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HR-LC-MS based metabolic profiling of *Fusarium solani* a fungal endophyte associated with *Avicennia officinalis*

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Abstract

Endophytic fungi form associations with plants, and often assist plant growth and produce bioactive metabolites, which confer resistance against plant pathogens and other stress factors. Mangrove plants being the flora of marshy and saline areas are highly tolerant to salinity and are rich in secondary metabolites. In this study, we isolated and identified *Fusarium solani* a fungal endophyte from leaves of mangrove, *Avicennia officinalis.* This endophyte was studied for metabolic profiling and further identification of bioactive compounds using HR-LC-MS based metabolomics. The fungal extract showed the presence of several anti-cancer compounds like 3-Pyridylacetic acid, Aloe-emodin, Antipyrine, Mitoxantrone, Sulfabenzamide etc. This metabolomic study highlights the potential of mangrove associated endophytic fungi for the production of industrially and medicinally important secondary metabolites.

Keywords – Endophytic fungi – Metabolomics – Mangroves – Secondary metabolites

Introduction

Endophytic fungi internally colonize plants without any apparent adverse effect, forms mutualistic association and occur ubiquitously in plants (Schulz et al. 2002). They produce a plethora of compounds with antimicrobial, plant growth-promoting and plant-stress alleviating properties (Strobel 2003). The fungal endophytes associated with mangrove plants are unique and are of special interest. Mangroves inhabit a special ecosystem, constituting saline marshy areas, which are the transition zone between marine and terrestrial habitats (Debbab et al. 2013). Similarly, mangroves harbor different fungal endophytes like marine, freshwater and soil-borne fungi (Ananda & Sridhar 2002).

Mangroves associated endophytic fungi represent the second largest ecological group of marine fungi (Sridhar 2004). About 200 species of fungal entophytes are isolated and identified (Liu et al. 2007). Endophytic fungi are less investigated and unexplored group which received considerable attention after they were found to protect their host against pathogens by secreting bioactive metabolites. Endophytes serve as potential source of novel natural products for exploitation in medicine, agriculture, and industry (Strobel 2003, Debbab et al. 2013). Endophytes

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produce novel and diverse bioactive metabolites such as terpenoids, steroids, quinones, phenols, coumarins etc., which can act as antimicrobial, anticancer, and antiviral agents (Suryanarayanan et al. 2009, Arya et al. 2019). The fluctuations in pH, salinity, oxygen level and nutrients in mangrove ecosystem are responsible for the synthesis of diverse chemical compounds having biotechnological potential. It is believed that mangrove associated entophytes play an important role in the synthesis of these compounds (Suryanarayanan et al. 2011).

In this study, our objective was to assess the diversity of endophytic fungi isolated from leaves of *Avicennia officinalis* collected from different geographic localities and investigate the metabolite profile of one of the selected common fungus *Fusarium* for further identification of bioactive compounds using HR-LC-MS. As most of the naturally-derived compounds have antimicrobials and antioxidants potential, we also aimed to investigate the antimicrobial and antioxidant activity of the fungal extract.

Materials & Methods

Collection of host plants

The endophytic fungus used in this study was isolated from the leaves of *Avicennia officinalis*. Healthy leaves of mangrove were collected from the Sea shoreline of Dive agar (18°10'40.75"N, 72°59'4.09"E and Shrivardhan (18° 2'44.45"N, 73° 1'49.51"E) (Fig. 1). The leaves were collected and stored in sterile polythene bags and brought to the laboratory.

Fig. 1 – Collection of plant material from the sea shoreline of Diveagar, Shrivardhan and Dapoli for endophytic fungi isolation.

Isolation of endophytic fungus

To remove unwanted fungal propagules from the leaf surface, leaves were washed thoroughly under running tap water for 2-3 min. The small pieces of leaves (~0.5cm of diameter) were cut from the midrib portion using a flame-sterilized surgical blade (Ezra et al. 2004). Then the leaf

pieces were surface sterilized by immersing in 70% ethanol for 1 min, followed by 4% sodium hypochlorite (v/v) for 2 min, and finally washed in sterile water for 1 min and then rinsed in sterile filter paper. Each piece placed in Petri plates containing potato dextrose agar (with chloramphenicol 150 mg/L). The plates sealed with parafilm strips and incubated in BOD incubator at 27°C for 20 days. The Petri plates monitored every day for the growth of endophytic fungi. The fungi that grow out from the tissues were isolated and stocked. The cultures were maintained on PDA slants at 4°C for the further screening process.

