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Isolation, characterisation and bioactivities of macro fungal isolates from Western Ghats of Courtallum Hills

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Abstract

In the present study, four macro fungal isolates were collected from tropical evergreen forest of Courtallam Hills, Western Ghats, Tamil Nadu, India. The macro fungal isolates were named as MS1, MS2, MS3 and MS4 at the time of collection. According to the preliminary screening of antimicrobial activity, isolate MS3 was taken for further bioactivities studies. Based on morphological characteristics, the isolate MS3 was identified as *Ganoderma* sp. at genus level. The *Ganoderma* sp. MS3 was grown in agar media to study the radial growth rate. The maximum radial growth was observed in PDA medium at 30°C and pH 5.5. The ethyl acetate extract of fruiting bodies of *Ganoderma* sp. MS3 had an effective antimicrobial activity against bacterial and fungal pathogens. The maximum inhibition zone of 31.2 mm was observed against *Staphylococcus aureus* (MTCC 3160) and minimum inhibition zone of 27.1 mm was observed against *Escherichia coli* (MTCC 4296).

Key words - Isolation – *Ganoderma* sp. – Human Pathogens – Macro fungi

Introduction

Nowadays human health problems caused by bacteria and fungi are increasing along the world. An intensive search for newer and effective antimicrobial agent is needed. The need for new and useful natural products to provide assistance and relief in all aspects of the human condition is ever growing and even challenging. The search for new drugs from fungi started with the discovery of penicillin (Fleming 1929), a potent antibiotic against Gram-positive bacteria, which was produced by *Penicillium notatum*. A further milestone in the

history of fungal products for medicinal use was the discovery of the immunosuppressant cyclosporine which is produced by *Tolypocladium inflatum* and *Cylindrocarpon lucidum* (Dreyfuss *et al* 1976). Further, fungi also have been shown to be one source of a variety of useful natural products. They were common in nature and considered as good antimicrobial agents (Lindequist *et al* 2005; Abad *et al* 2007; Muhsin *et al* 2011). As of 2010 approximately half a million natural products were known of which 60000 - 80000 are estimated to be of fungal origin.

Ganoderma species are regarded as macro fungi because the carpophores are visible enough to be seen with naked eyes. Although, the real organism comprises of intercillary microscopic bodies which could not be visualized with ordinary eyes. (Zoberi 1972; Jonathan 2002). The genus *Ganoderma* contains about 400 species and is member of the Ganodermataceae, characterized by unique double walled Basidiospores (Smith & Sivasithamparam 2003). The genus is distributed throughout the world, but is particularly diverse in the tropics (Kleinwachter *et al* 2001). The fruiting body has been used as traditional medicine for anticancer in China and reported to have diverse bioactivities like antitumor (Kim *et al*1998), anti-virus (Jeong *et al* 1999), immuno-stimulation (Nishitoba *et al* 1998) and various medical purposes. There appears to be limited information available that reports the antimicrobial properties of *Ganoderma* species. More exciting potential exist in the wild and unexplored part of the world for discovery of novel macro fungi, their biology and their potential usefulness. In the present study, the macro fungal isolates were isolated from Western Ghats of Courtallum Hills and their extracts were screened for antimicrobial activity against bacterial and fungal pathogens.

Materials and Methods

Isolation of macro fungi

Collection

Various fruiting bodies of macro fungal species were collected from tropical evergreen forest of Courtallum Hills, Western Ghats, Tamil Nadu, India. The collected fruiting bodies were placed in paper bags after removal of excess moisture. At the time of collection the morphological characters such as size, shape, colour and habitats whether it is decomposed wood or soil where they are picked were recorded. The fruiting body samples were stored at

4^{o}C and preserved in paper bags in air tight conditions with small amount of 1-4 para dichlorobenzene in porous packets to keep them insect free for further investigations.

Cultivation of macro fungi

The fruiting bodies of the macro fungi were washed thoroughly with sterile distilled water and were thereafter aseptically broken with aid of a sterile forceps. A small piece of 2 × 2 mm of the fruiting body was aseptically transferred onto plates containing PDA with 50 µg/mL of streptomycin to suppress bacterial growth. The plates were incubated at 30°C for three weeks for the development of macro fungi. The fungi growing out from the fruiting bodies were subsequently transferred onto fresh PDA plates without antibiotics.

