International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 4 July-Aug 2021, pp: 1373-1380 www.ijprajournal.com ISSN: 2249-7781

GC-MS Analysis of Bioactive compounds from Endophytic fungi *Mucor circinelloides* and it's Antimicrobial study

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Date of Submission: 01-08-2021 Date of Acceptance: 18-08-2021

ABSTRACT: Medicinal plants are significant source for isolation of endophytic fungi. Endophytic fungi reside in the intracellular regions of plants nourished by plant mutually, fungi provide bioactive metabolites which can play crucial role in plant defence system. Fungi can produce bioactive compounds with diverse activities and structure. The intention of study was to isolate and identify endophytic fungi from fruit part of M*orinda citrifolia* and determine their biological activities. Molecular identification was carried out by studying internal transcribed spacer primers (ITS1/ITS4). The experimental result reveals that M. *circinelloides* has shown remarkable antibacterial activity against Gram-negative bacteria. i.e., E. coli, such as Salmonella typhi, Proteus vulgaris and Gram positive bacteria Staphylococcus aureus, Micrococcus luteus. M. *circinelloides* has shown 16 mm of inhibition zone against Staphylococcus aureus and 10.5 mm of inhibition zone Micrococcus luteus. Whereas it has shown considerable antifungal activity against C. albicans (zone diameter 9.0 mm) and C. glabrata (7.0 mm). The diverse group of substances such as alkaloids, steroids, terpenoids, coumarins, phenols were extracted from endophytic fungi. GC-MS analysis of fungal extract indicated 32compounds with diversified structure.

Keywords: M. *circinelloides*, Antimicrobial activity, Bioactive compounds, GC-MS analysis.

I. INTRODUCTION

There is an increasing trend in number of people throughout the world suffering health problems caused by drug resistant bacteria, cancers, viruses and fungi is a cause for alaram [1]. There is an urgent need to search for novel antibacterial compounds for treatment of diseases due to emergence of multi drug resistant microorganisms [2]. More intensive research for novel antimicrobial compounds to deal with these diseases is now underway. Endophytes, especially fungi adapted to live in plant tissue without causing visible disease symptoms are a novel source of potentially useful medicinal plants [3]. Medicinal plants provide a suitable and cogenial environment for colonization of endophytes and recognised as best source for synthesis of novel secondary metabolites of pharmaceutical importance. There is a large scope exist for the recovery of novel fungal species, general and bio vazs from the medicinal plants. According to an estimation approximately 1.5 million fungal species exists in the world [4], [5]. In recent times, considerable knowledge has been gained on physiology and ecology of endophytic microorganisms [6]. Endophytes are little explored share of fungal diversity [7]. In view of biotechnology, endophytic fungal secondary metabolites that has been proven useful for discovery of novel drugs [8]. Endophytic fungi associated with tradational medicinal plants are crucial source for search of novel bioactive compounds [9]. Endophytic fungi harboured by higher plants are associated with the pharmacologically important products, in this context, the aim of this work was to characterize bioactive compounds from endophytic fungi M. *circinelloides* associated with M*orinda citrifolia* isolated from Pakhal forest of Telangana and study their antimicrobial activities against some pathogenic microorganisms. In recent years GC-MS (Gaschromatography-Mass spectrometry) being used as prominent technique to analyze secondary metabolites produced by endophytic fungi, hence the bioactive compounds were analyzed by GC-MS analysis.

II. MATERIALS AND METHODS

Sample collection, isolation and characterization

The fruit part of M*orinda citrifolia* collected from Pakhal forests of Telangana. After collection the fruit was washed by distilled water. The surface of the fruit was sterilized by

International Journal of Pharmaceutical Research and Applications
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consecutive immersion in 70% ethanol for 30 seconds, sterile distilled water for 2 seconds, 4 min with 11% aqueous sodium hypochlorite followed by several washing steps with sterile distilled water [10]. The fruit pieces were placed on potato dextrose agar, supplemented with 50µg/ml streptomycin and incubated at room temperature for 7 days at 27+2ºc. Developing fungal colonies were sub cultured on PDA plates under aseptic conditions to isolate pure cultures of the fungi.

