JOURNAL OF PURE AND APPLIED MICROBIOLOGY, June 2013. Vol. **7**(2), p. 1027-1033

Antimicrobial Evaluation of Fluorescent *Pseudomonas* sp. Inhabiting Medicinal Plant *Annona squamosa* L.

Syed Baker and S. Satish*

Herbal Drug Technological Laboratory, Department of Studies in Microbiology, University of Mysore, Manasagangotri, Mysore - 570 006, India.

(Received: 22 July 2012; accepted: 17 September 2012)

The present study was designed and executed to screen bacterial endophytes from medicinal plant *Annona squamosa* L. The obtained isolates were evaluated for antimicrobial activity, based on the higher inhibitory activity further studies were carried out. Among the isolated endophytes, fluorescent bacteria expressed significant activity against the all test pathogens which was the subject of the present investigation. Based on the 16s rRNA and biochemical tests, the bacterium belonged to the genus *Pseudomonas* sp. Crude ethyl acetate extract of the isolate was evaluated for antimicrobial activity against clinically important Gram positive and Gram negative bacteria via disc diffusion assay, minimal inhibitory concentration and bioautography. MIC concentration of the ethyl acetate extract varied from 0.19 μg mL^{-1} to 25.0 μg mL^{-1} against the test pathogens. Further purification and characterization of crude ethyl acetate extract will be promising enough to reveal any novel metabolites of pharmaceutical importance.

Key words: Bacterial endophytes, antimicrobial activity, fluorescent *Pseudomonas* sp.

The accelerating haunt for new antibiotics has upsurge due to increasing drug resistant microorganisms to existing drugs, the appearance of life-threatening viruses and the tremendous increase in the incidence of fungal infections has been a major concern across the globe, which in turn resulted for new scientific discoveries to combat existing problem. Plant source expressed promising results but slow growing rate and harvesting of rare endangered species pose a risk and imbalance to biodiversity of plants[1]. Chemical diversity bearing pharmaceutical potential has reached beyond the plant kingdom by dwelling alternative resource of microorganisms which forms a huge diversity in nature and has generated more attention and interest in recent decades. Among the richness of microbial world encompasses a plethora of endophytic entities occupying utterly millions of unique biological niches in higher plants at various, many times unusual, environments. The term *endophyte* (Gr. *endon*, within; *phyton*, plant) was first coined by de Bary (1866) and has become deeply embedded in the literature ever since. At present, endophytic microorganisms are defined as "microbes that colonize living, internal tissues of plants without causing any immediate, overt negative effects"[2]. These endophytes can mimic the chemistry of their respective host plants and produce almost similar bioactive natural products or derivatives that are more active than those of their respective host[3].

Perusal of studies reported so far envisioned that endophytes forms ware house of biologically active compounds. Modern technologies have opened new avenue on endophytic research for highly sustainable and economically feasible novel natural products at large scale via fermentation compared to any other sources which are presumed to push forward the frontiers in drug discovery research. Ongoing global efforts to discover antimicrobial compounds from endophytic plethora have yielding significant

* To whom all correspondence should be addressed. Tel.: +91 821 2419734(Off); +91 9448323129 (Cell); E-mail:satish.micro@gmail.com

promising results. Research on endophyte has yielded valuable compounds bearing antimicrobial properties such as antifungal, antibacterial and antiviral agents. So far reported antimicrobial compounds belong to several structural classes like alkaloids, peptides, steroids, terpenoids, phenols, quinines, and flavonoids[4]. *Annona squamosa* L. (custard apple) is reputed to possess several medicinal properties by curing various diseases such as skin infections, diarrhea, dysentery, urinary tract infections and as antitumor agents as it possesses potent bioactive principles in all its part[5]. The present study aims to screen potent bacterial endophytes inhabiting medicinal plant *Annona squamosa* L. to evaluate antimicrobial activity of isolated endophytes against clinically important and phytopathogenic microorganisms.

MATERIALS AND METHODS

Chemicals

All the media and chemicals employed in the present investigation were procured from reputed international firms such as M/s Hi media and Sigma Aldrich .Where as solvents were procured from M/s Merck.

