International Journal of Pharmacy and Biological Sciences
ISSN: 2321-3272 (Print), ISSN: 2230-7605 (Online)
IJPBS™ | Volume 8 | Issue 4 | OCT-DEC | 2018 | 200-205
Research Article | Biological Sciences | Open Access | MCI Approved| ज्ञान-विज्ञान विमुक्तये
|UGC Approved Journal |

GCMS ANALYSIS OF BIOACTIVE COMPOUNDS IN ETHYL ACETATE EXTRACT OF EARTHWORM GUT *Streptomyces fulvissimus* (Jenson, 1930)

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ABSTRACT

GC-MS chromatogram of the Ethyl acetate extract of earthworm gut actinomycetes (Streptomyces fulvissimus) clearly showed fifteen peaks indicating the presence of fifteen bioactive compounds. The identification of the bioactive compounds was based on the peak area, retention time and molecular formula. The results reveal the presence of Phthalic acid, di-(1-Hexen-5-yl) Ester (12.97%) , 2- Decen-1-ol,(E) (1.04%) , Octadecanoic acid, Methyl Ester (1.16%), 9- Octadecanoic acid (Z) (13.70%) , 2-(Isobutoxycarbonyl) benzoic acid (3.75%), 1- Hexadecanol (0.78%) , 7- Hexadecenoic acid, Methyl Ester (Z)- (0.72%), Pentadecanoic acid, 14-Methyl- M, (0.19%), 9-12 Octadecadienoic acid (Z,Z)- (40.06%), Decanoic acid (18.33%) , Hexadecanoic acid, 1-(Hydroxymethyl)-1,2 – Ethanediyl (1.69%), 8- Hexadecenal, 14-Methyl-, (Z)- (0.79%), (Z)-4-Decen-1-ol, Pentafluoropropionate (1.41%), 0-Cyclohexyl N-Ethyl N-Propyl (0.16%), 1,2-Benzenedicarboxylic acid (3.25%). The spectrum sketch out of GC-MS confirmed the presence of 15 components with the retention time 15.551, 18.711, 19.162, 19.581, 19.724, 20.817, 20.999, 21.225, 21.225, 21.489, 21.725, 23.478, 23.765, 26.809, 26.850, 28.658 min respectively. The bioactive compounds recognized through GC-MS analysis showed many biological activities.

KEY WORDS

Streptomyces fulvissimus, Earthworm gut, Gas Chromatography Mass Spectroscopy, and Bio-active compounds.

INTRODUCTION

History had shown that discovery of novel antimicrobial agents have often times come from natural sources (Chin *et al.,* 2006, Ganesan, 2008). These natural products having novel skeletons have been found to possess important biological activities and producing a significant number of therapeutic agents in clinical all around the world. They even serve as template for the synthesis of synthetic and semi-synthetic drugs. These discoveries involve the screening of microorganisms and plants from nature, using various techniques (Newman and Cragg, 2012).

Actinomycetes perform significant biogeochemical roles in terrestrial soils and are highly valued for their unparalleled ability to produce biologically active secondary metabolites. Totally 22,500 bioactive secondary metabolites have been reported, out of which 16,500 compounds show antibiotic activities. Out of the 22,500 total bioactive secondary metabolites, 10,100 (45%) are reported to be produced by actinomycetes in which 7630 from *Streptomycetes* and 2470 from rare actinomycetes. A search of recent literature revealed that atleast 4607 patents have been issued on actinomycete related products and processes (Berdy, 2005). Members of the genus Actinomycetes

especially *Streptomyces* sp. have been recognized as a prolific producer of useful bioactive metabolite with a broad spectrum of activities. *Streptomyces* have many vital bioactive compounds with high commercial values and are able to produce a wide variety of antibiotics and extra-cellular enzymes (Narendhran *et al.,* 2014).

Most of the microbial bioactive compounds discovered so far originated from actinomycetes accounting for about two-third of antibiotics, including those in clinical uses. Actinomycetes are the most economically and biotechnologically worthwhile microorganisms (Baltz, 2005; Naine *et al.,* 2011; Raja and Prabakarana, 2011). They have produced a wide range of secondary metabolites of various medical importances such as antibiotics, antifungal, antiprotozoal, antiviral, anticholesterol, antihelminth, anticancer, and immunosuppressant. Among the 140 described Actinomycetes genera, only a few are responsible for the over 10,000 bioactive compounds in clinical use.