III. PHENOTYPIC AND GENOTYPIC IDENTIFICATION OF ENDOPHYTIC FUNGI

The isolated strains of endophytic fungi were primarily identified by morphology of colonies and their spore characteristics by microscopic study of examination Both molecular and morphological techniques were use to identify the endophytic fungi DNA was isolated from the fungi sample provided and analysed in agarose gel. Fragment of 5.8S rDNA was amplified using ITS1 and ITS4primers in PCR method and amplification was analysed in agarose gel. PCR product was purified to remove the impurities that hamper the sequencing reaction. DNA sequencing was accomplished on BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of the PCR amplicon was generated from forward and reverse sequence data using BioLign/BioEdit software. The consensus sequence was searched in NCBI - non redundant nucleotide (nr)database using Basic Local Alignment Search Tool (BLAST). The BLAST result was sorted based on percentage of identity; top 10 organism sequences were retrieved. Clustal-W program in MEGA-X was used toper form multiple sequence alignment of top 10 BLAST hits and query sequence. Phylogenetic tree was predicted in MEGAX software.

IV. ANALYSIS OF BIOACTIVE PRODUCT USING TLC

Extraction of secondary metabolites performed according to the [11]. The fungal culture was grown in conical flasks containing PDB for 21 days. Cultured broth was filtered and mycelium was separated and filtrates were extracted three times with equal volume of ethyl acetate. Similarly, methanolic extracts were also prepared and were evaporated at certain reduced pressure at 40-45ºc using a rotary evaporator to obtain crude extracts. Concentrated endophytic fungal extract was spotted on TLC plate and was placed in a chamber poured with Hexane : Ethylacetate (9:1). TLC analysis was done using UV light at a wavelength of 250nm. Then plate was sprayed with anisaldehyde. The fractions were successfully separated with particular Rf value. These fractions of fungal extracts were collected in vials.

V. SCREENING OF PHYTOCHEMICALS PRODUCED BY ENDOPHYTIC FUNGI

Preliminary analysis of phytochemicals of crude extract of endophytic fungi was carried out for the presence of different metabolites such as alkaloids, flavonoids, tannins, phenols, terpenoids, coumarins, quinines, saponins, anthraquinones using standard procedure described by [12],[13].

VI. ANTIBACTERIAL ASSAY

Antibacterial activity was performed against human pathogens Gram negative bacteria (E. coli MTCC 723, Salmonella typhi MTCC 733, Proteus vulgaris MTCC 3384) and Gram positive bacteria Staphylococcus aureus MTCC 3381, Micrococcus luteus MTCC 1541) by agar well diffusion method. Nutrient agar plates were prepared and wells were made by using gel puncture. Test bacterial culture was swabbed uniformly under aseptic conditions on the surface of agar as to prepare a lawn culture. This was kept for 5 minutes and allowed for solidification before making the wells. One ml of fungal filtrate was mixed with one ml of solvent was loaded in the wells using micropipette and one well was loaded with the respective solvent as control. Plates were incubated for over night at 37ºc. The zone of inhibition was observed around the well.

VII. ANTIFUNGAL ASSAY

The isolated fungal strains were tested for their potential antifungal activity against two fungal strains Candida albicans ATCC 90028 and Candida glabrata ATCC 90030 by agar well diffusion method.

VIII. GAS CHROMATOGRAPHYMASS SPECTROMETRY ANALYSIS OF FUNGAL EXTRACTS

The crude extract of M. *circinelloides* was subjected to GC-MS analysis to identify the bioactive compounds. GC-MS was performed at IICT Hyderabad in 7890B GC with 5977A MSD, Agilent Technologies, USA. The instrument was set an initial temperature of 70ºc and maintained temperature for 2 min. At the end of this period the

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 4 July-Aug 2021, pp: 1373-1380 www.ijprajournal.com ISSN: 2249-7781

overall temperature raised up to 280°c, at the end of an increase of 5°c/min and maintained for 9 min. Injection port temperature was ensured as 260°c and Helium flow rate was 1mL/min. The ionization voltage was 70ev. The samples were injected in split mode as 10:1. Mass spectral scan range was set at 40-600 (m/z).

IX. IDENTIFICATION OF PHYTOCHEMICAL COMPOUNDS

The mass spectrum of compounds in the sample was obtained by electron ionization. The compounds were identified by comparision of their mass spectral fragmentation patterns with those data provided by National Institute of Standard and Technology (NIST 14) Library. The name, retention time, area%, and structure of bioactive compounds were ascertained.

