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Research Article

INFLUENCE OF SOLID MEDIA ON GROWTH OF MYCELIA AND ANTIBACTERIAL ACTIVITY OF WILD MACROFUNGI, *MACROCYBE GIGANTEA*

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ABSTRACT

The present study was focused on wild pantropical macrofungi, *Macrocybe gigantea* (Massee) Pegler & Lodge, collected from Agumbe rain forests of Karnataka, India. The macrofungal tissues was subjected to mycelial culturing in four different solid nutritive media viz., Potato Dextrose Agar, Malt Extract Agar, Sabouraud's Dextrose Agar and Czapek Dextrose Agar. The study also evaluated the *invitro* antibacterial activity of ethyl acetate extract of mycelial filtrates obtained from Potato Dextrose Broth cultures of *M. gigantea*. The crude extracts were tested against five Gram-negative bacteria using agar well diffusion assay. Our data showed that among the soild media tested, the mycelium grown in Potato Dextrose Agar recorded the maximum growth followed by Malt Extract Agar. The crude extracts had inhibition against *Salmonella typhi*, *Escherihia coli*, *Klebsiella pneumoniae*, *Pseudomonas syringae* and *Xanthomonas campestris.* The maximum inhibition was against *S.typhi*. The results revealed that the extracts had the potent bioactive compounds with antibacterial property.

Keywords: Wild macrofungi, mycelial culture filtrate, ethyl acetate extract, antibacterial activity

INTRODUCTION

The mycelium known as "the neurological network of nature"[1] representing the vegetative lifecycle of mushrooms have huge impact on human health and nutrition. The mycelium develops for years, secreting complex extracellular enzymes inorder to compete with the hostile environment of certain species of bacteria, viruses and other fungi. It also develops certain potent bioactive compounds to protect itself [2]. The mushroom fruiting bodies are enriched with important medicinal compounds including beta-glucans, triterpenoids, ergosterols and various other secondary metabolites. Some studies have already shown that the mycelial biomass of different medicinal mushrooms possess pharmacologic properties comparable to those of mushroom fruiting bodies [3, 4, 5].

Rainforests are repositories of various life forms. Interestingly, most of the life forms are unnoticed, particularly those on the rainforest floor. The giant sized macrofungi *Macrocybe gigantea* (Massee) Pegler & Lodge[6] was collected from Agumbe rainforest. More fruiting bodies of *M. gigantea* are found during continuous rain with an average relative humidity of 70% and temperature range between 25 - 28 º C. *M. gigantea* is a common edible mushroom which grows in regions of high temperature and humidity and distributes in subtropical rain forests in Africa and Asia [7]. In India, this macrofungus has been documented from three states, viz., Kerala, Karnataka and West Bengal[8,9,10]. This Basidiomycetes fungus belongs to the family Catathelasmataceae.

In our previous work it was shown that the ethanol extracts of fruiting bodies of *M.gigantea* clearly revealed the presence of 50 metabolites, which includes fatty acid methyl esters (FAMEs) like methyl palmitate, methyl linoleate, methyl oleate and a steroid, ergosterol relatively in high percentage, all of which are reported to have several industrial applications. It is understood that for the survival of a mushroom in its natural environment, requires antimicrobial compounds. If the antimicrobial compounds could be isolated from the macrofungal fruiting bodies with strong activities, then it is guessed that same can be isolated from the mycelia also. Around 15% of the bioactives are presently derived from extracts of mycelia, another small percent from culture filtrates [11].

Herein, the present work is intended to determine the efficacy of ethyl acetate extract of *M.gigantea* mycelial culture broth against five Gram-negative bacteria. The work was also aimed to determine the most suitable media for the growth of mushroom. The results obtained from this study might be of valuable information with antibacterial compounds and is hoped to introduce this macrofungi to the mushroom growing industry in future.

MATERIALS AND METHODS

Experimental design

The experiments were conducted in Mycology Laboratory, Department of Post Graduate Studies and Research in Applied

Botany, Kuvempu University in Shivamogga, Karnataka during post rainy season 2017(June – August).

Raising mycelial culture

The basidiocarps of *M.gigantea* was collected from wild and transported to laboratory and cultured aseptically with appropriate precautions on Potato Dextrose Agar (PDA) media. The mycelial culture was obtained by tissue culture technique[12]. The surface of the pileus was cleaned by rubbing carefully using a fine brush in order to remove the foreign debris. The fresh fruiting body was then rinsed in distilled water and surface sterilized with rectified spirit for 30 seconds. The excess of moisture content was removed by using sterilized blotting sheets. The pileus was teared into two halves and the innermost tissue placed on petridish containing PDA media aseptically, incubated at 25ºC for pure culture.