Culture identification

The fungal isolates were identified based on its morphological and reproductive characters using standard identification manual (Dugan et al. 2008).

Submerged Fermentation

The pure culture isolated by the above method was grown in Potato dextrose broth. The flasks were incubated in the shaker-incubator at 200 rpm for 5 days. The mycelium was aseptically transferred into 1000 mL Erlenmeyer flasks containing 300 mL PDB medium and incubated at 28 ± 1°C for 30 days under stationary conditions.Then the mycelium and filtrate were separately subjected to solvent extraction and used for metabolic profiling.

Authentication of endophytic fungi

The endophytic fungi isolate was identified to the species level based on the morphological features from National Fungal Culture Collection of India (NFCCI) Pune.

Molecular identification of endophytic fungi

To further validate the morphological identification, molecular identification was performed as per Ezra et al. (2004). The Fungal DNA was isolated by using XcelGen fungal genomic DNA isolation kit. DNA quality was estimated on 1% agarose gel. To perform Polymerase chain reaction (PCR), the ITS primer sets were ITS4 Forward GGAAGTAAAAGTCGTAACAAGG and ITS5 Reverse CAGACTT(G/A)TA(C/T)ATGGTCCAG. The polymerase chain reaction was performed using Eppendorf master cycler. PCR conditions were denaturation of DNA at 95°C for 3 min. then 40 cycles of PCR consisting of denaturation at 95°C for 1 min, primer annealing at 55°C for 45 sec. and extension at 72°C for 1 min. The sample was subjected to an additional extension at 72°C for 10 min at the end of the PCR cycles. PCR product was mixed with the loading buffer (DNA loading dye) with Gel Red stain and loaded on 1.4% Agarose gel and electrophoresis was done using 1X TAE buffer (Tris base acetic acid and EDTA).The fungal PCR product was then identified based on its nucleotide sequence from Eurofins Genomics India Pvt. Ltd. Bengaluru, India.

HR-LC-MS based metabolic profiling

For LC-MS analysis, 10 gm of dried fungal biomass was extracted in 100 ml HPLC grade methanol. The sample was sonicated for 5 mins and sent to IIT-Powai, India for High-resolution Mass Spectrophotometry (HR-LC-MS). ThevAgilent Technologies, TOF/Q-TOF Mass Spectrometer (Model- G6550A) with Dual AJS ESI as ion source with following details: Injection volume- 3 µl, Flow- 0.3 $ml.min^{-1}$, Mobile Phase consisted of A) 0.1% Formic acid and, B) 90% Acetonitrile + 10 % H_2O + 0.1% Formic acid, Min range (m/z) 125, Max range (m/z) 1000, Scan rate (spectra/sec) 1.00. The peaks were identified using the inbuilt library of LC-MS based on mass.

Antimicrobial activity

For the antimicrobial study, nutrient agar culture media were used with various incubation conditions. Nutrient agar plates were inoculated with standardized inoculum of the test microorganisms: *Escherichia coli* and *Staphylococcus aureus*. The filter paper discs (about 6 mm in diameter), containing the test compound (Amoxycillin) and suspension of *Fusarium* fungal

filtrate at the desired concentration, were placed in the middle of the plate agar surface. The Petri plates were incubated under suitable conditions. Antimicrobial agent that diffuses into the agar and inhibits germination and growth of the test microorganism is considered for measuring the diameter of inhibition growth zone.

Antioxidant activity (2, 2, Diphenyl -1-picryl hydrazyl (DPPH) Assay

Radical scavenging activity of *Fusarium* extract against free DPPH (2, 2-Diphenyl-1-picrylhydrazyl) (Sigma–Aldrich) was determined spectrophotometrically.Various concentrations of *Fusarium* extract (10, 20, 30, 40, 50, 75 and 100 $mg.mL^{-1}$) and standard (Gallic acid) were used and 1 ml of DPPH (1 mM) dissolved in methanol.The mixture was vortexed and incubated in the dark for 30 minutes at room temperature and then absorbance of stable DPPH was recorded at 517 nm. The DPPH (containing no sample) was used as a control prepared using the same procedure. The activity was expressed as the percentage of inhibition that was calculated using the equation of DPPH radical scavenging activity (%) = (Ac - As)/Ac x 100.

