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A Novel Drug from Endophytic Microbes from Indian Medicinal Plants

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ABSTRACT

Endophytic microbes normally present in the intracellular space of plants especially stems, petioles, roots and leaves of plants causing no external appearance of their presence and have typically unnoticed. In this present study, six endophytic bacterial and three endophytic fungal isolates were isolated from different medicinal plants. From the isolates, active secondary metabolites were screened. The compounds showed broad-spectrum activity against Gram-positive, Gram-negative organisms and showed a minimum zone of inhibition. Based on the antimicrobial and antibiotic resistance pattern the metabolites (ethyl acetate and methanol solvent) from Bacillus sp. Fusarium sp. were analysed by GC-MS. In this major compounds reported in ethyl acetate extract of bacillus sp. include 1, 1-dimethylethyl and methanol extract of fusarium sp. have a large amount of dotriaconate present. Both these compounds have been used as antimicrobial and antioxidant activity against various microbes. The result concluded that the endophytes could be potential for the development as pharmaceutical agents.

Keywords— *Endophytic Microbes, Indian Medicinal Plants, Column chromatography, GC-MS.*

INTRODUCTION

Endophytic microbes commonly live in the intracellular space of stems, petioles, roots and leaves of plants causing no outward manifestation of their presence and have typically gone unnoticed. The association of these microorganisms with higher plant range from mutualistic-symbiotic and commensal to borderline latent pathogenic (Cacabelos and Pathoepigenetics, 2019). Furthermore, the endophytes typically represent only a small percentage of the total plant biomass (Strobel and Daisy 2003). The endophytic microbes present within cell walls and intercellular regions and they may colonize seeds, fruits and flowers, among other tissues (Johnston-Monje and Raizada, 201, De Melo-Pereira *et al.*, 2012, Compant *et al.*, 2011). It is known that endophytes are located in the outside of plasma membrane (Koskimäki et al., 2015).

From the bacillus sp. family, different types of endophytes were screened, these compounds mainly act as various antimicrobial agents (Khianngam *et al.*, 2013). Some of the Endophytic bacteria, along with rhizospheric bacteria contribute to plant growth. Microbial secondary

metabolites are now increasingly being used against disease previously treated only synthetic drugs as anti-inflammatory, hypotensive, anti-tumour, anticholesterolemic etc., Moreover, new microbial metabolites are being used in non-medical fields such as agriculture, with major herbicide, insecticide, plant growth regulator and environmentally friendly herbicides and pesticides as well as antiparasitic agents (Balba, 2007).

The antimicrobial drugs not only used for drugs for mankind but also as food industries (food preservatives) to control food borne diseases eg. endophytic fungus *Xylaria* sp. YX- 28 isolated from *Ginkgo biloba* L. was identified as 7-amino-4-methyl coumarin. The compound presented broad-spectrum inhibitory activity against several foodborne and food spoilage microorganisms and also suggested to be used as a natural preservative in food (Wu *et al.,* 2008).

Some of the endophytes are very importance to improve crop yields, by inducing plant growth and immune response, this could help the agriculture sectors (Pandey *et al.,* 2018). The present study was aimed to screen the endophytes from medicinal plants and produce their novel bioactive compounds, investigate the antimicrobial and chemical compounds using appropriate methods.

MATERIALS AND METHODS

Sample Collection

Three plants *viz., Ocimum tunuiflorum, Calotropis gigantae, Cactus* were collected from SNMV college campus, Coimbatore, Tamilnadu, Plant samples were washed in running tap water to remove soil particles, leaves were then washed in the solution (Sterile distilled water for 1 minute, ethanol (70%) for 30 seconds, sodium hypochloride (3%) for 4 minutes, ethanol 70% for 30 minutes, sterile distilled water for 6 minutes.

Isolation of Endophytes

The plant samples (small fragments) were inoculated in nutrient agar medium and potato dextrose agar medium and incubated at 37^{0}C for 3-4days. After incubation colonies were screened based on the morphology **(**Anjum and Chandra, 2015)

Identification of the isolates

The isolates were identified based on the phenotypic characterization (Gram staining, Wet mound technique) and biochemical methods (IMVIC Test)

Production of secondary metabolites

The bacterial and fungal cultures are inoculated in an appropriate liquid broth (Nutrient broth for bacteria and Potato dextrose broth for fungi). Both media are placed into shaker for fermentation (Bacteria for 2 days and fungi for 7 days). Then the cultures were filtered by using Whatman no 1 filter paper. The whole broth was extracted with 1:1 volume of ethyl acetate. The layers were separated. The organic layer was considered as secondary metabolites it's used for further test.