Test strains

Fourteen different strains of human and phytopathogenic microorganisms *viz*., *Bacillus subtilis* (MTCC 121), *Candida albican* (MTCC 183), *Escherichia coli* (MTCC 7410), *Proteus mirabilis* (MTCC 245), *Salmonella typhi* (MTCC 733), *Shigella flexnerri* (MTCC 731), *Staphylococcus aureus* (MTCC 7443), *Staphylococcus epidermidis* (MTCC 435), *Vibrio parahaemolyticus* (MTC 451), *Xanthomonas campestris* (MTC 7908), *Fusarium verticilloides, Aspergillus niger* and *Aspergillus flavus* were employed to evaluate the antimicrobial activity of ethyl acetate extract. Test cultures were procured from IMTECH-MTCC Chandigarh India.

Sample collection

Plant material such as leaves, stem and bark were randomly collected from healthy plants from Mysore, Southern India. The materials were collected in a sterilized polybags and transported to laboratory within two hours before processing. Collected plants material were thoroughly washed under running tap water then were immersed in a double distilled water containing 50 µg/ml of cycloheximide for 60 mins (minutes) to suppress the growth of fungal endophytes[6].

Surface sterilization

Plant material were subjected to surface sterilization under aseptic condition by immersing in 3.15% sodium hypochlorite for 5 mins and then followed by ethanol 70% for 30 seconds. In every step of the surface sterilization procedures the plant material were washed in sterile double distilled water. Outer tissue of surface sterilized plant material were removed with sterilized scissor scalpel, later carefully excise into 0.5-1.0 cm tissue blocks of plant material and placed on surface of nutrient agar supplemented with 250 µg/ml of cycloheximide and incubated for 48hrs (hours) to observe colonies of endophytic bacteria[6,7]. To assess whether the method employed for surface sterilization was accurate, we individually assayed the sterile distilled water in which tissues were washed after being placed in bleach and ethanol and aliquots were plated on to nutrient agar which served as control plate.

Primary screening of endophytic bacterial isolates for antimicrobial activity

Endophytic bacterial colonies obtained were pure cultured and maintained for further studies. Isolates were subjected to primary screening for antimicrobial activity by dual culture method for fungi where in the isolates were streaked perpendicular across the test pathogenic fungi and incubated[8] and agar over lay method for bacteria where in endophytic isolate were point-inoculated and incubated for 3-days. Later colonies were inactivated by inverting the lower lid with 1-5ml chloroform for 40 mins. The inactivated colonies were overlaid with 5 ml of sloppy soft agar with 0.6% nutrient agar seeded with the test organisms. Zones of inhibition around the colonies were recorded after 24 hrs at 30°C [9]. In the present study fluorescent endophytic colony that showed fluorescence at 365 nm was selected for further evaluating potent antimicrobial activity and was cultured on an optimized media for the growth and production of antimicrobial metabolite.

Optimizing parameters for the growth and antimicrobial production

Endophytic fluorescent strain grown via, fermentation process for large scale production by inoculating the active growing cells into

sterilized nutrient broth with 10 % of glycerol and incubated at 28°C with 120 rpm and aerated with 40% diluted oxygen and maintained at pH 7.0 for 72 hours. The turbidity of the bacterial growth was measured by estimating the optical density through spectrophotometer at regular intervals.

Identification of isolated strain by biochemical tests and molecular techniques

Colonies with characteristic pigment was tested biochemically and identified using the Bergey's manual of Systematic Bacteriology. Molecular characterization was carried out by following procedure three milliliters of fresh cultures of the isolates in Luria broth was used for DNA extraction according to Genomic DNA was used as a template in a polymerase chain reaction (PCR) with the primers forward primer 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse primer 5' -GGTTACCTTGTTACGACTT-3' used for amplifying 16S rRNA for molecular characterization[10].

