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Evaluation of Antibacterial Potential of *Daldinia Concentrica* from North Eastern Region of India

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ABSTRACT

Morphological, bio-chemical and antibacterial potential of an ascomycetous fungus, *Daldinia concentrica,* was evaluated. It was collected from dead, decaying log of *Mimusops elengi* tree and isolated by plating in potato dextrose medium. Macroscopic and microscopic features of the stromata were studied. The crude methanol extract of the stromata (30 mg/ml) exhibited significant antibacterial activity against *Escherichia coli* (MTCC-40), *Pseudomonas aeruginosa* (MTCC-7093), *Klebsiella pneumoniae* (MTCC-661), *Enterobacter aerogenes* (MTCC-111) and Gram positive bacteria *Staphylococcus aureus* (MTCC-7443), *Bacillus subtilis* (MTCC-121). *Bacillus subtilis* was the most sensitive organism to the methanol extract of this fungus with 41.33 mm zone of inhibition. Gas chromatographic–mass spectral analysis of stromatal methanol extract revealed the presence of 19 compounds. The present investigation indicated the potential antibacterial activity of native *D. concentrica,* which could be useful for pharmaceutical application.

Keywords: *Daldinia concentrica*; Antibacterial; GC-MS; Stromata

1. INTRODUCTION

Daldinia concentrica also known as cramp balls, is an ascomycetes fungus of the family Xylariaceae with large globose stroma. The fungus is widely distributed throughout the world, mostly in temperate region. The fruit bodies appear singly or scattered on the dead, decaying logs and can be recognised very easily. The fungus is used in traditional medicine in Yorubaland, South Western Nigeria in the treatment of Pneumonia and other bacterial infections[1-3]. It is also used traditionally for curing skin irritation and wound healing[4]. The genus *Daldinia* contains a variety of compounds which contributes to biological activities. Several studies had been carried out to identify these compounds and lead to the discovery of twenty new compounds[5-7]. The antimicrobial potential of some edible and inedible mushroom has been confirmed in earlierstudies[8-9]. Out of the 1.5 million species of mushroom reported globally, only 50 % of these have been characterised sofar[10-11].

Mushrooms are not only good sources of nutrients, it can also be used as medicine. The rapid emergence of multidrug resistant bacterial strains to synthetic drugs, threaten public health day by day. Moreover long-term application of synthetic medicines results in several adverse effects. Therefore there is a need to explore natural resources for new and safer, antimicrobial agents. Mushrooms are rich sources of biologically active compounds, which can be explored for this purpose.

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Assam, situated in the north east of India is part of the Indo-Burma biodiversity hotspot. High humidity and rainfall is one of the major factor for the growth of varieties of wild mushrooms. The subtropical climate with average rainfall 1500 mm per year, maximum temperature 35 ^{O}C - 38 ^{O}C in summer and 6 °C - 8 °C in winter, makes this region a hub for the growth of many macrofungi.

Although several studies have been carried out on diversity of macrofungi from Assam, no effort has been made scientifically to characterise its potentialities and preserve these wild economically important species[12-13]. Keeping in view the limited reports from this region, the current investigation was done to explore the bioactive potential of native medicinally important fungus *D. concentrica.*

2. MATERIALS AND METHODS

2.1 Collection and Isolation

The macrofungus *D. concentrica* was collected from decaying log of *Mimusops elengi* L. tree of sonitpur district of Assam (India) during the month of August 2018 (26° 39′ 4.3848″ N and 92°47′ 1.7268″E). During the survey, fruiting bodies of *D. concentrica* were photographed at their natural habitat and collected in plastic bags for laboratory work. Some of the collected fruiting bodies were preserved in kew cocktail solution for future reference. Detailed macromorphological and micromorphological study was carried out in the laboratory. The fungus was confirmed as *D. concentrica*[14].

2.2 Morphological Characterisation

All relevant external and internal macroscopic features such as colour, size, shape of the stromata were observed. A portion of internal tissue mounts on a glass slide, in 5% KOH stained with 1% congo red were prepared to observe microscopic features (Zeiss AX10). All measurements (macroscopic and microscopic) were made with the help of ImageJ software. Pure culture was isolated, on potato dextrose agar (PDA) medium amended with 0.01% Streptomycin, from the collected fruiting body by standard procedure and maintained at 4 ^{O}C. Cultural characteristics such as color, texture, growth rate, of the isolate grown on PDA was studied.

