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ANTI-INFLAMMATORY EFFECT OF MARINE FUNGI *NEUROSPORA CRASSA* AND *MEYEROZYMA GUILLIERMONDII*

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**ABSTRACT**

Marine fungus produces certain compounds which gives them the property of anti-inflammatory reaction. This paper reports the anti-inflammatory activity of marine derived fungus *Neurospora crassa* and *Meyerozyma guilliermondii*. These organisms were isolated from the leaves of mangrove plant and identified by ITS sequencing. The fungal broth culture was prepared in potato dextrose broth and extracted using ethyl acetate for evaluating anti-inflammatory effect.
INTRODUCTION

Marine-derived fungi are a rich source of structurally new natural products with a wide range of biological activities\(^1\). The general increased interest in marine and estuarine habitats in the last decades has led to an increase in studies on marine fungi \(^2\). Marine fungi are those that grow and sporulate in the oceans, in brackish waters and in estuarine habitats. Fungi are found in all marine environments from the high water line down to the deep sea \(^3\). Uniqueness of marine fungi and their potential is: i) fungi associated with or endophytic fungi in marine algae, seagrasses and mangroves and their implications ii) fungi associated with marine invertebrates especially corals and sponges and their potential towards production of bioactive molecules iii) fungi from extreme environments such as the deep sea with elevated hydrostatic pressure and low temperatures, hypersaline waters of the Dead Sea and anoxic or hypoxic (oxygen deficient) sediments from the marine environment\(^4\). Several endophytic fungi have been isolated and cultured from the mangrove plants \textit{Rhizophora apiculata} and \textit{Dendrophthoe falcate} \(^5\). Several secondary metabolites are reported from fungi isolated from marine detritus. A polyketide metabolite, obionin-A was isolated from the liquid culture of the marine fungus \textit{Leptosphaeria obiones}, a halotolerant ascomycete obtained from the salt marsh grass \textit{Spartina alterniflora}. These workers also reported two novel compounds helicascolides A and B from the Hawaiian mangrove ascomycetous fungus \textit{Helicascus kanaloanus} \(^6\). Several pharmaceutical companies are engaged in bioprospecting marine extreme environments\(^4\).

Inflammation, a fundamental protective response, can be harmful in conditions such as life-threatening hyper sensitive reactions to insect bites, drugs, toxins and in chronic diseases such as rheumatic arthritis, atherosclerosis, lung fibrosis and cancer\(^7\). Inflammation can also accelerate cancer and chronic inflammation is regarded as an essential factor for the progression of the neoplastic process\(^8\). Acute and chronic inflammations are complex processes that can be induced by several means, and anti-inflammatory agents exert their effects through different modes of action \(^9,10\). Formalin-induced paw oedema is one of the most suitable test procedures to screen chronic anti-inflammatory agents, as it closely resembled human arthritis\(^11\). According to Archer and Peberdy\(^12\) and Gouka \textit{et al.} \(^13\), \textit{Aspergillus} fungi are excellent as receptor cells to express and secrete proteins of therapeutic importance. The biochemical properties of the anti-inflammatory factor was reported by Zucchi \textit{et al.} \(^14\) were purified and identified. In 1996, Zucchi \textit{et al.} \(^14\) developed the RT1 and RT2 \textit{Aspergillus nidulans} strains, which produced an anti-inflammatory factor. Anti-inflammatory substances isolated from natural products such as the extracts from \textit{Pongamia pinnata} leaves \(^15\) and ReN1869 \(^16\), has been largely used. In the present study two marine fungi were isolated from mangrove leaves and identified as \textit{N. crassa} and \textit{M. guilliermondii} using ITS primer. The identified fungi were cultured in potato dextrose broth and anti inflammatory effect was checked invitro.
MATERIALS AND METHODS

Isolation of fungi:

The mangrove leaves were collected, washed with sterile sea water and grinded using distilled water and seawater in 1:1 ratio in a mortar and pestle under aseptic conditions. 1ml of the above was mixed with 10 ml of sterile water (distilled water: seawater; 1:1) to get dilution 10⁻¹ aseptically. The serial dilution was repeated till 10⁻⁶. From each dilution plating was done in sabouraud’s agar and potato dextrose agar by spread plate technique. The plates were then incubated at 27°C for 5 days. After 5 days, the plates were examined and the pure culture was isolated on pure agar plate.

Preparation of fungal broth culture:

The pure culture isolated by the above method was grown in sabouraud’s dextrose broth. The flasks were incubated in the shaker – incubator at 200rpm for 5 days. Then the mycelium and the filtrate were separated using whattman filter paper no 1.

Preparation of extract:

The filtrate of each fungus was extracted several times with ethyl acetate, (v/v) in a separating funnel. The solvent extracted from filtrate were evaporated under vacuum at 50°C till dryness and dissolved in respective solvents to form the crude extract and tested for bioassays.

