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IN VIVO AND IN VITRO PROPORTIONAL ANTIMICROBIAL ACTIVITY IN KARANJ (PONGAMIA PINNATA): AN IMPERATIVE LEGUMINOUS TREE

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ABSTRACT

The present study was undertaken to determine the anti-microbial potential of the methanol extract of different plant parts of Pongamia Pinnata in vivo and in vitro. All the extractions were tested against different gram positive and gram negative bacteria and fungi to find out their antimicrobial activity using agar well diffusion and micro dilution method for fortitude the MIC value. The maximum antimicrobial activity of root extract was observed against E. coli and F. oxisporum while in leaf extract maximum antimicrobial activity was observed against S. aureus, B. cereus and A. solani. However, Callus extracts showed mild to moderate inhibitory effects against E. cloacae, P. aeruginosa, A. flavus and R. stolonifer. Besides these, the maximum antimicrobial activity of stem extract was observed only against A. niger. Flucanazole and Ampicillin standards were used as positive controls respectively for fungi and bacteria in this study.

KEYWORDS Pongamia Pinnata, MIC, Anti-microbial, Flucanazole, Ampicillin.

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 sources; many of these isolations were based on the uses of the agents in traditional medicine. This plant-based, traditional medicine system continues to play an essential role in health care, with about 80% of the world's inhabitants relying mainly on traditional medicines for their primary health care (Owolabi et al., 2007). According to World Health Organization, medicinal plants would be the best source to obtain a variety of drugs. Therefore, such plants should be investigated to better understand their properties, safety and efficacy (Nascimento et al., 2000).

Plant products and their active constituents played an important role in plant disease control by combating growth and development of pathogens and including resistance in plants. Plant based natural constituents can be derived from any part of the plant like bark, leaves, flowers, roots, fruits, seeds etc. (Gordon and David, 2001). As these plants and their products are known to posses various secondary metabolites, which showed significant inhibitory effect against the growth of pathogens, therefore, the plant and their products should be utilized to combat the disease causing pathogens. Thus the wide spectra of antimicrobial research is geared towards the discovery and development of noval antibacterial and antifungal agents. The demand for plant based medicines, health products, pharmaceuticals, food supplement and cosmetics etc. are increasing in both developing and developed countries. Since these natural products are non-toxic, have less side effects and easily available at affordable prices they are recognize worldwide (Kalia, 2005).

Pongamia is a genus having one species only Pongamia pinnata (L.) [Syn. Pongamia glabra (Vent); Derris indica (Lamk.)] which belongs to family Leguminosae and sub-family Papilionaceae (Merra et al., 2003). It is a medium sized glabrous, perennial tree grows in the littoral regions of South Eastern Asia and Australia (Satyavati et al., 1987). In spring, Pongam is at its finest when the showy, hanging clusters of white, pink, or lavender, pea-like, fragrant blossoms appear, the clusters up to 10 inches long. These beautiful blossoms and the glossy, nearly evergreen leaves imparipinate, shiny, young and deep green, leaflets 5-9, the terminal leaflet larger than the others, help make Pongam a favorite for use as a specimen, shade, or windbreak. It is a preferred species for controlling soil erosion and binding sand dunes because of its dense network of lateral roots. Root, bark, leaves, flower and seeds of this plant also have medicinal properties and traditionally used as medicinal plants. All parts of the plant have been used as crude drug for the treatment of tumors, piles, skin diseases, wounds and ulcers (Tanaka et al., 1992). In the traditional system of medicines, such as Ayurveda and Unani, the Pongamia pinnata plant is used for anti-inflammatory, anti-plasmodial, anti-nonciceptive, anti-hyperglycamic, anti-lipidperoxidative, anti-diarrhoeal, anti-ulcer, anti-hyperammonic and antioxidant (Chopade et al., 2009).

Therefore, the presents study was planned to observe the antimicrobial activity of methanolic extracts of different plant parts of Pongamia pinnata against selected microorganisms in vivo and in vitro.

MATERIAL AND METHODS

Collection of plant material

Plant of Pongamia pinnata was collected from the campus of University of Rajasthan, Jaipur. Specimens were compared with the voucher specimens at Herbarium of Department of Botany, University of Rajasthan, Jaipur. Different plant parts (root, stem, leaves, seeds) and in vitro callus was also taken for the further studies.

