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Molecular Characterization of Antagonistic Bacteria, *Pseudomonas otitidis* from Insect gut, Short horned Grasshopper

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The aim of the present work is to investigate bioactive metabolites from gut bacteria of short horned grasshoppers. In this present study the bacteria are isolated from the gut of short horned grasshoppers and screened for antagonistic activity. To test for antagonistic effect the gut isolated bacteria were tested against four human pathogenic bacteria *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Escherichia coli*, *Vibrio parahameolyticus* and one yeast like fungus *Candida albicans*. It was observed that gut associated *Pseudomonas* sp had antagonistic activity against yeast like fungus *Candida albicans* and *Escherichia coli*. The most potent strain was subjected for 16srRNA gene sequencing in order to identify the antagonistic bacteria. Phylogenetic tree has been constructed and our strain matches with *Pseudomonas otitidis* (99.1%). *Pseudomonas otitidis* has found to possess antimicrobial activity and the active compounds isolated can be utilized as antibiotics.

Key words: Antagonistic bacteria, short horned grasshoppers, 16srRNA, *Pseudomonas otitidis*.

A microbial natural product remains one of the most important sources of bioactive compounds for the pharmaceutical industry. Even though there is shift towards other sources such as computer based molecular modeling and manipulation of biosynthetic gene clusters, the pharmaceutical pipeline remains filled with traditional, microbial derived natural products. Microorganisms remain nature's best chemists and one of the best sources of novel, biologically active organic molecules (Paul *et al*., 2002; Chellaram *et al*., 2015; Prem Anand *et al*., 2011and Sherif *et al*., 2008). Antibiotics are actually rather easy to discover, but few are of medical or commercial value. Some are used commercially other than for treating disease for instance a supplement in animal feed. Many antibiotics are toxic to humans or lack any advantage over antibiotics already in use. More than half of antibiotics which are in use today are produced by *Streptomyces* species, filamentous bacteria that commonly inhabit soil (Balick *et al*., 1997)

In the pharmacology of natural products, a wide range of useful drugs have been isolated from plants and animals (Chellaram and Petterson Edward, 2009). These drugs include analgesics, antibiotics, and anti-inflammatory drugs and so on. But insects host is an environmental natural product having potential biomedical applications, many of which differ from other organisms. Insect gut provides a huge resource bank for the discovery of Novel compounds (Prem Anand *et al*., 2013). The natural compounds are usually about 1000 Dalton, with existing drug properties. Insects harbors a wide variety of antagonistic bacteria producing bioactive substances which are being isolated and characterized with great promise for the treatment of human diseases. However the traditional screening of soil and marine

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microorganisms as the source of antimicrobial products provided no results, which became responsible for the current lack of effective antibiotics (Benerji, 1992; Chellaram *et al*., 2011 and Kesavan and Chellaram, 2014). This has led to an urge into a new discovery of antimicrobial agents from alternative sources to find new pharmaceutical products. The search for bioactive compounds from insect gut microorganisms has been intensively done during last decade (Janos, 2005).

The aim of the present work is to investigate bioactive metabolites from gut bacteria of short horned grasshoppers which are abundantly seen in terrestrial environment. With a preface effort, the crude extract and partial purified fractions of the grasshoppers were tested against human pathogens, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Escherichia coli*, *Vibrio parahameolyticus* and *Candida albicans*. The precise antimicrobial compound was partially purified by column chromatography and through HPLC (High Performance Liquid Chromatography).

MATERIALS AND METHODS

Sample collection

Short horned grasshoppers (Phylum: *Arthropoda*) are collected by using butterfly nets from various places in and around Vel tech Multitech Dr.Rangarajan Dr.Sakunthala Engineering College, Avadi Chennai. Four different types of samples were collected. Based upon the external morphology the samples were differentiated.

Preparation of media

Six different types of medium were used for isolation of bacteria from the gut of grasshoppers. Since no one medium supports the growth of all types of bacteria, we used Nutrient agar, Actinomycetes agar, Luria-bertani agar, starch casein agar, soya bean casein digest agar, and Peptone agar.

Isolation of the gut associated bacteria

Grasshoppers were transferred aseptically into the lab, where the gut of grasshoppers was removed using sterile blade and forceps. The gut part was completely crushed with sterile distilled water in mortar.50 -100µl of sample was inoculated into each medium. The inoculated samples were incubated at room temperature for 24 –48 hrs. Morphologically different colonies were selected randomly. The collected samples were further sub cultured on appropriate medium. All the isolated samples were stored at 4°C. The isolated colonies were subjected to macroscopic observation which includes color, consistency, surface texture, appearance and opaqueness. The gut associated isolates were tested for antagonistic effect by double agar overlay method.

