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A COMPARATIVE STUDY OF GCMS ANALYSIS OF BIOACTIVE COMPOUND ISOLATED FROM MARINE ALGAE-DERIVED ENDOPHYTIC FUNGI

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Abstract
The present investigation was focused to screen the phytochemical constituents of ethyl acetate extract of endophytic fungi isolated from seven different types of green algae and brown algae. These isolated endophytic fungi were purified and identified by 18 S RNA sequencing and were deposited in NCBI. The identified organism was subjected for bioactive extraction using ethyl acetate. The bioactive compounds obtained from five different fungi were analyzed using GCMS and the mass spectrum of each compound was matched with NIST and Wiley 8 Library. Totally 66 compounds were identified during the period of study out of which 26 phytocompounds were from *Curvilariaplatzii*, 14 from *Chaetomium perlucidum*, 15 from *Corynascussepedonium*, 11 from *Talaromycesaurantiacu* and 4 from *Scopulariopsisgracilis*, respectively. The major compounds registered during the period of study were Pyrrolo[1,2-a]pyrazine-1,4-dione, Hexahydro-3-(2-methylprop; Cyclo(L-prolyl-L-valine); (2S,6R)-2,6-Dibutyl-4-Methylpiperidine, Phthalic acid, 5- methylhex-2-yl butyl ester; Phthalic acid, Hex-3-yl isobutyl ester,Bis (2- ethyl methyl phthalate) , Octadecane,3 ethyl 5 (2- ethyl butyl) and Pyrole,17- pentatriocontene. Out of five fungal organisms, C*urvilariaplatzii* showed a large number of bioactive compounds in ethyl acetate extraction and they are of high therapeutic value in the medical field and has various industrial applications.

Keywords: Endophytic fungi, phytoconstituents, GCMS analysis, chromatogram, therapeutic value, 18 S RNA sequences.

Introduction

All living organisms in the world are abundant with natural chemical compounds which are potent source for the discovery of new therapeutic agents[1]. Many dangerous health problems arises worldwide due to various factors like Multi Drug Resistance (MDR), cancers, viruses, and fungi which are cause of unease conditions[2]. As a result, there is a critical need to search for novel therapeutic drug compounds to treat various diseases. The World Health Organization (WHO) recommended traditional medicine as a safe remedy for all microbial and nonmicrobial diseases[3].Many chemically synthesized drugs used to treat many diseases has serious and severe side effects (different from person to prson) when compared to disease[4]. In developing countries, nearly 70 – 80% of the world's population uses herbal based medicines for many primary infections[5,6]. Marine algae are rich in unidentified secondary metabolites and in addition, the endophytic

microorganism which are residing in the algae has the ability to produce similar metabolites of the host or different metabolites[7]. The identification of secondary metabolites from endophytic microorganism is an important role in drug and therapeutic medicineproduction[8]. The microorganism exhibits maximum production of secondary metabolite (Intracellular / Extracellular origin) during the log and stationary phase of its growth cycle[9]. Various organic solvents like ethyl acetate, chloroform, and ethanol were used[10] to extract these intracellular and extracellular secondary metabolites which enhances the activity of secondary metabolites[11]. GCMS is the combination of separation Gas Chromatography(GC) and identification of compounds Mass Spectroscopy(MS) from the complex crude extract which is an ideal method for both quantitative and qualitative analysis of both volatile and non-volatile compounds. The present study is aimed to identify the secondary metabolites present in the ethyl acetate extract of endophytic fungi isolated frommarine algae.[12]

MATERIALS AND METHOD

Collection of algae

Fresh algal samples were collected from Thoonithurai, Mandapam, Rameswaram, India on 4thDecember 2021. Brown and green algae samples were observed and seggregatedby external appearance and color. Then the algal samples were collected and transferred into sterile plastic containers with sea water and kept in ice box during transportation uptolaboratry. The algal samples were surface washed to remove all the debris and dirt externally using sea water. The collected algae were identified and processed immediately for endophytic isolation as per standard protocol. The identification of macro algae was carried out in Prof. P. Jayaraman, Plant Anatomy Research Centre, Chennai and placed in the Herbarium and the voucher No PARC/2022/4822.