Preparation of extract

The fresh plant samples root, stem, leaf and seeds of Pongamia pinnata were collected and washed individually under running tap water to remove soil particles and other dirt. The leaf was air dried in the laboratory at room temperature for 15 days. While the stem, root, seeds and callus samples were dried at 60˚ C for 2 days in an oven. The dried all samples were ground well into a fine powder in a mixer grinder. The powder was stored in air sealed polythene bags at room temperature before extraction. A fixed weight (25 gm) of powdered material was soxhlet extracted in 250 ml of 70 % methanol for 72 hours. Each mixture was stirred at 24 hour interval using a sterile glass rod. At the end of extraction each extract was passed through Whatman No.1 filter paper, and evaporated under vacuum. All extracts were stored at 4^{o}C in a refrigerator until screened.

Callus induction

Internodes were surface sterilized by 1 % Teepol for 15 min followed by 0.1 % mercuric chloride for 10 min, and then rinsed thoroughly with sterile distilled water. The internodes were inoculated in the MS medium (Murashige and Skoog, 1962) fortified with different concentrations of NAA, BAP and TDZ. The pH of the media was adjusted to 5.8 before autoclaving. All media were autoclaved at 1.06 kg cm-2 and 121ºC for 15 min. The cultures were incubated in growth room at temperature of 25 ± 2 °C and 16-h photoperiod. 20 replicate cultures were established and each experiment was repeated twice and the cultures were observed at regular intervals.

Test Organisms

All the microbial strains (bacteria and fungi) of human pathogens used in the antimicrobial bioassay were procured from Institute of Microbial Technology (IMTECH), Chandigarh.

Bacteria

Pure cultures of all experimental bacteria viz. Gram-negative bacteria such as Escherichia coli (MTCC 1652), Enterobacter cloacae (7097); the Gram-positive bacteria such as Bacillus cereus (MTCC 4317), Staphylococcus aureus (MTCC 3160) and pseudomonas aeruginosa (MTCC 4676) were maintained on nutrient agar (Hi-media) in Institute of Applied Sciences and Biotechnology (Chemind Biosolutions Laboratory), Jaipur. Each bacterial culture was further maintained on the same medium after every 48 hours of transferring and stored at 4^{o}C before use in experiments.

Fungi

Pure cultures of all experimental fungi viz. Aspergillus niger (MTCC 282), A. flavus (MTCC 2456), Alternaria solani (MTCC 2101), R. stolonifer (MTCC 2591) and F. oxisporum(MTCC 6659) were maintained on Potato Dextrose Agar (PDA) (Hi-media) and the cultures stored at 4 ^{o}C and sub-cultured once a month in the Institute of Applied Sciences and Biotechnology (Chemind Biosolutions Laboratory), Jaipur.

Media preapration and its sterilization

For agar well diffusion method (Bauer et al., 1996), antimicrobial susceptibility was tested on solid (Agar-agar) media in petri plates. For bacterial assay nutrient agar (NA) (40 gm/L) and for fungus PDA (39 gm/L) was used for developing surface colony growth. The minimum inhibitory concentration (MIC), the minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) values were determined by serial micro dilution assay (Akinyemi, 2005). The suspension culture, for bacterial cells growth was done by preparing 2% Lauria Broth (w/v), and for fungus cells growth, 2.4% (w/v) PDB (Potato dextrose broth) was taken for evaluation. All the media prepared was then sterilized by autoclaving the media at (121°C) for 20 min.

Preparation of plates

Prepared agar was then allow the sterilized and then to cool to 50 °C in a water-bath. Pouring of about 20 ml agar into pre-labeled sterile Petri dishes was made. They were then allowed to set at room temperature and were dried so that no drops of moisture remain on the surface of the agar.

Agar well diffusion

Antibacterial and Antifungal activities of the plant extract were tested using Well diffusion method. Nutrient agar (NA) and Potato Dextrose Agar (PDA) plates were swabbed (sterile cotton swabs) with 8 hour old - broth culture of respective bacteria and fungi. Wells were made on the agar surface with 6mm in diameter and about 2 cm a part punctured in the culture medium using sterile cork borers. The plates were then turned up side down and the wells were labeled with a marker. Stock solution of each plant extract was prepared at a concentration of 1 mg/ml in methanol. Each of the wells was filled with 100 µl of the plant extracts using sterile syringe. Only one well was filled with 100 µl of methanol which served as a negative or a positive control, respectively. The plates were incubated at 37 ºC for 24 hours for bacterial and 25 ºC for 48 hours for fungal activity. The plates were observed for the zone clearance around the wells.

