], anti-inflammatory, ant androgenic [8].Different parts of *Vitex negundo* Linn., have been used in traditional Indian medicines as nervine sedative are of high value as constituents of ayurvedic preparations such as Vishagarbha thaila is widely used to treat rheumatism in India [9]. Fresh aromatic leaves are reportedly useful in rheumatism and to relieve pain [10]. It is widely used in Chinese herbal medicines. It is the second most important plant in chronic bronchitis and cold. The leaves of plant are astringent, febrifuge, sedative, tonic and vermifuge [11]. Chloroform extract of defatted seed of *V.negundo* L., showed anti-inflammatory activity [12]. It also possess potent mosquito repelling activity [3], anti tumor activity [11].

The aim of present study is to investigate the antimicrobial activity of *Vitex negundo* L., extracts in order to use it as a possible source for new antimicrobial substances against important human pathogens.

MATERIALS AND METHODS

Different parts of *Vitex negundo* L., (leaf, stem, root, fruit, and flower) 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.

Test Pathogens

Eight pathogens, in total were screened which include four bacteria,viz., *Escherichia coli* (MTCC no.46), *Staphylococcus aureus* (MTCC no 87), *Proteus mirabilis* (MTCC no. 425), *Pseudomonas aruginosa* (MTCC no.1934) and four fungal strains, viz., *Aspergillus flavus* (MTCC no.277), *Aspergillus niger* (MTCC no. 282), *Candida albicans* (MTCC no.183), and *Trycophyton mentegrophyte* (MTCC no. 7687). The pathogens were procured from IMTECH (Chandigarh, Punjab, India). Bacterial strains were grown and maintained on Muller-Hinton Agar medium, while fungi were maintained on Sabouraud Dextrose Agar medium.

Preparation of Extracts:

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 [14]. 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 5). The extracts were stored at 4°c and were re-suspended in their respective solvents to get 10mg/ ml for antimicrobial assay.

Antimicrobial assay

Disc diffusion assay [15] was performed for screening. MH agar and SD agar base plates were seeded with the bacterial and fungal inoculums respectively (inoculum's 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 & 2) 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 [16] 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 3 & 4). 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 at which test extract can be diluted without loosing 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 [17] (Table 5).

RESULTS & DISCUSSION

Antimicrobial potency of flavonoids (free and bound) were assessed by inhibition zone (Figure 1&2), activity index (Table 1 & 2), Minimum inhibitory concentration and minimum bactericidal/ fungicidal concentration (Table 3& 4). Quantity of extracts per gram of plant material was also calculated (Table 5). In the present investigation total 10 extracts were tested, among which 8 extracts showed antimicrobial activity. Two extracts were found inactive against all the test pathogens at chosen concentration.

Table 1: Inhibition zone and Activity index of extracts of *Vitex negundo* Linn. against different bacteria.

Microorganisms		*E.coli*		*S.aureus*		*P.mirabilis*		*P.aeruginosa*	
Plant Part	**Extract**	**IZ**	**AI**	**IZ**	**AI**	**IZ**	**AI**	**IZ**	**AI**
Leaf	E_1	-	-	15.33	0.747± 0.007	-	-	7.66	0.383± 0.017
	E_2	15.66	1.203± 0.027	-	-	9.66	0.36± 0.013	11.33	0.57± 0.015
Stem	E_1	-	-	-	-	-	-	-	-
	E_2	29	2.22± 0.043	-	-	13.66	0.520± 0.010	11.5	0.57± 0.015
Root	E_1	-	-	-	-	-	-	-	-
	E_2	-	-	11	0.52± 0.017	11	0.42± 0.023	16	0.80± 0.029
Flower	E_1	-	-	-	-	-	-	-	
	E_2	45	3.462± 0.00	37.66	1.78± 0.070	22.33	0.853± 0.058	16	161.183± 0.044
Fruits	E_1	-	-	-	-	-	-	-	-
	E_2	-	-	-	-	-	-	-	-

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.coli* (13mm), *S.aureus* (21mm), *P.mirabilis* (26mm*), P.aeruginosa* (20mm).