Results

Isolation and identification of endophytic fungi

Fungal endophyte, *Fusarium solani* was isolated from *Avicennia* leaves and cultured on Potato Dextrose Agar media (Fig. 2A). The fungal isolate was identified and authenticated at National Fungal culture collection of India (NFCCI)-A AgharkarResearch Institute, Pune.The fungus was authenticated as *Fusarium solani* (Mart.) Sacc. Family- Nectriaceae using morphological and molecular methods, .The fungus was found to show slightly curved hyaline microconidia in culture plate (Fig. 2B).

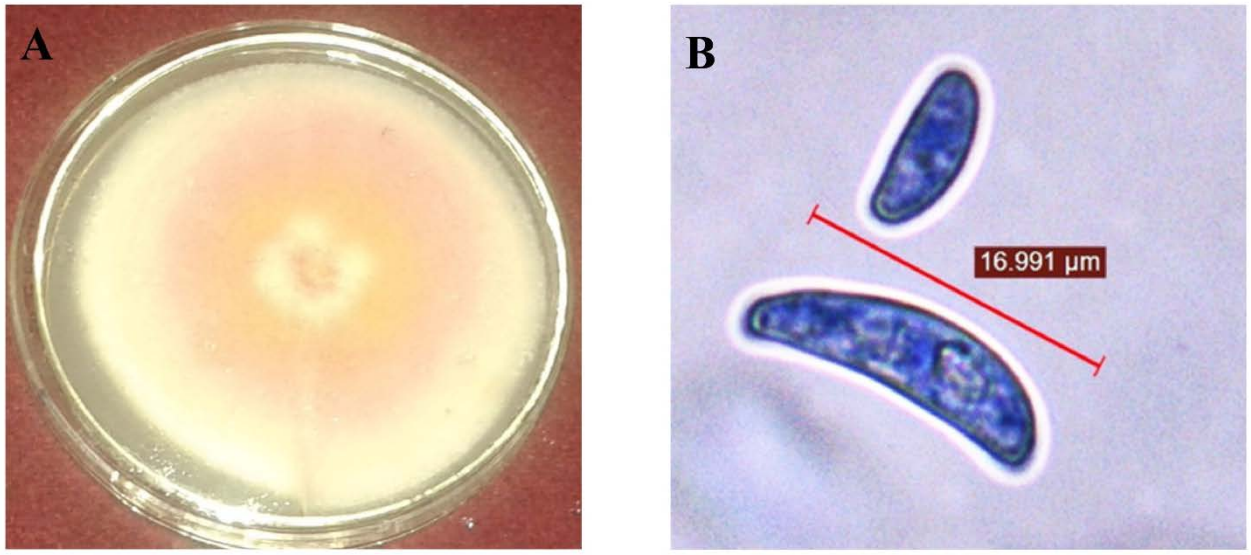

Fig. 2 – A Pure Culture of *Fusarium solani* endophytic fungi isolated from *Avicennia.* B Microconidia.

LC-MS based metabolic profiling

High throughput metabolic profiling of methanolic extract of *Fusarium* mycelium using HR-LC-MS was done for the identification of some important metabolites (Fig. 3). Based on the available literature, 38 annotated metabolites identified have shown bioactivity (Fig. 4). The anti-cancerous compounds included 3-Pyridylacetic acid, Aloe-emodin, Antipyrine, Mitoxantrone, Sulfabenzamide. Antioxidant metabolites were 2, 4, 6-Trimethylacetophenone Imine and Daidzein. Anti-inflammatory metabolites were Anabasamine, Desethylhydroxychloroquine and Mometasone

Furoate. The Antimicrobial metabolites were, Antipyrine, Dihydrodeoxystreptomycin, Mometasone Furoate, Phenylacetic acid and Phenylpyruvic acid.

