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In Vitro Antimicrobial Activity of Medicinal Plant *Pongamia pinnata*

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ABSTRACT

Object: This study was undertaken to investigate the *in vitro* antimicrobial activity of *P. pinnata* seed against human pathogens. **Method**: Petroleum ether, alcoholic and chloroform extracts of *P.pinnata* seeds were prepared and their antimicrobial properties were evaluated by agar well diffusion method. MIC, MBC/MFC and cell viability test were determined by agar dilution method. Various fractions were showed antibacterial activity against *Escherichia coli, Salmonella typhi, Proteus mirabilis, Pseudomonas aeruginosa, Bacillus subtilis* and antifungal activity against *Candida albicans* and *Aspergillus fumigates* **Results:** The result indicated that all of the extracts exhibited antimicrobial properties. The highest potential was observed with petroleum ether fraction of *P. pinnata* seed. MIC values of all fractions ranging from 40µg/ml to 7 mg/ml. Petroleum ether fraction showed the maximum inhibition against fungi as compared to bacteria. Bactericidal concentration of petroleum ether was found to be 120 µg/ml for *E. coli*, *P. aeruginosa* and *P. mirabilis* and 150µg/ml for *B. subtilis* and *S. Typhi.* 70 µg/ml MFC was observed for *C. albicans* whereas this extract showed only fungistatic activity against *A. fumigatus.* As concentration of the extract increased number of bacterial cells also decreased. No change in cell number of bacteria beyond 60µg/ml concentration was observed. **Conclusion:** The experiment confirmed the efficacy of *P. pinnata* seed extracts as natural antimicrobials and suggested the possibility of employing them in drugs for the treatment of infectious diseases caused by the test organisms.

INTRODUCTION

The term antimicrobial is used for any substance, which prevents infection in plants, animals or humans by either being toxic to the source of infection i.e. bacteria, fungus, virus and parasites or by inhibiting the influence of infectious toxins produced by these pathogens on a cell-tissue or organs [1]. Medicinal properties of several plants and their extracts have been studied with the goal to discover new chemical classes of antimicrobial that could resolves problems such as development of resistant microorganisms, side effects of modern drugs [2,3,4] and emerging new diseases [5,6,7]. With the increase of antibacterial resistance to antibiotics, interest has been generated to investigate the antimicrobial effects of different extracts against a range of bacteria, to develop other classes of natural antimicrobials useful for infection control [8]. Thus plant extracts are promising natural antimicrobial agents with potential applications in pharmaceutical industries for controlling of pathogenic bacteria.

Pongamia pinnata (L.) Pierre (Fabaceae), a medium sized glabrous tree, found throughout India [9]. This plant different part has been recommended as a remedy for various ailments [10]. The seed and seed oil of this plant have been used for treating various inflammatory and infectious diseases such as leucoderma, leprosy, muscular and particular rheumatism [11]. The leaves of this plant are laxative, anthelmintic and cure piles, wounds and other inflammations [12]. Aqueous extract of the leaves are used as a medicated bath for relieving rheumatic pains and for cleansing ulcers in gonorrhea and scrofulous enlargement [13, 14]. Anti-inflammatory activity of *P. pinnata* root and seed extracts has been reported [15, 16]. This plant has various ethnopharmacological properties but there is no report available in the literature on the screening of antimicrobial activity of different solvent extracts of *P. pinnata* seed extract against human pathogen. Therefore, study investigated the antimicrobial potential of *P. pinnata* seed extracts against human infective microorganisms with the potential use of natural antimicrobial agents in pharmaceutical industries.

MATERIALS AND METHODS

Collection of Plant materials

The seeds of *P. pinnata* were collected from college campus of MLS University, Udaipur, Rajasthan, India identified by Prof. N.C. Aery on the basis of morphological features and the voucher specimen present at the Department of Botany.

