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Evaluation of inhibitory potential of locally found plants in North-Eastern India on clinically isolated pathogens

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ABSTRACT

*Plant extracts are a valuable source of novel antibacterial compounds to combat pathogenic isolates. Increasing concern for antimicrobial resistance in infectious agents and antibiotic side-effects makes it mandatory to search for alternatives with greater efficacy and lesser side effects. The aim of the study was to evaluate in vitro bactericidal activity of five locally found plants, in dependence on time and concentration against clinical isolates of multidrug-resistant bacteria. Plant material (*F. geniculata* (leaves), *B. diffusa* (whole plant), *M. indica* (seed), *P. pinnata* (seed) and *C. tora* (leaves)) were extracted and dissolved in methanol for its further antibacterial evaluation. Bacterial strains were isolated from clinical samples (*E. coli*, *K. pneumonia*, *P. aeruginosa* and *S. aureus*). Minimum inhibitory concentration (MIC) for each plant extract was measured by broth dilution method. Time-kill assay was carried out at doses equivalent of MIC/2, MIC and MICx2, to estimate changes in the growth curve of investigated pathogens. Percentage inhibition was monitored for all four examined microorganisms. Minimum inhibitory concentration recorded variably ranging between 1 – 6 mg/ml for all five plant extracts. Suppressed growth of *E. coli* and *S. aureus* was recorded during complete period of investigation i.e. at 0, 2, 4, 6, 24 h by all plant extracts except for *Madhuca indica* seed extract which showed strong microbial inhibitory potential against *S. aureus* but was weak against other pathogens. *P. aeruginosa* was not very susceptible to any of the extracts. It was evident from the study that methanolic extract of all five plants possess antimicrobial activity.*

Key words: Plant extract, Antimicrobial, Multidrug-resistant bacteria, Time-kill assay

INTRODUCTION

Many researchers around the globe consider that natural products are rich source of novel bioactive compounds and could be an alternative to allopathic medicines [1]. Plants are ample source of a wide variety of secondary metabolites, such as; alkaloids, terpenes, flavonoids and tannins, all of which are known to possess medicinal property [2]. For many centuries plants or part of plants are used for wound healing and as antiseptic. Antibiotics are a foundation in the treatment of bacterial infections. Suitable dose prescription is essential to improve outcomes and to avoid the emergence of resistant organisms [3,4]. Antibiotic consumption in itself is a primary risk factor for the development of antibiotic resistance.

Many herbal medicines are used as antibiotics specifically in developing countries. For many years these medicines are used in native medicinal system for different disorders/infections such as gastrointestinal (colic, indigestion, loss of appetite, and diarrhea), respiratory (sore throats, cough, and bronchial problems), inflammatory, and

cardiovascular disorders [5,6]. Herbalism prescribes many plants to treat bacterial infections, however, these remedies are not well investigated for its potential role in antimicrobial activity.

Indian herbal remedies are still widely accepted over allopathic medicinal system in developing and underdeveloped states of India. Some of these remedies are claimed as better replacement for antibiotics. The aim of the current study was to assess the overall *in vitro* bactericidal activity of five locally found plants, traditionally known for its medicinal properties, in dependence on time and concentration against clinical isolates of multidrug-resistant bacteria. To the best of our knowledge, this is the first study dealing with the evaluation of novel microbiological characteristics of methanolic extracts of *Ficus geniculata* (leaves), *Boerhaavia diffusa* (whole plant), *Madhuca indica* (seed), *Pongamia pinnata* (seed) and *Cassia tora* (leaves).

MATERIALS AND METHODS

Plants material

Plants were collected from localities of rural Ranchi, Jharkhand, India, authenticated in the Department of Botany, University of Ranchi, Ranchi, India. Table 1 lists medicinal plants, parts of plant used for extraction and the specimen voucher number. Seeds/leaves/whole plant were dried under shade and crushed to prepare a semi-solid pulp mixture. The mixture (50 g) was extracted with methanol solvent (500 ml) by using Soxhlet extractor for 48-52 h. After complete extraction, the methanol solvent was evaporated by using rotary evaporator (Yamato, Rotary Evaporator, Model-RE 801) under reduced pressure to obtain methanol crude extract. Crude extracts were filtered separately through Whatman No. 41 filter paper to remove particles. The particle free crude extract was evaporated completely by using rotary evaporator under reduced pressure to obtain dry crude extracts. The residue left in the separatory funnel was re-extracted twice follow the same procedure and filtered. The combined extracts were concentrated and dried by using rotary evaporator under reduced pressure. Methanol was used as a solvent for further antibiotic evaluation.

