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Evaluation of Biological Activity of Secondary Metabolites of *Neurospora crassa* from Machilipatnam Sea Water

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ABSTRACT

The aim of present study is to study the anti-bacterial and fungal activities of *Neurospora crassa*, which is isolated from the marine sample collected from Machilipatnam coast of Andhra Pradesh, India. The fungus was isolated on PDA Media amended with chloramphenicol. Fungus was identified as, *N. crassa* by morphological and microscopic observations. Isolated fungus was inoculated into liquid medium for secondary metabolites production. Crude extract is characterized for biological activities. The TLC is performed to identify and purify the sub fractions in the extract. The same were tested against different pathogenic bacteria and fungi to determine the antibacterial and anti fungal activities. The study revealed that, among the fungal species used *Neurospora crassa* was found to be active against all bacterial and fungal strains tested. The collected crude extract of *N. crassa* showed 24 mm diameter zone against *Aspergillus flavus*, at concentration of 200 µg and the best activity was observed at a concentration of 200 µg against *E. coli* and *Streptococci* which showed 18 mm diameter. The TLC *Neurospora crassa* crude extract revealed 7 fractions. The selected organism which produces the compounds contains the biological activities, which include, anti-bacterial and anti fungal activities. Hence, we conclude that the compounds have high efficiency of anti microbial activity, which needs extensive attention in terms of new therapy. *Neurospora crassa* may lead to the discovery of pharmaceutically valuable products.

Key words: Antibacterial and antifungal activity, morphological observation, TLC-purification, *Neurospora crassa*

INTRODUCTION

Microbial secondary metabolites have provided numerous pharmaceutical agents ranging from antibiotics to immunosuppressive compounds. Synthesis of these low molecular weight compounds is not required for normal growth of the microbes, however these compounds may provide several benefits to the organism. Fungi have the ability to produce a plethora of secondary metabolites, typically dependent on the stage of development of the fungus and environmental factors ranging from nutrient concentrations to light and temperature (Coleman *et al.*, 2011). The marine environment is an extremely diverse reservoir of life and across the range of organisms, there is a virtually untapped source of structurally unique natural products. Marine fungi are predicted to be a valuable resource in the search and discovery of novel natural products. Marine microorganisms, such as; bacteria, fungi and microalgae have proven to be a rich source of structurally novel and biologically active secondary metabolites (Blunt *et al.*, 2004). Hence, they are considered to be bioactive. These include potentially effective therapeutic agents with antiviral,

antibacterial and antitumor properties produced by invertebrates from the classes Porifera, Cnidaria, Mollusca, Echinodermata, Bryozoa and Urochordata. Previous studies have also suggested that some bioactive compounds isolated from marine organisms have been shown to exhibit anticancer, antimicrobial, antifungal or anti-inflammatory and other pharmacological activities (Samuel *et al.*, 2011). Fungi produce a vast range of secondary metabolites. Some of these are high value products with pharmaceutical applications such as *Penicillin*. Marine microorganisms have become an important source of pharmacologically active metabolites. Bioactive compounds from marine fungus have extensive use in the treatment of many diseases and these compounds act as the templates for synthetic modification. Several molecules isolated from various fungi are currently involved in the advanced stage of clinical trials. Several of the fungal secondary metabolites produced by selected strains of these fungi are capable of producing eliciting toxicity in animals. Marine fungi have proved to be a rich source of new biologically natural products (Jensen and Fenical, 2000). Because of their particular living conditions, salinity, nutrition, higher pressure, temperature variations, competition with bacteria, viruses and other fungi, they may have developed specific secondary metabolic pathways compared with terrestrial fungi. Recently, *Neurospora crassa* was isolated from POME dump sites from Pedavegi, E.G. Dist, A.P, India (Suseela *et al.*, 2014). A number of antibiotics have been obtained from the culture broths of filamentous fungi to date. Recent investigations on marine filamentous fungi looking for biologically active secondary metabolites indicate the tremendous potential of them as, a source of new medicines (Hina *et al.*, 2015). Apart from secondary metabolite potential screening our lab also reported the ability of the almighty fungus, *Neurospora crassa* in removing metal ions from marine water (Anupama *et al.*, 2014). More specifically marine fungi are also believed to be prolific resources of natural products. Unlike the terrestrial fungi, which were initially exploited for drug delivery. Marine-derived fungi are a rich source of structurally new natural products with a wide range of biological activities. Marine fungi have attracted great attention as considerable resources only, since the late 1980. Furthermore, it was reported that the corresponding chemistry of marine fungi was structurally diverse and related to that of terrestrial fungi (Holler *et al.*, 2000). In the present study, we aim to isolate secondary metabolites and to assess their biological activity from the *Neurospora crassa* isolated from machilipatnam sea water.