Radial growth rate measurements

Radial growth rate was determined by following the method of Lonergan *et al* (1993). Briefly, radial growth rate measurements were performed in triplicate on the different fungal colonies grown on PDA. PDA was used in preference to MEA because of its translucent nature, which enabled the growing edge to be seen and measured clearly. Growth assays were performed on all the plates by measuring the radius (minus the plug radius) of the fungal colony in mm. The measurements were taken from four different points and the average radial growth was recorded. Measurements were taken at 24 h interval over a period of 4 d to 14 d. A light box fitted with a magnifying glass was used to facilitate viewing and enable accurate measurement of the fungi. Mycelial growth rate was calculated by using the following formula.

$$\frac{\{[G(12) - G(d10) + G(d10) - G(d8) + [G(d8) - G(d6)] + [G(d6) - G(d4)]\}}{4}$$

Where: G (d12) is equal to the average mycelial growth (in mm) on day 12 and G (d10) is equal to the average mycelial growth (in mm) on day 10. If the mycelial growth had reached the perimeter of the plate before day 14, then day 12 was used as the starting point for the calculation.

Microscopic analysis

Each specimen was examined for morphological characteristics of asci, ascospores, and other structures of taxonomic value. Spore dimensions were determined for 50 spores. Lacto phenol cotton blue and distilled water were used as mounting media for microscopy. Dried

materials were rehydrated in 3% aqueous KOH. Photography was carried out with a light microscope and binocular microscope attached with computer (COSLAP). The cultivated macro fungi were identified based on the morphological characteristics.

Identification of macro fungal isolate

After screening of bioactivities the selected macro fungal isolate was identified to genus level at Mycological Lab and samples were deposited at the herbarium of Department of Botany, VHNSN College, Virudhunagar, Tamil Nadu, India.

Extraction of bioactive compounds

The air-dried fruiting bodies were made into fine powder. For extraction, 200 mL of ethyl acetate was taken into a conical flask containing 20 g of powdered fruiting bodies. This was allowed to stand for 24 h with intermittent agitation (150 rpm) and filtered through Whatman No.1 filter paper. The residue was then extracted with two additional 200 mL of solvents as described above. The combined ethyl acetate extract was concentrated by evaporation under reduced pressure at 45°C using rotary vacuum evaporator. The dried extract was dissolved in 10% dimethyl sulfoxide (DMSO) and stored at 4°C for further study.

Antimicrobial activity

Test microorganisms

Staphylococcus aureus (MTCC 3160), *Escherichia coli* (MTCC 4296), and *Candida albicans* (MTCC 3018) were purchased from the Microbial Type Culture Collection (Chandigarh, India) and which is used to evaluate the antimicrobial activity.

Agar well diffusion method

The agar plate diffusion assay (Perez *et al* 1990) was used to evaluate the antimicrobial activity against test microorganisms. For bacteria, 100 µl of bacterial liquid culture, in an exponential growth phase, was spread onto the surface of Muller Hinton agar plate. Immediately, 100 µl of partially purified fraction of the fungal isolate was loaded onto the well. The culture was incubated at 30°C for 18 h and the zone of inhibition was measured. Ethyl acetate (100 µl) was used as positive control for antibacterial activity.

For fungi, 100 µl of fungal culture/spore was spread out onto the surface of potato dextrose agar medium. Immediately, 100 µl of partially purified fraction was loaded onto the well. The

fungal culture was incubated at 30^{o}C for 48-72 h and the zone of inhibition was measured around the well. Ethyl acetate (100 µl) was used as positive control. Experiments were performed in triplicate and the antimicrobial activity was expressed as the average of diameters of the inhibition zone (in mm) produced by the macro fungal extract.

Results

Collection of macro fungal isolates

Fruiting bodies of the macro fungal species were collected from tropical evergreen forest of Courtallam Hills, Western Ghats, Tamil Nadu, India. At the time of collection, they were named as MS1, MS2, MS3 and MS4 based on typical morphological characteristics. All the three except MS3 macro fungal fruiting bodies were collected from the partially decaying wood, whereas MS3 was collected from the living tree (Fig. 1).

Figure 1. Fruiting of macro fungal species isolated from Southern Western Ghats, Courtallum Hills-Tamilnadu (**a)** MS1, **(b)** MS2 **(c)** MS3 and **(d)** MS4.

Preliminary screening of antimicrobial activity of macro fungal isolates

In order to screen the significant bioactive of macro fungi, the ethyl acetate extracts of four macro fungal isolates was tested against human bacterial pathogens. The preliminary screening results showed that the isolate of MS3 only have antimicrobial activity against bacterial pathogens such as *E. coli, S. aureus* and *C. albicans* (Table 1). Whereas, the antimicrobial activity was completely absent in the macro fungal isolates MS1, MS2 and

MS4. Therefore, the MS3 alone was selected for further morphological and antimicrobial studies.