Most susceptible organism in the investigation was *C. albicans* against which most of the plant extracts showed inhibition zone which were persistent as compared with the standard, and best activity was observed by bound flavonoids of flowers with (IZ) of 24 mm , (AI) 1.71± 0.081 and MIC 0.039 mg/ml was recorded. Among all the tested extracts, bound flavonoid extract of flowers was found to be the most potent extract which showed pronounced antimicrobial activity against *E.coli* (IZ= 45 mm, AI= 3.46± 0.000), *S.aureus* (IZ=37.66mm, AI=1.78±0.070), *P.mirabilis* (IZ=22.33mm, AI=.853±0.058), *P.aeruginosa* (IZ=23.66mm, AI=1.183± 0.044), *C.albicans* (IZ=24mm, AI= 1.71± 0.081)and against *A.flavus* (IZ=23mm, AI= 1.28±) which was much higher and stable as compared to standard drug (figure 8). Free flavonoid of leaf showed best antimicrobial activity against *S.aureus* (IZ=15.33mm, AI= 0.747± 0.007) whereas bound flavonoid of leaf showed satisfactory bioactivity against *E.coli* (IZ= 15.66mm, AI= 1.203± 0.027), *P.mirabilis* (IZ=9.66mm, AI= 0.36± 0.013), *P.aeruginosa* (IZ=11.5mm, AI= 0.57±0.015), *A.flavus* (IZ=11.5mm, AI=0.64) and *C.albicans* (IZ=11.5mm, AI= 0.57±0.015). Bound flavonoid of stem was more active than free flavonoid as it showed excellent bioactivity against *E.coli* (IZ= 29mm, AI= 2.22± 0.043). Bound flavonoid of root showed good activity against *C. albicans* (IZ= 14.66mm, AI= 1.043±0.126) and *A.flavus* (IZ=12.5, AI=0.69) with MIC value of 0.312 mg/ml. In the complete study *A. niger*, and *T. mentegrophyte* were found quite resistant against all the tested extracts.

Table 2: Inhibition zone and Activity index of extracts of *Vitex negundo* Linn. against different Fungi

Microorganisms		*A.flavus*		*A.niger*		*T.mentagrophyte*		*C.albicans*	
Plant Part	**Extract**	**IZ**	**AI**	**IZ**	**AI**	**IZ**	**AI**	**IZ**	**AI**
Leaf	E_1	-	-	-	-	-	-	8.66	0.617± 0.023
	E_2	11.5	0.64± 0.023	-	-	-	-	11.5	0.57± 0.015
Stem	E_1	9.5	0.53± 0.010	-	-	-	-	7.16	0.15± 0.010
	E_2	-	-	-	-	-	-	7.66	0.54± 0.023
Root	E_1	-	-	-	-	-	-	7.5	0.53± 0.010
	E_2	12.5	0.69± 0.023	-	-	-	-	14.66	1.043± 0.126
Flower	E_1	-	-	-	-	-	-	11.66	0.82± 0.023
	E_2	23	1.28± 0.015	-	-	-	-	24	1.71± 0.081
Fruits	E_1	-	-	-	-	-	-	-	-
	E_2	-	-	-	-	-	-	-	-

IZ= inhibition zone in mm (mean value: including 6mm diameter of disc).

AI= activity index (IZ developed by extract/ IZ developed by standard), ±=SEM. (-)= no activity

E1= free flavonoids, E2= bound flavonoids

IZ of standard drug candid v6 against *C.albicans* (14mm*), A.flavus* (18mm)

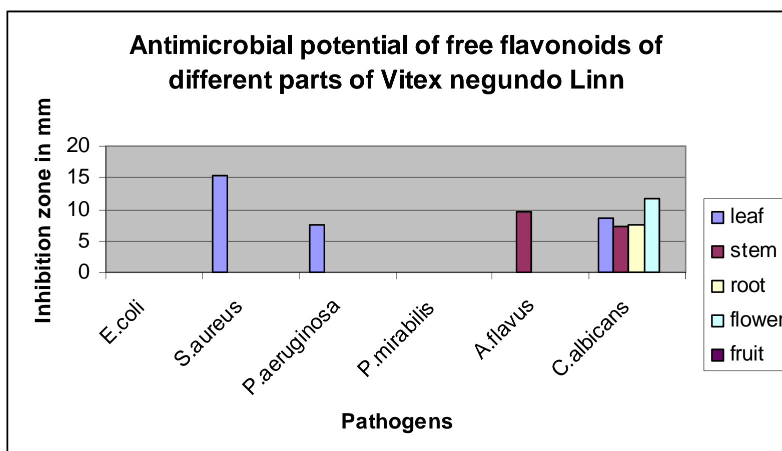


Fig. 1: Inhibition zones of free flavonoids of *Vitex negundo* Linn.