2.3 Preparation of Solvent Extracts

Fresh sample of the fungus cut into bits, oven dried at 40 °C for 24 h and ground aseptically into fine powder usinga blender. Methanol was used for extraction of the sample in the ratio of 1:10 (w/v). Extraction was done at ambient condition for 48hours and filtered through Whatman filter paper No.1. The process was repeated twice. The filtrate was dried at 40 °C in a hot air oven and stored at 4 ^{O}C for future use to evaluate the antimicrobial activity. The extract was prepared according to Jonathan[15], *et al.* with slight modifications.

2.4 Antibacterial Bioassay

2.4.1 Microbial Strains

Total six pathogenic bacteria, two Gram positive *B. subtilis*, *S. aureus* and four Gram negative bacteria *E.aerogenes, E.coli, K. pneumoniae* and *P. aeruginosa* were taken as inoculum to study the antimicrobial efficacy of the crude extract.

2.4.2 Agar Well Diffusion Method

In vitro antibacterial efficacy was ascertained by agar well diffusion method[16]. The test organisms, revivified in nutrient broth medium (NB) by incubating for 18 h at 37 ^{O}C were considered as inoculum. Turbidity of the inoculum was adjusted to 0.5 McFarland standard. Agar plates were inoculated by streaking the test organisms evenly with the help of sterile cotton swabs. After 20 min, wells of 6mm diameter were made in the agar plate with the help of sterile cork borer. The extracts was dissolved in 2.5 % dimethyle sulphoxide (DMSO) for preparation of different concentration and employed for the experiments. 50 µl of each solvent extract of the test sample (conc. 30 mg/ml) was dispensed into the wells asceptically and incubated for 24 h at 37 °C. Antibacterial efficacy of the extract was ascertained by measuring the diameter of zone of inhibition (ZI) around the well in millimeters after 24 h of incubation. In the control plate 2.5% DMSO and distilled water was dispensed as negative control.

The fungicidal or fungistatic property of the extract was observed by inoculating plugs of PDA medium taken out from the zone of inhibition area and re-incubated in fresh media and incubated for their growth. Plates were examined after 96 h according to Kariba[17], *et al*.

2.4.3 Minimum Inhibitory Concentration

Minimum inhibitory concentration (MIC) was obtained by broth dilution technique. Each tube of potato dextrose broth was inoculated with inoculum (0.5 McFarland standard) of test bacteria and incubated for 24 h at 37 °C. The least concentration which showed no turbidity was recorded as MIC. The experiment was performed in triplicate for each species.

2.5 Gas Chromatographic –Mass Spectral Analysis

The crude extract was dissolved in HPLC grade methanol and analysed by Gas Chromatographic –Mass Spectral Analysis (GC-MS). The GC-MS system consisting of an aglient 9890 B gas chromatogram and a mass selective detector 5977A with HP 5ms column. A sample was injected in a splitless mode. Scan range was 50 Amu - 550 Amu. Inlet temperature was kept at 250 °C and the effluent of the GC column was introduced into the source of the MS via a transfer line at 280 °C. Ionisation voltage was 70 eV and ion source temperature was 230 °C. Helium was used as the carrier gas at the rate of 1.0 ml/min. Chemical compounds were identified by their retention time and comparing with the mass spectrum data available at National Institute of Standard and Technology (NIST) library. Initial temperature of the oven was set at 50 °C with 20 °C per minute rate, increased to 250 °C with 2 min hold.

2.6 Statistical Analysis

The bar graph and standard deviation of antimicrobial activity against bacterial pathogens was plotted and calculated with GraphPad Prism[5].

3. RESULTS

3.1 Morphology

Stromata was 3.9 x 2.4 x 2.7cm in size, oval or globose in shape, sessile, reddish brown in young stage and became black with age. Perithecia was small crowded, tubular, length ranged from 1.01 mm - 1.18 mm, width ranged from 0.23 mm - 0.33 mm. Tissue under the perithecial layer composed of altering concentric zones of black and silver bands. The black bands width ranged from 0.26 mm to 0.61 mm and the width of silver bands ranged from 0.52 mm - 1.33 mm. Width of the rings increases in size from the centre to the periphery. These concentric lines are the diagnostic feature of the fungus as shown in Fig. 1.