Preparation of extracts

P. pinnata seeds were dried in shade at room temperature and finely ground in an electrical grinder. The ground material was passed through sieve no. 240 so as to obtained powder of mesh size 60 which was used for preparation of extract. Reflux method of solvent extraction was used for successive separation of different organic constituents present in plant material [17, 18]. 40 gm of the air dried powdered material was extracted successively with 280 ml of respective solvents in a soxhlet extractor. The series of successive solvents used was as follows:

Petroleum ether → Chloroform → Ethanol (100%)

Residue of each extraction was dried in hot air oven at 45^0C before extracting with the next solvent. Fraction obtained with each solvent was concentrated in rotary evaporator and dried at room temperature. This fraction was weighed and percentage extractive was calculated in terms of fraction obtained from total dry weight of plant material.

Percentage Yield (%) = Dry weight of extract / Dry weight of plant material × 100

Microorganisms

Fungi *Aspergillus fumigatus, Candida albicans* and bacteria *E. coli, Bacillus subtilis, Salmonella typhi, Pseudomonas aeruginosa* and *Proteus mirabilis* were used to study the antimicrobial effect of plant extracts. Cultures of each bacterial strain were maintained on Luria broth (LB) agar medium at 4°C and fungal strains are maintain on potato dextrose agar medium.

Assay of antimicrobial activity assessment

Cup Well Method

Preliminary screening of antimicrobial activity of various fractions of *P. pinnata* seed was done by agar well diffusion method [19]. Inoculum was prepared by culturing bacteria on nutrient agar and potato dextrose agar for fungi. 0.l ml (10^6 cells/ml) of 24 hrs old bacterial cultures and 0.1 ml (10^4 cells/ml) spore suspension of 7 days old fungal culture were used for inoculation. 20 ml of the molten sterile nutrient agar was poured aseptically in sterilized Petri plates and was allowed to solidify at room temperature. Seeding of these plates was done by

spread plate method (Sharma, 2005). 8 mm size wells were bored in the solidified nutrient agar with the help of sterile cork borer and filled with 250µl of respective extract concentration. The plates were incubated at 37ºC for 24 hrs for bacteria and 72 hrs for fungi. The zone of inhibition developed around the well was measured by Himedia zone scale. Ciprofloxacin and Fluconazole were used as standard antibacterial and antifungal drugs respectively. DMF and distilled water control were also maintained simultaneously.

Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration of the extract was assayed by agar dilution method [20]. Stock solution was prepared by dissolving 100 mg dried extract in 10ml dimethylformamide (DMF) to get 10mg/ ml extract concentration. This stock solution was further diluted to get 10µg to 1mg, 2mg, 3mg-10mg/ ml extract concentrations respectively. 1ml test solution was added in the order of increasing extract concentration i.e. 10µg / ml to 10 mg/ ml respectively to culture tubes containing 9 ml sterile nutrient media and autoclaved at 121ºC and 15 psi for 20 minutes. Autoclaved tubes were inoculated with pure cultures of bacteria and fungi and incubated for 24 hrs for bacteria and 72 hrs for fungi. Tubes containing extract free medium were used as control. Positive control was maintained with DMF and a negative control was maintained with nutrient medium. Three replicates were maintained and the experiment was repeated three times.

Minimum fungicidal/bactericidal concentration (MFC/MBC)

Minimum fungicidal/bactericidal concentration was determined by inoculating treated fungi/bacteria on extract free medium and incubated at 37ºC. Fungi and bacteria were treated with 10 -200µg per ml extract concentration respectively was re-inoculated on extract free medium and incubated fungi for 72 h. and bacteria for 24 h. Concentration that prevent the growth of fungal/bacterial colony on the extract free medium was taken as MFC/MBC [19].

Effect of extract on cell viability of test bacteria

Inhibitory effect of extract was also studied as a function of increase or decrease in cell no. One ml of bacterial suspension containing $1x10^6$ cell/ml was inoculated in respective tubes containing increasing concentration of extract up to MIC concentration and incubated for 24 h after which change in cell number was measured by cell counting.

Total number of bacteria was calculated by following formula.

$$\text{Number of bacteria\ml} = X \times \frac{1}{0.00025} \times \text{ Dilution of sample [21]}$$

Where X =Average number of cell per small square

Statistical analysis

Data were expressed as the mean and standard deviation (SD) of the means and statistical analysis was carried out.

