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Evaluation of *in vitro* antimicrobial activity of different parts of *Bauhinia variegata* Linn.

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Abstract: Antimicrobial activity of methanolic extracts of leaf, bark and flower of *Bauhinia variegata* Linn. was studied against various standard reference bacterial and fungal strains and clinical isolates collected from various parts of India and abroad. The antimicrobial susceptibility was screened using serial dilution and disc diffusion methods. Ciprofloxacin and Griseofulvin were used as standard drugs. The results showed that the extracts were active against both bacteria (Gram positive and Gram negative) & fungi and amongst the three parts studied, the methanolic extract of bark showed comparatively higher antibacterial and antifungal activity than the other two parts studied. Preliminary phytochemical screening of the methanolic extract of the bark proved the presence of flavonoids, glycosides and saponins in it. The bark extract was active against almost all *Shigella* species, most of the *V. cholera* strains, *E. coli* 597, *E. coli* K88, *Enterobacter* AP596, *S. aureus, B. cereus, B. subtilis Pseudomonas putida* MTCC 2252, *Ps. aeruginosa* AP 585 NLF, *Proteus vulgaris* AP 679 NLF and all tested fungal species, except *Penicillium chrysogenum* MTCC 2725. The results of the study indicated that the bark of *Bauhinia variegata* possessed a broad spectrum of antrimicrobial activity.
Keywords: *B. variegata,* antimicrobial susceptibility, antifungal activity, disc diffusion, serial dilution techniques

INTRODUCTION

Medicinal plants are playing an important role in protecting against dreadful and dangerous microbial species. These plants are being used in various traditional systems due to having better immune potential and activity against numerous diseases, as compared to synthetic drugs. The medicinal activity may be slow with the plant extracts, but has permanent cure against various diseases [(1)]. One such plant *B. variegata* (family: Caesalpiniaceae) is presently being used for ailments such as sores, wounds and diarrhoea; the root is carminative and is used in dyspepsia and flatulence**.** A decoction of the root is reported to prevent obesity. The bark is astringent, tonic, anthelmintic and used in dysentery. A decoction of the buds is given in cough, piles, hematuria and menorrhagia [(2)].

This plant is commonly known as the Kachnar [(3)]. It bears fragrant flowers of pink colour from September to December. The bark powder of the plant is a major ingredient of the herbal tonic *Kanchanar guggul*, an ayurvedic remedy prescribed to increase the white blood cells [(4)].

The present investigation has been carried out to evaluate the antimicrobial activity of the methanolic extracts of leaf, bark and flower of *B. variegata* and to find out the mostly active part of the plant. This is in pursuance of the efforts to search for the active phytochemical(s) present in the mostly active part and responsible for such activity.

EXPERIMENTAL

Plant Material:

The leaves, bark and flowers of *B. variegata* were collected from the surrounding areas of Greater Noida in April, June and December, 2010, respectively. The plant was identified and authenticated by Botanical Garden of Indian Republic, Noida. A copy of the herbarium has been preserved in the Department of Pharmaceutical Technology, NIET, Greater Noida, for future reference. The parts were sun dried after washing and then ground to a coarse powder in a mechanical grinder.

Method of Extract Preparation:

The coarse powder of the leaves (24 gm) was extracted in a soxhlet apparatus with methanol and the solvent was removed by controlled evaporation under reduced pressure on a heating mantle at temperature below 60°C. A semisolid dark viscous crude extract (yield 5.41 % w/w) thus obtained was tested for its antibacterial and antifungal potentiality.

Similar extracts for bark (38.8 gm; yield 13.7 % w/w) and flower (42.7 gm; yield 38.36 % w/w) were obtained following the same method.

Test Microorganisms:

The various strains of bacteria and fungi were obtained as clinical isolates from various parts of India and abroad. All stock cultures of bacteria were maintained on nutrient agar slants and that of fungi on Sabouraud's Dextrose Agar (SDA) slants at 4°C prior to their use in antimicrobial tests.

Determination of Minimun Inhibitory Concentrations (MIC) of the Extracts Against Various Tested Microorganisms by Serial Dilution Technique:

Antibacterial activity [5]:

All the extracts were reconstituted with a minimum amount of dimethyl sulfoxide (DMSO). DMSO did not possess any antibacterial and antifungal activity of its own [6]. Calculated volumes of the stock solution was poured in molten nutrient agar medium (40-45°C) to prepare final volume of 30 ml each with dilutions of 5, 10, 25, 50, 100, 200, 400 and 800 µg/ ml of the extract. Nutrient agar plates containing equal volumes of solvent and no extract served as control.