Bacterial strains

Microorganisms used in this study were isolated from clinical samples at Laboratory of the One Point Diagnostic Centre at Ranchi, Jharkhand, India. Clinical isolates were: *Klebsiella pneumonia*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Identification of microorganisms was carried out by the use of their biochemical profiles as recommended in the manual of Bactériologie Medical [7].

Inocula preparation

The bacterial strains grown on nutrient agar at 37°C for 18 h were suspended in a saline solution (0.9%, w/v) NaCl and adjusted to bring a turbidity of 0.5 Mac Farland standard (10⁸ CFU/ml). To obtain the inocula, these suspensions were diluted 100 times in Muller Hinton broth to give 10⁶ colony forming units (CFU)/ml [8].

Minimum inhibitory concentration

The tubes containing peptone (10 g), NaCl (10 g) and Yeast extract (5 g) were prepared, autoclaved and respective concentrations of the samples were added. Each tube was inoculated with 100 µl of 18 h old cultures (10⁴ CFU). A control tube was added with inoculums without any sample was prepared along with a sterile media tube. All the tubes were incubated at 37°C (44°C for *E. coli*) on a shaker with 140 rpm for 24 h and the growth was measured at 660 nm. The % of inhibition was calculated by using the formula below

$$\% \text{ Inhibition} = 100 - \left[\frac{\text{OD of culture with sample (Test)}}{\text{OD of culture without sample (Control)}} \times 100 \right]$$

The MIC was defined as the lowest concentration of extract at which no colony was observed after incubation. So, the MIC was defined as the lowest concentration at which no visible growth was observed.

Time-kill assay

In order to assess the antibacterial property with MIC/2, MIC, and MICx2 concentration over time, growth profile curves were plotted [9]. A 18 h culture was harvested by centrifugation, washed twice with phosphate saline, and resuspended in phosphate saline. The final suspension was adjusted using the McFarland standard. Methanolic plant extracts were added to aliquots of 25 ml Mueller-Hinton broth (MHB) in a 50 ml Erlenmeyer flask (37°C) to achieve corresponding concentrations of MIC/2, MIC and MICx2 after addition of the inocula. One ml of inocula was added to all Erlenmeyer flasks. Finally, 1 ml portion was removed and the growth of respective microorganisms was monitored using this portion by measuring optical density at 660 nm using UV-Visible spectrophotometer (UV-9100; Ruili Co., Beijing, China). The growth of microorganisms was measured at 0, 2, 4, 6 and 24 h by the above method.

RESULTS

Inhibitory role of plant extracts

Growth of *E. coli*, *P. aeruginosa*, *S. aureus* and *K. pneumonia* was investigated at variable concentration of methanolic extracts of *F. geniculata*, *M. indica*, *B. diffusa*, *P. pinnata* and *C. tora*. Percentage inhibition was monitored for all four examined microorganisms. Minimum inhibitory concentration recorded variably ranging between 1 – 6 mg/ml for all five plant extracts. Inhibitory performance based on MIC for *P. pinnata*, *B. diffusa*, *F. geniculata* and *C. tora*; were better against *E. coli* and *S. aureus*, which was recorded as 0.8 and 1 mg/ml, 2 and 1 mg/ml and 1.5 mg/ml, respectively. Inhibitory role of all five plant extracts were only effective at highest concentration i.e. 5 – 6 mg/ml against *P. aeruginosa* and *K. pneumonia* (Table 2).

