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CHARACTERIZATION OF ENDOPHYTIC FUNGI FROM *PONGAMIA PINNATA* AND *GLIRICIDIA SEPIUM*

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ABSTRACT
Medicinal plants have enormous applications and more than 30% of total plant populations have been used for medicinal purposes. The medicinal plants like *Pongamia pinnata* and *Gliricidia sepium* contains a number of pharmaceutical compounds having anti-microbial, anti-tumor, wound healing properties. Combinatorial biosynthesis of these plant metabolites in microbial factories has tremendous commercial value. Endophytes are microbial populations (Bacterial, Fungal and Actinomycetes) inhabiting inside the specific plant parts such as leaves, roots, stem, flowers, cotyledons, etc. The fungal endophytes are being under research for their pigment production potential. Hence the textiles and dyeing industries were more focused on the eco-friendly dyes and dye fixatives. Other than the pigment production the fungal communities of the medicinal plants were synthesizing numerous compounds of therapeutic value. The current study focused on the isolation, identification of genera, production and identification of metabolites of endophytic fungi from *Pongamia pinnata* and *Gliricidia sepium*. This will open up a new trend in combinatorial biosynthesis as the isolation and purification of fungal metabolites is more advantageous than that of bacteria due to the post-translational modification of the metabolites.

KEYWORDS: Medicinal plants, *Pongamia pinnata, Gliricidia sepium,* Endophytic fungi, Pigments, Secondary metabolites, Combinatorial biosynthesis.

INTRODUCTION
Medicinal plants of more than 30% of total plant population have been used for medicinal purposes. Pongamia *pinnata* is an Indian Beech tree belong to the family Leguminoseae, is commonly known as Pongam tree. The incorporation of *Pongamia* plant parts is very common in Indian medicine practices. It has been used for several ailments, because all the plant parts of Pongamia contains several secondary metabolites of medicinal activity. *Gliricidia sepium* is a medium-sized leguminous tree, occurs in abundance in its native Mesoamerica belongs to family Fabaceae. Endophytes are broad genera of microorganisms reside in the different plant parts, which might be bacteria, fungi or actinomycetes. These inhabitants influence the quality and quantity of plant metabolites that may be responsible for certain characterizations of the host such as disease resistance, therapeutical uses, etc.

The plant secondary metabolites are of immense applications throughout different fields. The need of huge quantities of metabolites and difficulties in extraction of metabolites from plants opens up a new field called combinatorial biosynthesis. Combinatorial biosynthesis is a new field for the synthesis if plant derived compounds in microbial cells.

MATERIALS AND METHODS
Sample Collection: The plant samples, *Gliricidia sepium* and *Pongamia pinnata* for endophytes isolation were collected from Peringottukurissi, Palakkad, Kerala and Hindusthan College of Arts and Science, Coimbatore, Tamil Nadu respectively.

Surface Sterilization: The healthy explants (both roots and leaves) were chosen and surface sterilization was done as per Mani *et al*., 2015 with modifications.The selected roots were washed thoroughly under running tap water to remove soil followed by distilled water (3 minutes) and were kept in 70% Ethanol (2 minutes). Then treated with 5% Sodium hypochlorite (1 minute) followed by distilled water (1, 2, 5 and 5 minutes). The washed roots were again treated with 70% Ethanol (2 minutes), trimmed and washed with 0.1% Mercuric

chloride (1 minute) followed by serial wash in distilled water(1min, 2min, 5min and 5 mins) to remove Mercuric chloride. The leaves surface sterilized with tap water, 5% Sodium hypochlorite (2 minutes) and distilled water (1min, 2min, 5min and 5 mins).

Isolation of Fungal Endophytes: The surface sterilized explants were cut into 1cm length and inoculated into solidified Potato dextrose agar (PDA) with 100mg streptomycin. The endophytic fungal colonies were produced after 5 to 7 days of incubation at Room temperature.