MATERIALS AND METHODS

Materials: The marine water sample was collected from Machilipatnam Beach, Krishna district andhra Pradesh, India.

Test organisms

Bacteria used: *Escherichia coli*, *Bacillus* sp., *Salmonella* sp., *Streptococci* sp.

Fungi used: *Aspergillus flavus*, *Aspergillus niger*, *Trichoderma viridae*, *Aspergillus parasiticus*, *Rhizopus solani*, *Candida albicans*, *Penicillium citrilinum*.

Media used: Potato Dextrose Broth, Potato Dextrose Agar, Nutrient Broth, Rose Bengal chloramphenicol Agar containing Mycological peptone 5.0 g, Dextrose 10.0 g, Mono potassium Phosphate 1.0 g, Magnesium Sulphate 0.5 g, Rose Bengal 0.05 g, Chloramphenicol 0.10 g, Agar 15.0 g and filtered marine water.

Methodology

Sample collection: Marine water sample was collected into sterile water bottle. The samples were stored carefully and capped tightly till they reached the laboratory. The samples were processed in an isolation process using the dilution plate in the laboratory.

Isolation of marine fungi: The sample is diluted at 8 and 10 fold with autoclaved filtered seawater. An aliquots of 100 μ L from each folds of marine water sample were spread onto Rose Bengal-Chloramphenicol, Agar plates. Two replicates of rose Bengal-Chloramphenicol agar plates were used for each sample. After 2 days of incubation at 27°C, the inoculated rose Bengal-Chloramphenicol agar plates were examined daily for the presence of developing fungal growth using, a dissecting microscope at 20 $\times$ magnification. Distinct fungal colonies on the rose Bengal chloramphenicol agar plates were then transferred to new Sabouraud dextrose agar plates for further isolation and purification.

Microscopic observation: After incubation of slide culture, slides are stained with Lactophenol blue for microscopic examination. First the slides are observed under light microscope at 10 $\times$ resolution and then at 45 $\times$ and 100 $\times$ (oil immersion) for morphology of fungi.

Extraction of secondary metabolites

Preparation of potato dextrose broth media: Weigh 24 g of potato dextrose broth Hi-media and it was dissolved in 1000 mL of distilled water.

Sterilization: This prepared media was sterilized in an autoclave at 121°C, 15 lbs pressure for 15-20 min.

Culturing of the isolated fungi: Inoculate the pure culture of *Neurospora crassa* in the sterilized media and the culture flasks were incubated at 27°C for 15 days.

Extraction of secondary metabolites: After incubation ethyl acetate was added to the culture flasks and kept for 5 h minimal shaking is required for the dissolving of metabolites into ethyl acetate solvent.

Separation of metabolites: The metabolites, which are now dissolved in ethyl acetate solvent are separated by using, separating funnels. In the separating funnel add the media with ethyl acetate. To that, add some amount of ethyl acetate, shake well and allow it to settle for a few minutes. Two layers were observed. The bottom layer is discarded and the upper layers of ethyl acetate with metabolites are collected, which is called the organic layer. The washes were repeated for three times to extract the complete metabolites. This separated extract was Rota vapoured for the collection of crude extract.