Effect of different solidified nutritive media on mycelial growth

Various agar media like Potato Dextrose Agar (PDA), Malt Extract Agar (MEA), Sabouraud's Dextrose Agar (SDA) and Czapek Dextrose Agar (CZA) were used to study the growth of mycelia. The composition for those culture media were as follows: PDA: 200g potato, 20g dextrose, 15g agar in 1000ml distilled water, p^H - 5.6; MEA: 30g malt extract, 5g peptone, 15g agar in 1000ml distilled water, p^H - 5.6; SDA: 10g peptone, 40g dextrose, 15g agar in 1000ml distilled water, p^H- 5.6 and CZA: 30g sucrose, 2g sodium nitrate, 1g dipotassium phosphate, 0.5g magnesium sulphate, 0.5g potassium chloride, 0.01g ferrous sulphate, 15g agar in 1000ml distilled water, p^H-7.5. Media and petridishes (90mm diameter) were autoclaved at 121ºC for 20 min. The petridishes containing different media were inoculated with mushroom mycelial discs (1cm diameter) aseptically. The inoculated plates were incubated at 25±2º C and the mycelia growth expansion was observed for daily basis until the culture reached its maturity. Four replicates were maintained for each culture.

The mycelial morphology like texture and growth density was identified by visual observation after the complete colonization in petridishes. The mycelial texture was assessed in terms of cottony or woolly and the growth density as luxuriant, average or reduced.

Preparation of crude extracts from PD broth culture filtrate of *M.gigantea*

After complete colonization in various media, i.e., after attaining full growth, 5-6 actively growing mycelial discs of 6mm diameter were transferred to 100 ml of each PD broth in ten 250 ml Erlenmeyer flasks. The liquid medium (P^H- 6.6) contained glucose 2%, peptone 1% and yeast extract 2% [13]**.** The inoculated broth was incubated in both dark and light cycles for a period of 12 days under stationary conditions with intermittent shaking.

After 12 days of fermentation, a thick white mycelial mat developed (Figure 1) and the culture broth was filtered to separate out the mycelial mat from the culture filtrate. The filtrate was then filtered through Whatman no: 1 filter paper discs. The filtrates obtained were partitioned in ethyl acetate using a separating funnel, mixed and shaken well for 10min and kept until two clear immiscible layers were formed. Ethyl acetate which forms upper layer was extracted several times and the fractions were evaporated to dryness at room temperature using rotary flash evaporator. The yield of the culture filtrate extract was recorded. (Figure 2)

Bacterial strains

Antibacterial effects of the mycelial culture filtrate extracts of *M.gigantea* on 5 strains of Gram- negative bacteria were investigated.

1. *Escherichia coli* [MTCC – 1599]
2. *Klebsiella pneumoniae* [MTCC – 7028]
3. *Pseudomonas syringae* [MTCC – 1604]
4. *Salmonella typhi* [MTCC – 734]
5. *Xanthomonas campestris* [MTCC – 2286]

MTCC - Microbial Type Culture Collection, Chandigarh, India.

The bacterial strains of *E.coli*, *K. pneumoniae*, *P. syringae*, *S.typhi* and *X.campestris* were maintained on nutrient agar. The strains cultured were then subcultured and inoculated in nutrient broth, incubated at 37ºC for 24 h to reach the concentration of 10^6 cfu/ml.

***In vitro* antibacterial activity of crude extract by agar well diffusion method**

To determine the effects of mycelial culture filtrate against five bacterial strains, the nutrient agar plates were prepared, and the target bacteria were inoculated on to the media using sterile cotton swabs. Wells of 5mm diameter were made in each plate and then subjected to addition of mycelial extracts of varying concentrations in each well.

To study the antibacterial activity, the crude mycelial extracts were dissolved in dimethylsulfoxide (DMSO) to equal 100mg/ml as stock solution. Hundred microlitres of 25%, 50% and 100% concentrations of each culture filtrate extracts were then loaded into the wells and incubated at 35±2ºC against test bacterial strains. The wells loaded with Ciprofloxacin and DMSO served as positive and negative control respectively. The activity was evaluated by measuring the diameter of the inhibition zone (mm) obtained after 24 hours.