Extraction

After the fermentation process the culture broth was centrifuge to separate supernatant and cell pellet. Supernatant obtained was used for solvent extraction with ethyl acetate as a solvent. Equal volume of supernatant and ethyl acetate with ratio 1:1 was pooled in a separating funnel and shaken vigorously and allowed to settle. Organic phase was separated and collected the procedure was repeated 3 times to pool the organic phase. Later the extracted solvent was reduced under vacuum and residue obtained was dried and stored in vials at 4°C for further antimicrobial studies.

Evaluation of antimicrobial activity

Disc diffusion assay

Antibacterial activity was determined using the disc diffusion method pre-warmed MHA (Mueller-Hinton agar) plates were seeded with 10^6 CFU (colony forming unit) suspension of test bacteria. Crude ethyl acetate extract dissolved in ethyl acetate (1 mg/ml). 20 µl extract was impregnated onto sterile paper discs (6 mm diameter) and placed on the surface of inoculated agar plates. Plates were incubated at 37°C for 24 hrs. Antibacterial activity was expressed as the diameter of the inhibition zone in mm (millimeter) produced by the extracts. Ethyl acetate was used as negative control and gentamicin standard disc were used as positive control[11].

Minimum inhibition concentration

Minimum inhibitory concentration was determined by broth micro-dilution assay technique in 96 wells micro-titer plates in which 90 µl (micro-liter) overnight broth cultures of the each test organisms were seeded into the wells and 100 µl crude extract was added in each well at descending concentration starting from 1000 µg mL^{-1} to 1.95 µg mL^{-1} with alternative wells seeded with test microorganism and standard antibiotic gentamicin. The plates were incubated for 24hrs at 35±1 ºC and TTC (triphenyl tetrazolium chloride) was used as microbial growth indicator. Minimum inhibition concentration was determined as lowest concentration of the crude extract that inhibited the growth of the test organisms[12].

Bioautography

The TLC bioautographic agar assay, was carried out where in crude ethyl acetate extract was partially purified and separated by using TLC plates (Silica GF 60) with solvent system ethyl acetate and hexane at a ratio (2:1) later developed TLC plates were overlaid with 1-2 mm layer of soft medium BHI (Brain Heart Infusion agar 0.6%) containing 0.1 % (w/v) 2, 3, 5 triphenyltetrazolium chloride (tetrazolium red) with previous inoculated test organisms at a final concentration of 10^7 CFU/ml. The plates were placed in a sterile tray, sealed to prevent the thin agar layer from drying, and incubated at 37°C for 24 hrs the plates were run in duplicate[13].

RESULTS

Bacterial endophytic diversity inhabiting *Annona squamosa* L. has led us to report the endophytic fluorescent *Pseudomonas* sp. exhibiting antimicrobial activity against the test pathogens which attributed the assumption of endophytes being one of the rich sources of functional bioactive metabolites in nature. The use of cycloheximide for surface sterilization suppressed the growth of fungi resulted in obtaining only bacterial endophytic flora. The isolates obtained expressed different morphological characteristic, the preliminary screening of antimicrobial activity by dual culture method inhibited the growth of test fungal pathogen *viz*., *F. verticilloides, Aspergillus niger* and *A. flavus* with perpendicular streak of isolate across the test

Table 1. Biochemical characteristics of fluorescent *Pseudomonas* sp.

Biochemical tests	Results
Catalase	+
Oxidase	-
Lecthiniase	+
Gelatin hydrolysis	+
Starch hydrolysis	-
Argenine dihydrolase	+
Urease	+
Nitrate reductase	-
Growth on King's B medium	+
Growth on Cetrimide agar	+
Growth at 4°C	+
UV Fluorescence	+

pathogen. Agar over lay method for bacteria displayed clear zone in the lawn of test pathogenic bacteria laid over point inoculated endophytic isolate expressing the antimicrobial activity the former and latter methods displayed significant results with seven isolates exhibiting antimicrobial activity among which fluorescent strain expressed potent antimicrobial activity against all the test pathogens which was the subject of interest in present investigation. The colonial morphological trait of the fluorescent isolate was bright greenish color colony on the nutrient agar with fluorescent property when exposed to UV light. Microscopic observation and gram staining revealed the rod shape pink color bacterium proving to be Gram-ve.