Isolation of endophytic micro-organism associated with marine algae

The identified brown and green algae were washed with sterile water as per standard sterilization procedure. After completion of sterile water wash the samples were kept in filter paper to remove excess water on the surface of algae.

SURFACE STERLIZATION

The dried algal samples are rinsed with 70 % ethanol for 60 sec, followed by 0.4 % sodium hypochlorite for 30 sec to remove the epiphytic micro-organism from outer surface of algae. Final wash was collected in beaker after two washes with sterile distilled water to isolate and identify the endophytic microorganism from the respective algae. The water washed algae were placed in filter paper to remove excess water for 10 to 20 minutes using sterile blade and the surface samples were cut into fine pieces and they were placed in the petriplate containing Potato Dextrose Agar (PDA), Nutrient Agar, Actinomyces Agar,respectively. The mediawere prepared using sea water and antibodies such as streptomyces in case of PDA media (to reduce the bacterial growth) and nystatin in starch casein (agar to suppress fungi isolates) were added, respectively. Then the algal pressed plates are incubated for 7 days in dark condition at 28 $\pm 2^{0}$ C and AA media plates are incubated for 10 to 15 days in 37^{0} C. The colonies grown around the segments were isolated and sub cultured in slants for further studies. The pure endophyte culture were preserved in glycerol and photographed for colony morphological studies.

MOLECULAR IDENTIFICATION OF ENDOPHYTES

The fungal cultures were grown in PDA slant for 7 days at 28 $\pm 2^{0}$ C and after incubation, the fungal mat were removed and suspended in lysis buffer. The DNA isolation was done using Expure Microbial DNA isolation kit. After DNA isolation ITS 1 (5^{1} TCC GTA GGT GAA CCT GCG G 3^{1}) and ITS 4 (5^{1} TCC TCC GCT TAT TGATAT GC 3^{1}) primers were used for DNA amplification of the fungal genome .The pure PCR product were used for Sanger Sequencing in Reginal Facility for DNA fingerprinting, Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, India. From the raw

data (ATB file), the FASTA sequence collected and the Basic local alignment search tool (BLAST Analysis) were carried out for identification of fungal species.

GCMS

The ethyl acetate extract of endophytic fungi was identified into HP -5 column(30 m x 0.25 mm with 0.25 µm film thickness) Agilent technologies 6890 N JEOL GC Mate II GCMS Model. Following Chromatographic conditions were used, Helium as carrier gas, flow rate of 10^0 C / min; and column oven temperature was programmed as 50 ^{0}-250^0 C at a rate of 10^0 C / min injection mode. Following MS conditions were used to screen all the fungal end ophytecrude using computer searches on a National Institute Standard and Technology (NIST Ver 2.1) MS Data library.

RESULT AND DISCUSSION

The brown algae collected from Thoonithurai, Rameswaram were identified and authenticated by Prof. P Jayaraman, Plant Anatomy Research Centre, Tambaram, Chennai and preserved in the Herbarium for record. Then the identified algae were used to isolate the endophytic fungi using Potato Dextrose Agar and the grown endophytes were subculture in PDA slant for further analysis. Colony morphological studies were carried out using Lactophenol cotton blue staining (primary method of screening of fungal isolates). The purified culture was further screened for secondary metabolite production. The isolated endophytes were inoculated in Potato Dextrose broth and incubated at room temperature for 15 to 20 days. After incubation, the fungal mass separated from the PD broth and double volume of ethyl acetate were added in both fungal mat and the PD broth. The ethyl acetate was filtered from the broth and distillation was carried out to obtain crude. The fungal crude after distillation was analyzed to determine the bioactive compounds using TLC and GCMS from the respective crude. From the Fig. 1, the ethyl acetate crude of fungi showed more bands in the TLC using Hexane: Ethyl Acetate (2:3); (1:1) Dichloromethane: Methanol(2:3) and Ethyl acetate : chloroform(4.5:0.5)solvent system. The fungal crude extracts were chromatographed and Rf Value are calculated and tabulated in Table 1.