The molten nutrient agar media containing varying concentrations of each extract were poured aseptically in sterile Petri dishes (70 mm) to give sterile nutrient agar plates with varying dilutions of the extract. These plates were then kept in refrigerator at 4°C for 24 hours to ensure uniform diffusion of extract. The plates were then dried at 37°C for 1 hour before spot inoculation. One loopful of 18 hours bacterial culture of each tested strain was spotted on each quadrant by Checker Board technique [7]. The spotted plates were incubated at 37°C for 24 hours and MIC values were determined.

Antifungal activity [5]:

Calculated volumes of stock solutions of the extracts (reconstituted with minimum amount of DMSO) were dispersed in a series of McCartney bottles previously containing calculated volumes of sterile cooled molten SDA media (40-45°C) to prepare final volume of 5 ml each with dilutions of 50, 100, 200, 400, 800, 1000, 1500 & 2000 µg/ml. SDA plates containing equal volumes of solvent and no extract served as control.

These molten media with extracts were poured aseptically in sterile test tubes to give sterile SDA slants containing varying concentrations of the extracts [8]. The slants were then kept in refrigerator at 4^0C for 24 hours to ensure uniform diffusion of extract. Then the tubes were dried at 25°C for 1 hour before inoculation. One loopful of an overnight grown culture of each fungal strain (10^5 CFU/ml) was inoculated (by streaking) on the respective slants. These slants were then incubated at 25°C for 3-7 days and MIC values were noted.

Determination of Zone of Inhibition by Disc Diffusion Method [9, 10, 11]:

The stock solutions (each of 1 mg/ml) of both the methanolic extract of bark and the reference standard were prepared. From these stock solutions two sets of two dilutions (200, 400 µg/ ml) each of bark extract (solvent: DMSO) and the standard drug (solvents: sterile distilled water for Ciprofloxacin and acetone : dimethyl formamide :: 1:3 for Griseofulvin) were prepared. Sterile nutrient agar and SDA plates were prepared and incubated at 37°C and 25°C, respectively, for 24 hours to check for any contamination. Then each sterile agar plate was flooded with 18 hours liquid culture of each microbial strain, dried for 30 minutes at 37°C (for bacteria) and 25°C (for fungi). Sterile Whatman no. 1 filter paper discs (6 mm dia.) were soaked in two different concentrations of the crude extract and placed in appropriate locations on the quadrants as marked at the back of each Petri dish. The plates containing cultures of bacteria and fungi were then incubated at 37°C and 25°C, for 24 and 48 hours, respectively, and diameters of zones of inhibition were measured in mm. Similar procedure was adopted for the standard antibacterial and antifungal drugs, Ciprofloxacin and Griseofulvin, respectively.

Preliminary Phytochemical Screening:

Preliminary Phytochemical Screening of the various extracts of bark of *B. variegata* was performed following standard methods [12].

Determination of Mode of Antibacterial Action of the Methanolic Extract of Bark of *B. variegata* [13]:
A highly sensitive bacterial strain, *E. coli* 597, to the extract was grown in sterile nutrient broth medium overnight, 2 ml from which was added to 4 ml of sterile nutrient broth and incubated for 2 hours at 37°C, so that the culture attained logarithmic phase of growth. After 2 hours incubation the extract was added at a higher concentration than its MIC value against that particular strain. The number of colony forming units (CFU/ml) was determined at an interval of 2 hours up to 6 hours and then after 18 hours starting from zero hour.