Antibacterial activity: The *in vitro* antibacterial activity of the collected crude extract was studied against the bacterial strains, *Escherichia coli*, *Bacillus* sp., *Salmonella* sp., *Streptococci* sp., by agar cup diffusion method (Farag *et al.*, 1989) and the strains were obtained from the Krishna University, Machilipatnam. The ready-made Nutrient Agar (NA) medium (Himedia, 39 g) was suspended in distilled water (1000 mL) and heated to boiling until it dissolved

completely, the medium and Petri dishes were autoclaved at pressure of 15 lb/inc2 for 20 min. Agar cup bioassay was employed for testing antibacterial activity. The medium was poured into sterile Petri dishes under aseptic conditions in a laminar flow chamber. When, the medium in the plates solidified, 0.5 mL of (week old) culture of test organism was inoculated and uniformly spread over the agar surface with a sterile L-shaped rod. Solutions were prepared by dissolving in DMSO and different concentrations were made. After inoculation, cups were scooped out with 6 mm sterile cork borer and the lids of the dishes were replaced. To each cup, different concentrations of test solutions were added. Controls were maintained with DMSO. The plates were kept at 37°C for 48 h. Inhibition zones were measured and the diameter was calculated in millimeter. Three to four replicates were maintained for each treatment. Media Nutrient broth and Nutrient agar were procured from M/S media.

Antifungal activity: The *in vitro* antifungal activity of the collected crude extract was studied against the fungal strains, *Aspergillus flavus*, *Aspergillus niger*, *Trichoderma viridae*, *Aspergillus parasiticus*, *Rhizopus solani*, *Candida albicans*, *Penicillium citrinum* and by agar cup diffusion method. Controls were maintained with DMSO. The treated and the controls were kept at 27°C for 48 h. Inhibition zones were measured and the diameter was calculated in millimeter.

RESULTS

Morphological observation: Morphological appearance of the fungal sp. is a filamentous fungi, which grows rapidly on potato dextrose agar at 27°C and produces colonies, which later turn in to orange colour. The hyphae are septate and produce brown conidiophores.

The microscopic image and morphological characters were mainly compared by using, the methods of identification provided by Fungal Genetics Stock Center, School of Biological Sciences, University of Missouri, USA (<http://www.fgsc.net/neurospora/sectionB1.htm>), it was identified as *Neurospora crassa* (Fig. 1).



Fig. 1: Morphological characterization of the fungus

Microscopic morphology: Hyphal diameter is 8-15 μm , Compartment length 15-40 μm , Branch interval Range 0-600 μm , mode 50 μm , macroconidial diameter: 5-8 μm , micro conidial diameter 2.5-3.5 μm , Ascospore length and breadth 27-30 $\times$ 13-15 μm mean growth rate at 25°C: 3.7-4.0.

Neurospora crassa is a type of red bread mould of the phylum Ascomycota. The genus name, meaning “Nerve spore” refers to the characteristic striations on the pores. The first published account of this fungus was from an infestation of French bakeries in 1843 (Perkins and Davis, 2000). With the pioneering work of Beadle and Tatum leading to the famous one gene-one enzyme hypothesis and in 1958, to the Nobel Prize. This work brought the disciplines of genetics and biochemistry together. *Neurospora crassa* is being used as a model organism because, it is easy to grow and has a haploid life cycle (Osherov and May, 2001). *Neurospora* is one of the fastest-growing filamentous fungi, approximately 10 cm per day. Striated football-shaped ascospores. The genus name *Neurospora*, when literally translated means “Nerve spore”. This is the key diagnostic for the genus. Orange, because of its characteristic pinkish-orange colour due to carotenoid pigments (Rashmi *et al.*, 2003). The species *Neurospora crassa* has been the main choice for genetic research over the past 60 years. However, there are a number of different *Neurospora* species, such as; *N. intermedia*, *N. discreta* and *N. tetrasperma*. Cultures of many of these species are indistinguishable to the eye, their grouping into species is based on interbreeding.

Measurement of zones for anti fungal activity: Table 1 shows the activity of crude extract against different fungi. Crude extract is prepared in 50, 100, 150 and 200 μg and the activities was observed against *Aspergillus flavus*, at a concentration of 150 μg (zone diameter is 22 mm) and *Penicillium citrinum*, at a concentration of 150 μg (zone diameter is 20 mm). For *Aspergillus flavus*, *Penicillium citrinum*, the best activity was observed at a concentration of 200 μg (zone diameter is 24 mm). For, *Penicillium citrinum*, the best activity was observed at a concentration of 200 μg (zone diameter is 22 mm), which shows that the crude extract good activity against *Aspergillus flavus* and *Penicillium citrinum*.