RESULTS

Plant extract yield

The percentage extractive values of various fractions of *P. pinnata* seed are shown in Table No. 1. Petroleum ether fraction of *P. pinnata* seed yield maximum percentage extractive value as compared to others.

Antimicrobial Activity of plant Extract

Various fractions of *Pongamia pinnata* seed were investigated to evaluate their antimicrobial activity against human pathogenic bacteria and fungi using cup well method. Evaluation of antimicrobial activity of these plant extract was presented in Table No.2. Amongst the various fractions petroleum ether were found to be most inhibitory to *C. albicans.*19.3 mm, 17.0 mm, 14.1 mm and 11.3 mm, 14.1 mm, 12.2 mm, 21.3 mm and 22. 2 mm wide, zone was observed against *B. subtilis, S. typhi, E. coli* and *P. mirabilis A. fumigatus and C. albican*s respectively with petroleum ether fraction. Similarly, Chloroform fraction formed 18.0 mm 17.00 mm, 10.9 mm, 9.1 mm, 8.3 mm, and 8.1 mm wide zone against *A. fumigatus, C.albicans,* S. typhi, *B. subtilis, S. typhi, E. coli* and *P. mirabilis* respectively. Whereas 18.3 mm, 17.0 mm,12.3 mm, 10.0 mm, 6.8 mm, 7.2 mm and 7.0 mm wide inhibition zones were observed against *A. fumigatus, C. albicans, B. subtilis, S. typhi, E. coli, Pseudomonas aeurigenosa* and *P. mirabilis* respectively by alcohol fraction. Results revealed that maximum inhibition of all test organisms was brought about by petroleum ether fractions where chloroform and alcohol fractions exhibited moderate activity against all test organisms. Results of antimicrobial activity of various fractions can suggest that all fractions were most effective against test fungi as compared to bacteria.

Minimum Inhibitory Concentration (MIC) of *Pongamia pinnata* seed extract

According to the results given in Table No.3, the minimum inhibitory concentrations (MICs), defined as the lowest concentrations of various fractions of *P. pinnata* seed extract. Petroleum ether, chloroform, and methanol that resulted in complete growth inhibition of the tested pathogens were found to be in the range of 40 µg to 7 mg/mL. The polar extracts displayed significantly remarkable antibacterial activity against all test pathogens. *B. subtilis, P. mirabilis, E.coli, P.aeroginosa, S. typhi, C. albicans and A.fumigatus*with their respective. MIC values ranging from 40 µg/ml to 7 mg/ml. Petroleum ether extract showed greater antimicrobial effect as minimum inhibitory concentrations as compared to chloroform and methanol extracts. In this study test fungi, *A. fumigatus and C. albicans* were found to be more susceptible to plant extracts than bacteria. However, in all cases, the standard antibiotic streptomycin and antifungal fluconazole revealed mild antibacterial and antifungal effect against all the bacterial strains and fungal strain respectively tested as compared to the extracts.

MBC and MFC of petroleum ether fraction of *P. pinnata* seed

Results of bactericidal and fungicidal concentration of petroleum ether fraction of *P. pinnata* seed are given in Table No.4. Bactericidal concentration was found to be 120 µg/ml for *E. coli, P. aeuroginosa* and *P. mirabilis* and 150 µg/ml for *B. subtilis* and *S. typhi.* 70 µg/ml MFC was observed for *C. albicans* whereas this extract showed only fungistatic activity against *A. fumigatus.*

Effect of petroleum ether fraction of *P. pinnata* seed on cell viability of test pathogens

Results of effect of extract on cell numbers of test bacteria are given in Figure No.1. It is evident from results that as concentration of the extract increased number of bacterial cells decreased. No change in cell number of bacteria beyond 60 µg/ml concentration was observed.

In case of *E. coli* average cell number in control was 342.6 x 10^2 cells/ml, which was reduced to 2.66 x 10^2 cell/ml at MIC concentration. 454 x 10^2 cells per ml of *P. mirabilis* were observed in control but at MIC concentration it decreases to 8.6 x 10^2 cell/ml.