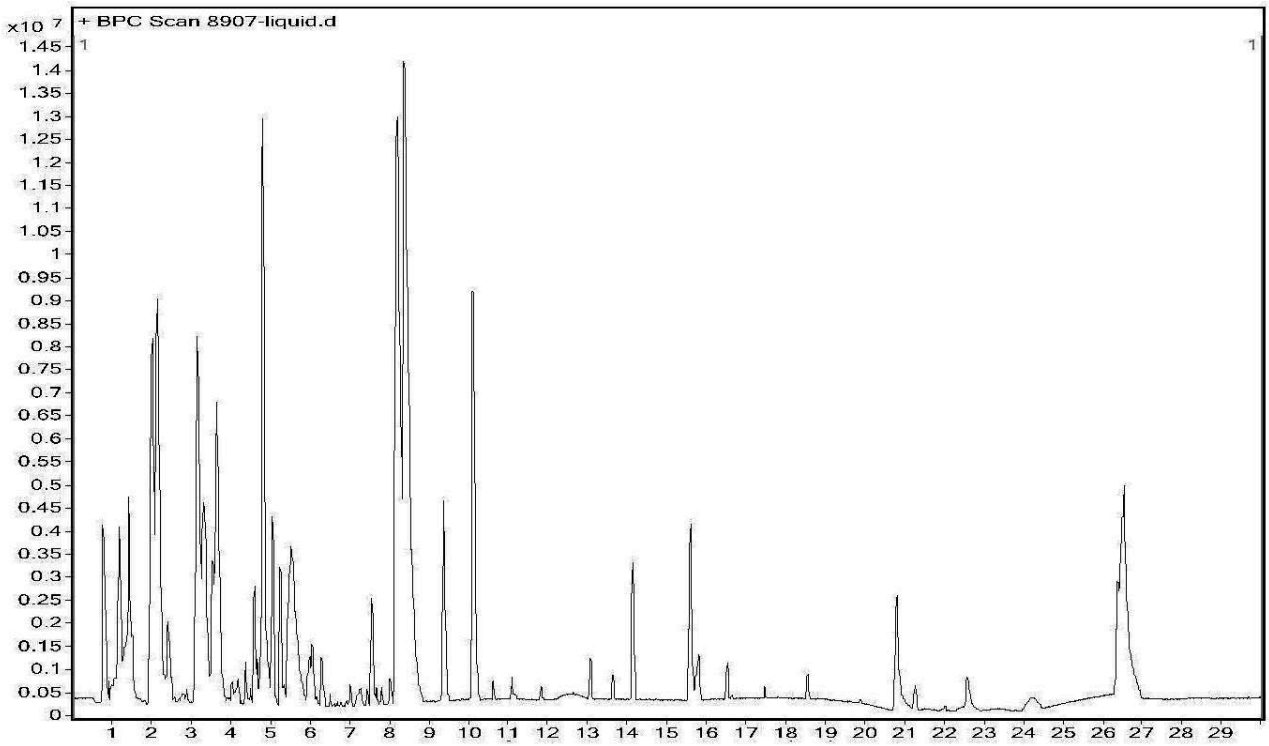

Fig. 3 – Chromatogram of LC-MS based metabolite profiling of *Fusarium solani.*

Antimicrobial activity

The results showed that *Fusarium* extracts exhibited high antibacterial activity against Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria. The concentration of 250ug/100ul was found effective against both the bacteria. Gram-negative bacteria i.e. *E. coli* and *S. aureus* exhibited inhibition zones, whereas Gram-positive bacteria showed smaller inhibition zone, compared to the antibiotic control. *Fusarium* significantly inhibited the growth of *E.coli* and *S. aureus* (Fig. 5). *Fusarium* extract showed strong antibacterial activity and may be considered as an alternative to antibiotics with potential to deal with multi-drug resistant bacteria.

Antioxidant Activity

The fungal extract showed significant DPPH free radical scavenging activity (Fig. 6). Different concentrations of the extract ranging from 10 to 100 µg ml^{-1} showed an increasing trend in the % inhibition.

Discussion

Mangrove ecosystem has become a valuable resporce for the isolation of endophytic fungi with considerable bio-potential. As many as 39 endophytic fungi were isolated from leaf tissues of *Rhizophora apiculata* and *R. mucronata* (Suryanarayanan et al. 1998). In another study, 35 species of fungal endophytes were isolated from roots of *Avicennia officinalis, Acanthus ilicifolius, R. mucronata* and *Sonneratiacaseolaris* (Ananda & Sridhar 2002). In the Northeast Brazil, mangroves like *Avicennia schaueriana, Rhizophora mangle* and *Laguncularia racemosa* were explored for the isolation of 40 species (Costa et al. 2012). Different fungi were isolated from leaves, bark and woody tissue of *Kandelia cande l.* in Hong Kong (Pang et al. 2008). Using root and stem tissues of *Bruguiera sexangula, Ceriopstagal, R. stylosa, R. apiculata* from the south coast of China, 38 species of fungi were reported (Xing & Guo 2011).