Molecular characterization and Identification of elite fungi by ITS sequencing

The fungi were grown in culture in potato dextrose broth at room temperature in the dark for 48 to 72 hours. The genomic DNA was isolated and the ITS region of 5.8sRNA was amplified using primer ITS1 TO 5’ TCCGTAGGTGAACCTGCGG 3’ and primer ITS5 5’ TCCTCCGCTTATTGATATGC 3’ (White et al., 1990) and sequenced using automated sequencer.

Anti inflammatory activity

The HRBC membrane stabilization has been used as a method to study the anti-inflammatory activity. The blood was collected from median cubital vein of healthy volunteers and mixed with equal volume of sterilized Alsever solution (2% dextrose, 0.8% Sodium citrate, 0.05% citric acid and 0.42 Sodium chloride in water). The blood was centrifuged at 3000 rpm and packed cells were washed with isosaline (0.85% Sodium chloride, pH 7.2) and a 10% v/v suspension was made with isosaline. The assay mixture contained the drug (at various concentrations), 1ml phosphate buffer (0.15M, pH7.4), 2ml of hyposaline (0.36%) and 0.5ml of HRBC suspension.
Indomethacin was used as the reference drug. Instead of hyposaline 2ml of distilled water was used as control. All the assay mixtures were incubated at 37°C for 30 min and centrifuged. The haemoglobin content in the supernatant solution was estimated using spectrophotometer at 560nm. The percentage haemolysis was calculated by assuming the haemolysis produced in the presence of distilled water as 100%. The percentage of HRBC membrane stabilization or protection was calculated by using the formula,

\[
\text{Percentage protection} = 100 - \left(\frac{\text{OD of drug treated sample}}{\text{OD of control}}\right) \times 100
\]

RESULTS
In the present study 2 fungi were isolated and identified from the mangrove plant leaves. The ITS region is now perhaps the most widely sequenced DNA region in fungi. It has typically been most useful for molecular systematics at the species level, and even within species (e.g., to identify geographic races). In the present study the DNA was isolated from the positive strain which showed synthesis of silver nanoparticles and the ITS region of 5.8s rRNA was amplified using specific primers ITS 1 and ITS4 and sequence was determined using automated sequencers. Blast search sequence similarity was found against the existing non redundant nucleotide sequence database thus, identified the fungi and the percentage of similarity between the fungi and database suggests it as novel strains. Thus, the novel strains were named as *Neurospora crassa* VB2, *Meyerozyma guilliermondii* VB7 and made publically available in Gen Bank with an assigned accession numbers.

In the present investigation the HRBC membrane stabilizing potency of fungal extracts was carried out. From the results obtained for the *in vitro* studies it was observed that all the extracts exhibited excellent membrane stabilizing activity. Among all the fungal extracts, *N. crassa* exhibited excellent membrane stabilizing activity with 93% for 1mg/ml when compared to *M. guilliermondii*. Flavonoids and steroids are known to show remarkable anti-inflammatory activity by inhibiting the cox and lox systems (17,18). Some works showed the correlation between presence of flavonoids and their membrane stabilizing ability(19). The anti-inflammatory effect of three extracts may be attributed to the presence of flavonoidal and saponin entities. The main action of the anti-inflammatory agent is the inhibition of the cyclooxygenase system, which is responsible for the conversion of arachidonic acid to prostaglandinsG 2 to PGH2 along with peroxidation, which is associated with formation of the longchannels in the membranes. The opening of the channel occurs due to the release of chemical mediators. The extra cellular activity of these enzymes is said to be related to acute and chronic inflammation(20). NSAIDS acts by inhibiting the lysosomal enzymes or by stabilizing the lysosomal membrane. Since HRBC membranes are similar to the lysosomal membrane components, the prevention of hypo tonicity-induced HRBC lysis was taken as measure of anti-inflammatory activity of drugs. The Indomethacin drugs act either by inhibiting these lysosomal enzymes or by stabilizing the lysosomal membrane (21).
In conclusion, from the present study it could be interpreted that extracts of *N. crassa*, *M. guilliermondii* possess anti-inflammatory activity which may be governed by multiple mechanism such as inhibition of 1) histamine, 2) cyclooxygenase and 5-lipoxygenase products (PGE2 and LTB4) and 3) scavenging of various free radicals and their generation. These areas of research remain significant, particularly in view of the development of techniques enabling culture of mangrove fungi on large scales. Thus secondary metabolites of mangrove fungi have a promising potential to be included specifically in anti-inflammatory drug discovery program.

<table>
<thead>
<tr>
<th>Concentrations mg/ml</th>
<th>Neurospora crassa</th>
<th>Meyerozyma guilliermondii</th>
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<tbody>
<tr>
<td>0.5mg/ml</td>
<td>36%</td>
<td>21%</td>
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<tr>
<td>0.6mg/ml</td>
<td>45%</td>
<td>33%</td>
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<tr>
<td>1.0mg/ml</td>
<td>93%</td>
<td>64%</td>
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REFERENCES