Morphological Characterization of Endophytic Fungal Isolates: The different fungal colonies were chosen according to its morphology, colour and pattern of colony formation. Nine colonies were selected and named according to the type of organism, source, explant and isolate number. The fungal isolates from leaf of *P. pinnata* were named as FPPL1, FPPL2 and FPPL3, and those from roots were named as FPPR1, FPPR2 and FPPR3 and the isolates from leaf of *G. sepium* were named as FGSL1 and FGSL2, from roots were named as FGSR1. The selected colonies were sub-cultured in Potato Dextrose Broth several times for purification and coverslip culturing of each isolates were done for staining by using Lactophenol Cotton Blue (LCB) Staining.

Synthesis of Pigments from Endophytic Fungal isolates: The pigment synthesis was done using 2% Malt Extract Broth according to Eskandrighadikolaii *et al.,* 2015 and incubated for 28^{o}C for 20 days with adequate shaking at orbital shaker.

Extraction of Fungal Pigments: The fungal metabolites were extracted according to the protocol by Velmurugan *et.al.,* 2010. The fungal mycelium is filtered using Whatmann No.1 filter paper and diluted the media with 60% of solvent (95% Ethanol). The mixture was kept in Rotary shaker at 180 rpm at 30^{o}C for 30 minutes followed by centrifugation at 3780 rpm for 15 minutes. The supernatant was recovered and the residue was resuspended and repeated the same procedure for extraction.

Confirmation of Fungal Pigments: The UV- Visible Spectrometry and Thin Layer Chromatography were used for the confirmation of fungal pigments.

UV- Visible Spectrometry: The optical Density of the fungal extract was measured at 300 to 600 nm wavelength.

Thin Layer Chromatography (TLC): The components of fungal extracts were separated by Thin Layer Chromatography using Ethyl acetate as solvent system, according to Eskandringhadikolaii *et.al.,* 2015. The crude fungal extracts were dissolved in Methanol: Acetone (1:1) and spotted on the TLC plate 2cm above the bottom and made into a concentration of 1-2µg. Solvent (Ethyl acetate) was poured in the chromatographic chamber and kept closed for some time to saturate chamber with solvent vapours. After placing the spotted TLC plate, the chamber was kept undisturbed for 15-20 minutes (till the solvent front reaches ¾ thof the plate). After the solvent reaches appropriate length the TLC plate was taken, air dried and sprayed with 10% Ethanolic sulphuric acid (10 ml of concentrated sulphuric acid is added side by side to 90 ml of ice cold ethanol) followed by heating at 105^{o}C until separated spots were visible. The Retention Factor (R_f) values determined by the following formula;

$$R_f \text{ Value} = \frac{\text{Distance travelled by the spot}}{\text{Distance travelled by the solvent front}}$$

DPPH Assay on Thin Layer Chromatography: For checking the presence of Anti-oxidants DPPH assay on TLC plate was done according to Eskandringhadikolaii *et.al.,* 2015. The spotted TLC plate was kept in the chromatographic chamber saturated with solvent Methanol: Formaldehyde (2:8) and the TLC plate with separated metabolites was dried and sprayed with 1, 1-diphenyl-2-picrylhydroxyl (DPPH) reagent and waited till spots with clear zone against a violet background is visible.

Fourier Transform Infrared Spectroscopy: The FTIR analysis of the extracts of selected fungal isolates such as, FPPL1, FPPR2 and FPPR3 was carried out to analyse the functional groups by Potassium bromide pellet method and the peak spectrum was recorded with Thermo Fisher Nicolet Impact 500 FT-IR Spectrophotometry using diffuse reflectance mode.

Gas Chromatography- Mass Spectroscopy: The vital compounds in the microbial extracts were detected by performing Gas Chromatography and Mass Spectroscopy in Shimadzu GC-MS- QP2010S, Column of Rxi-5Sil MS with 30 meter length, 0.25 mm ID and 0.25 µm thickness and the peaks were recorded by the software GC MS Solutions and compound identification was done by comparing with standard peaks of NIST 11 and WILEY 8 libraries.