Time-kill assay

Time based analysis of inhibition of *E. coli* by *F. geniculata*, *B. diffusa*, *P. pinnata* and *C. tora* were significantly greater at doses equivalent to MIC/2, MIC and MICx2. Suppressed growth of *E. coli* was recorded during complete period of investigation i.e. at 0, 2, 4, 6, 24 h except for *M. indica* for which growth was restricted till second hour of incubation later elevation was recorded in OD₆₆₀ (Figure 1). Growth of *P. aeruginosa* was almost unaffected by any of the doses i.e. MIC/2, MIC and MICx2. A gradual elevation was observed in OD₆₆₀ from 0 – 24 h for all five plant extracts. It was however notable that growth of *P. aeruginosa* was similar in response to all extracts except *F. geniculata* in which variation was observed in growth curve with respect to doses (Figure 2). Growth curve of *S. aureus* was similar for all five plant extracts used in this investigation. OD₆₆₀ observed for all plants extract were ranged between 0.1 – 0.4, which was significantly lower comparing to OD₆₆₀ measured for plants extracts against other microorganisms (Figure 3). Response of plants extracts against *K. pneumonia* was similar to as of response against *P. aeruginosa*. A gradual increase in microbial density was observed which was ranged between 0.1 – 1.5. However, restrained growth was observed in media containing extracts of *P. pinnata*, *C. tora* and *F. geniculata*. Antimicrobial performance of *P. pinnata* against *K. pneumonia* was better comparing to all other plant extracts (Figure 4).

DISCUSSION

Earlier studies have well documented evidences supporting medicinal properties of plant products [10]. Medicinal plants are widely used around the globe as traditional remedies against many diseases. These plants produce bioactive molecules that allow them to impede metabolic pathways of other organisms, thus, actively participate in self defense mechanisms and contribute to the resistance to diseases [11]. In addition, bactericidal activity of these bioactive compounds has been observed by researchers against many serious infectious organisms [12]. In the present study time kill analysis of methanolic extracts of *F. geniculata* (leaves), *M. indica* (seed), *B. diffusa* (whole plant), *P. pinnata* (seed) and *C. tora* (leaves) against *E. coli*, *S. aureus*, *P. aeruginosa* and *K. pneumonia* revealed, antibiotic potential at MIC/2, MIC and MICx2 equivalent doses.

In this study we have observed effective inhibition of microorganism by methanolic extract of all five plants extracts investigated. However, antibacterial performances of few selected extracts were remarkably great. Such as effect of *Madhuca indica* seed extract against *S. auerus*, it was completely efficient in inhibiting growth substantially. An earlier study reported that methanol extract of flowers, leaves, stem and stem bark of *M. indica* have antimicrobial activity [13].

Methanolic extract of *P. pinnata* seeds showed great antimicrobial property against *E.coli*, *S. aureus* and *K. pneumoniae*. It is to be noted that these three microbes are predominantly found in urinary tract infection and most of its strains are multi drug resistant [14,15,16]. *P. pinnata* is not well studied for its medicinal properties; however, many countries traditionally use the seed oil for wound healing and as antiseptic. More studies are required to evidently establish its method of inhibition of microbial growth.

Table 1: Details of types of extracts used from medicinal plants

Plant	Extract	Specimen voucher number
<i>Boerhaavia diffusa</i>	Whole plant	
<i>Pongamia pinnata</i>	Seed	
<i>Ficus genculata</i>	Leaves	
<i>Madhuca indica</i>	Seed	
<i>Cassia tora</i>	Leaves	

le 2: Microbial inhibitory role of plant extracts at various concentrations.