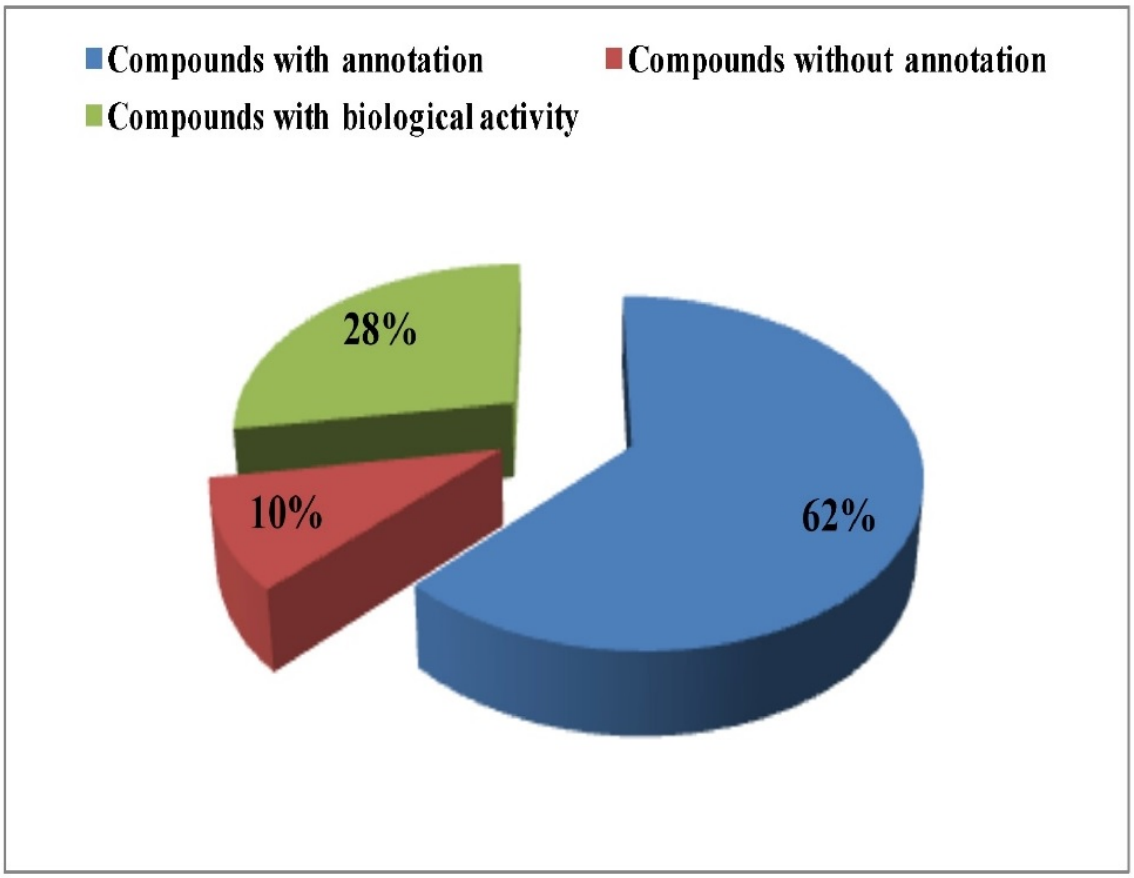

Fig. 4 – Classification of metabolites obtained from LC-MS based Metabolite profiling of *Fusarium solani* based on compounds with annotation, compounds without annotation and compounds with biological activity.