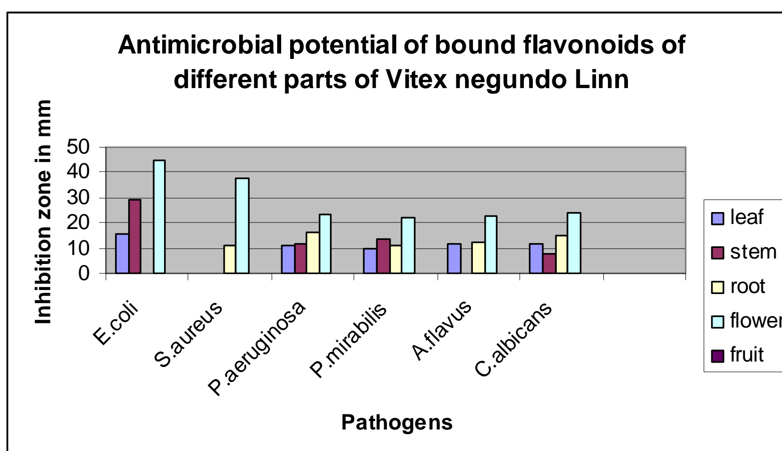
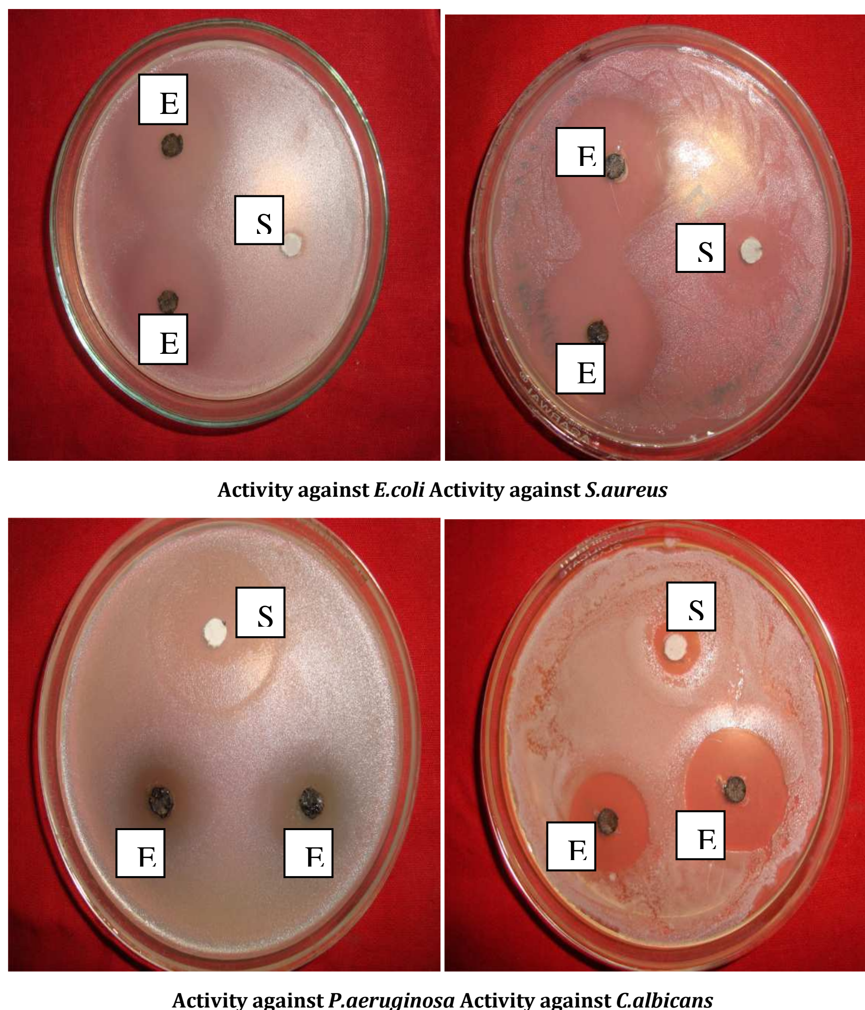


Fig. 2: Inhibition zones of Bound flavonoids of *Vitex negundo* Linn.



Activity against *E.coli* Activity against *S.aureus*

Activity against *P.aeruginosa* Activity against *C.albicans*

Fig. 3: **Pictures showing antimicrobial activity of bound flavonoids of flowers of *Vitex negundo* against different pathogens**

- Standard drug for bacteria is Streptomycin and for Candida is clotrimazole.
- Extracts are tested in duplicate
- E= Extracts
- S= Standard

MIC and MBC/MFC values (Table 3) were evaluated for those plant extracts, which were showing activity in diffusion assay. The range of MIC and MBC/MFC of extracts recorded was 0.39 – 1.25. In the present investigation lowest MIC value 0.039 mg/ml was recorded against *E.coli* and *S. aureus* whereas, against *P. aeruginosa* and *C.albicans* and *A.flavus* lowest MIC value 0.078 was recorded, indicating significant antimicrobial potential of test extracts. MIC and MBC/MFC values were found equal for four extracts of *V.negundo* showing their bactericidal or fungicidal nature, whereas in rest of the extract MBC/MFC values were higher than MIC values of the extracts, indicating bacteriostatic/ fungi static effects of the extracts.