RESULTS

Isolation of Fungal Endophytes: The Potato Dextrose Agar plates were inoculated with roots and leaves of *Pongamia pinnata* and leaves of *Gliricidia sepium* developed fungal growth after 5 days of inoculation. The plates inoculated with roots of *Gliricidia sepium* showed fungal growth after 10 days of inoculation.

Morphological Characterization of Endophytic Fungal Isolates: From the Lactophenol Cotton blue (LCB) staining the morphological characteristics of fungal isolates were studied and genera was identified as FPPL1 is *Torula sp.* (Cappuccino and Sherman. 2007),

FPPL2 is *Alternaria sp.* FPPL3 is *Fusarium sp.* (Yemeda *et.al*., 2015; Frazier and Westhoff. 1995), FPPR1 is *Alternaria sp.*, FPPR2 is *Penicillium sp.*(Sayyed *et.al.*, 2015; Yemeda *et.al*., 2015), FPPR3 is *Aspergillus sp.*, FGSL1 is *Fusarium sp.*(Yemeda *et.al.*, 2015; Frazier and Westhoff, Food Microbiology. 1995), FGSL2 is *Cladosporium sp.*, FGSR1 is *Candida sp* (Picture no.1 to 9).

Synthesis and Extraction of Pigments from Endophytic Fungal isolates: The fungal metabolites were synthesized in Malt Extract Broth were extracted with 95% Ethanol. The isolates FPPL1, FPPR1, FPPR2 and FPPR3 have shown distinguishable colours which indicated the pigment synthesis in the fungi.

UV- Visible Spectrometry: The colour intensity of extracts of the nine endophytic fungal isolates were read at 300-600 nm, which gives peaks at 399nm and absorbance of around 0 to 2. This confirmed the presence of pigment traces in the extracts.

Thin Layer Chromatography (TLC): The isolates FPPL1, FPPR2 and FPPR3 have given coloured spots and the Retention Factor value is calculated (Fig. 10).The R_f values of isolate FPPL1 is 0.698, FPPR2 is 0.446 and FPPR3 is 0.468.

Fourier Transform Infrared Spectroscopy: The spectrum revealing the presence of functional groups in the extracts of FPPL1, FPPR2 and FPPR3 were given in the Fig. 11, 12 and 13.

Gas Chromatography- Mass Spectroscopy: The peaks obtained from GC-MS of FPPL1. FPPR2 and FPPR3 were given in the Fig. 14, 15 and 16, and the compounds corresponding peaks identified were given in the Table 1, 2 and 3.

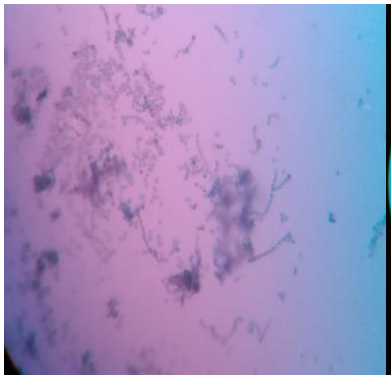

Figure 1: FPPL1.

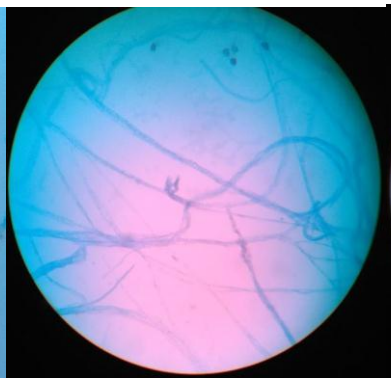

Figure 2: FPPL2.

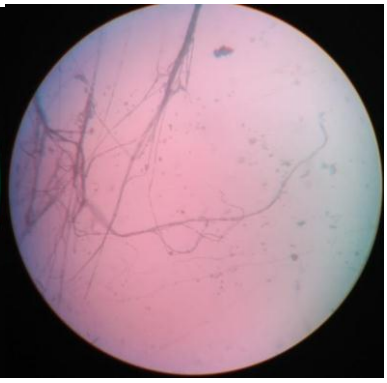

Figure 4: FPPR1.