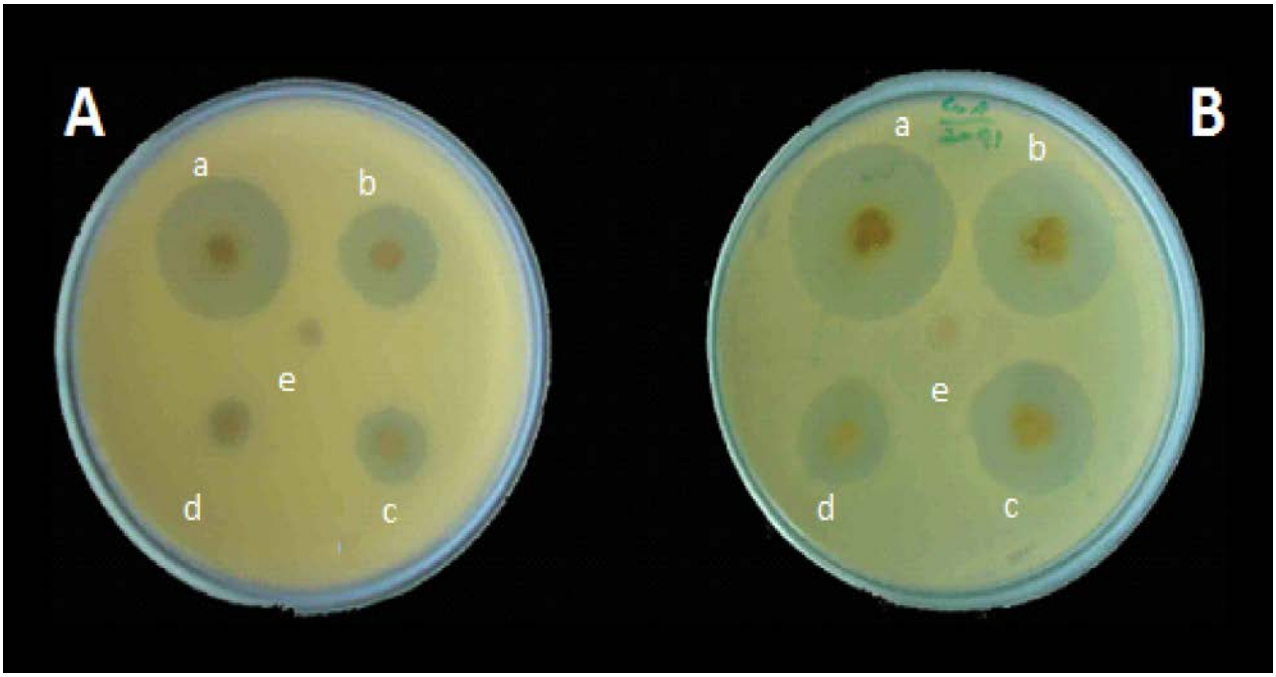

Fig. 5 – Antimicrobial activity of *Fusarium solani* against. A *E. coli.* B *S. aureus.* (a = Positive control (Tetracyclin), b = *Fusarium* (250µg/ml), c = *Fusarium* (100µg/ml), d = *Fusarium* (50µg/ml), e = Negative control).