The zone of inhibition was calculated by measuring the diameter of the inhibition zone around the well (in mm) including the well diameter. The readings were taken in three different fixed directions in all three replicates and the average values were tabulated.

Micro dilution method

The minimum inhibitory concentrations (MIC), MBC and MFCs were performed by a serial dilution technique using 96-well microtiter plates. The plant extracts were dissolved in methanol (1 mg/ml) and serial dilution of the extract with luria broth for bacterial culture and for fungus, potato dextrose broth medium with respective inoculum were used. The microplates were incubated for 72 hours at 28°C, respectively. The lowest concentrations without visible growth (at the binocular microscope) were defined as MICs.

The fungicidal concentrations (MFCs) were determined by serial sub-cultivation of a 2 µl into microtiter plates containing 100 µl of broth per well and further incubation 72 hours at 28°C. The lowest concentration with no visible growth was defined as MFC indicating 99.5% killing of the original inoculum. Methanol was used as a negative control, and commercial standards, Flucanazole and Ampicillin (Sigma), were used as positive controls (1–3000 µg/ml) respectively for fungi and bacteria. All experiments were performed in duplicate and repeated three times. The MBCs were determined by serial sub-cultivation of 2 µl into microtitre plates containing 100 µl of broth per well and further incubation for 72 hours. The lowest concentration with no visible growth was defined as the MBC, indicating 99.5% killing of the original inoculum. The optical density of each well was measured at a wavelength of 655 nm by Microplate reader (Perlong, ENM8602) and compared with the standards Ampicillin for Bacteria and Fluconazole (Hi-media lab, India) for fungi as the positive control. All experiments were performed in duplicate and repeated three times.

RESULTS

In the present investigation, the inhibitory effect of crude methanolic extracts of different plant parts from Pongamia pinnata were evaluated against both fungicidal and bacterial strains (gram positive and gram negative). In vitro and in vivo antimicrobial activity was determined using agar well diffusion method and micro dilution method summarized in Table 1 and 2. The activity was quantitatively assessed on the basis of inhibition zone and their activity index was also calculated along with minimum inhibitory concentration (MIC).

Callus Induction

MS medium supplemented with combination of NAA, BAP and TDZ along with additives like citric acid and ascorbic acid for callus induction. Internodes showed maximum callus formation on MS medium with combination of auxin NAA (6mg/L) and cytokinin BAP (1mg/L), TDZ (0.02mg/L) along with additives like citric acid (50mg/L) and ascorbic acid (100mg/L). Similar result of using auxins, cytokinin and additives for callus induction was also observed by Keresa et al., 2009 in Iris adriatica Trinajstić ex Mitić. However in contrast to this Amiri et al., 2011, reported that only one auxin 2,4-D is responsible for maximum callus growth in Datura stramonium L. The callus obtained from above observation was compact and creamish yellow colored. Callus obtained after 8 weeks of culture was further evaluated for antimicrobial activity.

Measurement of inhibition zone diameter

The largest zone of inhibition was observed for methanolic root extract against E.coli (22.4 ± 0.86 mm) and minimum was observed in stem (13.2 ± 0.31mm).Where as in the case of Enterobacter cloacae the largest zone of inhibition was shown by callus (19.5 ± 0.52 mm) and minimum by methanolic

seed extract (10.8 ± 0.21 mm). In Staphylococcus aureus and Bacillus cereus the largest zone of inhibition was observed by leaf extract (22.4 ± 0.23 mm) and (21.3 ± 0.62 mm) respectively but minimum was observed in seed (11.6 ± 0.13 mm) against Staphylococcus aureus and in callus (13.8 ± 0.16 mm) against Bacillus cereus. However, the largest zone of inhibition was observed for callus extract (21.8 ± 0.79 mm) and minimum was observed in stem (12.9 ± 0.66 mm) against Pseudomonas aeruginosa.(Fig.1)

The largest zone of inhibition was observed for methanolic stem extract (19.6 ± 0.73 mm) and minimum was observed in callus (9.6 ± 0.70 mm) against Aspergillus niger. In Aspergillus flavus and R. stolonifer the largest zone of inhibition was observed by callus extract (19.2 ± 0.45 mm) and (23.5 ± 0.22 mm) respectively, and minimum inhibition zone was observed in stem (13.6 ± 0.82 mm) ,(14.6 ± 0.39 mm) respectively for above both the fungus. However, in Alternaria solani highest zone of inhibition was found in leaf (20.4 ± 0.39 mm) and minimum zone in stem (11.6 ± 0.79 mm). In F. oxisporum the largest zone of inhibition was found in root (18.2 ± 0.55 mm) and lowest zone in seed extract (10.2 ± 0.16 mm).(Fig.2)