Purification of secondary metabolites

The secondary metabolites were purified by column chromatography using ethyl acetate as a eluting solvent. Further, different fractions are collected between 5 mins interval period.

Human pathogens

Human pathogens *viz., E.coli, Staphylococcus sp. Streptococcus sp. Proteus sp., Klebsiella sp. Pseudomonas sp.* were collected from PSG Hospital, Coimbatore was used for antimicrobial analysis.

Antimicrobial assay

Test bacterial pathogens such as *Escherichia coli, Pseudomonas sp., Klebsiella sp., Streptococcus aureus., Staphylococcus sp., and Proteus sp.,* were maintained in nutrient agar slants. The antimicrobial assay was carried out on Muller-Hinton agar medium which was sterilized and used for the experiment. The antimicrobial activity of the crude extract and different solvent extract (ethyl acetate and methanol) was performed against test bacteria. 1 ml of inoculum was swapped on Muller-Hinton agar plates then using the sterile cork borer 5 mm wells were made in the plates then, 20µl of crude ethyl acetate fraction was loaded, and ethyl acetate adds as a negative control. After incubation of 24 h at 37^{0}C, the plates were observed for the zone of inhibition and measured.

Antibiotic Resistance Assay

Test antibiotic discs are Azithromycin (50 mcg), Ampicillin (10 mcg), Tetracycline (30 mcg), Streptomycin (10 mcg), Kanamycin (30 mcg), Cefotaxime (30 mcg) were used for antibacterial activity. 1ml of isolated endophytic samples was swapped on Muller-Hinton agar and placed the antibiotic disc on the medium. After incubation of 24 h at 37^{0}C, the plates were observed for the zone of inhibition and measured.

GC-MS analysis

The bioactive extracts obtained from endophytes

were analysed through gas chromatography-mass spectroscopy (GC-MS) method. Injection volume had 1.5µl mixed within DMSO having a Split flow of 1.5 ml/min. The mass spectra had taken at 76 eV with a mass scan range from m/z 41-505 amu. The individual elements had recognized by comparing their mass spectra with those of standard using NIST (National Institute of Standards and Technology, U.S. Department of Commerce) compounds (Garcia *et al.,* 2012)

RESULT AND DISCUSSION

Screening and Isolation of Endophytes

In nearly 300,000 plant species that exist on the earth, each plant is host to one or more endophytes. Only a few of these plants have ever been completely studied relative to their endophytic biology. Consequently, the opportunity to find new and interesting endophytic microorganisms among myriads of plants in different settings and ecosystems is great (Strobel and Daisy, 2003). Endophytic microbes were colonized in different plant species but screening of endophytes is very difficult in laboratory condition (Tadych *et al.*, 2019).

In this present study, six endophytic bacteria were isolated from three medicinal plants (Viz., *Ocimumten uiflorum, Calotropis gigantae, Cactaceae)* based on the Colony morphology and Gram staining (Table 1). Endophytic microbes from various plant exist in different ecosystems. Plants have long provided mankind with a source of medicinal agents, with natural products once serving as the source of all drugs. Microbial extracts have been and continue to be a productive source of new biologically active molecules for drug discovery (Arunachalam and Gayathri, 2010).

Table 1: Phenotypic characterization of screened endophytic isolates (colony morphology and gram staining)

S. no	Name of the plant	Screened isolates	Colony morphology		Gram staining
			Color	Shape	
1	*Calotropis gigantea*	A1	White	Round scattered	(-ve) rod
		A2	Green	Round clustered	(+ve) rod
2	*Ocimum tenuiflorum*	B1	White	Round clustered	(-ve) rod
		B2	Green	Round scattered	(+ve) rod
3	*Cactaceae*	C1	White	Round clustered	(-ve) rod
		C2	Green	Round scattered	(+ve) rod

Phenotypic Characterization and Production of Secondary Metabolites from Endophytic Microbes

The isolates were identifying based on the biochemical characterization such as Indole, Urease, Triple Sugar Ion, Citrate Utilization (Table 2) and morphological characterization the strain name listed in table 3 fungal isolates were identified based on colony morphology and wet mount technique the strain name listed in table 4.