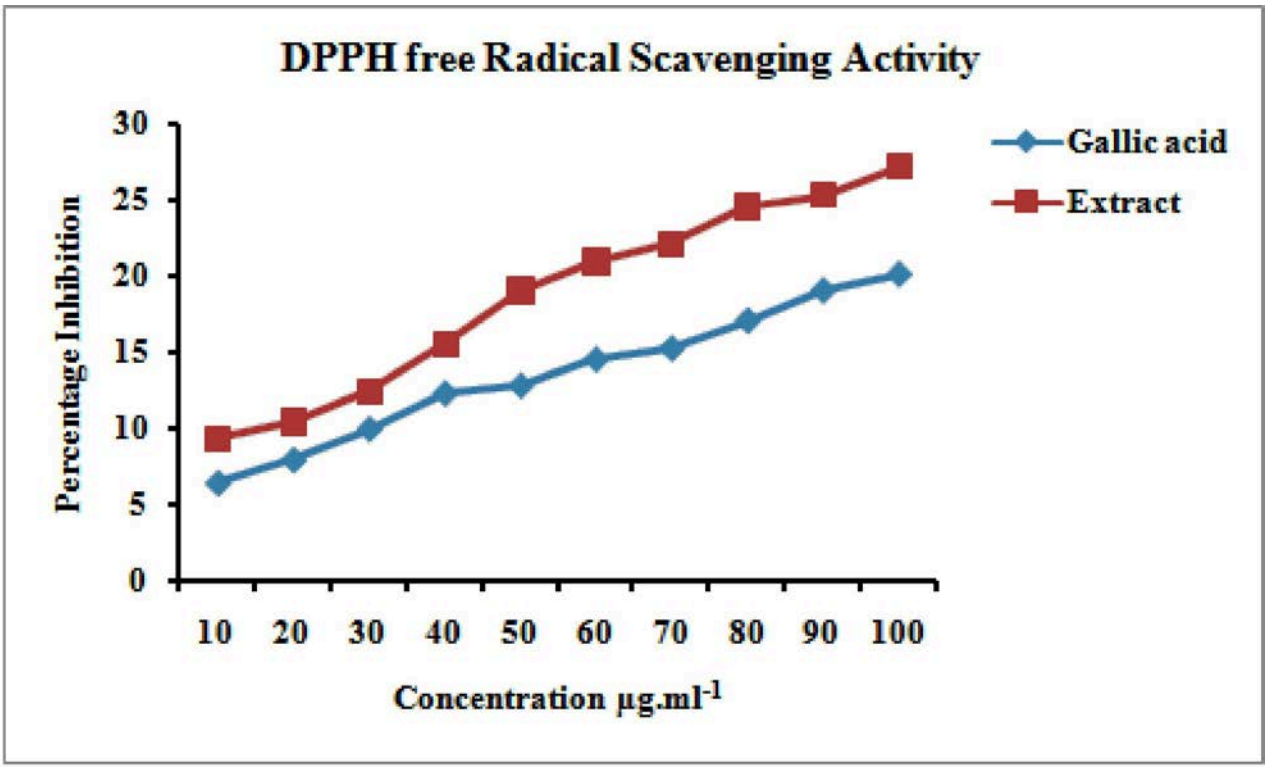

Fig. 6 – Antioxidant activity of *Fusarium solani*

Mangrove associated fungi have attracting great attention of researchers as they serve as a reservoir of secondary metabolites with high biological activities (Wang et al. 2014). Wang et al. (2014) have described the isolation of secondary metabolites and their structural elucidation and biological activities from mangrove associated endophytic fungi. Song et al. (2012) characterized mangrove associated endophyte *Xylaria* sp. BL321 and identified three new eremophilane sesquiterpenes which showed inhibitory activity on α-glucosidase. An endophytic fungus *Talaromyces flavus* was isolated from *Sonneratia apetala* showing the presence of four new norsesquiterpene peroxides (talaperoxides A-D). These compounds tested against human cancer cell lines MCF-7, MDA-MB-435, HepG2, HeLa and PC-3, revealed that compound Talaperoxide B and D have cytotoxicity against all human cancer cell lines (Li et al. 2011). An endophytic fungus *Pestalotiopsisclavispora*was isolated from *Bruguierasexangula*and evaluated for the presence of novel natural products. Three new triterpenoid derivatives, named (15α)-15-hydroxysoyasapogenol B (1), (7β, 15α)-7, 15-dihydroxysoyasapogenol B and (7β)-7,29-dihydroxysoyasapogenol B (Luo et al. 2011). Metabolic profiling of an endophytic fungus *Pestalotiopsis*sp isolated from *Rhizophora mucronata showed presence of cytosporones J–N, coumarins pestalasins A–E, alkaloid pestalotiopsoid A, cytosporone C, dothiorelone B (7), and 3-hydroxymethyl-6, 8-dimethoxycoumarin* (Xu et al. 2009).

The biological activities were classified in to major classes such as Antioxidants, Anti-inflammatory, Anti-microbial etc. (Table 1). Endophytic fungi *Halorosellinia* sp. and *Guignardia* sp. isolated from mangroves produced fourteen anthracenedione derivatives having anticancerous activity and the derivatives showed good inhibition of growth of KB and KBv200 cell (Zhang et al. 2010). *Kandelia candel* harboring fungus *Diaporthe* sp., produced compound, diaporthelactoneshowed cytotoxic activity against KB and Raji cell lines (Lin et al. 2005). Three compounds phomopsin A-C isolated from *Phomopsis sp* harboring on *Excoecaria agallocha* showed inhibitory effect on *Candida albicans* and *Fusarium oxysporum* (Huang et al. 2008).

Table 1 List of metabolites identified using HR-LC-MS based metabolic profiling and their biological activities.