3.2 Cultural Characteristics

Pure fungal colonies grown on PDA plates was woolly,

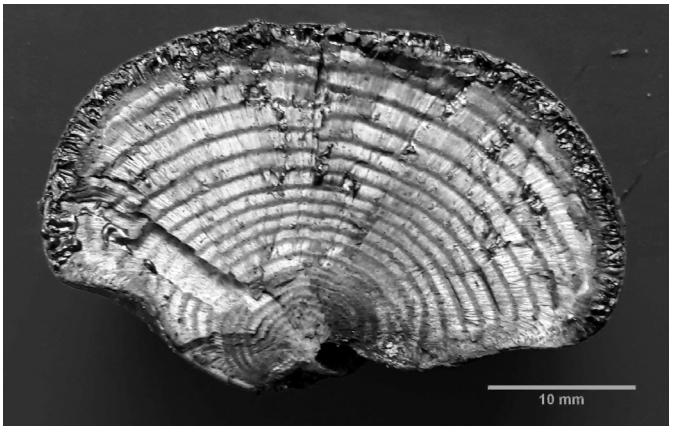

Figure 1. Internal structure of stromata.

wavy, radially furrowed, zonate and white in colour initially, later became concentrically zoned with alternating white and light grey bands. Peripheral and central zones were grey and light grey respectively. Reverse of colony showed black central zone, surrounded by yellowish orange zone followed by grey periphery with white margin as shown in Fig. 2. The isolate was fast growing with growth rate 15.26 mm per day. The exponential phase started from the third day and covered the entire petriplate within seven days. The ascus contained eight ascospores. The ascospore was ellipsoid, average length and width 12.31 ± 1.45 µm and 5.6 ± 0.41 µm, respectively as shown in Fig. 3.

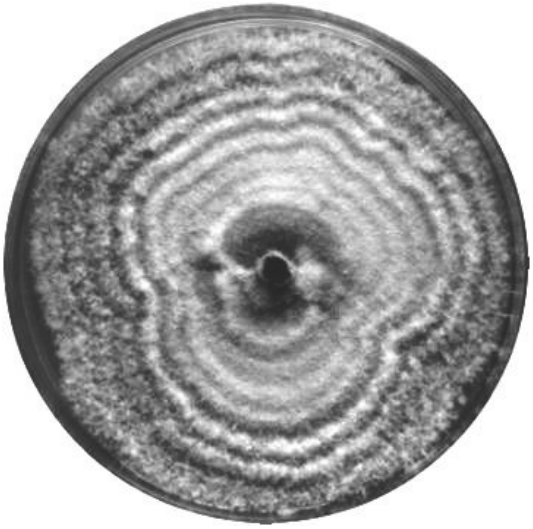

Figure 2. Isolate on PDA.

3.3 Antimicrobial Bio-assay

The antibacterial efficacy of methanol extract against six pathogenic bacteria as shown in Fig. 4 exhibited zones of inhibitions that ranged from 22.29±0.46 mm to 41±0.33 mm. Among the test pathogens *B. Subtilis* was the most susceptible, exhibiting an inhibition zone of 41±0.33 mm while *P. aeruginosa* was less susceptible exhibiting a inhibition zone of 22.29±0.46 mm. The sensitivity pattern of the test bacteria to the extract was found according the following decreasing order. *B. subtilis*(bs) > *E. coli* (ec) >*K.pneumoniae* (kp) >*S. aureus*(sa) > *E. aerogenes*(ea) > *P. aeruginosa*(pa). No growth of bacteria has been observed in the re-incubated plates after 96 h of incubation, suggesting fungicidal property of the methanol extract. *D. concentrica* methanol extract inhibited all the bacterial strains, with MIC for *B.subtilis, E. coli , K. pneumoniae, S. aureus, E. aerogenes* and *P. aeruginosa* at 780 µg, 780 µg, 390 µg, 390 µg, 500 µg and 1 mg/ml, respectively.