Table 3: MIC and MBC of extracts of *Vitex negundo* Linn. against different Pathogens

Microorganisms		*E.coli*		*S.aureus*		*P.mirabilis*		*P.aeruginosa*	
Plant Part	**Extract**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**
Leaf	E_1	-	-	0.156	0.312	-	-	1.25	1.25
	E_2	0.156	0.312	-	-	0.625	1.25	0.625	1.25
Stem	E_1	-	-	-	-	-	-	-	-
	E_2	0.039	0.078	-	-	0.312	0.625	0.625	1.25
Root	E_1	-	-	-	-	-	-	-	-
	E_2	-	-	0.312	0.625	0.625	1.25	0312	0.625
Flower	E_1	-	-	-	-	-	-	-	-
	E_2	0.039	0.039	0.039	0.078	0.156	0.312	0.078	0.156
Fruits	E_1	-	-	-	-	-	-	-	-
	E_2	-	-	-	-	-	-	-	-

MIC = Minimum Inhibitory Concentration (mg/ml)

MBC = Minimum Bactericidal (mg/ml)

Table 4: MIC and MBC of extracts of *Vitex negundo* Linn. against different Pathogens

Microorganisms		*A.flavus*		*A.niger*		*T.mentagrophyte*		*C.albicans*	
Plant Part	**Extract**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**
Leaf	E_1	-	-	-	-	-	-	0.312	0.625
	E_2	0.156	0.312	-	-	-	-	0.312	0.312
Stem	E_1	0.625	1.25	-	-	-	-	0.625	1.25
	E_2	-	-	-	-	-	-	0.625	0.625
Root	E_1	-	-	-	-	-	-	1.25	2.5
	E_2	0.312	0.312	-	-	-	-	0.156	0.312
Flower	E_1	-	-	-	-	-	-	0.156	0.625
	E_2	0.078	0.156	-	-	-	-	0.078	0.156
Fruits	E_1	-	-	-	-	-	-	-	-
	E_2	-	-	-	-	-	-	0.156	0.625

MIC = Minimum Inhibitory Concentration (mg/ml)

MFC = Minimum Fungicidal Concentration (mg/ml)

Amount of extracts isolated from plant parts and total activity (TA) was calculated and recorded (Table 4). Total activity indicates the volume at which extract can be diluted with still having ability to kill microorganisms. Bound flavonoid of flower of *V.negundo* showed high values of TA against *E.coli, S.aureus, P.aeruginosa, P.mirabilis* and *C.albicans* (figure 3), which proves the potential to inhibit the growth of the test microorganisms even at low concentrations. Maximum TA values calculated were 410.25 for *E.coli, S.aureus* and *C.albicans* and 205.12 for *P.aeruginosa* and 102.56 for *P.mirabilis.*

Table 5: Total activity of the extracts of *Vitex negundo* Linn

Plant Part	Extract	Quantity of extract mg/g d.wt	Total Activity mg/g					
			E.coli	***S.aureus***	***P.mirabilis***	***P.aeruginosa***	***A.flavus***	***C.albicans***
Leaf	E_1	9	-	57.69	-	7.2	-	28.84
	E_2	5	32.05	-	8	8	33.33	16.02
Stem	E_1	30.7	-	-	-	-	49.52	49.12
	E_2	6	153.8	-	19.23	9.6	-	9.6
Root	E_1	5.5	--	-	-	-	-	4.4
	E_2	6.5	-	20.83	10.4	20.83	20.97	41.66
Flower	E_1	23.5	410.25	-	-	-	-	150.64
	E_2	16	-	410.25	102.56	205.12	228.57	410.25
Fruit	E_1	5.4	-	-	-	-	-	-
	E_2	16	-	-	-	-	-	-

Total activity= Extract per gram dried plant part; MIC

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 [19]. Crude ethanol extract of fruit (seed) has also been examined for *in vitro* antifungal activity. [20] Petroleum ether, carbon tetrachloride and crude methanol extract of *V.negundo* has already tested for antimicrobial activity.[21] Pet ether, chloroform, water and water: ethanol extract of *Vitex* leaves have also been screened for antibacterial and antifungal activity. [22]

Screening of the plant under investigation (*V.negundo*) so far 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.

In the present study IZ, AI, MIC, MBC/MFC 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. It is worth mentioning that IZ of bound flavonoid of flowers against most of the tested pathogens, found to be more as compared to standard drugs.

In the light of the fact that microorganisms are becoming resistant against the drugs in use present investigation is of great significance as far as the future drugs are concerned and advocates the use of selected plant by the pharmaceutical industries for preparing flavonoids based antimicrobial drugs for resistant pathogens.

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