Metabolite	RT	Mass	*m/z*	Formula	Biological activity	Reference
2,4,6-trimethylacetophenoneImine	26.53	161.1193	162.1266	$C_{11}H_{15}N$	Antioxidant activity	Yuswan et al. 2015
2-amino-tetradecanoic acid	13.64	243.2184	244.2256	$C_{14}H_{29}NO_2$	Antifungal and antitumor	Guo et al. 2009
2R-aminohexadecanoic acid	15.59	271.2493	272.2565	$C_{16}H_{33}NO_2$	Cytotoxic potential	Ravi & Krishnan 2017
3-amino-2-naphthoic acid	3.655	187.0621	188.0693	$C_{11}H_9NO_2$	Antiallergy activity	Althuis et al. 1979
3-methoxy-4-hydroxyphenylglycol glucuronide	3.207	360.1035	383.0926	$C_{15}H_{20}O_{10}$	Norepinephrine metabolites	Elsworth et al. 1983
3-pyridylacetic acid	1.181	137.0468	138.054	$C_7H_7NO_2$	Inhibitors of androgen biosynthesis for prosthetic cancer treatment	Rowlands et al. 1995
4-hydroxy-6-methylpyran-2-one	3.169	126.0311	127.0384	$C_6H_6O_3$	Phytotoxic activity	Demuner et al. 2009
Aloe-emodin	7.566	270.0516	271.0589	$C_{15}H_{10}O_5$	Anticancer agent	Pecere et al. 2000
Anabasamine	1.407	253.1522	254.1595	$C_{16}H_{19}N_3$	Anti-inflammatory	Barbosa-Filho et al. 2006
Antipyrine	10.62	188.0962		$C_{11}H_{12}N_2O$	Anti-bacterial and anti-cancer activity	Muna et al. 2017
Capryloylglycine	1.273	201.135	202.1422	$C_{10}H_{19}NO_3$	Cosmetic treatment	Sparavigna et al. 2014
Daidzein	8.211	254.0564	255.0636	$C_{15}H_{10}O_4$	Antioxidant activity	Foti et al. 2005
Desethylhydroxychloroquine	13.076	307.1433	308.1504	$C_{16}H_{22}ClN_3O$	Anti-inflammation	Robinson et al. 2016
Dextroamphetamine	1.192	135.1021	158.0913	$C_9H_{13}N$	Acute psychologic and neuroendocrine effect	Schrantee et al. 2016
Dihydrodeoxystreptomycin	10.127	567.2863	568.2935	$C_{21}H_{41}N_7O_{11}$	Antifungal activity	Pawar et al. 2017
Dinorpromazine	11.099	256.1029	211.0853	$C_{15}H_{16}N_2S$	Inhibition of tumour necrosis	Bertini et al. 1991
Genkwanin	8.4	284.0669	285.0742	$C_{16}H_{12}O_5$	Melanoma B16F10 cell proliferation	Bouzaiene et al. 2016
Mitoxantrone	4.63	444.1969	467.186	$C_{22}H_{28}N_4O_6$	Anticancer activity	Shenkenberg & Von-Hoff 1986
Mometasone Furoate	1.071	520.1404	543.1293	$C_{27}H_{30}Cl_2O_6$	Antibacterial, Anti-inflammatory	Neher et al. 2008
Neuraminic acid	1.201	267.094	268.1022	$C_9H_{17}NO_8$	Binding Activity of Influenza A Viruses	Sauer et al. 2014
Oleamide	18.559	281.2701	282.2774	$C_{18}H_{35}NO$	Actions on blood pressure and core body temperature	Reséndiz et al. 2001
Phenylacetic acid	5.728	136.0541	159.0433	$C_8H_8O_2$	Antityrosinase and antimicrobial activities	Zhu et al. 2011

Table 1 Continued.

Metabolite	RT	Mass	*m/z*	Formula	Biological activity	Reference
Phenylethylamine	5.057	121.0908	144.0799	$C_8H_{11}N$	Analgesic effects	Mosnaim et al. 2014
Phenylpyruvic acid	1.239	164.0464	165.0537	$C_9H_8O_3$	Antimicrobial activity	Chaudhari & Gokhale 2016
Phthalic acid Mono-2-ethylhexyl Ester	15.794	278.1499	301.1391	$C_{16}H_{22}O_4$	Estrogen-antagonist activities	Ohtani et al. 2005
Succinoadenosine	2.457	383.1057	384.1128	$C_{14}H_{17}N_5O_8$	Fumarate Levels	Dennison et al. 2010
Sulfabenzamide	0.814	276.055	299.0443	C_{13} $H_{12}N_2O_3S$	Promotes autophagic cell death in T-47D breast cancer cells through p53/ DRAM pathway	Mohammadpour et al. 2012
Sulfinpyrazone sulfone	2.328	420.1248	443.1137	$C_{23}H_{20}N_2O_4S$	Inhibition of (S)-warfarin metabolism	He et al. 1995
Tranexamic acid	3.326	157.1092	158.1164	C8 H15 NO_2	Fibrinolytic activity of vein walls	Astedt et al. 1977
Tuberonic acid	6.064	226.1193	227.1266	$C_{12}H_{18}O_4$	Thioglucohydrolase activity	Sansenya et al. 2011

Conclusion

Endophytic fungi hold great potential for the synthesis of novel bioactive compounds of pharmaceutical, agricultural and industrial significance. The present study showed the isolation and characterization of endophytic fungi, *Fusarium solani* isolated from a mangrove, *Avicennia marina.* The presence of five anticancer and other bioactive metabolites in *Fusarium* highlighted its bioprospecting for pharmaceutical application. Further studied on the isolation, purification and characterization of these biologically active metabolites should pave way for their use for different functionalities.

Conflict of interest: All the authors confirmed that there is no conflict of interest.

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