Determination of MIC, MBC and MFC values

The minimum inhibitory concentration is defined as the lowest concentration which able to inhibit any visible bacterial and fungal growth on the culture plates (Shahidi, 2004; Prescott et al., 1999). The lowest concentration of the extract that completely inhibited micro-organisms growth (no turbidity) in comparison to control was regarded as MIC. Whereas, the MBC and MFC was determined by sub-culturing the test dilution (used in MIC) on to a fresh solid medium and incubated further for 24 hours. The concentration of plant extract that completely killed the bacteria and fungi was taken as MBC and MFC respectively. However, it was noted that most of the antimicrobial properties in different plant part extractions shows, MBC value that is almost two fold higher than there corresponding MICs (Omar et al., 2010).

Root extract of Pongamia Pinnata showed least MIC value i.e. 19.3 µg/ml against E.coli. While the callus extract was 28.4 µg/ml against Enterobacter cloacae and 30.9 µg/ml against P. aeruginosa. Where as in case of Staphylococcus aureus and Bacillus cereus the least MIC value i.e. 22.3 µg/ml and 26.5 µg/ml respectively for the both bacteria was observed in leaf extract.

Stem extract of Pongamia Pinnata showed least MIC value i.e. 11.2 µg/ml against Aspergillus niger while the leaf extract i.e. 8.9 µg/ml and the root extract 27.9 µg/ml was observed against Alternaria solani and F. oxisporum respectively. However, in case of Aspergillus flavus and R. stolonifer the least MIC value i.e. 8.9 µg/ml and 13.5 µg/ml respectively for the both fungi was observed in callus extract.

DISCUSSION

Many microorganisms, which cause damage to human health, exhibit drug resistance due to inadequate use of antibiotics. Thus, there is a need for the discovery of new substances from natural sources, including plants. Santo et al, (1995) repotted that the World Health Organization has indeed recognized medicinal plants as the best source for obtaining a variety of synthetic drugs. No doubt, some studies have identified and isolated the main active ingredients in the plants responsible for this antimicrobial activity (Oloke et al., 1988; Carson and Riley, 1995). However, the study on medicinal

plants will allow for the demonstration of their physiological activity and also catalyze many pharmacological studies that will lead to the development of more toxicity and high sensitivity especially towards the emerging microbial agents (Fabricant and Famsworth, 2001).

There is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action due to an alarming increase in the incidence of new and reemerging infectious diseases and development of resistance to the antibiotics in current clinical use (Bauer et al., 2003). The screening of plant extracts has been of great interest to scientists in the search for new drugs for greater effective treatment of several diseases (Dimayuga and Garcia, 1991). Therefore, plant extracts and phytochemicals with known antimicrobial properties can be of great significance in therapeutic treatments (Diallo et al., 1999; Rojas et al., 2006; Erdogrul, 2002).

In the present investigation methanolic extracts of the different plant parts of Pongamia pinnata were screened for their antimicrobial activities. Among the ten microbes tested (E. coli, E. cloacae, B.cereus, S. aureus, P. aeruginosa, A. niger, A. flavus, A. solani, R. stolonifer and F. oxisporum), extracts of the various plant parts showed antimicrobial activity against all the tested microorganisms. The maximum antimicrobial activity of root extract was observed against E. coli and F. oxisporum while in leaf extract maximum antimicrobial activity was observed against S. aureus, B. cereus and A. solani. However, Callus extracts showed mild to moderate inhibitory effects against E. cloacae, P. aeruginosa, A. flavus and R. stolonifer. Besides these, the maximum antimicrobial activity of stem extract was observed only against A. niger.

The antimicrobial activities of Pongamia pinnata plant parts have been studied earlier by many scientists (Simin et al., 2002; Ahmad et al., 2004; Brijesh et al., 2006; Kumar et al., 2007; Wagh et al., 2007; Bajpai et al., 2009; Kesari et al., 2009; Patil et al., 2010; Shanthi et al., 2011).