Antagonistic activity of the gut associated bacteria

The human pathogens, *Candida albicans, Vibrio parahameolytics, Klebisella pneumonia, Pseudomonas aerogenosa* and *Escherichia coli*. were obtained from NCC Culture

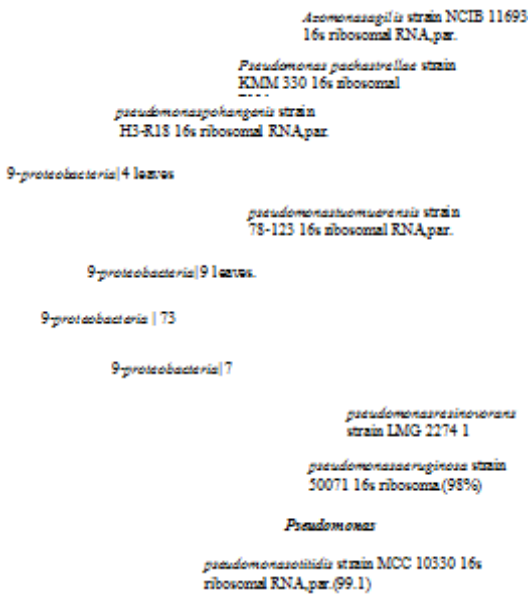

Fig. 1. Phylogenetic Tree- Max Seq Difference: 0.75

Fig. 2. Antagonistic activity against *Candida albicans*

Collection centre, Pune. The 24 hours of old isolates were spotted on the nutrient agar medium and incubated at room temperature for 16 hours. About 1000 µl of the test cultures were suspended in 100 ml of soft agar (0.75%) mixed vigorously and were pored immediately over the colonies of the antagonistic bacteria on the nutrient agar plates. The plates were then incubated at room temperature for 24 hours. The zone of inhibition around the macro-colonies of the antagonistic bacteria was measured.

Molecular characterization

For the molecular characterization, an effective bacterium was selected based on the antimicrobial assay. The selected bacterium strain was grown in appropriate medium and the DNA was extracted. The 16s rRNA genes were amplified by PCR using universal primer. The process of 16s rRNA Sequencing was carried out in Chromas biotech Bangalore.

Column purification using amberlite XAD 16

The supernatant was then subjected in the column chromatography. The column was initially packed with the amberlite XAD 16 polymer. The polymer is shipped as water wet product inhibited with sodium chloride and sodium carbonate salts to retard bacterial growth. These salts should be washed from the adsorbent prior to use and it is suggested to wash the adsorbent at a flow rate of 5-10 m/h. The supernatant is passed through the column; the adsorbed compound was eluted using Ethyl acetate and Ethanol. Ethyl acetate and Ethanol are used as a solvent system. Each fraction thus obtained was once again evaporated, concentrated and assayed for antimicrobial activity.

Column purification of the active crude extracts

The column fractions of the crude extract of potent strain against *Candida albicans* was eluted as, An aliquot of the isolated compounds