Neurospora crude extract is characterized by antibacterial activity. Crude extract is dissolved in DMSO. Crude extract is prepared in 50, 100, 150 and 200 μg and tested against, *Escherichia coli*, *Bacillus* sp., *Salmonella* sp., *Streptococci* sp., At 50 μg concentration show activity against *E. coli* (zone diameter is 16 mm), 100 μg showed best against *Streptococci* and *E. coli* (zone diameter is 18 mm), 150 μg show against *E. coli* (zone of diameter is 20 mm) and at 200 μg activity against *E. coli* (zone diameter is 22 mm) and *Streptococci* shows 18 mm diameter (Table 2).

Table 1: Anti-fungal activity of the crude extract of *Neurospora* sp.

Organisms	50 (μg)	100 (μg)	150 (μg)	200 (μg)
Control	-	-	-	-
<i>Aspergillus flavus</i>	18	20	22	24
<i>Aspergillus niger</i>	15	16	17	17
<i>Trichoderma viridae</i>	14	15	15	15
<i>Aspergillus parasiticus</i>	15	17	18	20
<i>Rhizopus solani</i>	13	15	16	16
<i>Candida albicans</i>	12	12	14	14
<i>Penicillium citrinum</i>	18	18	20	22

Table 2: Anti-bacterial activity of the crude extract

Concentrations (μg)	<i>Escherichia coli</i>	<i>Bacillus</i> sp.	<i>Salmonella</i> sp.	<i>Streptococci</i> sp.
Control	-	-	-	-
50	16	10	12	14
100	18	10	14	14
150	20	12	15	16
200	22	12	16	18