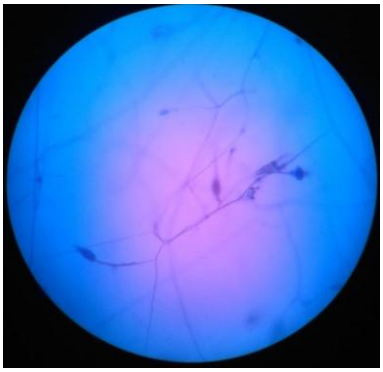

Figure 3: FPPL3.

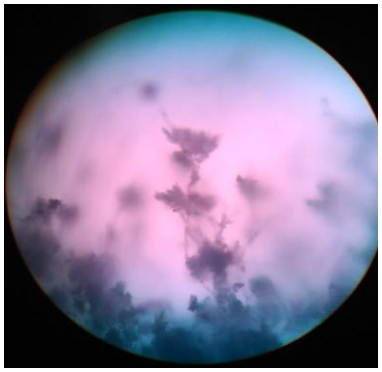

Figure 5: FPPR2.

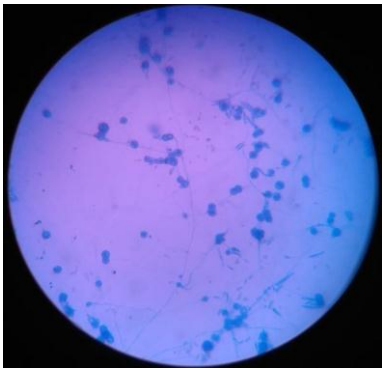

Figure 6: FPPR3.

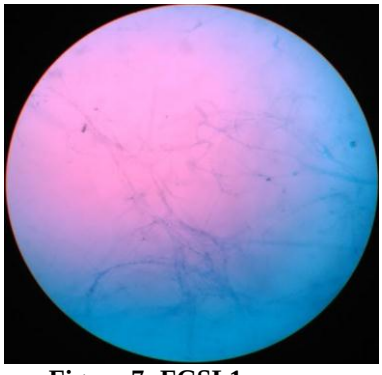

Figure 7: FGSL1.

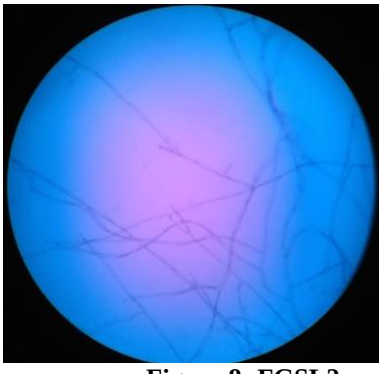

Figure 8: FGSL2.

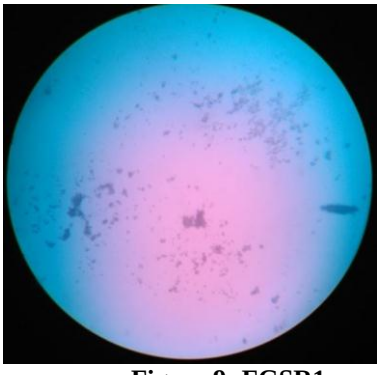

Figure 9: FGSR1.