Macro fungal isolates	Antimicrobial activity		
	E. coli	*S. aureus*	*C. albicans*
MS1	-	-	-
MS2	-	-	-
MS3	+	+	+
MS4	-	-	-

Table 1. Preliminary screening of antimicrobial activity of mcaro fungal isolates against human bacterial pathogens

- No Activity, + Activity Present

Morphological characteristics of the macro fungal isolate MS3

Macro fungal isolate MS3

Fruiting bodies were growing either singly or in groups which are typically seen emerging at the base and stumps of deciduous trees. They are yellow with white in colour, becoming dark orange at maturity (Fig. 1.c). The fruiting bodies are produced during spring season under natural condition. They may persist for several months or even years and can release spores continuously during this time.

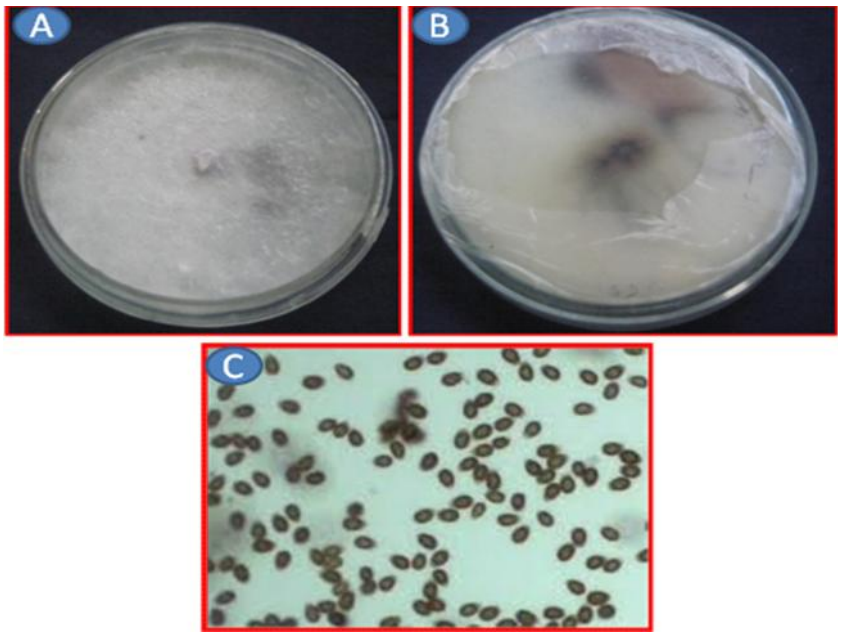


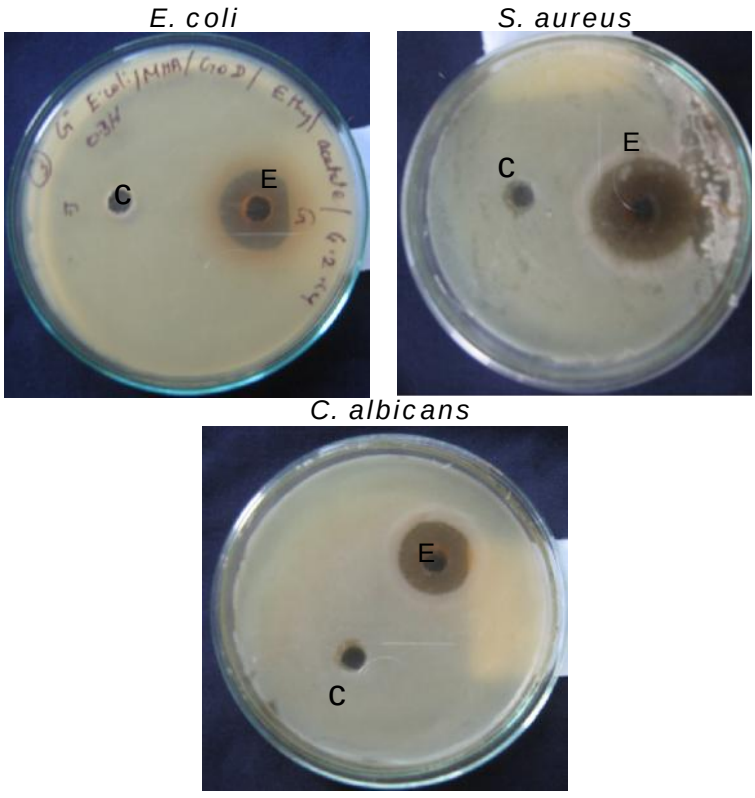
Figure 2. (**a**) Cultural morphology of MS3 on PDA plate dorsal view, (**b**) Cultural morphology of MS3 on PDA plate in ventral view and (c) Morphology of spore

The fungus also produced its fruiting bodies on agar medium (Fig. 2). The morphological characteristics of the macro fungal isolate MS3 was observed on PDA after 2 d of growth at 30°C. Growth rate is high, 5.8 - 7.5 cm/week, covering petriplate in 6 - 8 d. Mycelial mat was white colour in front side but it was whitish pink colour in back side of the plate. Multiple hyphae fuse together to form a network of threads called mycelium. Eventually, mycelium will grow to produce matured fruiting body.