Table 1: Bacterial inhibitory spectrum of the methanolic leaf extract of *B. variegata*

Bacteria	No. Tested	No. of bacteria inhibited by methanolic leaf extract at µg/ ml							
		5	10	25	50	100	200	400	>800
Shigella spps.	16		1	2			1		12
V. cholerae	11		1				3		7
E. coli	10		1	1			1		7
Enterobacter spps.	1						1		
S. typhii	1						1		
S. aureus	4						2	1	1
Bacillus spps.	3							1	2
Pseudomonas spps.	2								2
Klebsiella pnemoniae	1								1
Proteus vulgaris	1								1
Total number of strains	50	0	3	3	0	0	9	2	33

Table 2: Bacterial inhibitory spectrum of the methanolic flower extract of *B. variegata*

Bacteria	No. Tested	No. of bacteria inhibited by methanolic flower extract at µg/ml							
		5	10	25	50	100	200	400	>800
Shigella spps.	16			1	1	1	2	3	8
V. cholerae	11			1		1		7	2
E. coli	10		1				2	2	5
Enterobacter spps.	1						1		
S. typhii	1								1
S. aureus	4							3	1
Bacillus spps.	3							2	1
Pseudomonas spps.	2							2	
Klebsiella pnemoniae	1								1
Proteus vulgaris	1								1
Total number of strains	50	0	1	2	1	2	5	19	20

Table 3: Bacterial inhibitory spectrum of the methanolic bark extract of *B. variegata*

Bacteria	No. Tested	No. of bacteria inhibited by methanolic bark extract at µg/ ml							
		5	10	25	50	100	200	400	>800
Shigella spps.	16				3	2	2	3	6
V. cholerae	11			1	1	1		7	1
E. coli	10		1		1		1	2	5
Enterobacter spps.	1						1		
S. typhii	1								1
S. aureus	4			1				3	
Bacillus spps.	3							2	1
Pseudomonas spps.	2							2	
Klebsiella pnemoniae	1								1
Proteus vulgaris	1							1	
Total number of strains	50	0	1	2	5	3	4	20	15

Table 4: Antifungal activity of methanolic extract of the leaves of *Bauhinia variegata*

Sl. No.	Name of Fungi	Growth in SDA medium containing different concentrations of the methanolic leaf extract (µg/ml)							
		0*	100	200	400	800	1000	1500	2000
1	*Candida albicans* 5	+	+	+	+	±	±	-	-
2	*Aspergillus niger* MTCC 281	+	+	+	+	±	-	-	-
3	*Penicillium chrysogenum* MTCC 2725	+	+	+	+	+	+	-	-
4	*Phaenorochaete chrysporium* MTCC 787	+	+	+	+	±	±	±	±
5	*Candida albicans* ATCC 10231	+	+	+	+	+	±	±	±
6	*Ralstonia entropha* (GM3) MTCC1255	+	+	+	+	+	+	-	-

(+): Growth; (-): No growth; (±): Inhibited growth

Table 5: Antifungal activity of methanolic extract of the flower of *Bauhinia variegata*

Sl. No.	Name of Fungi	Growth in SDA medium containing different concentrations of the methanolic flower extract (µg/ml)							
		0*	100	200	400	800	1000	1500	2000
1	*Candida albicans* 5	+	+	+	+	+	±	±	±
2	*Aspergillus niger* MTCC 281	+	+	+	+	±	±	±	±
3	*Penicillium chrysogenum* MTCC 2725	+	+	+	+	+	+	+	+
4	*Phaenorochaete chrysporium* MTCC 787	+	+	+	+	+	+	+	+
5	*Candida albicans* ATCC 10231	+	+	+	+	±	-	-	-
6	*Ralstonia entropha* (GM3) MTCC1255	+	+	+	+	±	±	±	±

(+): Growth; (-): No growth; (±): Inhibited growth

Table 6: Antifungal activity of the methanolic extract of the bark of *Bauhinia variegata*

Sl. No.	Name of Fungi	Growth in SDA medium containing different concentrations of the methanolic bark extract (µg/ml)							
		0*	100	200	400	800	1000	1500	2000
1	*Candida albicans* 5	+	+	+	+	+	±	±	±
2	*Aspergillus niger* MTCC 281	+	+	+	+	±	±	-	-
3	*Penicillium chrysogenum* MTCC 2725	+	+	+	+	+	+	+	+
4	*Phaenorochaete chrysporium* MTCC 787	+	+	+	+	+	+	±	-
5	*Candida albicans* ATCC 10231	+	+	+	+	±	-	-	-
6	*Ralstonia entropha* (GM3) MTCC1255	+	+	+	+	±	-	-	-

(+): Growth; (-): No growth; (±): Inhibited growth

Table 7: Antibacterial activity of the methanolic extract of bark of Bauhinia *variegata* Linn. by disc diffusion method