3.4 Gas Chromatographic–Mass Spectral Analysis

Gas chromatographic–mass spectral (GC-MS) analysis of the test extract showed 19 peaks within the retention time 8.83 min and 14.248 min as shown in Fig. 5. Components detected from GC-MS analysis are shown in Table 1. Total 19 compounds were detected in the gas chromatography. Out of these five compounds were dominant with a total percentage of 65.9 %. Concentration of

Figure 3. Ascus with ascospores.

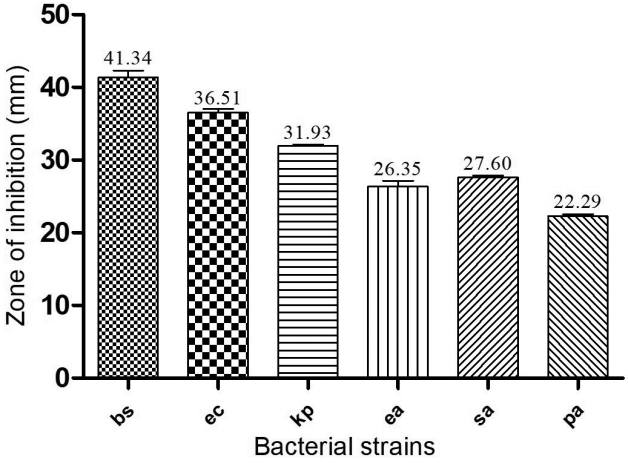

Figure 4. Efficacy of methanolic extract of *D. concentrica.*

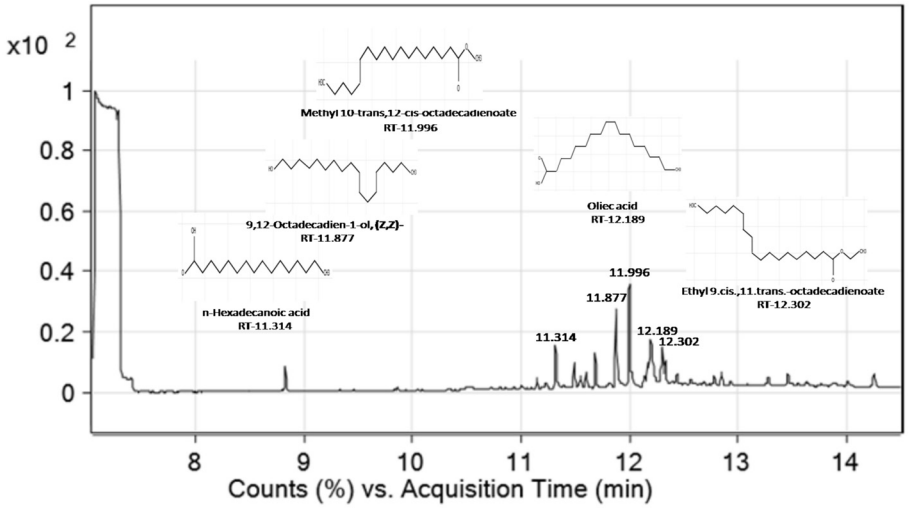

Figure 5. GC-MS chromatogram of methanolic extract.

Table 1. Compounds present in *D. concentrica* (GC-MS)