Results:

In present study, the fungal endophyte isolated from medicinal plant M*orinda citrifolia* was studied to evaluate the production of bioactive compounds, the plant was taxonomically identified and authenticated by Botanical Survey of India. The isolated endophytic fungi was identified morphologically and genotypically as M. *circinelloides.*

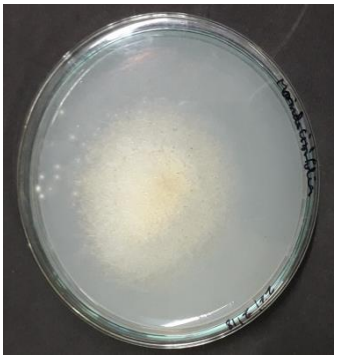

Fig :1 Fungi grown on PDA medium.

***Mucor circinelloides* under magnification.**

The result support the findings that fungal endophytes from medicinal plant M*orinda citrifolia*. The isolate MS1was identified as M. *circinelloides* and the morphological features including characteristic elongate and sympodially branched. The elongate sporangiophores have larger sporangia, floccose with growth pale grey to yellowish, brown at 37°c (Fig.1). The fungus was characterized by PCR amplification of 18 s rRNA gene using both forward and reverse ITS region. The amplification PCR product was found to be ~600bp for OMS and ~700bp for TR2. The blast analysis and multiple sequence alignment showed 96% identify with the sequence of Mucor strains and designated as M. *circinelloides*. The accession number was MZ185357.

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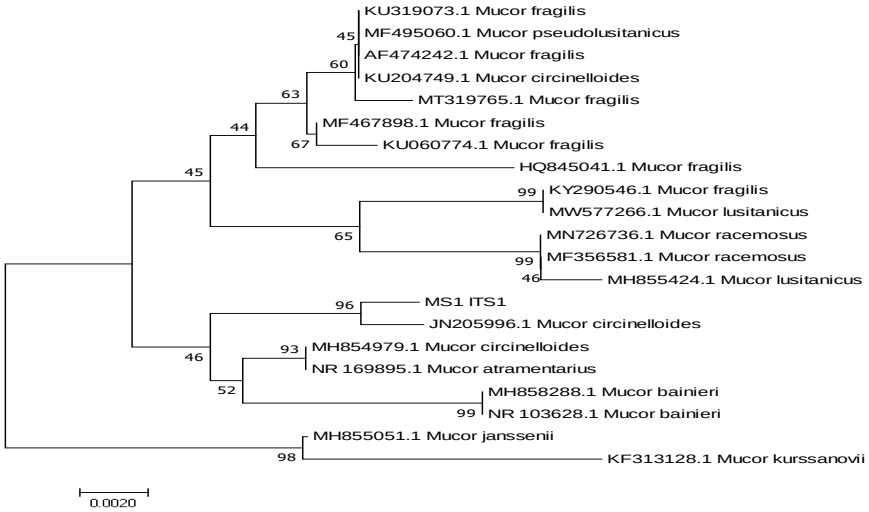

Extraction and Phytochemical analysis of bioactive compounds:

S.No	Phytochemical compounds	Presence or Absence
1	Alkaloids	+
2	Steroids	-
3	Terpenoids	+
4	Coumarins	+
5	Tannins	-
6	Quinones	-
7	Flavonoids	+
8	Phenols	+
9	Saponins	+

Table:1 Phytochemical Compounds

Fig: 2 TLC plate with Spot of endophytic extract

Thin layer chromatography was used for purification of crude extract of fungus by using hexane and ethyl acetate. Total three fractions were purified from TLC for detention of bioactive compounds with different Rf values 0.71, 0.67, 0.62. TLC plate which also absorbs 254 nm UV light to give dark spots against green flouroscence (Fig.2). TLC analysis of active compounds using spray detection reagents suggested that the active compounds may be those the have conjugated with double bond, aromatic rings comparative analysis of TLC profile of MS1 extract [14] with extract from the stem and leaves of host plant were differ, indicating that secondary metabolites produced by endophytic fungi may different forms. It has been revealed that M. *circinelloides* isolated from medicinal plant M*orinda citrifolia* were found to be able to produce different functional metabolites (Table-1). The inability for production of coumarins and quinones, steroids was observed in M. *circinelloides*. Although the isolate showed more or less efficiency (as observed from intensity of colour) for the production of alkaloids, flavonoids and tannins, terpenoids. Similar observations were reported by other researchers [15],[16],[17]. In addition the biology of endophytes may be influenced by many factors such as season, habitat, age and climatic bioactive compound synthesis conditions that could influence the host plant [18]. The current attempt was made to extract bioactive compounds against multi drug resistant bacteria and fungi for existing drugs.