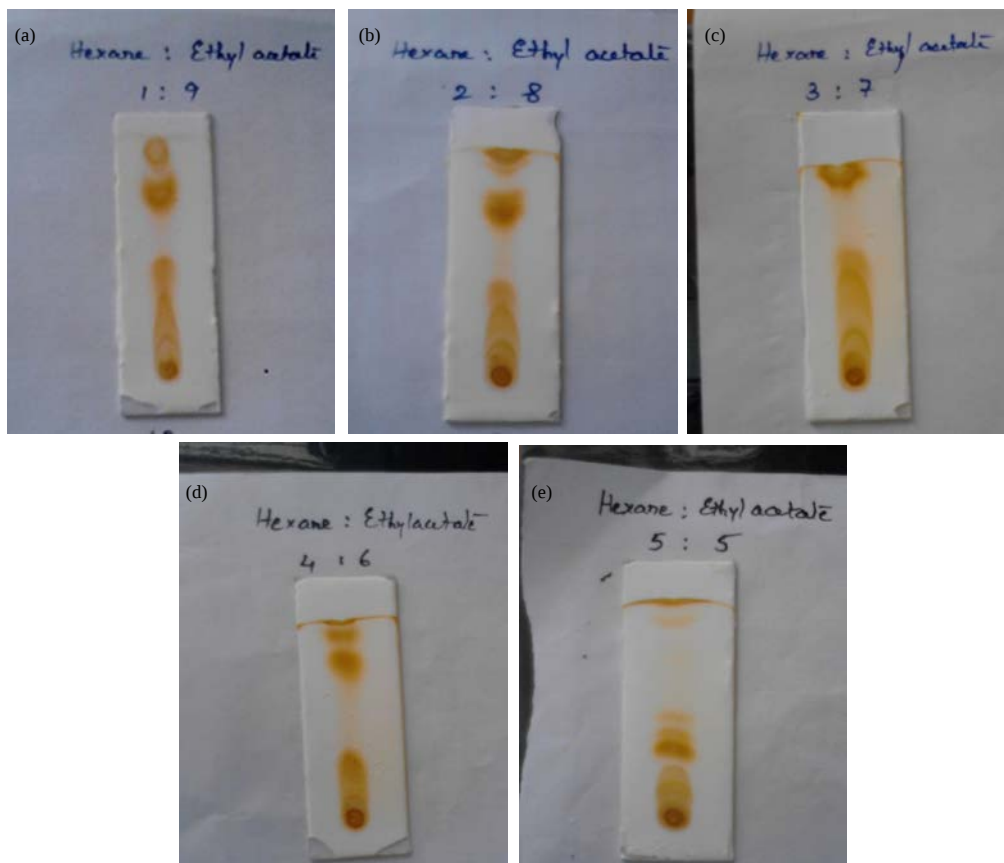


Fig. 2(a-e): Compounds separation using thin layer chromatography

Separation processes

Separation of compounds by Thin Layer Chromatography (TLC): The TLC is performed with hexane and ethyl acetate solvent in different proportions. After performing in all proportions, among them crude extract show 7-8 compounds in 5 (Hexane): 5 (Ethyl acetate). Hence, the crude extract is known to have 7-8 sub compounds. The following pictures shows the TLC plates with different concentrations with the migration if the compounds (Fig. 2).

DISCUSSION

Recently, the marine fungi have proved to be a rich source of new biologically natural products (Namikoshi *et al.*, 2002; Jensen and Fenical, 2000). Because of their particular living conditions, salinity, nutrition, higher pressure, temperature variations, competition with bacteria, viruses and other fungi, they may have developed specific secondary metabolic pathways compared with terrestrial fungi. A number of antibiotics have been obtained from the culture broths of filamentous fungi to date. Recent investigations on marine filamentous fungi looking for biologically active secondary metabolites indicate the tremendous potential of them as, a source of new medicines (Samuel *et al.*, 2011). By today the estimated 30004000 known fungal secondary metabolites have been isolated, possibly not more than 5000-7000 taxonomic species have been studied in this respect. Genera, such as; *Aspergillus*, *Penicillium*, *Fusarium* and *Acremonium* are among fungi highly capable of producing a high diversity of secondary metabolite (Dreyfuss and Chapela, 1994).

Fungal secondary metabolites constitute a wide variety of compounds, which either play a vital role in agricultural, pharmaceutical and industrial contexts, or have devastating effects on agriculture, animal and human affairs by virtue of their toxigenicity. Owing to their beneficial and deleterious characteristics, these complex compounds and the genes responsible for their synthesis have been the subjects of extensive investigation by microbiologists and pharmacologists (Awaad *et al.*, 2012; Zain *et al.*, 2009; Yu and Keller, 2005).

Marine derived fungi have been recognized as, a potential source of structurally novel and biologically potent metabolites and a growing number of marine fungi have been reported to produce novel bioactive compounds. Marine microorganisms became an important source of pharmacologically active metabolites. These organisms produced various types of metabolites, which are capable to inhibit other organisms for competing to the same ecological niche. Secondary metabolites are produced after active growth of the organism with structurally diversified compounds *Neurospora* sp is one of the marine fungi from which the secondary metabolites are produced.

This study was mainly aimed at the characterization of novel secondary metabolites with antibiotic activity against microbial pathogens resistant to antibiotics currently used in the clinical practice. The marine water sample is taken as source for the isolation of secondary metabolite for fungi. So far, literature shows, 25 species were identified. The secondary metabolite extracted was active against fungi and bacteria, the extract is more active against fungi especially *Aspergillus flavus*, with a zone diameter of 24 mm at a concentration of 200 µg) and *P. citrinum* with a zone diameter of 22 mm at a concentration of 200 µg). Bacteria at 200 µg activity against *E. coli* (zone diameter is 22 mm). From the Thin Layer Chromatography, it is known that it has nearly 7-8 compounds to be purified. Further the crude extract can be purified by using, the column chromatography. Secondary metabolites are produced by micro organisms and serve survival functions for the organisms producing them.

CONCLUSION

This is to conclude that the compounds have high efficiency of anti microbial activity, which needs extensive attention in terms of new therapy. *Neurospora crassa* may lead to the discovery of pharmaceutically valuable products.