Table 2. Antimicrobial activity measured as zone of inhibition at 20 µl of ethyl acetate extracts and standard antibiotic Gentamicin on pathogenic bacteria

Test organism	Zone of Inhibition(mm) Crude extract	Zone of Inhibition(mm) Standard
Bacillus subtilis (MTCC 121)	17 ± 1.5	27 ± 1.5
Candida albican (MTCC 183)	16 ± 1.5	23 ± 1.5
Escherichia coli (MTCC 7410)	19 ± 1.5	28 ± 1.5
Proteus mirabilis (MTCC 245)	12 ± 1.5	18 ± 1.5
Salmonella typhi (MTCC 733)	16 ± 1.5	25 ± 1.5
Shigella flexneri (MTCC 731)	30 ± 1.5	28 ± 1.5
Staphylococcus aureus (MTCC 7443)	21 ± 1.5	27 ± 1.5
Staphylococcus epidermidis (MTCC 435)	10 ± 1.5	20±1.5
Vibrio parahaemolyticus (MTCC 451)	11 ± 1.5	19 ± 1.5
Xanthomonas campestris (MTCC 7908)	12 ± 1.5	24 ± 1.5

Values are zone of inhibition in mean ± Standard deviation

Table 3. Colorimetric MIC (in µg mL^{-1}) determination using TTC against test bacteria

Test Pathogens	Minimum inhibition concentration (MIC) value of extract	Minimum inhibition concentration (MIC) value of Gentamicin
Bacillus subtilis (MTCC 121)	12.5	0.39
Candida albican (MTCC 183)	12.5	1.50
Escherichia coli (MTCC 7410)	12.5	0.19
Proteus mirabilis (MTCC 245)	12.5	3.12
Salmonella typhi (MTCC 733)	6.25	0.39
Shigella flexneri (MTCC 731)	0.19	0.19
Staphylococcus aureus (MTCC 7443)	1.56	3.12
Staphylococcus epidermidis (MTCC 435)	25.0	3.12
Vibrio parahaemolyticus (MTCC 451)	25.0	3.15
Xanthomonas campestris (MTCC 7908)	25.0	1.50

Various physiological parameters attributed towards the growth of fluorescent *Pseudomonas* and for the production of antimicrobial metabolites. The maximum growth of the isolate was attenuated at 36 hours under the optimized parameters and after 52 hours there was no further growth which resulted into decline phase [Fig. 1].

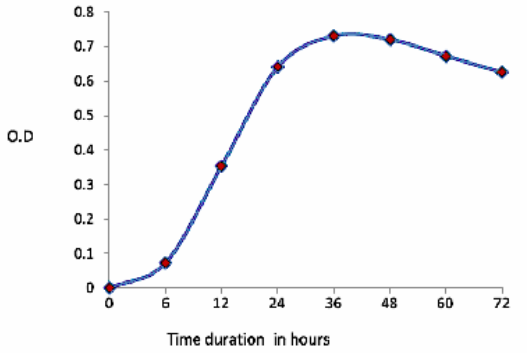

Fig. 1. Growth curve of the fluorescent *Pseudomonas* sp.

The production of green color fluorescent pigment in a broth was achieved after 12 hours under static condition. The production of fluorescent green color pigment was due to the iron-deficient and it was observed in present study as the production of green color pigment increased, the pH of the broth also increased simultaneously[14]. As the endophytes exist in a

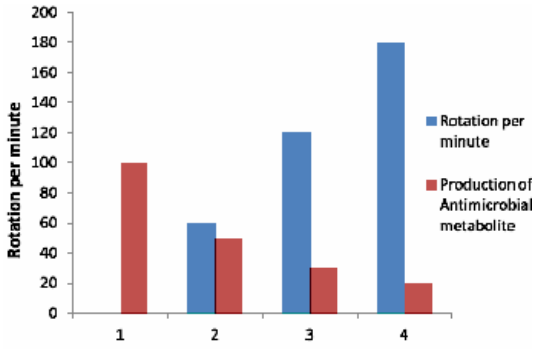

Fig. 2. Comparison between static and shaking condition influencing the production on antimicrobial metabolites

complex interaction with their host plants and isolating them from their native habitat might affect their metabolic capabilities hence production of antimicrobial metabolite was enhanced in the static condition compared to the shaking (figure-2)[15]. In addition, nutrient levels and the choice of fermentation as well as extraction techniques also influence the growth and overall production of antimicrobial metabolites.