Similarly number of *S. typhi* cells also decreased from 425.3.3 x 10^2 cells/ml in control to 9.6 x 10^2 cells/ml at MIC concentration. 672.6 x 10^2 cells/ml of *B. subtilis* were observed in control whereas this number was reduced to 8.6 x 10^2 cells/ml at MIC concentration. In case of *P. aeuroginosa* average cell no. in control is was 336x 10^2 cells per ml which was reduced 10x 10^2 cells/ml at 70 µg/ml. Similarly no. of cells of *C.albicans* and *A. fumigates* were also reduced beyond 60 µg/ml (Table No.5).

DISCUSSION

Problems of antibiotics resistance in both hospital acquired (nosocomial) and community acquired bacterial infections have made many antibiotics virtually obsolete. As the reports show no antibiotics can remain effective for too long. Logical way to combat the problem of microbial antibiotics resistance is to provide new antimicrobial agents. It is vital that research strategies be oriented towards discovery and development of novel antimicrobial agents. Many naturally occurring compounds found in plants, herbs, and spices have been shown to possess antimicrobial functions and serve as a source of antimicrobial agents against pathogens [22]. The main objective of the present study was to evaluate the ability of the plants extract to inhibit the growth of pathogenic bacteria with and without antibiotics and non-antibiotics drugs and to determine their ability to enhance the activity of antibiotics or non-antibiotics drugs.

In the present study, petroleum ether extract of *P. pinnata* seed is more effective against all test microorganisms than the chloroform and alcoholic extracts. Petroleum ether extract were more active against *A. fumigates* and *C. albicans* as compared to bacteria. Many workers have reported that various parts of *P. pinnata* showed antibacterial activity [23-25].

Badole *et al* (2011) [26] reported that *P. pinnata* seed oil exhibited antifungal activity against yeast and mold. Inhibition of tested pathogen is due to presence of sterols, fatty acids, furanoflavonoid; pongamol, pongapin, karanjin, rotenoid, flavone glycosides, flavonoid, triterpenes etc in *P. pinnata* [27-32], Triterpenes 118 is isolated from *P. pinnata* seed oil which showed strong antifungal activity [33].

Results of the present study indicate that inhibition of test microorganisms is concentration dependent. As concentration of the extract increased growth of test organisms also decreased. At higher concentration growth of test organisms was totally inhibited. This concentration dependent activity suggests that the active molecules act as extracellular signals. It can be

assumed that as the concentration of the extract increases the binding between ligands and receptor sites also increases which results in increased inhibitory effects [34].

The petroleum ether fraction of *P. pinnata* seeds shows antibacterial activity against all test organisms but is more active against gram-positive strain *B. subtilis* as compared to gram-negative strains. Lin *et al*.,[1999][35] reported that plant extracts are usually more active against gram-positive bacteria than gram-negative bacteria. Gram-negative bacteria have an effective permeability barrier, comprised of the outer membrane, which resists the penetration of amphipathic compounds and multidrug resistant pumps (MDRs), which extrude toxins across the barrier. It is possible that the apparent ineffectiveness of plant antimicrobial is largely due to this permeability barrier [36].

On comparison of antimicrobial activity of plant extracts with inhibitory effect of standard antimicrobials it can be said that the extract are more potent than their standard drugs as they showed significant inhibition of all test organisms some of which were found to be resistant to the standard drugs.

CONCLUSION

P. pinnata is an ethnomedicinal important plant. It is an enriching plant of flavanoids pongamol, pongapin, karanjin**,** triterpene**,** sterols and fatty acids. Due to these constituents, it showed antibacterial and antifungal activity. From future aspect, it can be used as antibacterial and antifungal ointment and lotion.