Thus from our findings, it is concluded that the bioactive principles responsible for the antimicrobial activities against these tested microorganisms should be isolated identified and elucidated its structure to develop a new lead of therapeutic interest to cure various human ailments. It also gives evidence that the extracts of the both plant species can be regarded as a promising source for antimicrobial drugs.

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Test Organisms		**Pongamia pinnata**					
Bacteria		**IZ / AI**	**Root**	**Stem**	**Leaf**	**Seed**	**Callus**
1	**E.coli**	**IZ**	22.4 ± 0.86	13.2 ± 0.31	18.9 ± 0.11	20.7 ± 0.52	16.3 ± 0.89
		AI	0.918	0.54	0.774	0.848	0.668
2	**E.cloacae**	**IZ**	12.4 ± 0.13	15.1 ± 0.19	18.4 ± 0.50	10.8 ± 0.21	19.5 ± 0.52
		AI	0.65	0.791	0.964	0.566	1.022
3	**S.aureus**	**IZ**	14.5 ± 0.39	19.3 ± 0.82	22.4 ± 0.23	11.6 ± 0.13	17.9 ± 0.62
		AI	1.418	1.528	1.773	0.918	1.417
4	**B. cereus**	**IZ**	19.4 ± 0.35	17.6 ± 0.19	21.3 ± 0.62	16.6 ± 0.12	13.8 ± 0.16
		AI	0.901	0.817	0.989	0.771	0.641
5	**P. aeruginosa**	**IZ**	17.4 ± 0.56	12.9 ± 0.66	20.9 ± 0.34	14.4 ± 0.30	21.8 ± 0.79
		AI	0.848	0.628	1.019	0.702	1.062

Fungi		IZ / AI	Root	Stem	Leaf	Seed	Callus
1	**A. niger**	**IZ**	14.6 ± 0.35	19.6 ± 0.73	15.7 ± 0.43	10.4 ± 0.55	9.6 ± 0.70
		AI	0.828	1.111	0.89	0.589	0.544
2	**A. flavus**	**IZ**	15.5 ± 0.24	13.6 ± 0.82	17.3 ± 0.45	18.5 ± 0.34	19.2 ± 0.45
		AI	0.908	0.796	1.013	1.083	1.124
3	**A. solani**	**IZ**	17.1 ± 0.62	11.6 ± 0.79	20.4 ± 0.39	17.6 ± 0.16	14.2 ± 0.19
		AI	0.79	0.536	0.942	0.813	0.656
4	**R. stolonifer**	**IZ**	18.3 ± 0.66	14.6 ± 0.39	20.9 ± 0.43	16.5 ± 0.29	23.5 ± 0.22
		AI	1.193	0.952	1.363	1.076	1.532
5	**F. oxisporum**	**IZ**	18.2 ± 0.55	17.9 ± 0.42	14.4 ± 0.69	10.2 ± 0.16	13.1 ± 0.82
		AI	2.068	2.034	1.636	1.159	1.488

Table: 1- Inhibition zone diameters in methanolic extract of different plant parts of Pongamia pinnata were measured by Agar well diffusion method (in mm).

IZ = Inhibition zone, AI = Activation index

AI (Activity index) = Inhibition area of test sample

Inhibition area of the standard

Microorganisms		**Pongamia pinnata**						
Bacteria		**MIC / MBC**	**Standard**	**Root**	**Stem**	**Leaf**	**Seed**	**Callus**
1	**E.coli**	**MIC**	20.1	19.3	49.3	32.4	26.4	40.2
		MBC	40.3	40.2	98.4	65.6	52.8	81.8
2	**E.cloacae**	**MIC**	33.2	52.2	46.2	41.3	58.2	28.4
		MBC	60.9	105.4	93.8	82.6	117	56.8
3	**S.aureus**	**MIC**	20.6	44.9	30.6	22.3	46.4	34.5
		MBC	40.1	89.2	61.4	45.4	93.4	69.9
4	**B. cereus**	**MIC**	23.5	29.6	32.2	26.5	39.4	49.2
		MBC	64.9	61.3	65.5	52.3	72.3	94.5
5	**P. aeruginosa**	**MIC**	22.01	40.3	55.2	39.8	50.7	30.9
		MBC	45.3	82.6	114	82.4	109	62.4