The isolate obtained was biochemically confirmed using Bergey's manual of systematic Bacteriology (Table 1) and molecular characterization with 16s rRNA amplification confirmed the endophytic bacterium belongs to *Pseudomonas* species.

The isolated endophytic fluorescent *Pseudomonas* exhibited broad spectrum antimicrobial activity against the test pathogenic microorganisms including MRSA strain, antimicrobial activity as determined by dual culture method displayed the inhibition of fungal pathogen and agar over lay assay for fungi and bacteria respectively. The crude ethyl acetate extract obtained from solvent extraction displayed antimicrobial activity by disc diffusion method exhibiting clear zone of inhibition against the test pathogens (Table 2). The antifungal activity was obtained by dual culture method against the phytopathogens *viz*., *Fusarium, Aspergillus niger* and *Aspergillus flavus*.

The Minimum Inhibition Concentration (MIC) value determined as the lowest concentration of the crude extract that inhibited any visible bacterial growth after 24 hrs of incubation. Minimal Inhibitory Concentration (MIC) of crude extract varied from 0.19 $\mu g/mL^{-1}$ to 25.0 $\mu g/mL^{-1}$ against the test pathogens (Table -3). Partially purified crude ethyl acetate extract by using TLC plates displayed various separated bands at different R_f value. Metabolites with antimicrobial activity were identified as clear inhibition zones with no growth of test pathogen onto a red-colored background. Two zones obtained with R_f - 0.35, 0.7 exhibited inhibition activity against the test pathogen.

DISCUSSION

Research on endophytic plethora is gaining enormous importance due to its potential source of bioactive compound with therapeutic value. Perusal of studies reported so far envisioned

that fluorescent *pseudomonas* isolated from various sources are known to produce antimicrobial compounds against various phytopathogens, thus acting as effective biocontrol agents, plant growth promoting substances and antimicrobial agents through several mechanisms *viz*., production of antibiotics, siderophores, HCN and competition for nutrients[16-18]. They could serve as promising bioinoculants for agricultural system to increase productivity since the action of such bacteria is highly specific, ecofriendly and cost-effective[19-21]. The preliminary results of present study confer that endophytic fluorescent *Pseudomonas* isolated from *Annona squamosa* in the present investigation displayed antimicrobial against the test pathogens. Hence with the obtained results the crude extract deserve further purification and characterization to discover any novel metabolite of pharmaceutical importance, which could be then produced at large scale.

CONCLUSION

The burgeoning interest on endophytic research have demonstrated significant progress in recent decades as one of the novel source of bioactive metabolites. Further research in this area will envisions some of the untapped metabolites bearing pharmaceutical importance.

ACKNOWLEDGMENTS

Authors are pleased to thank University Grant Commission for financial support and Department of Studies in Microbiology , University of Mysore for Providing infrastructure.