Thin Layer Chromatography analysis revealed the qualitative and quantitative identification of bioactive compound from the ethyl acetate extract. In *Corynascus sepedonium* crude extract Hexane: Ethyl acetate (2:3 ratio) solvent system gave lot of tailing bands in the TLC plate. In Hexane: Ethyl Acetate(1:1 ratio)and Ethyl acetate : chloroform (4.5:0.5 ratio)solvent system, the more number of tailing bandsfrom all the endophytic fungi.

Table 1 Five endophytic fungal strains identified by 18 s r DNA Sequencing

Closest Relative[a]	**Accession No[b]**	**% Identity[c]**
Corynascus sepedonium	ONO59588.1	99.78
Talaromyces aurantiacus	ONO59708.1	98.84
Amesia atrobrunnea	ONO63018.1	100
Curvularia platzii	ONO63065.1	99.77
Chaetomium perlucidum	ON350775.1	93.55

[a]Closest species which high % identity in BLAST Analysis,
[b]NCBI Gene bank accession number in website (http://www.ncbi.nlm.nih.gov/pubmed),
[c]Gen Bank accession no. of our strains deposited on NCBI website (http://www.ncbi.nlm.nih.gov/pubmed),[d] % identiy of strain based on BLAST Analysis

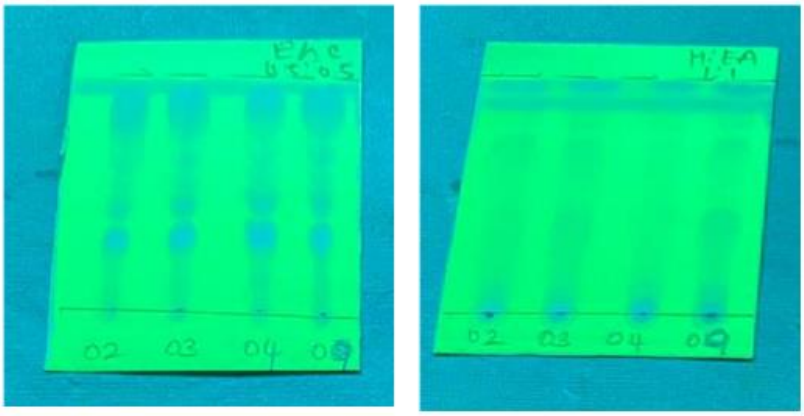

Fig. 1: TLC Chromatogram of endophytic Fungi isolated from marine algae

Table 2: Rf values of bioactive compound eluted from different mobile phases from different endophytic fungal ethyl acetate extracts

Name	Solvent Extraction	Mobile Phase	Ratio	Rf Value
Corynascus sepedonium	Ethyl Acetate	Dichlorom ethane : Methanol	9:1	0.2
Talaromyces aurantiacus				No Tailing Band
Amesia atrobrunnea				0.2
Curvularia platzii				No Tailing Band
Chaetomium perlucidum				0.2
Corynascus sepedonium	Ethyl Acetate	Hexane: Ethyl Acetate	1:1	0.425,0.475,0.6,0.675,0.7 5,0.8,0.85,0.925,0.925,0. 1,0.15
Talaromyces aurantiacus				0.2,0.375,0.525,0.825,0.2 ,0.12,0.25,0.45,0.87
Amesia atrobrunnea				0.075,0.125,0.225,0.375, 0.02,0.2,0.5,0.62,0.87
Curvularia platzii				0.02,0.12,0.25,0.75,0.87
Chaetomium perlucidum				0.25,0.75,0.87
Corynascus sepedonium	Ethyl Acetate	Ethyl Acetate: Chloroform	4.5:0.5	0.825,0.925
Talaromyces aurantiacus				0.002
Amesia atrobrunnea				0.85,0.925
Curvularia Platzii				0.02,0.375,0.45,0.5
Chaetomium Perlucidum				0.85