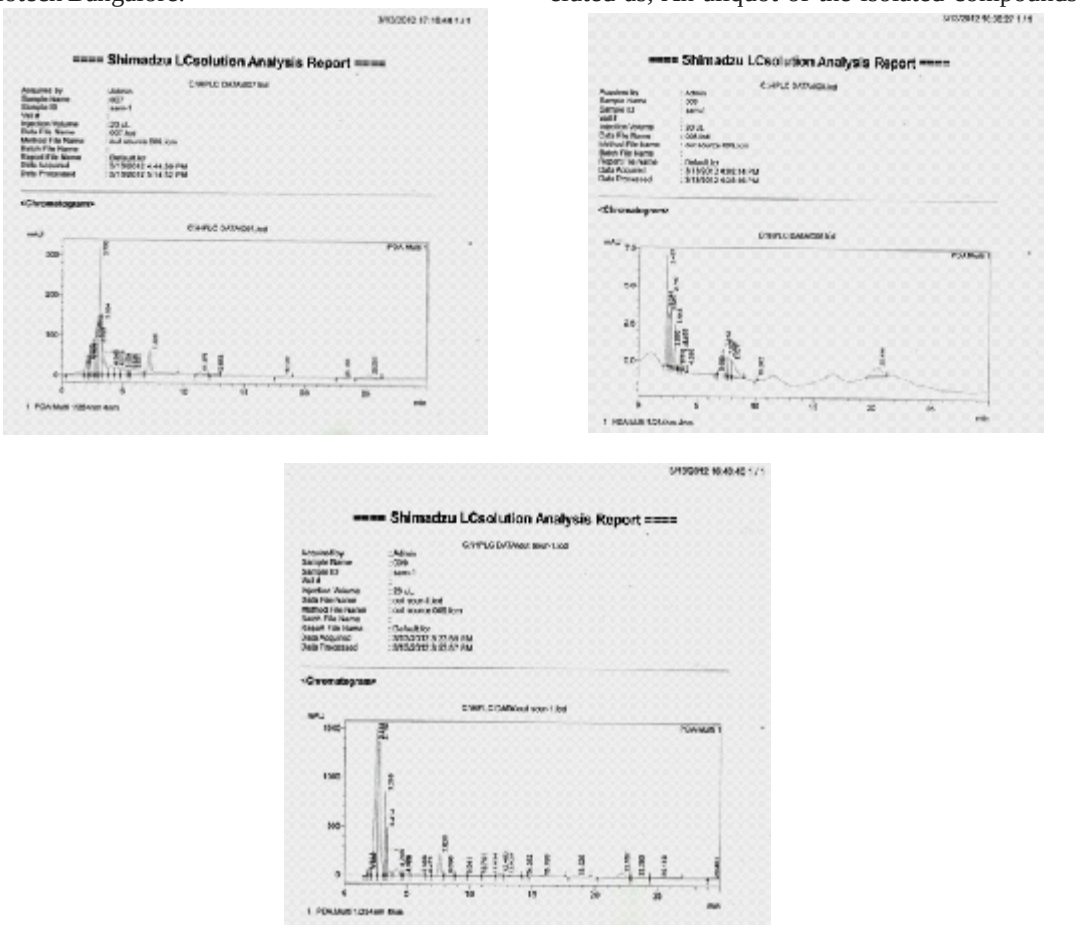

Fig. 3. HPLC Profile of the purified compounds (a, b and c)

(fractions 5-8, 9-10) were subjected to HPLC. Fractions from the separation will be obtained by using a fraction collector and antifungal and antibacterial activity will be carried out for all fractions against human pathogens.

High performance liquid chromatography

An aliquot of the isolated compounds (fractions 5-8, 9-10) were subjected to HPLC, hence the fractions were dried. Separation was then performed using c18 reverse phase column (Schizmadzhu).

RESULTS

Among the 122 bacterial strains 15% (22) of isolates were found to have antagonistic activity against most of the human pathogens. It was observed that strain 1 (*Pseudomonas otitidis)* inhibited the growth of *Candida albicans* (Fig. 2) and *E.Coli* to nearly 5mm and 4mm respectively. The strain that showed antagonistic activity against *Candida albicans* is further subjected for mass culturing in order to isolate the bioactive compounds. *P. otitidis* was selected for more studying on the basis of most average diameter of the zones of microbial inhibition in disk diffusion method against tested microorganisms. *Pseudomonas otitidis* was identified by performing 16s rDNA sequence. Morphological and physiological characteristics of the isolated bacterium were compared with data from Bergey's Manual of systemic bacteriology (Fig.1). Acetonitrile and water was used as an elutent at a flow rate of 1.0 ml/minute. The peaks are detected at 252nm in a UV-VIS detector. HPLC profile was detected for the *P. otitidis* (Fig.3). Fractions from the separator have to be collected by using a fraction collector and antimicrobial activity was carried out for all the fractions against human pathogens.

DISCUSSION

There are numerous potential bacteria which were isolated from different insects and characterized (Meylears *et al*., 2002 and Costa, 2011) but, antibiotic producing bacterium from the gut of short-horned grasshoppers has not been reported. There are more than 120 strains were isolated from this study and one strain of the potential was identified based on phenotypic and genotypic characteristics. Because of these characteristics, this species can be assigned to the species level as *P. otitids* which is more similar to *P. aeruginosa*. Antimicrobial agent from this organism shows an interesting spectrum of antimicrobial activity. Differences were observed in the susceptibility to the antimicrobial agent within different analyzed strains (Ebrahimipour *et al*., 2011 and Ramadan *et al*., 2008). This may be due to presence or absence of receptors for the absorption of bioactive compounds or to some mechanism of bacterial resistance. Some antimicrobial substances such as pyocyanin and pyoverdin have been described for *Pseudomonas sp*. Pyoverdin has a good antifungal activity against deleterious fungi and its maximum inhibition zone was against *A. niger* (Kjer *et al*., 2010 and Chellaram and Patterson Edward, 2009). Whereas, this study showed that the antimicrobial compound has the ability to inhibit dimorphic fungus *Candida albicans*.On the other hand, our strain showed a broad inhibitory spectrum against *Candida albicans* and other bacteria. The activity and stability of this antimicrobial agent in alkaline pH could be promising in potential clinical application due to mild alkalinity of human serum (7.4).