	<i>E. coli</i>				<i>P. aeruginosa</i>				<i>S. aureus</i>				<i>K. penumoniae</i>			
	Co nc. (m g/ml)	OD (660 nm)	% inhibi tion	MIC (mg/ml)	Conc. (mg/ml)	OD (660 nm)	% inhibi tion	MIC (mg/ml)	Conc. (mg/ml)	OD (660 nm)	% inhibi tion	MIC (mg/ml)	Conc. (mg/ml)	OD (660 nm)	% inhibi tion	MIC (mg/ml)
<i>Ficus genculata</i>	2	0	100	2	6	0	100	6	2	0	100		6	0	100	
	1.75	0.06	92.31		5	0.13	86.02		1.75	0	100	1.75	5	0	100	5
	1.5	0.16	79.49		4	0.52	44.09		1.5	0.39	54.65		4	0.28	56.92	
	1.25	0.38	51.28		3	0.93	0		1.25	0.61	29.07		3	0.33	49.23	
	1	0.75	3.85		2	0.93	0		1	0.83	3.49		2	0.62	4.62	
<i>Madhuca indica</i>	6	0	100	6	6	0.01	98.92	6	1	0	100	1	6	0	100	6
	5	0.12	84.62		5	0.11	88.17		0.9	0.16	81.40		5	0.29	55.38	
	4	0.22	71.79		4	0.19	79.57		0.8	0.39	54.65		4	0.48	26.15	
	3	0.53	32.05		3	0.58	37.63		0.7	0.64	25.58		3	0.56	13.85	
	2	0.77	1.28		2	0.92	1.08		0.6	0.85	1.16		2	0.65	0.00	
<i>Boerhaavia diffusa</i>	2	0	100	2	6	0	100	6	1	0	100	1	6	0	100	6
	1.75	0.16	79.49		5	0.24	74.19		0.9	0.09	89.53		5	0.24	63.08	
	1.5	0.39	50.00		4	0.49	47.31		0.8	0.24	72.09		4	0.41	36.92	
	1.25	0.52	33.33		3	0.72	22.58		0.7	0.48	44.19		3	0.54	16.92	
	1	0.77	1.28		2	0.92	1.08		0.6	0.79	8.14		2	0.63	3.08	
<i>Pongamia pinnata</i>	1	0	100		6	0	100	6	1	0	100	1	6	0	100	
	0.9	0	100		5	0.21	77.42		0.9	0.099	88.49		5	0	100	5
	0.8	0	100	0.8	4	0.32	65.59		0.8	0.24	72.09		4	0.22	66.15	
	0.7	0.23	70.51		3	0.59	36.56		0.7	0.51	40.70		3	0.49	24.62	
	0.6	0.42	46.15		2	0.86	7.53		0.6	0.84	2.33		2	0.63	3.08	
<i>Cassia tora</i>	2	0	100		6	0	100	6	2	0	100		6	0.01	98.46	6
	1.75	0	100		5	0.12	87.10		1.75	0	100		5	0.12	81.54	
	1.5	0	100	1.5	4	0.39	58.06		1.5	0	100	1.5	4	0.39	40.00	
	1.25	0.33	57.69		3	0.51	45.16		1.25	0.31	63.95		3	0.58	10.77	
	1	0.64	17.95		2	0.91	2.15		1	0.79	8.14		2	0.63	3.08	

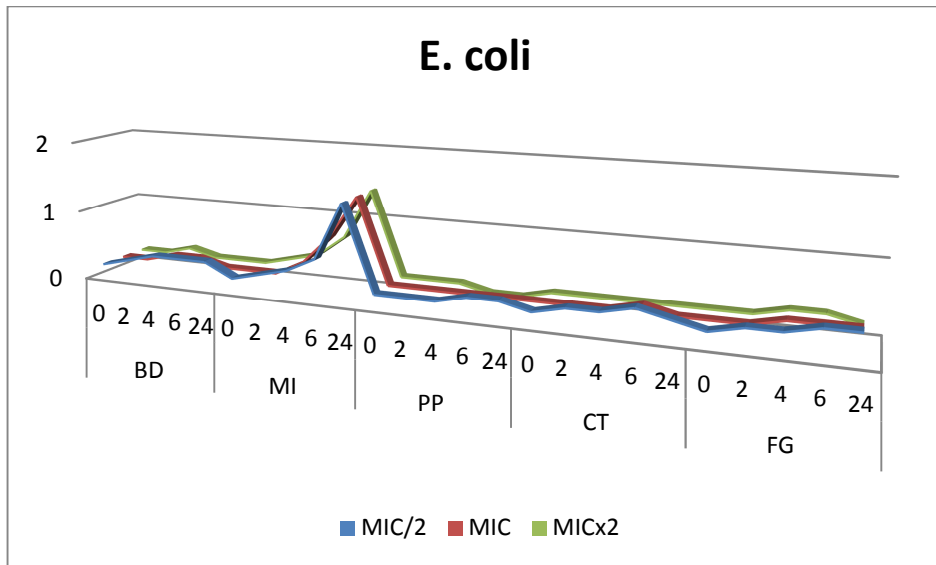


Figure 1: Time kill curve of *Boerhaavia diffusa* (BD), *Madhuca indica* (MI), *Pongamia pinnata* (PP), *Cassia tora* (CT) and *Ficus geniculata* (FG) against *E. coli* at 0, 2, 4, 6 and 24 h.