The technique Gas chromatography- mass spectrometry analysis was carried out to detect the bioactive compounds present in the actinomycetes ethyl acetate extracts. GC-MS is a highly effective and versatile analytical technique that combines the separation process of gas liquid chromatography, with the detection feature of mass spectrometry to identify different compounds within a test sample. Using these modern techniques, we can identify the bioactive compounds of screened actinomycetes easily with less duration.

MATERIALS AND METHOD

Sampling site

The earthworms were collected from two different sites in paddy field Thiruppampuram Village and Municipal Solid Waste dumping site at Kurikulam, Kumbakonam. The samples were collected digging and hand-sorted method as per the techniques of Edwards and Lofty (1977). Collected worms were washed in fresh water. The earthworms were stored in perforated polythene bags and were brought to the laboratory for their identification.

Preparation of bioactive compound extract

The most intense antagonistic activity of the Actinomycetes was selected and its antibacterial spectrum was tested against the pathogenic bacteria. The selected isolates were inoculated separately into 500 ml conical flask casein broth, and shaken at 28 ±2°C and 250rpm for seven days, after incubation the staling substances were filtered through filter paper (Whatman No.1) and then through Seitz filter (G5). The filtrates were transferred aseptically into the conical flasks and stored at 4°C for further assay an equal volume of ethyl acetate was added to the cell free culture filtrate.

Antibacterial assay

The sterilized nutrient agar medium was poured into each sterile petriplate and allowed to solidify. Using a sterile cotton swab, fresh bacterial cultures such as *Escherichia coli*, *Proteus vulgaris*, *Klebsiella pneumonia*, *Vibrio cholera*, *Staphylocoous aereus* and *Shigella bodyii* with known population count was spread over the plates separately. Six mm diameter well was made over the media inoculated with appropriate bacteria, and then supernatant of these actinomycetes culture 10µl was added into the each well. All the plates were incubated at 37º C for 24 – 48 hours. After the incubation period the results were observed and measured the zone of inhibition in diameter.

The extracts of best zone formation of *Streptomyces fulvissimus* (D12) isolates screened having various bioactivities in our previous studies were taken to identify its bioactive compound principles by GC-MS analysis. The *Streptomyces fulvissimus* (D12) which exhibited different biological activity were taken and mass cultivated, the culture filtrate is extracted with Ethyl acetate by solvent extraction method. The extracts were concentrated and subjected to GC-MS analysis.

GC-MS Analysis

Ethyl acetate extracts of *Streptomyces fulvissimus* species were analyzed by GC-MS method. GC-MS technique was performed by using GC Shimadzu QP2010 system and gas chromatograph interfaced to a Mass Spectrometer (GC-MS) equipped with Elite-1 fused silica capillary column. Helium gas (99.99%) was used as the carrier gas at a constant flow rate of 1.51ml/min and an injection volume of 2µl was employed (split ratio: 20). Injector temperature was 200°C; Ion-source temperature 200°C. The oven temperature was programmed from 70°C (Isothermal for 2 min.) with an increase of 300°C for 10 min. Mass spectra were taken at 70eV; a scan interval of 0.5 seconds with scan range of 40 -1000 m/z. Total GC running time was 35min (Rana and Salam 2014).

Identification of Compounds

Identification of components Interpretation of mass spectra of GC-MS was done using the database of

National Institute of Standards and Technology (NIST) having over 62,000 patterns. The mass spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST 08 library 8. The name, mass and structure of the components of the test materials were ascertained (Sudar and Justin Koil Pillai 2014).

RESULT AND DISCUSSION

Actinomycetes are usually of interest to Scientists and Industrialists because of their ability to produce useful products such as antibiotics, enzymes, pigments and vitamins (Goodfellow and Haynes, 1984). Thus it is obvious that Streptomyces is ubiquitous and adapted to diverse habitats, which vary widely in space and time, and also to diverse habitats, and also to diverse environmental condition. Gas chromatography–mass spectrometry (GC-MS) is an analytical method that combines the features of gas-chromatography and mass spectrometry to identify different substances within a test sample (Kell *et al.,* 2005).