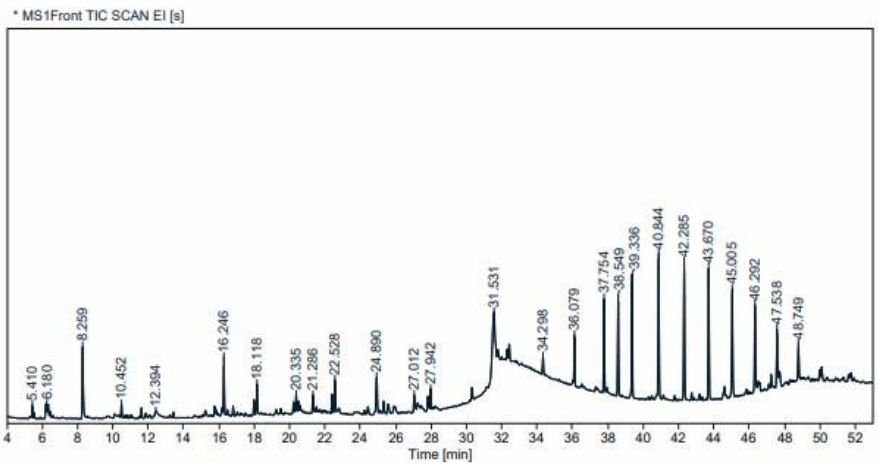

Fig.2: Chromatogram of endophytic Fungi *Corynascus sepedonium* of *Ceratophyllum submersum* L.

Table3: List of identified eluted compounds from GCMS Chromatogram using NIST Library of *Corynascus sepedonium*

Sl No	Peak	Name of the Compound	Peak Area %
1	5.410	Undecane	1.10
2.	6.180	1,2,3-Propanetriol, 1-acetate	2.66
3.	8.259	Naphthalene	4.20
4.	10.452	Dodecane, 2,6,11-trimethyl-	0.44
5.	12.394	Sorbitol	1.43
6.	16.246	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7methyl-4-methylene-1-(1-methylethyl	2.83
7.	18.118	Hexadecane	2.02
8.	20.335	Tetradecane, 2,6,10-trimethyl-	3.00
9.	21.286	Benzo[c]azepin, 7,8,9-trimethoxy-	1.74
10.	22.528	Tetradecane, 2,6,10-trimethyl-	1.85
11.	24.890	N-(2-Methylbutyl) undeca-(2E,4E)-diene-8,10diynamide	3.06
12.	27.012	1,2-Benzenedicarboxylic acid, butyl 8methylnonyl ester	1.38
13.	27.942	Eicosane	2.50
14.	31.531	9,12-Octadecadienoic acid (Z,Z)-s	11.22
15.	34.298	7-Methyl-Z-tetradecen-1-ol acetate	1.19
16.	36.079	Heptacosane	2.61
17.	38.549	Bis(2-ethylhexyl) phthalate	5.15
18.	39.336	Octacosane	6.82
19.	40.844	Heptacosane	7.77
20.	45.005	Hexatriacontane	6.29
21.	48.749	17-Pentatriacontene	2.59

In this present study ,some compounds like octadecane,3 ethyl 5 (2- ethyl butyl) and pyrole were reported in *Corynascussepedonium*, Table 2 and Fungi 3 have effective anti-microbial and anti-fungal agent. Anti inflammatory agent like 17- pentatriocontene are also present.