Antimicrobial activity

In present study, the antimicrobial activity of the ethyl acetate extract of fruiting bodies of *Ganoderma* sp. MS3 was investigated against bacterial and fungal pathogens by agar well diffusion method. The results showed that ethyl acetate extract had an effective antimicrobial activity against all the tested bacterial and fungal pathogens. The ethyl acetate extract produced maximum inhibition zone of 31.2 mm against *Staphylococcus aureus* (MTCC 3160), 29.3 mm against *Candida albicans* (MTCC 3018). Whereas, minimum inhibition zone of 27.1 mm was observed against *Escherichia coli* (MTCC 4296) in fig. 3. All these results were compared with the control.

Figure 3. Antimicrobial activity of fruiting bodies of *Ganoderma* sp. MS3 against human bacterial pathogens.



C-Control, **E**- Ethyl acetate extract

Discussion

Morphology of macro fungal isolate MS3

The morphological characteristic of the macro fungus was observed on PDA after one week of growth at 30°C. Growth rate is high, 5.8 - 7.5 cm/week, covering petriplate in 6 - 8 d. Colonies on PDA were circular, mycelial mat was raised at white in beginning state, sometimes grey and becoming whitish pink with age, dense, cottony aerial mycelia without visible conidial masses reverse bright whitish pink. Multiple hyphae fuse together to form a network of threads called mycelium. Eventually, mycelium will grow to produce matured fruiting body. These characteristics allowed the identification of the macro fungal isolate MS3 as *Ganoderma* sp. MS3. It was reinforced by using keys and morphological characters mentioned by Steyaert 1972; Ryvarden 2000. The genus *Ganoderma* species belong to the kingdom of fungi, the division of basidiomycota, the class of homobasidiomycetes, the order of aphylloporales and the family of polyporaceae (Chang 1995; Wasser & Wesis 1999). *Ganoderma* species are among those fungi that can thrive under hot and humid conditions and are usually found in subtropical and tropical regions (Moncalvoc & Ryvarden 1998).

Antimicrobial potential of *Ganoderma* isolate

Natural products play a dominant role in the discovery and development of drugs in the treatment of human diseases (Newman *et al* 2003). Throughout the history of drug development, natural products have provided a fundamental source of drugs for fighting infection, inflammation and cancer in humans. Macro fungi have been proved to be one of the most productive sources producing a large and diverse variety of secondary metabolites with significant bioactivities (Mugdha *et al* 2010).

In the present study, the ethyl acetate extract produced maximum inhibition zone of 31.2 mm against *Staphylococcus aureus*, 29.3 mm against *Candida albicans*. Whereas, minimum inhibition zone of 27.1 mm was observed against *Escherichia coli.* This result is similar to the findings of (Mothana *et al* 2003) who reported that the organic extracts of fruiting bodies of *Ganoderma* sp. showed an effective antibacterial activity against some selected gram negative bacteria. The observed inhibitory effect of *Ganoderma* sp. MS3 against *E.coli* is in line with the report of Smania *et al* (2007) who worked on the antimicrobial metabolites in *G. Lucidum.* Similarly, Keypour *et al* (2008) observed that the comparable antibacterial activity of a chloroform extract of *G. lucidum* against bacterial pathogens from Iran. Recently, Natsir

Djide *et al* (2014) investigated that the antibacterial activity of various extracts from the fruiting bodies of *Ganoderma lucidum* growing at *Samanea saman* trunk in *Indonesia*. Here, the antibacterial activities were very low (11.5 mm against *S. aureus*) when compared to present study.

From this study, it is clearly seen that the entire medicinal macro fungus *Ganoderma* sp. MS3 demonstrated high level of antimicrobial activities in against bacterial and fungal pathogens. These results affirm the claims of traditional herbalists in the world that *Ganoderma* species could be used to treat some bacterial and fungal infections of human beings.

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Conflict of interests

We declare that we have no conflict of interest.

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