The effective antibacterial compounds were obtained from ethyl acetate extraction method. The 10µl of bioactive crude D12 (*Streptomyces fulvissimus*) compounds were showed most active against all testing bacteria. Zone of inhibition of active strain showed (Table 1) and (Figure 1). Ethyl acetate crude extract were checked for their antibacterial activity by agar well diffusion method such as *Escherichia coli*, *Proteus vulgaris*, *Klebsiella pneumonia*, *Vibrio cholera*, *Staphylococcus aereus* and *Shigella bodyi* where two pathogens were found to be highly susceptible *Shigella bodyi* (2.8cm), *Proteus vulgaris* (2.7cm) in the isolates D12. The antibacterial crude compounds were extracted from Ethyl acetate extraction method. The Ethyl acetate extract of *Streptomyces fulvissimus* (D12) was subjected to GC-MS analysis.

Table 1 - Antibacterial Activities of Actinomycetes bioactive compound in Zone Formation (cm)

Sl. No.	Bacterial Pathogens	Zone of inhibition (cm)			
		D7	D12	D14	D15
1.	*Staphylococcus aureus*	-	2.2	0.2	0.3
2.	*Escherichia coli*	-	2.7	0.3	-
3.	*Klepsella pnemoniae*	0.2	2.4	0.1	0.2
4.	*Protease vulgaris*	-	2.8	0.5	0.5
5.	*Vibrio cholarae*	0.3	1.6	0.6	0.4
6.	*Shigella bodyii*	0.1	3.6	0.9	0.3

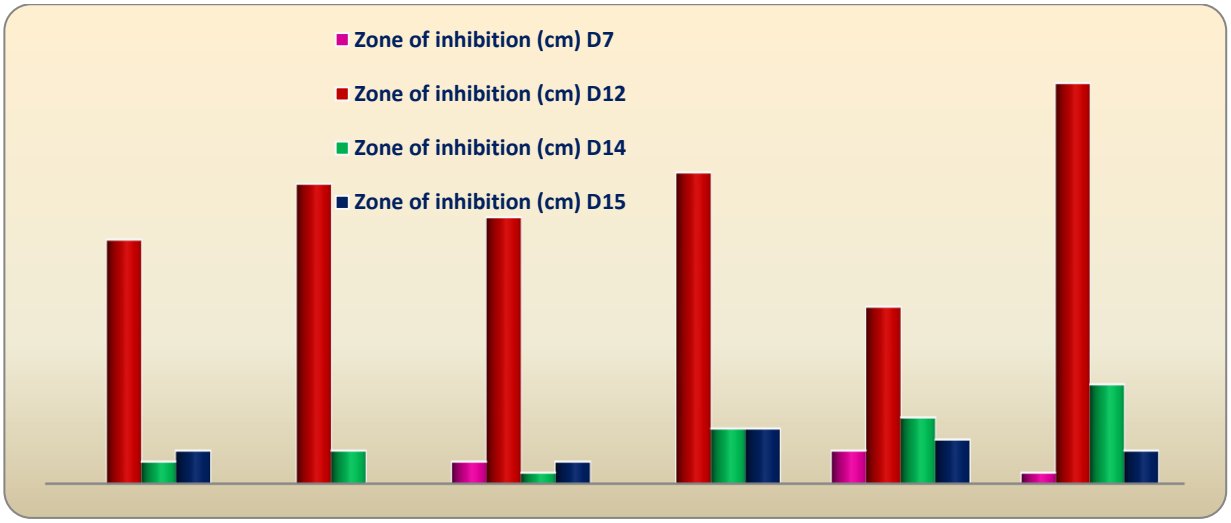

Figure 1. Antibacterial Activities of Actinomycetes bioactive compound in Zone Formation (cm)

GCMS analysis

Applications of GC-MS include drug detection, fire investigation, environmental analysis, explosives investigation, inorganic, biochemistry and identification of unknown samples. Additionally, it can identify trace in materials that were previously thought to have

disintegrated beyond identification. GC-MS has been widely heralded as a "gold standard" for forensic substance identification because it is used to perform a specific test. GC-MS instruments have been used for identification of hundreds of components that are present in natural and biological system (Sharma *et al.,* 2009).

GCMS chromatogram of the ethyl acetate extract of earthworm gut actinomycetes (*Streptomyces fulvissimus*) Figure 2 clearly showed fifteen peaks indicating the presence of fifteen bioactive compounds.