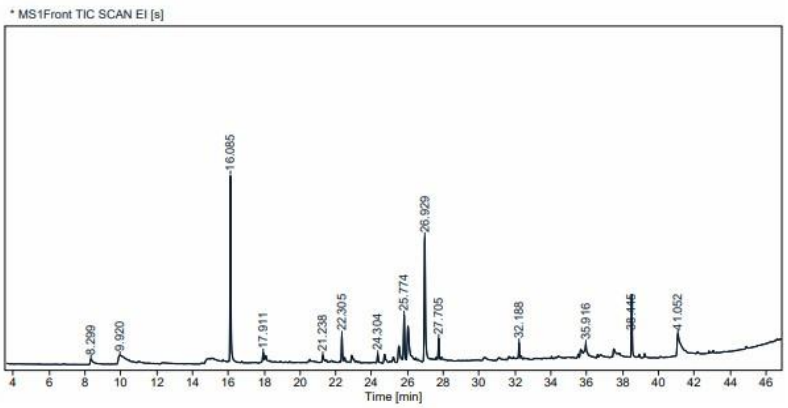

Fig.3: Chromatogram of endophytic Fungi *Talaromyces aurantiacus* of *Ceratophyllum submersum* L.

Table4: List of identified eluted compounds from GCMS Chromatogram using NIST Library of *Talaromyces aurantiacus*

Sl No	Peak	Name of the Compound	Peak Area %
1	8.299	Halfenprox	1.41
2.	9.920	Benzeneacetic acid	9.33
3.	16.085	2,4-Di-tert-butylphenol	19.78
4.	17.911	1-Eicosene	3.41
5.	21.238	Dodecanedioic acid	1.49
6.	22.305	1-Octadecanol	3.87
7.	24.304	1,2-Benzenedicarboxylic acid, butyl octyl ester	0.50
8.	25.774	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	11.08
9.	26.929	Dibutyl phthalate	23.28

10.	27.705	1-Eicosene	5.02
11.	32.188	Behenic alcohol	1.80
12.	35.916	1-Hexacosene	6.46
13.	38.445	Bis(2-ethylhexyl) phthalate	3.81
14.	41.052	Chloramphenicol	8.75

Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylprop; Cyclo(L-prolyl-L-valine); (2S,6R)-2,6Dibutyl-4-Methylpiperidine from endophytic Fungi *Curvularia platzii.* Table 4 has been previously reported as a effective multi drug resistant agent and its also specific to vancomycin resistant enterococci[13]. It acts as reducing power agent. They also act as a high potential antipsoriasis. They affect the aflatoxin production.

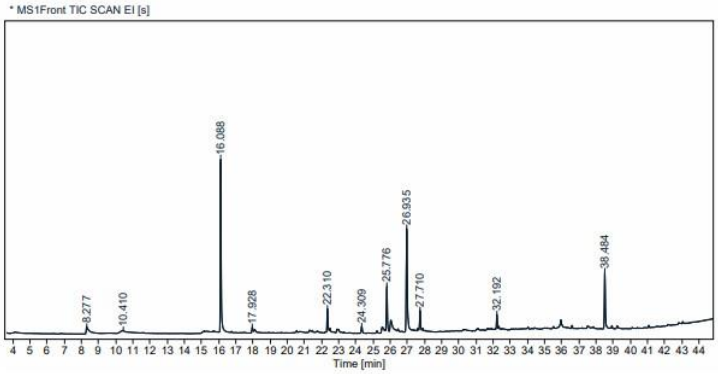

Fig.4: Chromatogram of endophytic Fungi *Amesia atrobrunnea* of *Ceratophyllum submersum* L.

Table5: List of identified eluted compounds from GCMS Chromatogram using NIST Library of *Amesia atrobrunnea*