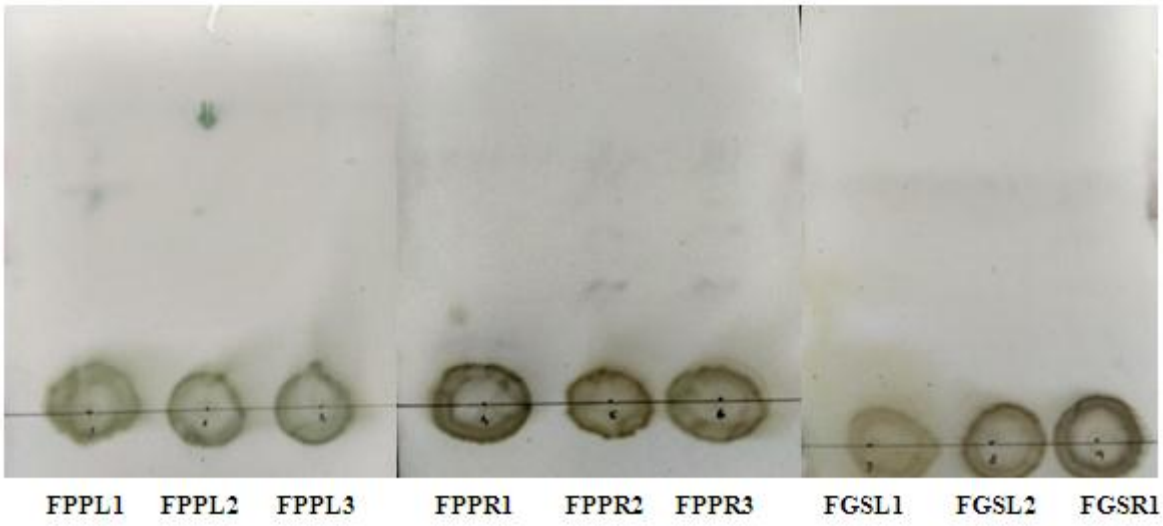

Figure 10: The TLC of Fungal Extracts.

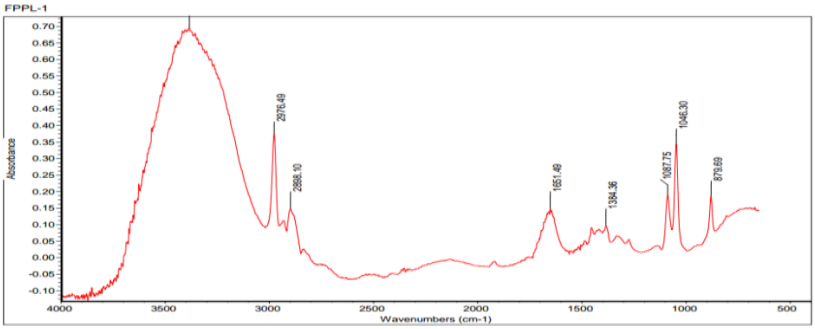

Figure 11: FTIR Peaks of FPPL1 Extract.

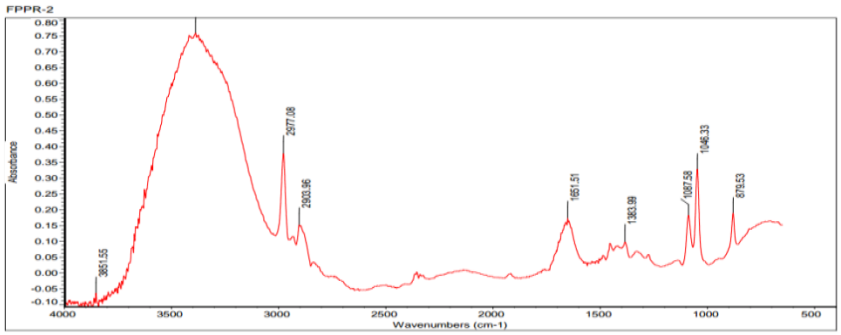

Figure 12: FTIR Peaks of FPPR2 Extract.

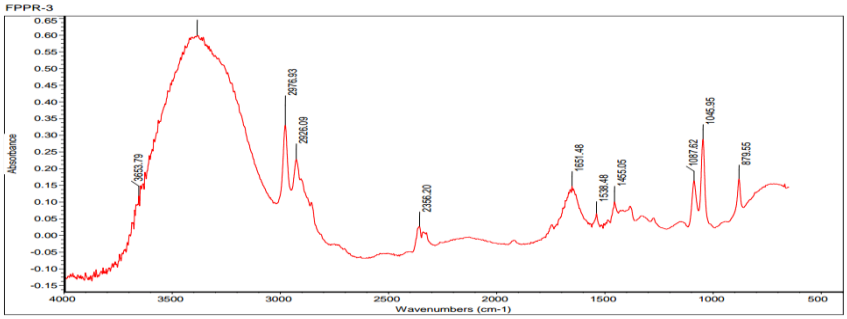

Figure 13: FTIR Peaks of FPPR3 Extract.