Table 2: Biochemical test and screened isolates

S.no	Test	A1	A2	B1	B2	C1	C2
1	Indole test	-ve	-ve	-ve	-ve	-ve	-ve
2	Urease test	+ve	+ve	+ve	+ve	+ve	+ve
3	Trible sugar-ion test	-ve	+ve	-ve	+ve	-ve	+ve
4	Citrate utilization test	+ve	+ve	+ve	+ve	+ve	+ve

Note: +ve; Positive: -ve ; Negative

Table 3: Showed identified endophytic isolates (Bacteria) based on phenotypic characterization

S.No	Test strain	Name of the Strain
1	A1	*Pseudomonas sp.*
	A2	*Bacillus sp.*
2	B1	*Pseudomonas sp.*
	B2	*Bacillus sp.*
3	C1	*Pseudomonas sp.*
	C2	*Bacillus sp.*

Table 4: Showed identified endophytic isolates (fungi) based on colony morphology and wet mount

S.no	Name of the plant	Sample Name	Morphology color	Starin
1	*Calotropis gigantea*	F1	Pale to pink	*Fusarium sp.*
2	*Ocimum tenuiflorum*	F2	Green with light white	*Penicilliu m sp.*
3	*Cactaceae*	F3	Pale brown	*Aspergillus sp.*

In the present study, the natural bioactive products were extracted from the selected isolates, the yields may vary from one bacterial isolate to another. The bioactive yield difference could be qualified to the chemical composition (metabolites) and the genetic composition of the isolates. Different isolates produce a host of a different number of compounds/metabolites; these metabolites also have different molecular weights causing the difference in yields (Newman *et al.,* 2003).

Antimicrobial activity

Antibacterial assay of endophytic bacterial secondary metabolites was tested against 6 human pathogens the results were given in table 5. The natural bioactive products were subjected to antimicrobial activity, Most of the bacterial extracts exhibited activity against the six test organisms in this maximum zone of inhibition (1.8 mm) found against *Staphylococcus* sp. (Table 4) endophytic fungal metabolites showed maximum zone of inhibition (1.9 mm) against *Klebsiella* sp. (Table 5). The difference in the *in vitro* activity among the various extracts could be due to the production of either a broad-spectrum antimicrobial or several compounds with different activities (Foldes *et al.*, 2000). The study conducted by Omura (1992), he concluded that the differences in levels of antagonism are dependent on the concentration of the active substances. Antibiotic resistance assay showed the bioactive compounds from *Bacillus sp* and *Fusarium sp.* showed more resistance against all the antibiotics (Table 6). The zones of inhibition of the bioactive fractions may be similar to the standard drugs, but if they have different modes of activity, this would still make them promising (Fatope, 1995). However, there are six samples are have the zone of inhibition against these standard organisms as well. It is possible that there were novel compounds from the test isolates and this could be promising agents to replace drugs which resistance has developed. The most of the natural products from endophytes are antibiotics, anticancer agents, biological control agents, antivirals, anti-diabetic agents and other bioactive compounds by their different functional roles (Guo *et al.,* 2008).

Table 5: Antimicrobial assay of screened endophytic bacterial secondary metabolites against human pathogens (well diffusion method)

S.NO	ORGANISM NAME	A1 (cm)	A2 (cm)	B1 (cm)	B2 (cm)	C1 (cm)	C2 (cm)
1	***E.coli***	1.2	0.7	1.0	1.0	1.2	0.8
2	***Staphylococcus sp.***	1.3	1.1	1.2	1.3	1.8	1.4
3	***Streptococcus sp.***	1.6	1.2	1.4	1.2	1.2	1.3
4	***Proteus***	0.9	0.8	0.9	0.6	1.3	0.8
5	***Klebsiella sp.***	0.8	0.6	0.7	0.7	1.0	0.6
6	***Pseudomonas sp.***	0.7	0.8	0.7	0.9	1.2	0.8

Table 6: Antimicrobial Assay of Screened Endophytic Fungal Secondary Metabolites against Human Pathogens (Well Diffusion Method)

S.NO	ORGANISMS NAME	*Aspergillus sp.*, (cm)	*Fusarium sp.*, (cm)	*Penicillium sp.*, (cm)
1	***E.coli***	1.2	2.8	0.9
2	***Staphylococcus sp.***	1.4	1.6	1.2
3	***Streptpcoccus sp.***	1.6	1.5	1.3
4	***Proteus***	1.4	1.4	1.4
5	***Klebsiella sp.***	1.3	1.9	1.2
6	***Pseudomonas sp.***	1.5	2.4	1.2