Sl No	Peak	Name of the Compound	Peak Area %
1	8.455	Halfenprox	2.55
2.	16.090	2,4-Di-tert-butylphenol	14.03
3.	21.237	3-Buten-2-ol,3-methyl-4-(2,6,6-trimethyl-2-cyclohexen-1yl)-; (4aR,5S)-1-Hydroxy-4a,5-dimethyl-3-(propan-2ylidene)-4,4a;Buflomedil	2.23
4.	22.320	1-Eicosene; 1-Nonadecene; 1-Tetracosene	2.75
5.	24.315	1,2- Benzenedicarboxylicacid,Butyl octyl ester; Pthalicacid,Hex- 3 -yl isobutylter,Phthalicacid,isobutyl octadecyl ester	0.52
6.	25.488	Pyrrolo [1,2 – a] pyrazine-1,4-dione, hexahydro- 3-(2methylprop cyclo(L-Prolyl –L - Valine) ; 5-Azacytosine ,N,N,N – trimethyl -	4.26
7.	25.77	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	7.65
8.	25.993	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3- (2-methylprop; Cyclo(L-prolyl-L-valine); (2S,6R)-2,6-Dibutyl-4-Methylpiperidine	9.58
9.	26.936	Dibutyl phthalate; Phthalic acid, butyl hept-4-yl ester; Phthalic acid, butyl hex-3-yl ester	18.32
10.	27.71	1-Eicosene; 1-Tetracosene; 1-Hexacosene	2.64
11.	32.19	Behenic alcohol; 1-Tricosanol; 2-Hexadecanol	2.87
12.	35.922	2-Hexadecanol; 17-Pentatriacontene; 1-Hexacosene	6.27
13.	37.468	β-N-Acetylneuraminic acid, methyl ester-2-methyl-8,9methyl; β-N-Acetylneuraminic acid, methyl ester-2- methyl-7,9-methyl; 1-(2,4-Dichloro-phenyl)-N'hydroxycyclo propane carboxamidin	3.96
14.	38.476	Bis(2-ethylhexyl)Phthalate; Bis(2-ethylhexyl) Phthalate; Phthalic acid, di(2-propylpentyl) ester	9.16
15.	41.044	Chloramphenicol; Acetamide, 2,2-dichloro- N-[2-hydroxy-1-(hydroxymethyl)-2-(4)]	13.18

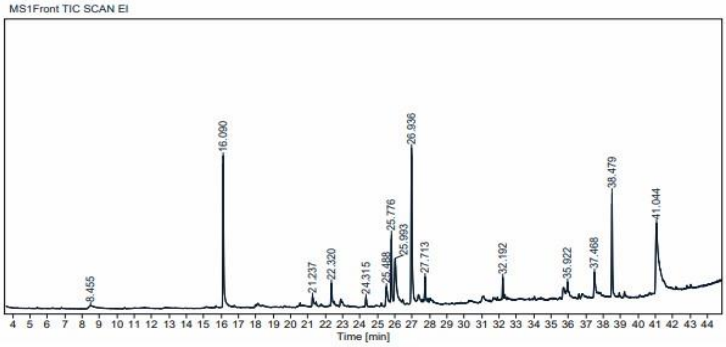

Fig.5: Chromatogram of endophytic Fungi *Curvularia platzii* of *Ceratophyllum submersum* L.

Table 6: List of identified eluted compounds from GCMS Chromatogram using NIST Library of Curvularia platzii

Sl No	Peak	Name of the Compound	Peak Area %
1	8.277	Boldione; 11α Hydroxyprogesterone; Halfenprox	2.84
2.	10.410	Benzeneacetic acid, hexyl ester; Pentyl phenylacetate; Benzeneacetic acid, decyl este	3.69
3.	16.088	2,4-Di-tert-butylphenol	28.91
4.	17.928	1-Hexadecanol; Dichloroacetic acid, 4- hexadecyl ester; 2-Hexadecanol	2.86
5.	22.310	E-15-Heptadecenal; 1-Nonadecene; 1-Octadecene	4.61
6.	24.309	Phthalic acid, 5- methylhex-2-yl butyl ester; Phthalic acid, hex-3-yl isobutyl ester	1.60
7.	25.776	7,9-Di-tert-butyl-1- oxaspiro(4,5)deca-6,9- diene-2,8-dione	9.65
8.	26.935	Dibutyl phthalate	24.08
9.	27.710	1-Eicosene; 1-Tetracosene; 1-Nonadecene	6.25
10.	32.192	1-Eicosene; 1-Tricosanol; 1-Dodecanol, 2-octyl-	4.18
11.	38.484	Phthalic acid, di(2- propylpentyl) ester; Bis(2-ethylhexyl) phthalate	11.33