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Table: 3 Antibacterial activity of endophytic fungal extract:

Endophytic fungi	Streptomycin	Solvents	Zone of Inhibition in mm				
			E. coli	S. aureus	S. typhi	P. vulgaris	M. luteus
Mucor circinelloides	50µg/ml	Ethyl acetate	16.6±0.13	10.8±0.00	9.24±0.08	9.60±0.00	10.53±0.01
		Methanol	16.6±0.04	5.57±0.01	5.17±0.00	10.10±0.00	6.10±0.01
		DMSO	-	-	-	-	-

Table: 4 Antifungal activity of endophytic fungal extract:

Endophytic fungal extract	Zone of inhibition with mm	
	C. albicans	C. glabrata
MS1	9.00±0.001	7.00±0.005
Fluconazole	12.00± 0.00	12.00±0.000
DMSO	-	-

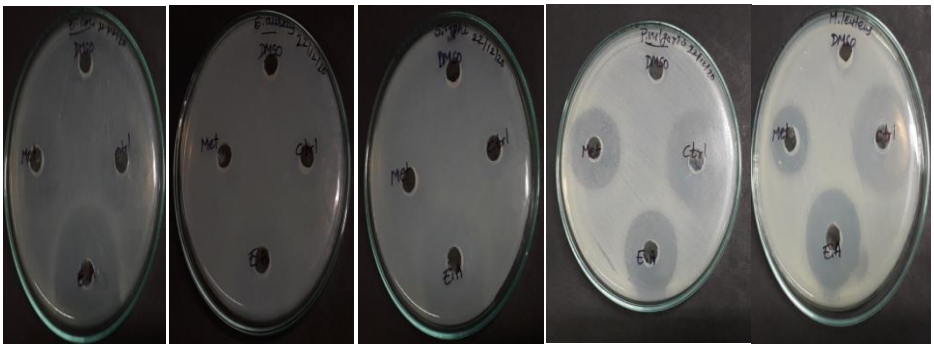

Fig:3Antibacterial activity of endophytic fungal extract.

To screen the antimicrobial activity agar well diffusion method was employed using standard protocol of clinical laboratory prescribed by national committee [19]. Endophytic fungus isolated from fruit extract of M*orinda citrifolia* was cultivated in PDA for 21 days and extraction was done using ethylacetate and methanol with the aim of obtaining more secondary metabolites. The screening of antibacterial activity of this extract against E. coli (MTCC- 723) S. aureus (MTCC-3381), S. typhi (MTCC-733), P. vulgaris (MTCC-3384), M. luteus (MTCC-1541) has been performed. Based on the screening results, it is known that, ethyl acetate and methanol extracts inhibit the growth of E. coli remarkably with a diameter of 16.6 mm whereas ethyl acetate extract has also inhibited the growth of S. aureus 10.8mm, S. typhi 9.24mm, Proteus vulgaris 9.60mm and M. luteus10.53mm with a zone of diameter respectively (Table….3).

Antifungal activity of endophytic fungal extracts

Ethylacetate extract of endophytic fungi was tested for antifungal activity by agar well diffusion method. The extract from M. *circinelloides* was active against candida albicans and candida glabrata with 9.0 mm and 7.0 mm zone of inhibition respectively (Table-4). The antifungal activity exhibited by endophytic fungi indicates presence of secondary metabolites screened by

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Volume 6, Issue 4 July-Aug 2021, pp: 1373-1380 www.ijprajournal.com ISSN: 2249-7781

fungi may contribute to defence mechanism of plant against pathogens as organic compounds from endophytic fungi may be used as biological control agents for preventing plant diseases. In future there is a scope to exploit these secondary metabolites in development of sprays that have antifungal activity against infections [20].