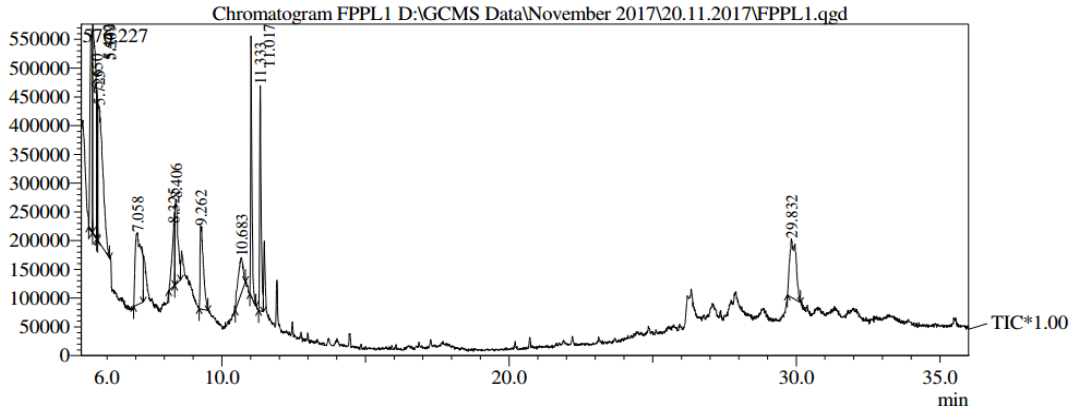

Figure 14: GC-MS Peaks of FPPL1 Extract.

Table 1: Peak Report of FPPL1.

Peak#	R.Time	Area	Area%	Height	Height%	Name	Base m/z
1	5.442	1910771	10.57	338708	12.38	UNDECANAL, 2-METHYL-	58.05
2	5.509	2854000	15.79	363282	13.28	PENTANAL, 2-METHYL-	58.05
3	5.650	496207	2.75	260256	9.52	1,1-Bis[aziridyltrimethylamine]	58.05
4	5.725	2820782	15.61	237429	8.68	PIMELIC ACID-CARBOXY-D2	55.05
5	7.058	1863469	10.31	125609	4.59	Orcinol	124.10
6	8.325	719720	3.98	108471	3.97	METHYL 2-HYDROXYETHANESULFONATE #	80.10
7	8.406	1065207	5.89	147863	5.41	GUANOSINE	57.05
8	9.262	1147796	6.35	144453	5.28	o-Orsellinaldehyde	151.05
9	10.683	783696	4.34	61535	2.25	3-Deoxy-d-mannoic lactone	57.05
10	11.017	1445735	8.00	452594	16.55	.beta.-Resorcylic acid, 3,6-dimethyl-, methyl ester $$ Atraric acid	136.10
11	11.333	1416411	7.84	392337	14.34	Resorcylic acid,.gamma., 4-methyl-,methyl ester	150.05
12	29.832	1546762	8.56	102634	3.75	DODECANOIC ACID, 1,2,3-PROPANETRIYL ESTER	57.05
		18070556	100.00	2735171	100.00		