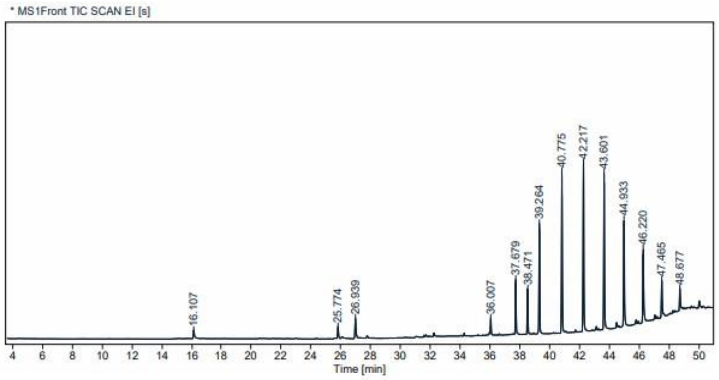

Fig.6: Chromatogram of endophytic Fungi *Chaetomium perlucidum* of *Ceratophyllum submersum* L.

Table7: List of identified eluted compounds from GCMS Chromatogram using NIST Library of Chaetomium perlucidum

Sl No	Peak	Name of the Compound	Peak Area %
1	16.107	2,4-Di-tert-butylphenol	1.40
2.	25.774	7,9-Di-tert-butyl-1- oxaspiro(4,5)deca-6,9- diene-2,8-dione;	1.74
3.	26.939	Dibutyl phthalate; Phthalic acid, butyl hept4-yl ester; Dibutyl phthalate	2.58
4.	36.007	Heptacosane; Octacosane; Tetracosane	1.60
5.	37.679	Pentacosane; Octacosane; Heptacosane	5.29
6.	38.471	Bis(2-ethylhexyl) phthalate; Phthalic acid, di(2- propylpentyl) ester	4.22
7.	39.264	Hexacosane; Octacosane	10.31
8.	40.775	Octacosane; Heptacosane; Triacontane	14.93

9.	42.217	Octacosane; Hexatriacontane	16.20
10.	43.601	Hexatriacontane; Octacosane; Nonacosane	15.21
11.	44.933	Hexatriacontane; Heptacosane; Tetratetracontane	11.10
12.	46.220	Hexatriacontane; Tetratetracontane; Heptacosane	8.19
13.	47.465	Tetratetracontane; Hexatriacontane; Heptacosane	4.04
14.	48.677	Octadecane, 1,1'-[1,3- propanediylbis(oxy)]bis; Octadecane, 3-ethyl-5-(2- ethylbutyl)-; Stearic acid, 3- (octadecyloxy)propyl ester	3.17

Phthalic acid, 5- methylhex-2-yl butyl ester; Phthalic acid, hex-3-yl isobutyl ester from endophytic Fungi 4, Table5 also reported as a plasticizer in nitrocellulose lacquers, the role of plasticizer is to soften PVC during bioplastic formation.Bis (2- ethyl methyl phthalate) act as a solvent in ink preparation[14]. It also act as pesticides and plasticizers.

CONCLUSION

The present investigation, marine algae derived endophytic fungi from mandapam, Rameshwaram are rich in potential bioactive compounds.Out of five fungal organisms Table 7, *curvilariaplatzii* showed a large number of bioactive compounds in ethyl acetate extraction and they are of high therapeutic value in the medical field and has various industrial applications.Further investigation and purification of bioactive compound helps to identify their pharmaceutical potential.

ACKNOWLEDGEMENT

We acknowledge Vinayaka Mission Research Foundation, Salem, Tamil Nadu, India for providing facilities and funds to carry out our research.

CONFLICT INTEREST

The authors declare that there is no conflict of interest.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the NCBI Bank, Accession numbers: ONO59588.1; ONO59708.1; ONO63018.1; ONO63045.1; ONO63065.1; ON350775.1

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