Statistical analysis

The values expressed as mean±SD for various treatments against five strains of bacteria. The data recorded were subjected to statistical analysis. A one-way ANOVA test and Dunnett's post analysis ($p< 0.05$) were used to determine the significant difference if any between the treatments.

RESULTS AND DISCUSSION

Raising Mycelial culture

The mycelial culture was initiated after seven days of tissue inoculation (Figure 3). As the mycelial growth was slow, full maturity was achieved on 14th day (Graph 1).

Effect of different solidified nutritive media on mycelial growth

It is evident from the results that the mycelial growth varied depending on the media employed. Inoculation of mycelia in PDA recorded the fastidious growth. It was observed that among the four media used to study the growth characteristics of mycelia of *M.gigantea*, luxuriant growth was found in mycelial discs inoculated in PDA(10mm/day) followed by MEA(8mm/day) (Figure 4). The mycelium inoculated in SDA and CZA took longer time to complete the growth. SDA(2mm/day) showed average mycelial growth, whereas CZA (1mm/day) observed reduced development of mycelia (Graph 1). The luxuriant growth of the mycelia in PDA may be due to the supply of required

nutrient content that the macrofungi obtained. As the mycelia grew well on PDA, it can be inferred that PDA can be widely used for mushroom culture. The PDA contains 1.5% agar, 2% glucose, with nitrogen, phosphorus, vitamins and micronutrients being derived from a crudely filtered extract of macerated potatoes.

In case of incubation period, mycelial run was highest in PDA and MEA. In both the media the mycelial growth initiated on 6th day prior to inoculation. CZA media showed slowest mycelial run (13th day). The cottony texture was observed in mycelia inoculated with PDA, MEA and SDA.

Under *in vitro* conditions, the mycelial growth rate performed best on P^H 5.6 and reduced growth rate in case of media (CZA) above p^H level 7. The media with p^H 5.6 were PDA, MEA and SDA. P^H had significant influences on the growth of fungi and grows better between pH 5 and pH 6.

***In vitro* antibacterial activity of mycelial extracts by agar well diffusion method**

The PD broth cultured mycelial extract of wild basidiomycetes mushroom *M.gigantea* was investigated to evaluate the antibacterial activity against five Gram-negative bacteria using agar well diffusion method. Evaluation of antibacterial activity revealed varied results and is recorded in the Table 1. and illustrated in Figure 5. The results revealed that the culture filtrate extract was potentially effective in suppressing the bacterial growth of *S.typhi*, *E.coli* and *X.campestris* and moderately effective against *K. pneumoniae* and *P.syringae*.

The results obtained from the present study revealed that the extract inhibited five Gram-negative bacteria suggesting potential antibacterial activity. The mycelial culture filtrate extract of *M.gigantea* exhibited satisfactory results by inhibiting all the tested bacterial strains. The antibacterial property may be attributed to the presence of bioactive compounds. It should be noted that this study is one of the few works reported so far on the antibacterial activities of PD broth mycelial extract of *M.gigantea*.

Table 1: Antibacterial activity of mycelial culture filtrate extract of *M.gigantea*

	Zone of Inhibition in mm (Mean±SD)					
Sl.No.	**Bacterial strains**	**EAE 25%**	**EAE 50%**	**EAE 100%**	**Standard Ciprofloxacin (1mg/ml)**	**Control DMSO**
1	*E. coli*	18.1±0.17	18.73±0.11*	28±0**	18±0**	0±0
2	*K. pneumoniae*	18±0	18.43±0.11*	28.33±0.28**	28.16±0.28**	0±0
3	*P. syringae*	0±0	0±0*	0±0**	28.6±0.34**	0±0
4	*S. typhi*	18.56±0.11	28±0*	28.43±0.11**	18.36±0.11**	0±0
5	*X. campestris*	8.76±0.05	18.13±0.11*	18.63±0.23**	18.43±0.11**	0±0
One way ANOVA	P value	0.0034	0.0034	0.0034	0.0034	0.0034
	F value	5.622	5.622	5.622	5.622	5.622

EAE: Ethyl acetate extract; All the values were replicated four times;
One-way ANOVA significance level P<0.05, Significant - **, *