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Table No. 1: Percent Extractive Value *P. pinnata* seed

Sr. No.	Plant part	Petroleum ether	Chloroform	Alcohol
1	Seed	40.3	12.5	20.2

Table No. 2: Antimicrobial Activity of *Pongamia pinnata* seed Extract

Sr. No.	Microorganisms	Zone of Inhibition (mm)				
		Plant Extract			Standard control	
		PE	AE	CE	ciprofloxacin	Fluconazole
1.	*P. mirabilis*	11.3±0.20	7.0±0.15	8.3±0.10	5.0±0.15	--
2	*s. typhi*	17.0±0.20	10±0.15	10.9±0.05	9.0±0.11	--
3	*B. subtilis*	19.3±0.15	12.3±0.10	8.1±0.15	11.00±0.10	--
4	*E. coli*	14.1±0.15	6.8±0.10	8.1±0.15	5.2±0.13	--
5	*P. aeruginosa*	12.2±0.11	7.2±0.20	9.1±0.11	6.7±0.14	--
6.	*A. fumigatus*	21.3±0.15	18.3±0.1	18.0±0.15	--	3.5±0.12
7.	*C. albicans*	22.2±0.15	17.0±0.15	17±0.5	--	5.5±0.2

PE= Petroleum Ether Extract, AE= Alcoholic Extract, CE= Chloroform Extract

Table No. 3: Minimum Inhibitory Activity of *Pongamia pinnata* Seed Extract

Sr. No.	Microorganisms	Minimum Inhibitory Concentration (MIC)				
		Plant Extract			Standard control	
		PE	ME	CE	ciprofloxacin	Fluconazole
1.	*P. mirabilis*	80 µg/ml	5 mg/ml	1 mg/ml	100 µg/ml	--
2	*S. typhi*	80 µg/ml	7 mg/ml	1.5 mg/ml	200 µg/ml	--
3	*B. subtilis*	60 µg/ml	3 mg/ml	1 mg/ml	100 µg/ml	--
4	*E. coli*	60 µg/ml	5 mg/ml	1 mg/ml	100 µg/ml	--
5	*P. aeruginosa*	70 µg/ml	5 mg/ml	1.5 mg/ml	200 µg/ml	--
6.	*A. fumigatus*	40 µg/ml	3 mg/ml	2 mg/ml	--	100 µg/ml
7.	*C. albicans*	50 µg/ml	4 mg/ml	1.5 mg/ml	--	100 µg/ml

PE= Petroleum Ether Extract, AE= Alcoholic Extract, CE= Chloroform Extract

Table No. 4: MIC and MFC/MBC of Petroleum Ether Fractions of *P. pinnata* Seed

Sr. No.	Test Microorganism	MIC(µg/ml)	MFC/MBC(µg/ml)
1	*P. mirabilis*	80	120
2	*S. typhi*	80	150
3	*B. subtilis*	60	150
4	*E. coli*	60	120
5	*P. aeruginosa*	70	120
6	*A. fumigatus*	40	Fungistatic
7	*C. albicans*	50	70

Table No. 5: Effect of Petroleum Ether Fraction on Cell viability of Test Microorganism