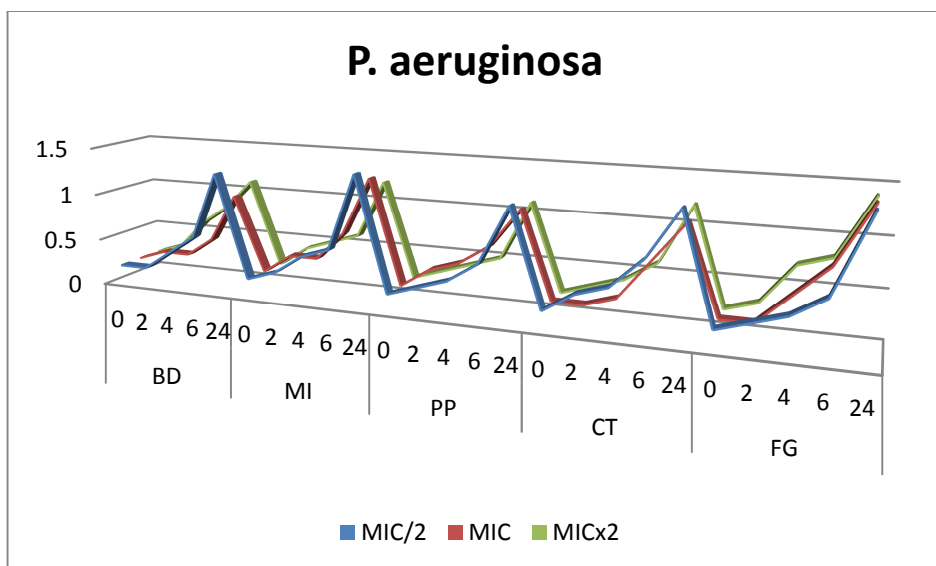


Figure 2: Time kill curve of *Boerhaavia diffusa* (BD), *Madhuca indica* (MI), *Pongamia pinnata* (PP), *Cassia tora* (CT) and *Ficus geniculata* (FG) against *P. aeruginosa* at 0, 2, 4, 6 and 24 h.

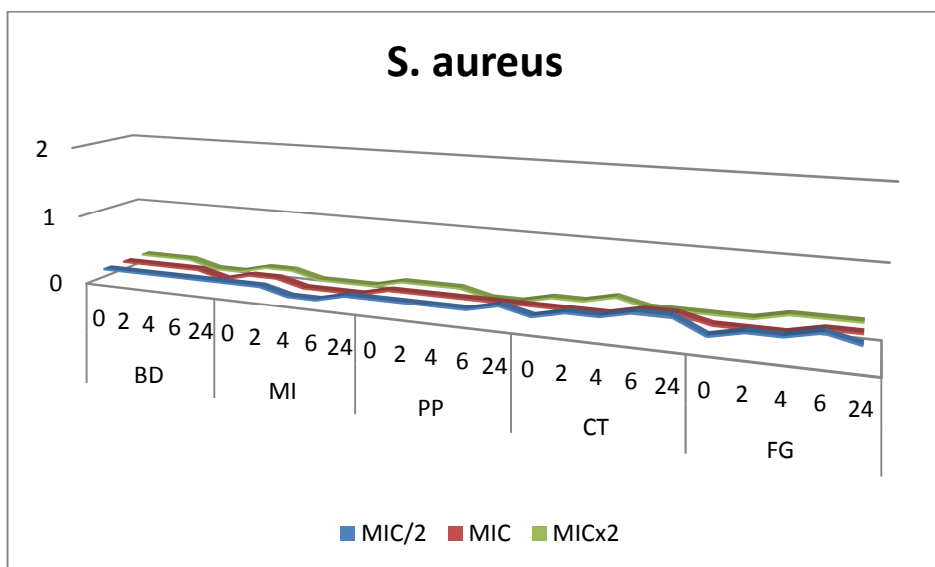


Figure 3: Time kill curve of *Boerhaavia diffusa* (BD), *Madhuca indica* (MI), *Pongamia pinnata* (PP), *Cassia tora* (CT) and *Ficus geniculata* (FG) against *S. aureus* at 0, 2, 4, 6 and 24 h.