REFERENCES

1. Nicolaou, K.C., Yang, Z., Liu, J.J., Ueno, H., Nantermet, P.G., Guy, R.K., Claiborne, C.F., Renaud, J., Couladouros, E.A., Paulvannin, K., Sorensen, E.J. Total synthesis of taxol. *Nature*., 1994: **367**: 630-634.
2. Stone, J.K., Bacon, C.W., White J.F. An overview of endophytic microbes: endophytism defined, Microbial endophytes, NewYork: Marcel Dekker Inc, 2000; 3-29.
3. Owen, N.L., Hundley, N. Endophytes the chemical synthesizers inside plants. *Sci Prog*., 2004; **87**: 79-99.
4. Strobel, G.A. Endophytes as sources of bioactive products. *Microbes. Infect*. 2003; **5**: 535-544.
5. Xiao, L., Yao, J., Huang ., Zhong, H,Z., Wen, J, S., Xiao, M, Q., Yue, M, S. Endophytes from the pharmaceutical plant, *Annona squamosa L.*: isolation, bioactivity, identification and diversity of its polyketide synthase gene. *Fungal. Divers*., 2010; **41**: 41-51.
6. Zin, N.M., Loi C.S., Sarmin, N.M., Rosli, A.N. Cultivation-dependent characterization of endophytic actinomycetes. *Res. J. Microbiol*., 2010; **5**: 717-724.
7. Webster, N.S., Wilson, K.J., Blackall, L.L., Hill, R.T. Phylogenetic diversity of bacteria associated with the marine sponge *Rhopaloeides odorabile*. *Appl. Environ. Microbiol*., 2001; **67**: 434-444.
8. Raubitschek, F., Dostrovsky, A. An antibiotic against dermatophytes, derived from *Bacillus subtilis*. *Dermatol*., 1950; **100**: 1-4.
9. Hayakava, M., Yoshida, Y., Limura, Y. Selective isolation of bioactive soil actinomycetes belonging to the *Streptomces violaceusniger* phenotypic cluster. *J Appl Microbiol*., 2004; **56**: 21-25.
10. Liu, H.M., Dong, D.X., Peng, H. S., Zhang, X.H., Xu, Y.Q. Genetic diversity of phenazine- and pyoluteorin-producing *pseudomonads* isolated from green pepper rhizosphere. *Arch Microbiol*., 2006; **185**: 91-98.
11. Radji, M., Sumiati, A., Rachmayani, R., Elya B. Isolation of fungal endophytes from *Garcinia mangostana* and their antibacterial activity. *Afr J Biotechnol*., 2011; **10**: 103-107.
12. Ellof, J.N. A sensitive and quick micro plate method to determine the minimal inhibitory concentration of plant extracts for bacteria. *Planta Med* ., 1998; **64**: 711-713.
13. Mattana, C.M., Satorres, S.E., Sosa, A., Fusco, M., Alcaraz, L.E., Antibacterial activity of extracts of *acacia aroma* against methicillin-resistant and methicillin-sensitive *staphylococcus. Braz. J. Microbiol*., 2010; **41**: 581-587.
14. Meyer, J.M. The Fluorescent Pigment of *Pseudomonas fluorescens: Biosynthesis*, Purification and Physicochemical Properties. *J. Gen. Microbiol.,* 1978; **107**: 319-328.
15. Maria, G.L., Sridhar, K.R., Raviraja, N.S. Antimicrobial and Enzyme Activity of Mangrove Endophytic Fungi of Southwest Coast of India. *J. Agric. Biotechnol.,* 2005; **1**: 67-80.
16. Benizri, E., Courtade, A., Picard, C., Guckert, A. Role of maize root exudates in the production of auxins by *Pseudomonas fluorescens. Soil Biol. Biochem*., 1998; **30**: 1481-1484.

17. Gutterson, N., Ziegle, J.S., Warren, G.J. Genetic determinants for catabolic induction of antibiotic biosynthesis in *Pseudomonas fluorescens* HV37a. *J. Bacteriol*., 1988; **170**:380-385.
18. Kloepper, J.W., Leong, J., Teintze, M., Schroth, M.N. *Pseudomonas* siderophores: A mechanism explaining disease suppressive soils. *Curr. Microbiol*., 1980; **4**: 317-320.
19. Elad, Y., Chet, I., Bakker, R. Increased growth response of plants induced by Rhizobacteria antagonistic to soil borne pathogenic fungi. *Plant and Soil*., 1987; **98**: 325-330.
20. Suryakala, D., Maheshwaridevi, P.V., Lakshmi, K.V. Chemical characterization and in vitro antibiosis of siderophores of rhizosphere fluorescent *pseudomonads*. *Indian J. Microbio*., 2004; **44**: 105-108.
21. Defago, G., Berling, C.H., Borger, U., Keel, C., Voisard, C. Suppression of black rot of tobacco by a *Pseudomonas* strain: Potential applications and mechanisms, In: *Biological Control of Soil Borne Plant Pathogen*, In. Horny, D. edn,