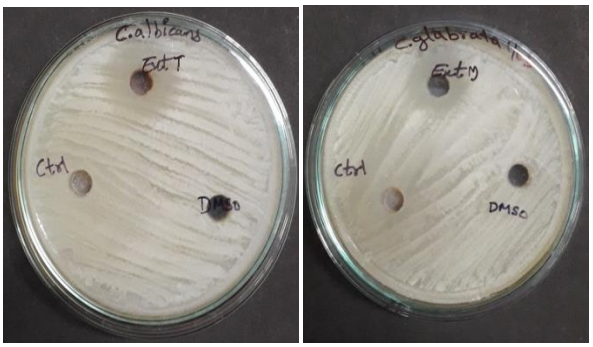

Fig: 4 Antifungal activity of M. *circinelloides* extract.

GC-MS analysis of bioactive compounds:

Peak	R.Time	Area	Area%	Height	Height%	Name
1	2.617	11109282869	53.28	281	56.20%	Hydrazine, 1,1-dimethyl
2	2.806	6936426617	33.27	83	0.37%	Nitrousoxide
3	3.072	954178830	4.58	303	16.07%	Formic acid hydrazide
4	3.412	334773240	1.61	2482	10.43%	Toulene
5	3.640	64955217	0.31	8231	13.93%	Isobutyl acetate
6	3.854	54154116	0.26	3953	39.44%	2-Methyl-tetrahydropyran
7	4.082	61710346	0.30	3289	51.67%	3-penten-2-one,4Methyl
8	4.536	158709916	0.76	4318	12.98%	Pentanoicacid
9	4.700	17149657	0.08	8340	20.96%	2-Pentanone,4hydroxy4methyl
10	4.887	176636094	0.85	1008	16.92%	Dimethyl sulfoxide
11	5.016	32738618	0.16	4361	27.94%	Butanoic acid, 3-methyl
12	5.155	110436471	0.53	13795	31.71%	1Butanol, 2-methyl acetate
13	5.192	65207194	0.31	13795	32.79%	1 Butanol, 2-methyl acetate
14	6.985	12172839	0.06	33688	9.94%	Di isopropanol nitrosamine
15	6.985	9899655	0.05	155951	16.76%	Cyclotetrasiloxane, octamethyl
16	7.793	11335808	0.05	5986	25.52%	Benzene,1-flouro-3methyl
17	8.929	28316742	0.14	18063	39.93%	2-Cyclopenten-1-one 2,3,4,5 tetramethyl
18	10.040	15403617	0.07	61862	79.11%	5-Tetradecane(E)
19	11.075	12528835	0.06	34671	17.58%	Acetic acid,2-phenyl ethylester
20	12.830	22016092	0.11	61855	88.72%	2-Tetradecene (E)
21	13.764	55951824	0.27	30426	5.05%	N-Ethyl-2-cerebethoxyazetidie
22	14.421	49455757	0.24	70634	14.60%	2,4 Di-tert-butylphenol
23	15.040	35316126	0.17	64986	47.38%	Dodecanoic acid
24	15.305	22872794	0.11	87833	81.71%	Cetene
25	17.249	69276635	0.33	91420	57.68%	Tetradecanoic acid
26	17.552	202375599	0.10	117465	27.21%	Tetra decanoic acid, ethyl