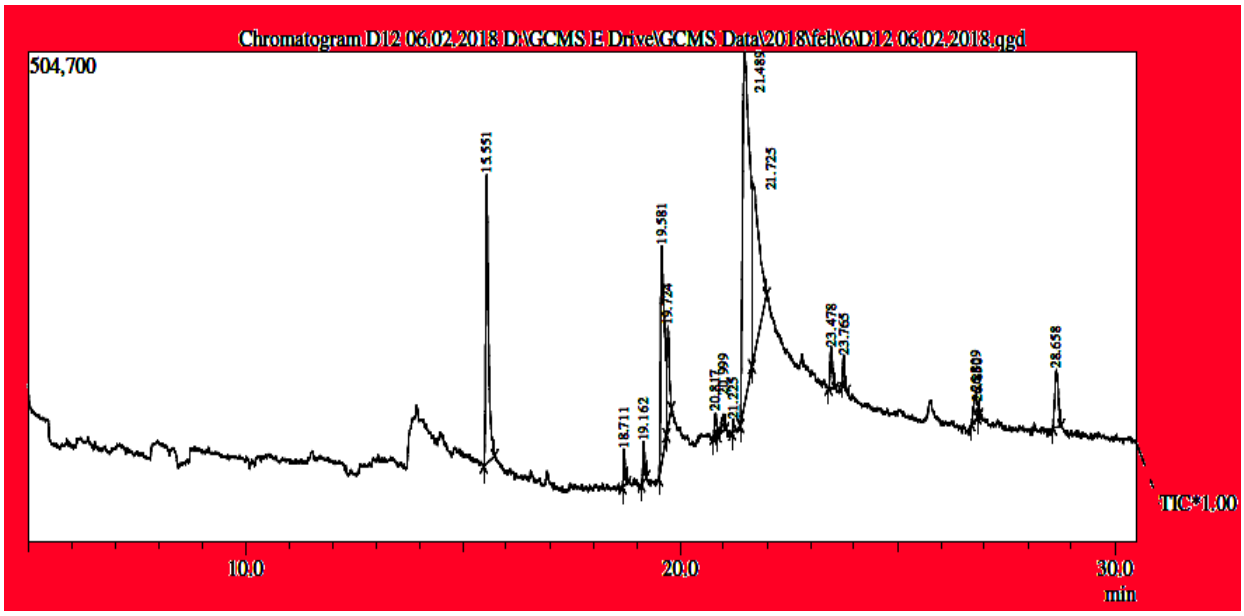

Figure 2. The GCMS Chromatogram of ethyl acetate extract of earthworm gut *Streptomyces fulvissimus*

The identification of the bioactive compounds was based on the peak area, retention time and molecular formula. The compound name with its molecular formula, Retention time, Peak area and % Peak area were represented Table 1. The results reveal the presence of Phthalic acid, di-(1-hexen-5-yl) Ester (12.97%) , 2- Decen-1-ol,(E) (1.04%) , Octadecanoic acid, Methyl Ester (1.16%), 9- Octadecanoic acid (Z) (13.70%) , 2-(Isobutoxycarbonyl) Benzoic acid (3.75%), 1-Hexadecanol (0.78%) , 7- Hexadecenoic acid, Methyl ester (Z)- (0.72%), Pentadecanoic acid, 14-Methyl- M, (0.19%), 9-12 Octadecadienoic acid (z,z)- (40.06%), Decanoic acid (18.33%) , Hexadecanoic acid, 1-(hydroxymethyl)-1,2 – ethanediyl (1.69%), 8-Hexadecenal, 14-methyl-, (Z)- (0.79%), (Z)-4-Decen-1-ol, Pentafluoropropionate (1.41%), 0-Cyclohexyl N-Ethyl N-Propyl (0.16%), 1,2-Benzenedicarboxylic acid (3.25%). The spectrum sketch out of GC-MS confirmed the presence of 41 components with the retention time 15.551, 18.711, 19.162, 19.581, 19.724, 20.817, 20.999, 21.225, 21.225, 21.489, 21.725, 23.478, 23.765, 26.809, 26.850, 28.658 minutes respectively which is shown in Figure 2 and Table 2. The bioactive compounds recognized through GC-MS analysis showed many biological activities are listed in Table 3.