Sr. No.	Concentration (µg/ml)	*E. coli*	*B. subtilis*	*S. typhi*	*P. mirabilis*	*P. aerogenosa*	*C. albicans*	*A. fumigatus*
1	**control**	342.6x10^2 ±0.57	**672.6x10^2** ±0.57	**425**x10^2 +0**.22**	454x10^2 ±1.0	336x10^2 ±1.52	550.6x10^2±1.5	245.3x10^2 ±1.52
2	**10 µg/ml**	175.3x10^2 ±0.57	455.6x10^2 ±0.57	254.3x10^2±1.52	309.6x10^2±0.57	123.6x10^2±0.57	218.3x10^2±1.52	151.66x10^2±1.15
3	**20 µg/ml**	132.33x10^2±1.15	243.3x10^2 ±1.52	95.6x10^2 ±0.57	152.3x10^2±1.52	79.66x10^2±0.57	132.6x10^2±1.52	120±1.00
4	**30 µg/ml**	125.3x10^2 +0.57	225.3x10^2 ±0.57	66.6x10^2 ±0.57	74x10^2± 1.73	71x10^2± 1.00	95x10^2± 1.00	72±1.00
5	**40 µg/ml**	72.6x10^2± 0.57	190.66x10^2±1.15	43.6x10^2 ±1.15	36.6x10^2 ±1.52	48.66x10^2±0.57	55.3x10^2 ±1.15	52.3±0.57
6	**50 µg/ml**	32.33x10^2 ±1.52	71x10^2±1.0	25.6x10^2 ±1.15	11.33x10^2±1.15	30.33x10^2±0.57	36x10^2± 1.0	30.6±0.57
7	**60 µg/ml**	20.6x10^2± 1.15	19.6x10^2± 0.57	9.6x10^2± 0.57	9.6x10^2± 0.57	23.66x10^2±1.52	22.3x10^2 ±1.52	9.66±0.57
8	**70 µg/ml**	2.66x10^2± 0.57	8.6x10^2±0.57	9.6x10^2± 0.57	8.6x10^2± 0.57	10x10^2± 00	10.6x10^2 ±1.15	2.33±0.57
9	**80 µg/ml**	2.66x10^2± 0.57	8.6x10^2±0.57	9.6x10^2± 0.57	8.6x10^2± 0.57	10x10^2± 00	10^2±1.00	2.33±0.57
10	**90 µg/ml**	2.66x10^2± 0.57	8.6x10^2±0.57	9.6x10^2± 0.57	8.6x10^2± 0.57	10x10^2± 00	**2.2±0.12**	2.33±0.57
11	**100 µg/ml**	2.66x10^2± 0.57	8.6x10^2±0.57	9.6x10^2± 0.57	8.6x10^2± 0.57	10x10^2± 00	**2.2±0.12**	2.33±0.57

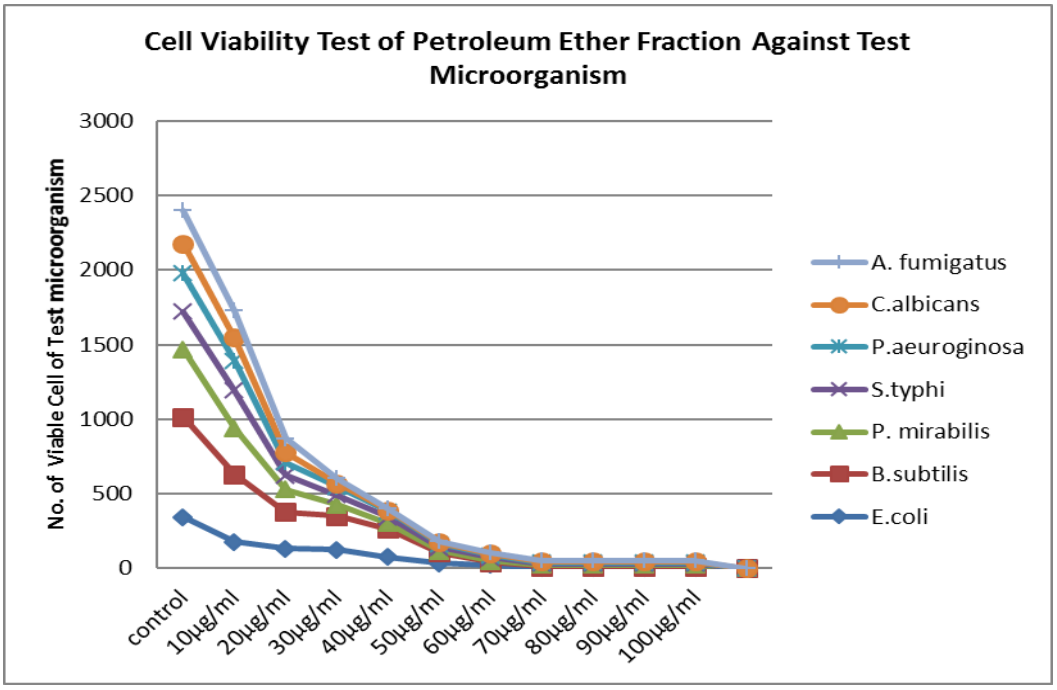

Figure No. 1: Cell Viability Test of Petroleum Ether Fraction against Test Microorganism