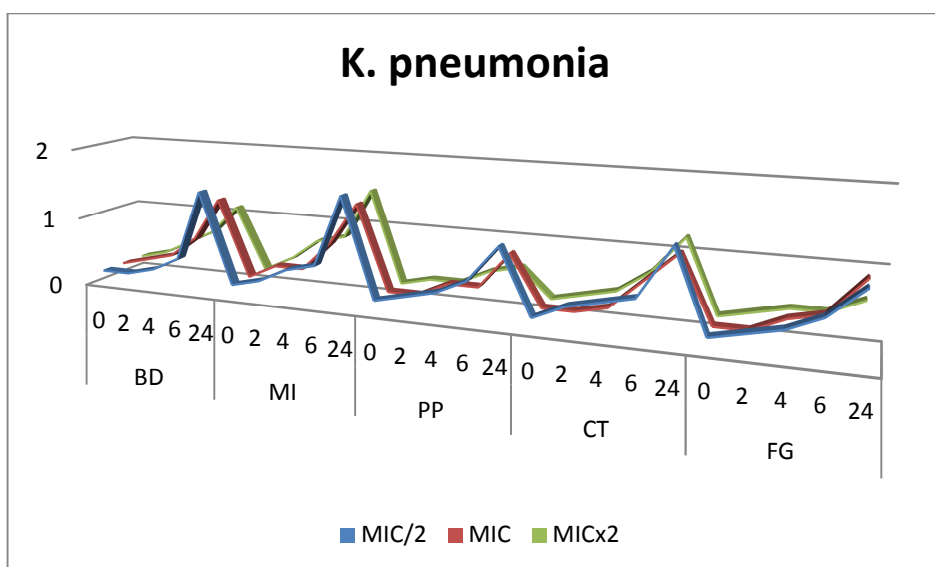


Figure 4: Time kill curve of *Boerhaavia diffusa* (BD), *Madhuca indica* (MI), *Pongamia pinnata* (PP), *Cassia tora* (CT) and *Ficus geniculata* (FG) against *K. pneumonia* at 0, 2, 4, 6 and 24 h.

Antimicrobial activity of *C. tora* leaf extract and *B. diffusa* whole plant extract have been mentioned in earlier studies [17,18]. Our study also revealed strong antimicrobial activity against *E. coli* and *S. aureus* for both extracts; however, their activity was weak against *P. aeruginosa* and *K. pneumonia*. A study by [19] also claimed presence of antimicrobial and antioxidant property of *Cassia* Sp (2009). Studies on antimicrobial property of *F. geniculata* leaf extract is limited, our study is first to claim its action against *E. coli* and *S. aureus*.

Based on the obtained results, all compounds assessed as active according to MIC values. The time-kill assay suggested that investigated plants extracts completely inhibited the growth of *E. coli* and *S. aureus* and they also exhibited prolonged antibacterial activity against the *P. aeruginosa* and *K. pneumonia* as determined by time-kill curves. Antimicrobial activities against *E. coli* and *S. aureus* were similar for all plants extracts. It was therefore hypothesized that these two strains were more susceptible to bioactive compounds present in the examined herbal

antibiotics. Largely, *P. pinnata* seed extracts was regarded as best among all extracts and effective at all doses i.e. MIC/2, MIC and MICx2. Nevertheless, more study is required to ascertain its doses as *P. pinnata* is considered to be toxic causing serious nausea and vomiting. Its constituents such as triglycerides and its disagreeable taste and odor also pose obstacle in way of making it a suitable antibiotics against many infections.

CONCLUSION

It was evident from the study that investigated methanolic extracts of plants or parts of plants positively have microbial growth inhibitory potential. In particular, the bactericidal effects were more prominent against *E. coli* and *S. aureus*, than against *P. aeruginosa* and *K. pneumonia*.

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