Fungi		MIC / MBC	Standard	Root	Stem	Leaf	Seed	Callus
1	**A. niger**	**MIC**	9.3	32.3	11.2	25.4	19.9	40.6
		MFC	20.4	65.9	24.8	51.8	39.2	84.9
2	**A. flavus**	**MIC**	10	24.2	39.4	16.9	32.4	8.9
		MFC	20.3	49.5	73.9	34.2	65.2	17.5
3	**A. solani**	**MIC**	36.3	47.7	53.2	32.4	42.6	37.8
		MFC	72.4	94.2	107.1	64.1	85.3	74.9
4	**R. stolonifer**	**MIC**	11.7	29.4	43.2	19.9	35.8	13.5
		MFC	23.9	61.6	88.4	39.3	71.9	27.2
5	**F. oxisporum**	**MIC**	30.7	27.9	38.2	40.5	49.5	44.3
		MFC	57.6	54.3	79.9	82.3	98.4	89.3

MIC = Minimum inhibition oncentration

MBC = Minimum bacterial oncentration

MFC = minimum fungicidal oncentration

Table: 2- MIC values obtained in methanolic extract of different plant parts of Pongamia pinnata were measured by Micro dilution method (in µg/ml).

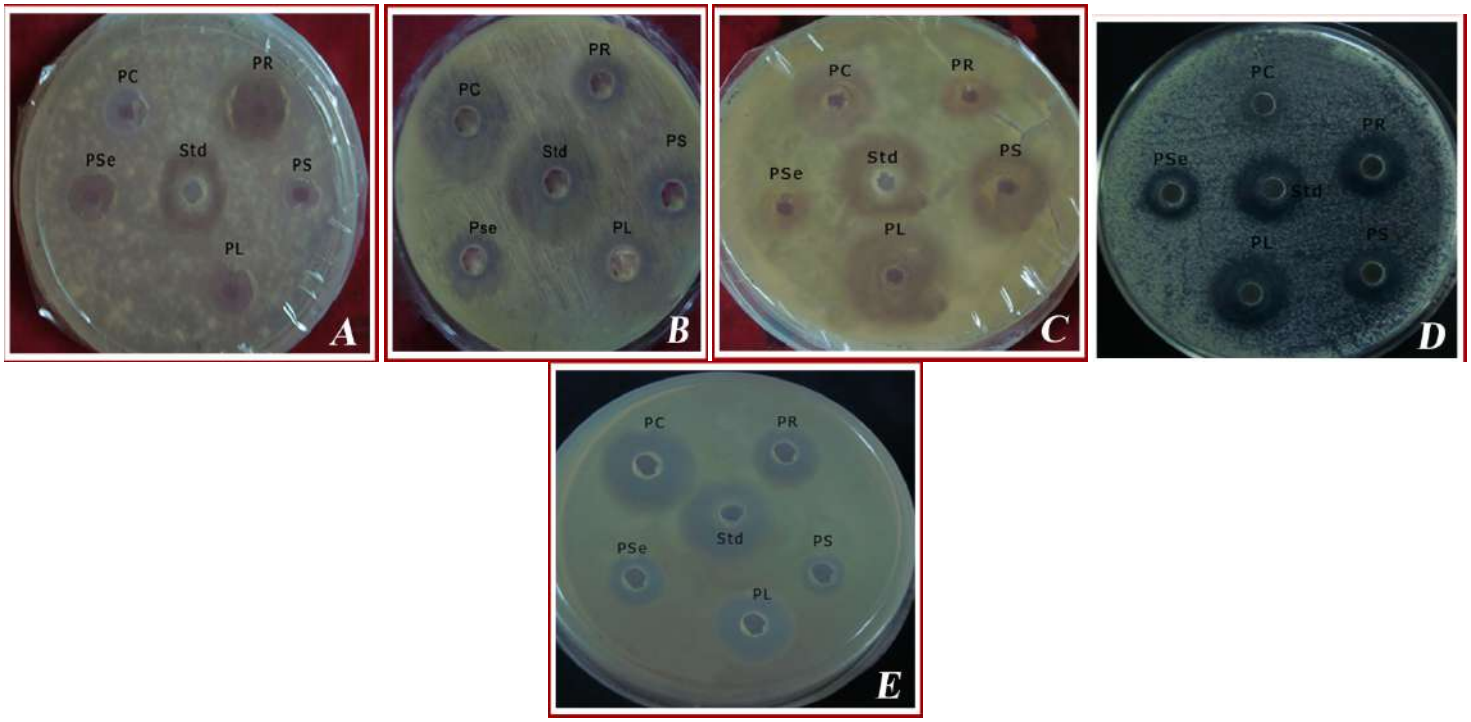

Fig:1 Antimicrobial activity of different plant parts of Pongamia pinnata against (a) E.coli (b) E.cloacae (c) S.aureus (d) B. cereus (e) P. aeruginosa