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REFERENCES

- Anupama, M., T.V. Kumar and J.N.L. Latha, 2014. Growth and metal removal by *Neurospora crassa* from marine water. Int. J. Biol. Pharmaceut. Res., 5: 22-26.
- Awaad, A.S., A.J.A. Nabilah and M.E. Zain, 2012. New antifungal compounds from *Aspergillus terreus* isolated from desert soil. Phytother. Res., 26: 1872-1877.
- Blunt, J.W., B.R. Copp, M.H.G. Munro, P.T. Northcote and M.R. Prinsep, 2004. Marine natural products. Nat. Prod. Rep., 21: 1-49.
- Coleman, J.J., S. Ghosh, I. Okoli and E. Mylonakis, 2011. Antifungal activity of microbial secondary metabolites. Plos One, Vol. 6. 10.1371/journal.pone.0025321

- Dreyfuss, M.M. and I.H. Chapela, 1994. Potential of Fungi in the Discovery of Novel, Low Molecular Weight Pharmaceuticals. In: The Discovery of Natural Product with Therapeutic Potential, Gullo, V.P. (Ed.). Butterworth-Heinemann, London, pp: 49-80.
- Farag, R.S., Z.Y. Daw, F.M. Hevedi and G.S.A. El-Baroty, 1989. Antimicrobial activity of some Egyptian spice essential oils. *J. Food Protect.*, 52: 665-667.
- Hina, M.I., S. Dhanapal and D.S. Sekar, 2015. Studies on antibacterial activity of some fungi collected from K.R.P Dam, Krishnagiri (TN). *Int. J. Eng. Res. Manage.*, 2: 1-2.
- Holler, U., A.D. Wright, G.F. Matthee, G.M. Konig, S. Draeger, H.J. Aust and B. Schulz, 2000. Fungi from marine sponges: Diversity, biological activity and secondary metabolites. *Mycol. Res.*, 104: 1354-1365.
- Jensen, P.R. and W. Fenical, 2000. Marine Microorganisms and Drug Discovery: Current Status and Future Potential. In: Drugs from the Sea, Fusetani, N. (Ed.). Karger Medical and Scientific Publishers, Basel, Switzerland, ISBN-13: 9783805570985, pp: 6-29.
- Namikoshi, M., K. Akano, H. Kobayashi, Y. Koike, A. Kitazawa, A.B. Rondonuwu and S.B. Pratasik, 2002. Distribution of marine filamentous fungi associated with marine sponges in coral reefs of Palau and Bunaken Island, Indonesia. *J. Tokyo Univ. Fish.*, 88: 15-20.
- Osharov, N. and G.S. May, 2001. The molecular mechanisms of conidial germination. *FEMS Microbiol. Lett.*, 199: 153-160.
- Perkins, D.D. and R.H. Davis, 2000. Evidence for safety of *Neurospora* species for academic and commercial uses. *Applied Environ. Microbiol.*, 66: 5107-5109.
- Rashmi, K., J.N.L. Latha, T.N. Sowjanya, P. Kiranmayi, M.V. Enugopal, C.P.S. Menon and P.M. Mohan, 2003. Colonization of cruciferous plants by *Piriformospora indica*. *Curr. Sci.*, 85: 1672-1674.
- Samuel, P., L. Prince and P. Prabakaran, 2011. Antibacterial activity of marine derived fungi collected from south east coast of Tamilnadu, India. *J. Microbiol. Biotechnol. Res.*, 1: 86-94.
- Suseela, L., M. Anupama, B. Prudhvilal, T.V. Narasaiah and J.N.L. Latha, 2014. Isolation and charecterization of lipase producing fungi from palm oil mill effluent obtained from Pedavegi, A.P., India. *Int. J. Biol. Pharma. Res.*, 5: 559-565.
- Yu, J.H. and N. Keller, 2005. Regulation of secondary metabolism in filamentous fungi. *Annu. Rev. Phytopathol.*, 43: 437-458.
- Zain, M.E., A.S. Awaad, A.A. Razak, D.J. Maitland, N.E. Khamis and M.A. Sakhawy, 2009. Secondary metabolites of *Aureobasidium pullulans* isolated from Egyptian soil and their biological activity. *J. Applied Sci. Res.*, 5: 1582-1591.