CONCLUSION

This study showed that the isolated bacterium *Pseudomonas otitids* has excellent antimicrobial activity against the *Candida albicans* and *E.coli*. The activity of the compound has to be tested against other human pathogenic bacteria in order to make it for wide range of antibiotics. It is evident that *P. otitidis* constitutively produces a novel compound which may be the substance responsible for antimicrobial activity. High antimicrobial effects of the bacterium are evident, further structure, functional group elucidation by using Nuclear Magnetic Resonance (NMR), Infra-red (IR) of the compound is necessary in order to use it for pharmacological purpose.

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REFERENCES

1. Balick, M.J and P.A. Cox. Plants, People and Culture: The Science of Ethnobotany. New York: W.H. Freeman, 1997.
2. Banerji, A. Biotechnological potential of Natural products, *Bioelectrochemistry Bioenergetics*. 1992: **27**:1057-1065.
3. Chellaram C and J.K.P. Edward. Anti-inflammatory potential of coral reef associated gastropod, *Drupa margariticola*. *Indian J. Sci. Technol*., 2009: **2** (2): 75-77.
4. Chellaram, C and J.K. Patterson Edward. Antimicrobial activity of the marine mollusk *Nassa costata* from the Tuticorin coastal waters, Southeastern India. *Asian J. Microbiol. Biotech. Environ. Sci*., 2009: (1): 41-48.
5. Chellaram, C., T. Prem Anand, D. Kesavan, G.Priya, M. Chandrika and C. Gladis. Enhanced cultivability of antagonistic bacterial strains from soft coral *Sinularia* sp., Gulf of Mannar, Southeastern India. *African J. Microbiol. Res*., 2011: **5** (12): 1521-1526.
6. Chellaram, C., P.Raja and K. Diraviya Raj. Medicinal Properties of Green Seaweeds, *Ulva fasciata* (Delile, 1813) and *U. reticulate* (Forsskal, 1777*). Int. J. Res. Pharmaceuti. Sci*., 201: **6** (1): 1-6.
7. Costa, R.R. Microbial Diversity in the larval gut of field and laboratory population of the sugarcane weevil sphenophorus levis (coleopteran curculionidae). *Gen. Mol. Res*., 2011: **10**(4): 2679-2691.
8. Ebrahimipour, G., A. Moradi., M. Mehrdad., H.Alaee and A. Marzban. Evaluation of antimicrobial substance produced by a bacterium isolated from *Parmacella iberica*. *Jundishapur J. Microbiol*., 2011: **4**(3): 131-140.
9. Janos, B. Bioactive Microbial Metabolites, an overview. *J. Antibiotic*. 2005: **58**(1): 1–26.
10. Kesavan, D and C. Chellaram. Antioxidant Activities of Methanolic Extract of Leaves of *Givotia rottlariformis* Linn. *Int. J. ChemTech Res*., 2014: **6** (9): 4228-4234.
11. Kjer, J., A. Debbab., A.H. Aly and P. Proksch. Methods for isolation of marine derived entophytic fungi and their bioactive secondary products. *Nature Protocols*. 2010: **5**: 479-490.
12. Meylears, K., A. Cerstiaens., E. Vierstraete., G. Baggerman., C.W. Michiels., A.D. Loof and L. Shcoofs L. Antimicrobial compounds of low molecular mass are constitutively present in insects: characterization of â-Alanyl-Tyrosine. *J. Current Pharmaceut. Design*. 2002: **8**: 99-110.
13. Paul, R.J., J.M. Tracy and W. Fenical. The true potential of the marine microorganism. *Current Drug Discovery.* 2003: **12**: 17-19.
14. Prem Anand., T., C. Chellaram and C. Felicia Shanthini. Isolation and Screening of Marine Bacteria Producing Antibiotics against Human Pathogens. *Int. J. Pharma Bio Sci*. 2011: **2**(4): 1-15.
15. Prem Anand, T., C. Chellaram and C. Felicia Shanthini and R. Sivakumar. Analysis on Inducement of Antibiotic Production in Marine Bacteria. *J. Chem. Pharmaceut. Res*., 2013: **5**(5): 236-240.
16. Ramadan, M.F., M.M.S.Asker and Z.K. Ibrahim. Functional bioactive compounds and biological activities of spirulina platens are lipids. *Czech. J.Food Sci*. 2008: **26**(3): 211-222.
17. Sherif, E., A.E.E. Ru., L. Wenhan and P. Peter. Methods for isolation, purification and structural elucidation of bioactive secondary metabolites from marine invertebrates. *Nature Protocols.* 2008: **3**(6): 35-39.