Abbreviations: (Std- Standard, PL- Pongamia leaf, PS- Pongamia stem, PR- pongamia root, PC- pongamia callus, PSe- Pongamia seed)

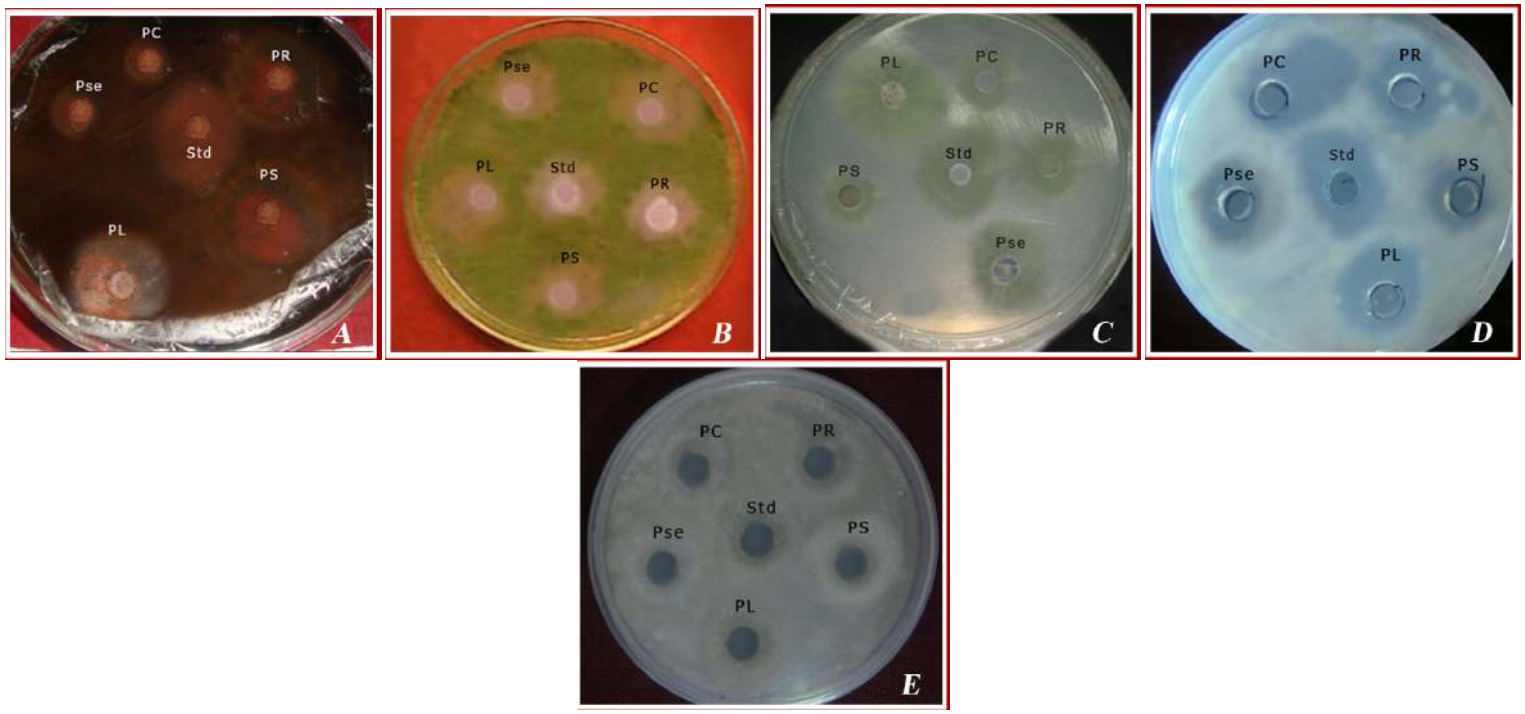

Fig:2. Antimicrobial activity of different plant parts of Pongamia pinnata against (a) A. niger (b) A. flavus (c) A. solani (d) R. stolonifer (e) F. oxisporum

Abbreviations: (Std- Standard, PL- Pongamia leaf, PS- Pongamia stem, PR- pongamia root, PC- pongamia callus, PSe- Pongamia seed)