Table 2 – Bioactive compounds identified in ethyl acetate extract of earthworm gut actinomycetes (*Streptomyces fulvissimus*)

Sl. No.	Peak Area (%)	RT (min)	Name of the Compound	Nature of the Compound	Molecular formula	Molecular Weight
1.	12.97	15.551	Phthalic acid, di-(1-hexen-5-yl) ester	Ester	C20H26O4	330
2.	1.04	18.711	2- Decen-1-ol, (E)	Fatty acid	C10H20O	156

3.	1.16	19.162	Octadecanoic acid, Methyl Ester	Stearic acid	C19H38O2	298
4.	13.70	19.581	9- Octadecanoic Acid (Z)	Oleic acid	C18H34O2	282
5.	3.75	19.724	2-(Isobutoxycarbonyl) benzoic Acid	-	C12H14O4	222
6.	0.78	20.817	1- Hexadecanol	Alkenes	C16H34O	242
7.	0.72	20.999	7- Hexadecenoic acid,methyl ester (Z)-	Palmittic acid	C17H32O2	268
8.	0.19	21.225	Pentadecanoic acid, 14-Methyl-, M	Fatty acid methyl ester	C17H34O2	270
9.	40.06	21.489	9-12 Octadecadienoic acid (Z, Z)-	Linoleic acid	C18H32O2	280
10.	18.33	21.725	Decanoic acid	Stearic acid	C10H20O2	172
11.	1.69	23.478	Hexadecanoic acid, 1-(hydroxymethyl)-1,2 - ethanediyl	Fatty acid	C35H68O5	568
12.	0.79	23.765	8- Hexadecenal, 14-methyl-, (Z)-	Alkenes	C17H32O	252
13.	1.41	26.809	(Z)-4-Decen-1-ol, pentafluoropropionate	Fatty acid	C13H19F5O2	302
14.	0.16	26.850	0-Cyclohexyl N-Ethyl N-Propyl	-	C12H23N2O2P	258
15.	3.25	28.658	1,2-Benzenedicarboxylic acid	Aromatic	C24H38O4	390

Table 3 - Biological activities of bioactive compounds identified in Ethyl acetate extract of earthworm gut actinomycetes (*Streptomyces fulvissimus*)

Sl. No.	Compound	Biological Activity
1.	Phthalic acid, di – (hexen-5yl) ester	Anti-hyper cholesterol
2.	2- Decen-1-ol, (E)	Acidifier
3.	Octadecanoic acid, Methyl Ester	Antimicrobial and anti-inflammatory
4.	9- Octadecanoic acid (Z)	Flavor, Cancer preventive, anti-inflammatory
5.	2-(Isobutoxycarbonyl) benzoic Acid	Pigments, aroma compounds
6.	1- Hexadecanol	Antibacterial, antifungal, antioxidant activity
7.	7- Hexadecenoic acid,methyl ester (Z)-	Antioxidant, hypocholesterolemic, antiandrogenic, hemolytic, Alpha reductase inhibitor.
8.	Pentadecanoic acid, 14-Methyl-, M	Antioxidant
9.	9-12 Octadecadienoic acid (Z, Z)-	Anti-inflammatory and anti-antherogenic properties, hepatoprotective, antihistaminic, hypocholesterolemic, anticancer
10.	Decanoic acid	Perfumes, antiseptics, fungicides, flavor compounds and corrosion inhibitors for antifreeze
11.	Hexadecanoic acid, 1-(hydroxymethyl)-1,2 – ethanediyl	Cosmetics, antioxidant
12.	8- Hexadecenal, 14-methyl-, (Z)-	Anti-inflammatory, antioxidant
13.	(Z)-4-Decen-1-ol, pentafluoropropionate	No activity reported
14.	0-Cyclohexyl N-Ethyl N-Propyl	No activity reported

15.	1,2-Benzenedicarboxylic acid	Preventing many diseases such as anti (hypertension, hypercholesterol)

***Source: Dr.Duke's: Phytochemical and Ethnobotanical Database**

CONCLUSION

In the present study, fifteen bioactive compounds have been identified from the earthworm gut actinomycetes (*Streptomyces fulvissimus*) by using Gas Chromatography - Mass Spectrometry (GC - MS) analysis. The presence of these bioactive compounds justifies the use of this earthworm gut actinomycetes for various purposes by traditional practitioners. The investigation concluded that the stronger extraction capacity of Ethyl Acetate extract could have been produced number of active constituents responsible for many biological activities. So that those might be utilized for the development of traditional medicines and further investigation needs to elute novel active compounds from the earthworm gut actinomycetes which may be created a new way to treat many incurable diseases including cancer. The future success of the pharmaceutical industry depends on the identification of new compounds with novel activities or directed to more specific targets. The rapidly growing amount of secondary metabolite gene clusters identified and characterized provides new genetic tools for the generation of novel compounds by combinatorial biosynthesis.

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Received:06.08.18, Accepted: 06.09.18, Published:01.10.2018

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