Tabble 7: Antibiotic Resistance Assay against Pathogen (Disc Diffusion Method)

S.No	Test strain	*Azithromycin (cm)*	*Streptomycin(cm)*	*Tetracycline (cm)*	*Kanamycin (cm)*	*Cefotaxime (cm)*	*Ampicillin (cm)*
1	*Pseudomonas sp.,*	0.1	1.5	1.3	1.5	2.3	1.7
2	*Bacillus sp.,*	0	0	0	0	0.1	0.2
3	*Pseudomonas sp.,*	1.5	1.7	2.3	1.3	2	1.5
4	*Bacillus sp.,*	0	0.4	0.2	0.3	0.5	0.5
5	*Pseudomonas sp.,*	1.4	1	1.3	0.7	2.5	1.6
6	*Bacillus sp.,*	1.2	1.2	1.5	0.5	2.3	2
7	*Fusarium sp.,*	0	0	0	0	0	0
8	*Penicillium sp.,*	0.2	0.1	0.1	0.8	0.8	0.5
9	*Aspergillus sp.,*	0.7	0	0	0.2	0.9	0.7

GC-MS analysis

It would be of interest to find out which useful group is accountable for the bioactivity and also whether any of them is a novel compound with an antimicrobial activity which desired make it a hopeful candidate for the production of new antimicrobials. In the present study ethyl acetate and methanol was used as extraction solvent since it is the most efficient method for the obtainment of fungal and bacterial secondary metabolites. The ethyl acetate and methanol extracts were characterized and identified by GC-MS analysis. The spectrum of the unidentified element was compared with the spectrum of the known components stored in the library. The active principles with their retention time (Rt), molecular formula, molecular weight and concentration percentage (area %) are represented in Fig.2 and 3 and Tables 7 and 8.

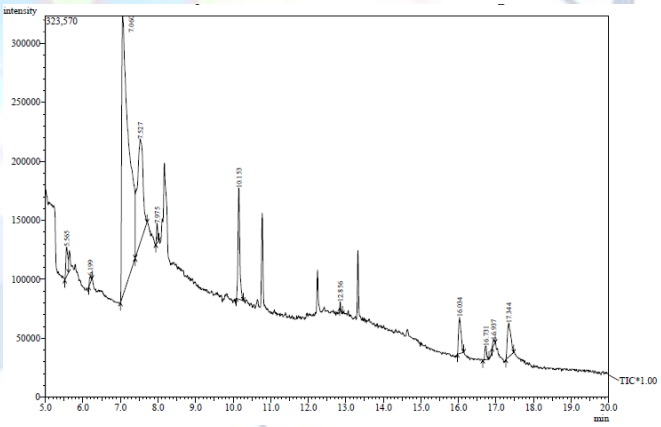

Fig. 1. GC-MS chromatogram of ethyl acetate extract of *Bacillus* sp.

Table. 7. Compounds identified in the ethyl acetate extract of *bacillus* sp. by GC-MS analysis

Peak Report TIC

Peak#	R.Time	I.Time	F.Time	Area	Area%	Height	Height%	A/H	Mark	Name
1	5.565	5.517	5.617	92910	1.95	24890	4.53	3.73		BENZENE, ETHYL-
2	6.199	6.158	6.242	18195	0.38	5510	1.00	2.84	MI	1,3-CYCLOPENTADIENE, 5-ETHEN
3	7.060	7.000	7.392	2960403	62.03	237732	43.22	12.45		Oxime-, methoxy-phenyl-_
4	7.527	7.392	7.708	965898	20.24	88038	16.01	10.97	V	4-NITROPHTHALAMIDE
5	7.975	7.942	8.025	37315	0.78	16658	3.03	2.22	MI	o-Methoxy-.alpha.,.alpha.-dimethylbenz
6	10.153	10.083	10.275	315822	6.62	94401	17.16	3.35		Benzaldehyde, 2,5-bis[(trimethylsilyl)o
7	12.856	12.842	12.917	3281	0.07	6275	1.14	0.53	MI	2-Carbamoylphenoxyacetic acid
8	16.034	15.975	16.142	141321	2.96	30896	5.62	4.57		1(3H)-Isobenzofuranone, 3a,4,5,7a-tetr
9	16.731	16.650	16.817	37713	0.79	11695	2.13	3.22	MI	,3-DIPHENYL-1-((TRIMETHYLSILY
10	16.937	16.900	16.983	16028	0.34	5162	0.94	2.84	MI	1,7,7-TRIMETHYLBICYCLO[2.2.1]H
11	17.344	17.267	17.475	183728	3.85	28785	5.23	6.38		Phenol, 3,5-bis(1,1-dimethylethyl)-
				4772614	100.00	550042	100.00			