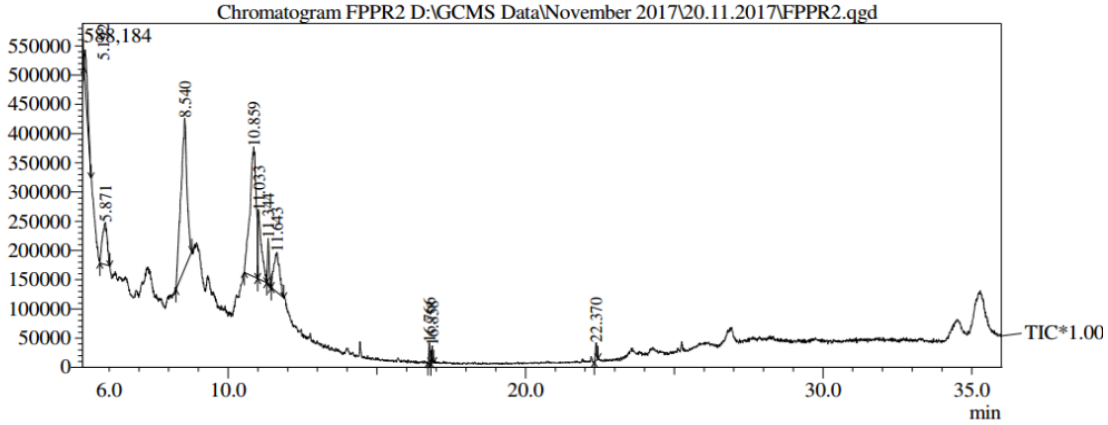

Figure 15: GC-MS Peaks of FPPR2.

Table 2: Peak Report of FPPR2.

Peak#	R.Time	Area	Area%	Height	Height%	Name	Base m/z
1	5.192	539919	5.09	64501	6.62	3-Acetoxy-3-hydroxypropionic acid, methyl ester	103.05
2	5.871	792498	7.47	72474	7.43	1-Propanol, 3-methoxy-2-(methoxymethyl)-2-methyl-	55.05
3	8.540	3677473	34.67	257334	26.40	GUANOSINE	57.05
4	10.859	3243444	30.58	222951	22.87	3-Deoxy-d-mannoic lactone	57.05
5	11.033	961517	9.06	116449	11.95	BETA-RESORCYLIC ACID, 3,6-DIMETHYL-, METHYL ESTER	136.10
6	11.344	272741	2.57	78748	8.08	o-Orsellinic acid, ethyl ester	150.05
7	11.643	892382	8.41	68382	7.01	Hydrazinecarboxylic acid, (1-ethylpentylidene)-, methyl ester	112.05
8	16.766	79522	0.75	34046	3.49	Cyclooctene, 3-methyl-	67.05
9	16.858	58025	0.55	29493	3.03	7-D2-METHYLENECYCLOHEXANE	55.05
10	22.370	89553	0.84	30476	3.13	INDOLO[2,3-A]QUINOLIZINE-1-METHANOL, 1,2,3,4,6,7,12,12B-OCTAHYDRO-, ACETATE (ESTER), CIS-	239.10
		10607074	100.00	974854	100.00		

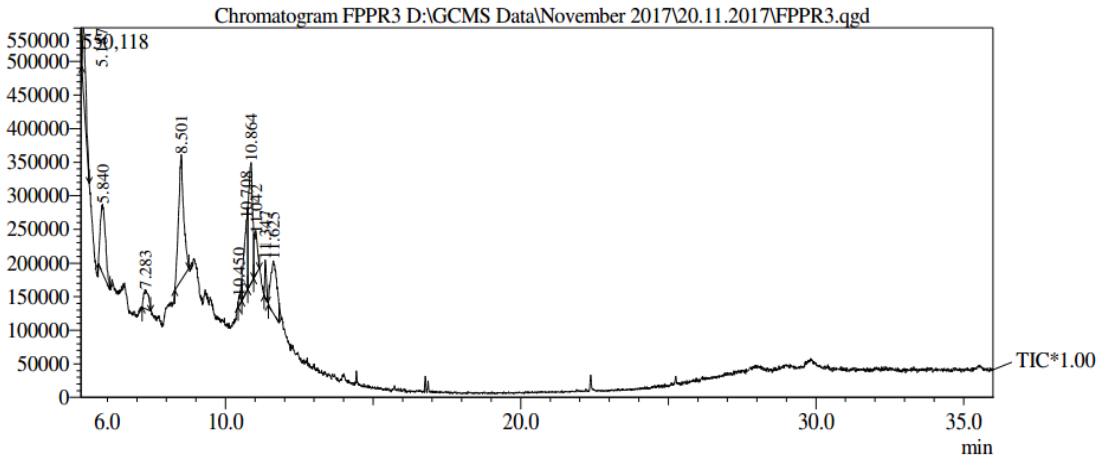

Figure 16: GC-MS Peaks of FPPR3.