Sl. No.	Name of Bacteria	Average zone of inhibition[a] of tested bacteria on different concentrations of the standard drug and methanolic bark extract (µg/ml)			
		S_{200}	S_{400}	B_{200}	B_{400}
1	*V. cholerae* 1341	16	18	8.5	10
2	*S.aureus* 381	11	12	7.5	9
3	*Sh.soneii* 1	10	13	6.5	9
4	*Sh.soneii* DN3	12	13	9	13
5	*Sh.flexneri type* 6 BCH 999	21	23	17	18

Where,
a: zones of inhibition are diameters in mm (means of three replicates) including diameter of the disc (6 mm)
Standard drug taken: Ciprofloxacin
S_{200}: Concentration of the standard drug is 200 µg/ ml.
S_{400}: Concentration of the standard drug is 400 µg/ ml.
B_{200}: Concentration of the extract is 200 µg/ ml.
B_{400}: Concentration of the extract is 400 µg/ ml.

Table 8: Antifungal activity of the methanolic extract of bark of *Bauhinia variegate* Linn. by disc diffusion method

Sl. No	Extract	Average zone of inhibition[a] of tested fungi[b]					
		CA^1	CA^2	PC^1	PC^2	AN	RE
1	Bark[c]	20	14	18	13	15	11
2	Gris[d]	16	14.33	12.22	12.00	11.22	11.00

a: zones of inhibition are diameters in mm (means of three replicates) including diameter of the disc (6 mm)
b: CA^1 *Candida albicans* 5; CA^2 *Candida albicans* ATCC 10231; PC^1 *Penicillium chrysogenum* MTCC 2725; PC^2 *Phaenorochaete chrysporium* MTCC 787; AN *Aspergillus niger* MTCC 281; RE *Ralstonia entropha* (GM3) MTCC1255
c: concentration of extract = 40 mg / ml
d: concentration of griseofulvin = 5 mg / ml

Table 9: Preliminary Phytochemical screening of various bark extracts of *Bauhinia variegata*

S. No	Plant constituents	Petroleum ether extract	Chloroform extract	Acetic acid extract	Acetone extract	Methanol extract	Aqueous extract	Benzene extract
1	Alkaloids	-	+	-	+	-	-	-
2	Carbohydrates	+	-	-	-	-	-	-
2	Glycosides	-	-	+	-	+	+	-
3	Saponins	-	+	+	-	+	-	-
4	Phenolic compds & Tanins	-	-	-	-	-	-	-
5	Flavonoids	-	-	-	+	+	+	-
6	Phytosterols	-	-	-	-	-	+	-

Table 10: Mode of antibacterial activity of the methanolic extract of *Bauhinia variegata* Linn. bark against *E. coli* 597

Time (hrs)	CFU count/ ml for the extract
0	9.5×10^6
2	9.3×10^4
4	8.4×10^3
6	8.1×10^2
18	0