Compound	RT	Formula	Peak area %
3-((3R)-2,3-Dimethyltricyclo [2.2.1.02,6]heptan-3yl)propanal	8.83	$C_{12}H_{18}O$	3.5
2,3-Dimethylquinolin-4(1H)-one	10.513	$C_{11}H_{11}NO$	2.7
n-Hexadecanoic acid	11.314	$C_{16}H_{32}O_2$	6.4
Hexadecanoic acid, ethyl ester	11.483	$C_{18}H_{36}O_2$	3.7
Butyl 4,7,10,13,16,19-docosahexaenoate	11.545	$C_{26}H_{40}O_2$	1.6
Phenanthrene, 7-ethenyl1,2,3,4,4a,4b,5,6,7,9,10,10a-dodecahydro1,1,4a,7-tetramethyl-, [4aS(4a. alpha.,4b.beta.,7.beta.,10a.beta.)]	11.601	$C_{20}H_{32}$	2.1
Methyl 5,9-heptadecadienoate	11.683	$C_{18}H_{32}O_2$	4.5
9,12-Octadecadien-1-ol, (Z,Z)-	11.877	$C_{18}H_{34}O$	13.6
Methyl 10-trans,12-cis-octadecadienoate	11.996	$C_{19}H_{34}O_2$	20.0
Cyclopropanebutanoic acid, 2-[[2-[[2-[(2pentylcyclopropyl)methyl]cyclopropyl]methyl]cyclo propyl]methyl]-, methyl ester	12.139	$C25H42O_2$	2.2
Oleic Acid	12.189	$C_{18}H_{34}O_2$	18.5
Ethyl 9.cis.,11.trans.-octadecadienoate	12.302	$C_{20}H_{36}O_2$	7.4
Ethyl Oleate	12.327	$C_{20}H_{38}O_2$	3.3
Dasycarpidan-1-methanol, acetate (ester)	12.565	$C_{37}H_{76}O$	1.6
1-Heptatriacotanol	12.677	$C_{37}H_{76}O$	0.9
E,E,Z-1,3,12-Nonadecatriene-5,14-diol	12.777	$C_{19}H_{34}O_2$	1.6
1-Heptatriacotanol	13.272	$C_{37}H_{76}O$	1.8
11,14-Eicosadienoic acid, methyl ester	13.453	$C_{21}H_{38}O_2$	1.8
Bis(2-ethylhexyl) phthalate	14.248	$C_{24}H_{38}O_4$	2.8

Methyl 10-trans,12-cis-octadecadienoate (20 %) was highest, followed by Oleic Acid (18.5 %),9,12-Octadecadien-1-ol,(Z,Z)-(13.6 %), Ethyl 9.cis.,11.trans.-octadecadienoate (7.4 %) and n-Hexadecanoic acid (6.4 %).

4. DISCUSSION

Mushrooms have been used as medicine to cure several diseases long before the onset of modern medicine. Antibacterial activity of macrofungi have been reported in earlier studies.

Methanol extract of *D. concentrica* was found to exhibit notable antibacterial activity against all tested pathogenic bacteria. These results were supported by earlier study that reported by Kavitha[18], *et al*. for *E. coli*, *P. aeruginosa. S. typhi*, *S. aureus and S. mutans.* Similar observations was also reported by Jonathan[3] on *D. concentrica* chloroform and ethanol extract against *S. aureus, E. coli* and *P. aerugino*[19]. Although antimicrobial property of *D. concentrica* extract was evaluated in earlier studies[18-19] it is worthwhile to investigate the same species in order to find out any variations in their activity from different geographical locations.

According to Danielli[20], lower the MIC, the more sensitive the extract[20]. The low MIC value of the extract against all the pathogenic bacteria in the present study, implies the potentiallity of *D. concentrica* against pathogenic bacteria. This observation suggests that the native *Daldinia* species could be a natural source of antibacterial agent against the infection caused by these strains. GC-MS analysis of methanol extract showed the presence of some biologically potent components. The antimicrobial potency of the extract may be due to the presence of these compounds. Among these compounds n-Hexadecanoic acid ethyl ester, n-Hexadecanoic acid, Oleic acidare reported as potent antibacterial agent[21-23]. n-Hexadecanoic acid ethyl ester also has anti-tumour activity[24]. The anticancer cytotoxic activity of n-Hexadecanoic acid against human fibroblast cells has been reported in earlier studies[25]. Oleic acidhave anti-inflamatory, antiandrogenic, cancer preventive, dermatigenic, hypocholesterolemic, anemiagenic, insectifuge activity[26]. Toxicity study of *D. concentrica* solvent extracts has also been carried out on human tumour cell line and animal model[27-28]. Some other compounds were also identified in GC-MS analysis, which may have notable biological activity.

To the best of our knowledge this is the first report on the native *Daldinia* species of Assam (NE India) revealing the antibacterial and GC-MS profiling.

5. CONCLUSIONS

The ethnic tribes of Assam used macrofungi as a source of food and medicine. The findings of this study, scientifically validates the use of this fungus in the traditional system of medicine. The species *D. concentrica* could be a natural source

of novel antibiotic in the near future. Therefore detailed study and the awareness for conservation of this indigenous species is necessary before it is wiped out, due to over exploitation and destruction of natural habitat.

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In the current study, he edited the final manuscript.