Table 3: Peak Report of FPPR3.

Peak#	R.Time	Area	Area%	Height	Height%	Name	Base m/z
1	5.187	762182	8.31	95407	10.47	ETHANOL, 2,2-DIETHOXY-	103.05
2	5.840	1217769	13.27	102861	11.29	1-Propanol, 3-methoxy-2-(methoxymethyl)-2-methyl-	55.05
3	7.283	330143	3.60	28823	3.16	2-Hexenedioic acid, 2-methoxy-, dimethyl ester	71.00
4	8.501	2263635	24.67	187074	20.54	GUANOSINE	57.05
5	10.450	124126	1.35	14037	1.54	HEPTANOIC ACID, 6-OXO-, ETHYL ESTER	127.05
6	10.708	1015785	11.07	107473	11.80	1,10-Dimethyl-4,7,13-trioxa-1,10-diazacyclopentadecane	118.05
7	10.864	1640784	17.88	179980	19.76	3-Deoxy-d-mannoic lactone	57.05
8	11.042	478039	5.21	59748	6.56	Coumarine, 3,4,5,6-tetrahydro-6,8a-epidioxy-4a-methyl-	130.10
9	11.347	207742	2.26	56888	6.25	.beta.-Resorcylic acid, 6-methyl-, ethyl ester	150.00
10	11.625	1135038	12.37	78519	8.62	DL-MENTHONE	112.05
		9175243	100.00	910810	100.00		

DISCUSSION

The structural and cultural characteristics of isolated fungi indicated that the isolates FPPL1, FPPL2, FPPL3, FPPR1, FPPR2, FPPR3, FGSL1, FGSL2 and FGSL3 genera might be *Torula sp., Alternaria sp., Fusarium sp., Alternaria sp., Penicillium sp., Aspergillus sp., Fusarium sp*., *Cladosporium sp*. and *Candida sp.* As per the work done by Velmurugan *et al*., 2010; Yemeda *et al*., 2015 and Eskandaringhadikolaii *et al*., 2015, the genera identified were common inhabitants of the medicinal plants. The TLC of Fungal extracts revealed the presence of coloured compounds in isolates FPPL1, FPPR2 and FPPR3 as it shows coloured band for each sample. These isolates were identified as *Torula sp., Penicillium sp.* and *Aspergillus sp.* The TLC performed by Eskandaringhadikolaii*et al*., 2015, identified 11 bioactive compounds from 15 isolates of *Gliricidia sepium, Canna indica* and *Gardenia jasminoides.* The UV-Visible spectroscopy data of all fungal extracts formed single peak in the wavelength of 399 nm which corresponds to the UV-A region of the spectra and these trace compounds may be carotenoids according to the work done by Stafsnes *et al*., 2010. According to the observations done by Sayyed and Majumder., 2015 from the study of pigment producing fungi, the FTIR analysis data shows the presence of alcohols, phenols and ketones, which were also present in the extracts of fungal isolates FPPL1, FPPR2 and FPPR3. The Gas Chromatography- Mass Spectrometry of extracts FPPL1, FPPR2 and FPPR3 shows the presence of variety of alkaloids, phenols and carboxylic acids, and compounds of industrial value and dye fixatives with Fluorogen activity.

CONCLUSION

The modified surface sterilization protocol for fungi from different explants (Leaf and Root) of *Pongamia pinnata* and *Gliricidia sepium* were shown to be effective as the control did not show any microbial colonies. The Endophytic fungal genera identified were common inhabitants of the medicinal plants. The TLC of Fungal extracts revealed the presence of pigments in the selected three isolates (FPPL1, FPPR2 and FPPR3). The UV-Visible spectroscopy data of all fungal extracts formed single peak in the wavelength of 399 nm which corresponds to the UV-A region of the spectra. According to the work done by Stafsnes *et al*., 2010, the peaks may be corresponds to Carotenoid family. The FTIR and GC-MS data revealed the presence of fluorogenic substances, fixatives and corresponding functional groups.

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