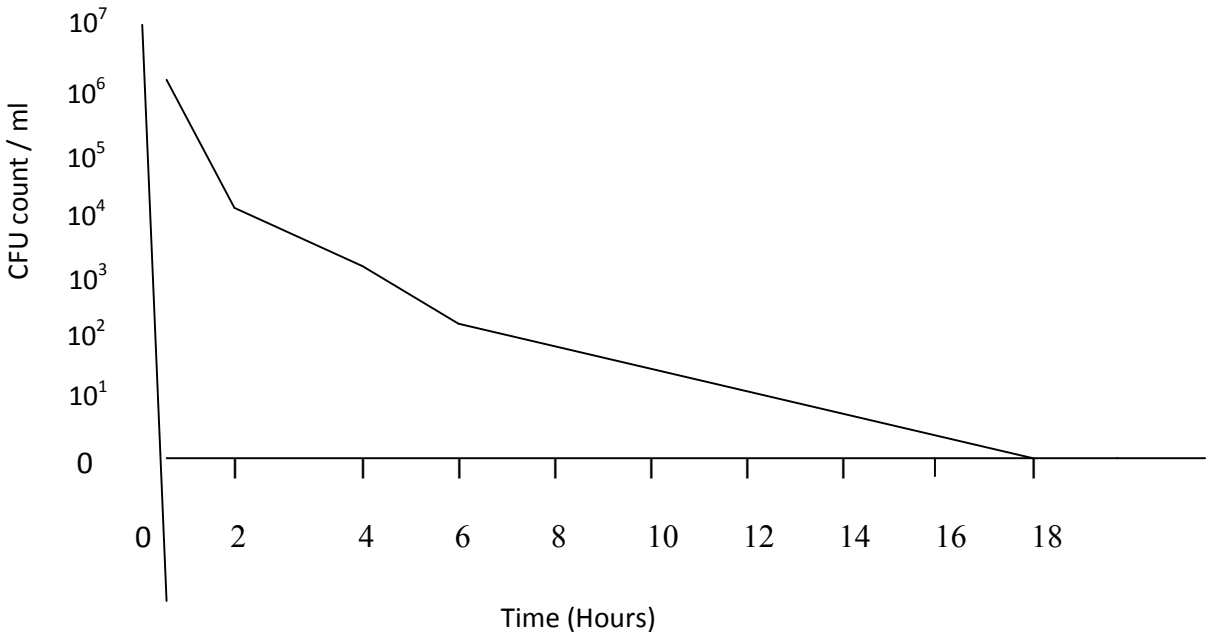

Fig 1: Graphical representation of the mode of antibacterial activity of the methanolic bark extract of *B. variegata* Linn.

RESULTS AND DISCUSSION

The extracts demonstrated antimicrobial activity against both bacteria and fungi, the methanolic extract of the bark proving to be the most active one amongst the three, followed by the flower and the leaf extracts (Table 1-6). The bark extract was active against almost all *Shigella* species, most of the *V. cholera* strains, *E. coli* 597, *E. coli* K88, *Enterobacter* AP596, *S. aureus, B. cereus, B. subtilis, Pseudomonas putida* MTCC 2252, *Ps. aeruginosa* AP 585 NLF, *Proteus vulgaris* AP 679 NLF and all tested fungal species, except *Penicillium chrysogenum MTCC 2725*. The flower extract had shown moderate activity against a few *Shigella* species, *V. cholerae* strains, *E. coli* 597, *Enterobacter* species AP 596, *S. aureus* strains, *Ps.strains*, *B.subtilis* MTCC 441, *B.cereus* MTCC 1305 and all fungal species except *Phaenorochaete chrysporium MTCC 787*. Most of the tested microorganisms were found to be resistant to the methanolic extract of leaf of *B. purpurea*.

The test organisms used in this study are associated with different human infections. From a clinical point of view, *Klebsiella pneumoniae* is the most important member of the *Klebsiella* genus of Enterobacteriaceae family and it is emerging as an important cause of neonatal nosocomial infection [(14)]. *E. coli* causes septicemias and can infect the gall bladder, meninges, surgical wounds, skin lesions and the lungs, especially in debilitate and immunodeficient patients [(15)]. Infection caused by *Salmonella typhimurium* is a serious public health problem in developing countries and of constant concern for the food industry [(16)]. The bark's activity against both Gram negative and Gram positive bacteria and tested fungi is an indication that the plant can be a source of bioactive substances that could have a broad spectrum of antimicrobial activity. The fact that the bark extract is active against a large number of microorganisms is also an indication that it can be a source of very potent antimicrobial agent that

can be used against drug resistant microorganisms prevalent in hospital environments.

The results of the disc diffusion study of both the antibacterial and antifungal activities of the methanolic extract of bark of *B. variegata* have been shown in Table 7 and 8.

Preliminary phytochemical screening of the various extracts of bark of *B. variegata* proved the presence of glycosides, saponins and flavonoids in it (Table 9).
The methanolic extract of the bark of *B. variegata* Linn. was proved to be bactericidal in its mode of action (Table 10; Fig. 1).

CONCLUSION

Methanolic extract of bark of *B. variegata* Linn. in this study has demonstrated a broad-spectrum of antimicrobial activity against a large number of both Gram positive and Gram negative bacteria and tested fungi. The broad-spectrum antibacterial activity of the plant extract may be possibly due to the presence of flavonoids, glycosides and saponins in it. Bioactive substances isolated from this plant can therefore be employed in the formulation of antimicrobial agents for the treatment of various bacterial and fungal infections including gonorrhea, pneumonia, eye infections and mycotic infections.
Isolation, identification and purification of these phytoconstituents and determination of their respective antimicrobial potencies and toxicological evaluation with the view to formulating novel chemotherapeutic agents should be the future path of investigation.

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