Figure 1. Flasks containing the mycelial mat on liquid medium

Figure 2. Crude extracts of mycelial culture filtrates

Figure 3. Mycelial growth around inoculated part of mushroom tissue

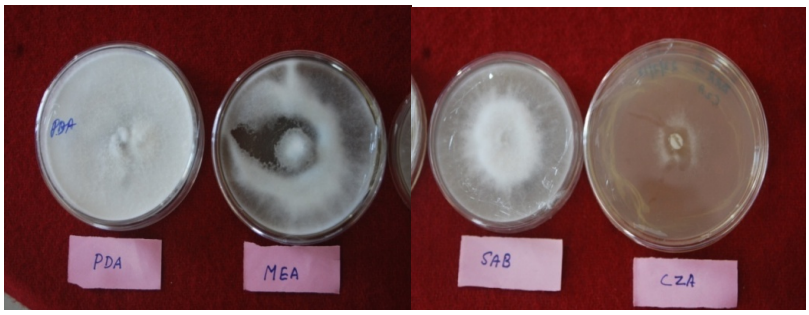
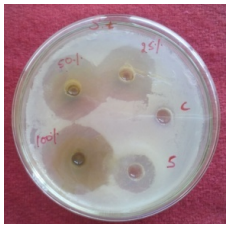
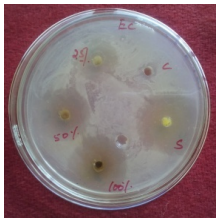


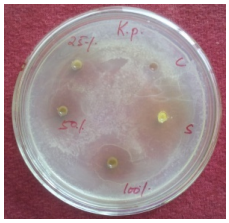
Figure 4: Mycelial culture in a) PDA, b) MEA, c) SAB, d) CZA



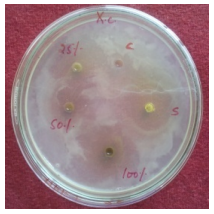
Salmonella typhi



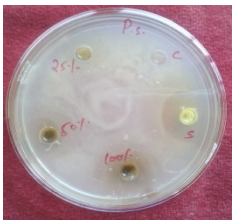
Escherichia coli



Klebsiella pneumoniae

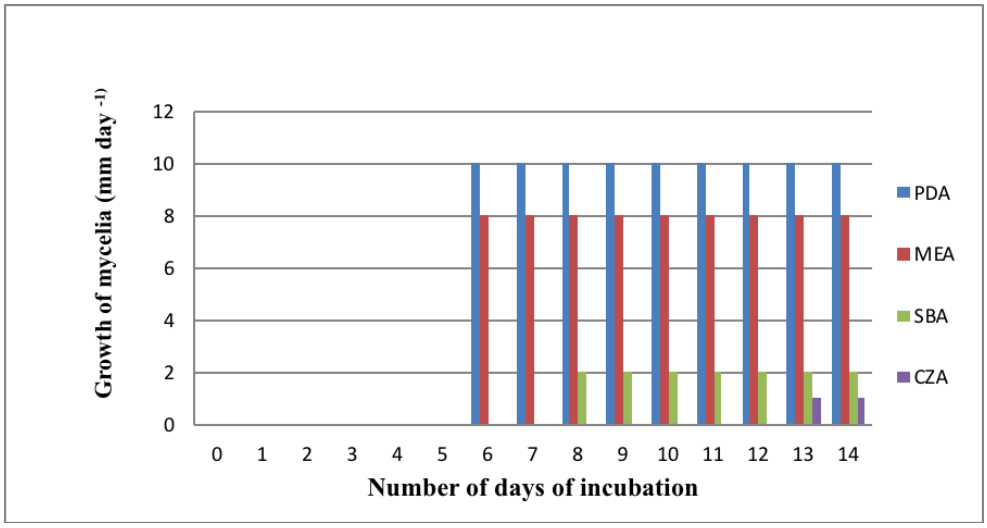


Xanthomonas campestris



Pseudomonas syringae

Figure 5: Growth inhibition of some Gram-negative bacterial strains caused by mycelial culture filtrate extract of *M.gigantea*



Graph 1: Effect of growth of mycelia on different solidified media

CONCLUSION

Monitoring of antimicrobial resistance using natural resources is now one of the core areas to discover potent biologically active compounds of pharmaceutical importance. The research findings on comparative evaluation of nutritive media for mycelial growth and the *in vitro* results of PD broth cultured extracts of *M.gigantea* for preliminary antibacterial screening will support the *in vivo* studies in future. More biochemical studies are needed to know in-depth knowledge about the individual compound responsible for those miraculous properties.

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