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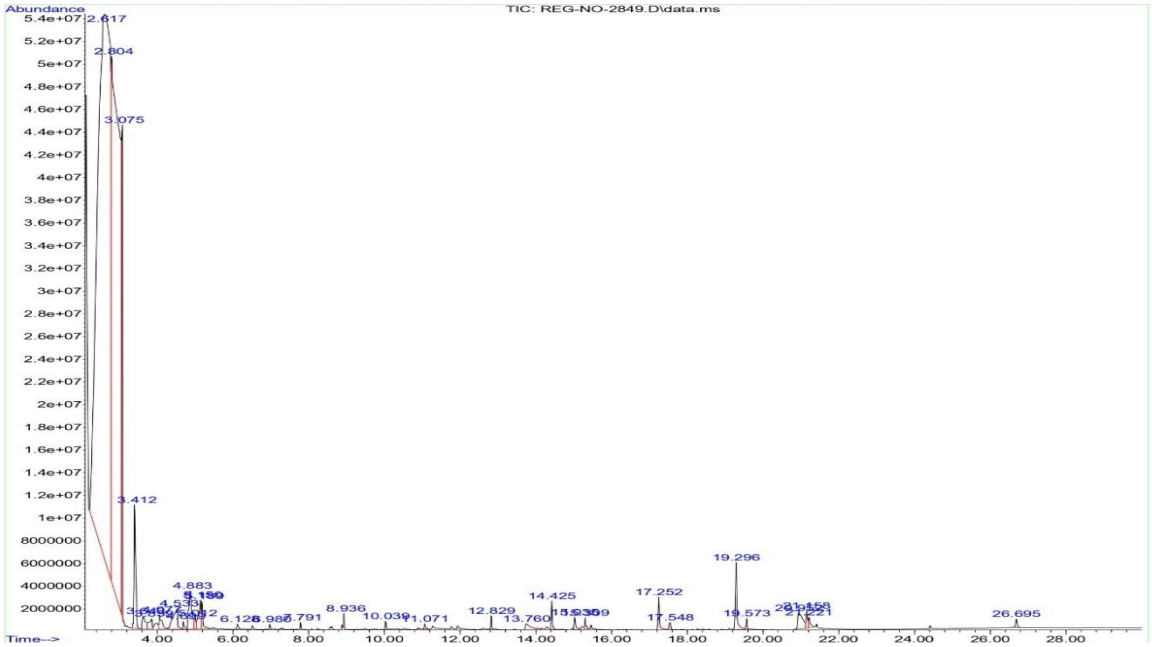
						ester
27	19.294	168959705	0.81	117419	66.50%	n-Hexadecane acid, ethyl ester
28	19.572	19907381	0.10	144309	22.38%	Hexa decanoic acid, ethyl ester
29	20.948	124885298	0.60	140139	62.48%	Octadecanoic acid (2,2)
30	21.162	39126683	0.19	167367	72.83%	Linoleic acid, ethyl ester
31	21.225	34535387	0.17	165642	39.08%	9,12,15, Octadecatrienoic acid, ethyl ester(z,z,z)
32	26.692	21479280	0.10	243225	15.54%	Squalene

The fractions of endophytic fungal extract were analysed using a Gas chromatography-Mass spectrometer (GC-MS) at IICT Hyderabad, which showed retention time, area% and Height% of several compounds were tabulated (Table…3). The GC-MS results of crude extract of endophytic fungi reveals that major bioactive compounds of M. *circinelloides* are Hydrazine 1,1- dimethyl (53.28%), Nitrous oxide (33.27%), Formic acid hydrazine (4.58%), Toulene (1.61%) compounds showed highest area% (Fig. graph) and have antimicrobial activities. GC-MS analysis of metabolites from endophytic fungus Phlogocanflusthysiflorus have shown the presence of Phenol1,2 4 – bis (1,1- dimethyl), 1, hexadecane, 1,hexadecanal, hexadecenoic acid, 1-nonadecane. The compounds produced by endophytic fungi could be the significant source for therapeutic purpose [21]. GC-MS analysis of metabolites from endophytic fungi Polycarpacaecorynbosalarns also showed bioactive compounds such as 5- hydroxy methyl furfural (26.68%) 2-chlorophenyl isothiocyanate (11.10) in root and n- Hexadecanoic acid (14.28) compounds have shown high peak areas which mainly indulge in the anti-inflammatory properties [22]. According to the above research findings, it may be concluded that the experimental strain M. *circinelloides* has potency to produce secondary metabolites, which have significant antimicrobial activity.



Thus, crude extract of experimental strain has the capacity to produce secondary metabolites having antimicrobial activity.

CONCLUSION:

In recent times the endophytic fungi gaining more biotechnological interest, they may be used in pharmaceutical industry. The bioactive

compounds derived from endophytic fungi have immense medicinal value for the betterment of human life. In this research work the crude extract of endophytic fungal extracts showed considerable amount of antibacterial and antifungal activity against human pathogens (E. coli, S. aureus, S. typhi, P. vulgaris, M. luteus and candida albicans and candida glabrata) and fungal extract has different types of phytochemicals. The presence of bioactive compounds were further identified by using GC-MS and shown presence of different antimicrobial compounds. The current research concluded that M. *circinelloides* has high efficiency to produce bioactive compounds which may be used in pharmacology and medicine.

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