The GC-MS analysis of *bacillus* sp. extract revealed that the 1,1 dimethyethyl (17.344 RT), trimethylsily (16.731 RT), Isobenzofuranone (16.034 RT). Most of the identified compounds possessed many biological properties (Table 7)

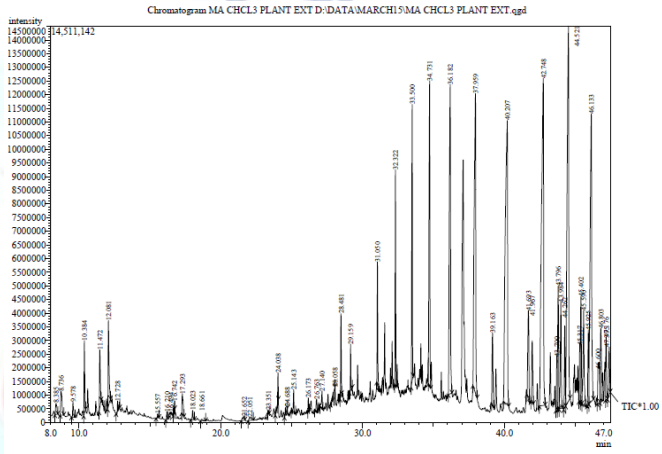

Fig. 2. GC-MS chromatogram of methanol extract of *Fusarium* Sp.

Table. 8. Compounds identified in the methanol extract of *Fusarium* sp. by GC-MS analysis

Peak#	R.Time	I.Time	F.Time	Area	Area%	Height	Height%	A/H	Mark	Name
34	40.207	39.942	40.317	113608823	11.84	10406222	6.31	10.92		TETRACONTANE
35	41.693	41.592	41.792	17348632	1.81	3231296	1.96	5.37		TETRACONTANE
36	41.967	41.875	42.067	11230261	1.17	2261249	1.37	4.97		Hexatriacontane
37	42.748	42.467	42.883	123146629	12.84	11970300	7.26	10.29		HEXACOSANE
38	43.700	43.633	43.742	8262952	0.86	1954490	1.18	4.23		TRICOSANE
39	43.796	43.742	43.867	19222098	2.00	4521281	2.74	4.25	V	TETRACONTANE
40	43.984	43.867	44.083	18285241	1.91	3885358	2.36	4.71	V	Hexatriacontane
41	44.262	44.183	44.325	11083621	1.16	2993311	1.81	3.70		Octacosanol
42	44.521	44.325	44.633	106491764	11.10	13724268	8.32	7.76	V	HEXACOSANE
43	45.317	45.250	45.358	9119383	0.95	2061118	1.25	4.42		TRICOSANE
44	45.402	45.358	45.475	15714672	1.64	3895506	2.36	4.03	V	TETRACONTANE
45	45.590	45.475	45.733	18626707	1.94	3467902	2.10	5.37	V	Hexatriacontane
46	45.925	45.842	45.967	11195176	1.17	2717210	1.65	4.12		1-EICOSANOL
47	46.133	45.967	46.283	80904786	8.43	10522921	6.38	7.69	V	TETRACONTANE
48	46.600	46.542	46.742	12263771	1.28	1256565	0.76	9.76		1-Triacontanol
49	46.803	46.742	46.892	15136178	1.58	2694512	1.63	5.62	V	1,37-Octatriacontadiene
50	47.176	47.133	47.275	9352938	0.98	2295718	1.39	4.07	V	Dotriacontane
51	47.395	47.275	47.475	8296140	0.86	1753667	1.06	4.73		Octadecane, 3-methyl-
				959257773	100.00	164961348	100.00			

The GC-MS analysis of *Fusarium* sp. extract revealed that the more amount of octadecane (47.395 RT), Dotriacotane (47.176 RT), 1,37 octatricontadiene (46.803 RT), triacontanol (46.600 RT), tetracontane (46.133) (Table 8). Besides, a high percentage of these compounds may have more biological activities against broad pathogens. A similar result was also obtained by other workers (Abdel-Hady *et al.,* 2016). Based on the present study we concluded that the endophytic compounds have wide-spectrum antibacterial activity, so this novel compounds may be used as a natural antimicrobial agent for human infectious diseases.

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