SCREENING ENDOPHYTIC FUNGI FOR PRODUCTION OF PICEATANNOL, A NOVEL INHIBITOR/DISRUPTOR OF α-SYNUCLEIN

A THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT OF THE DEGREE OF

Master of Technology
In
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By

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CERTIFICATE

This is to certify that the thesis entitled "***Screening endophytic fungi for production of piceatannol a novel inhibitor/disruptor of α-synuclein***" being submitted by **Ms. Jessica (Roll No-601604004)** in partial fulfillment of the requirements for the award of degree of Master of Technology in Biotechnology, Thapar Institute of Engineering and Technology, Patiala, Punjab is a bonafide work carried out under the supervision and conception of Dr. Sanjai Saxena and that no part-of this thesis has been submitted for the award of any other degree.

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CANDIDATE'S DECLARATION

I hereby declare that the work being presented in the thesis entitled " ***Screening Endophytic Fungi For Production Of Piceatannol, A Novel Inhibitor/Disruptor Of α-synuclein*** " in partial fulfillment of the requirements for the award of degree of Master in Biotechnology, Department of Biotechnology, Thapar Institute of Engineering and Technology, Patiala is my own laboratory work during the period of July 2017 to July 2018, under the conception and supervision of **Dr. Sanjai Saxena,** Professor, Department of Biotechnology (DBT), Thapar Institute of Engineering and Technology, Patiala I have not submitted the matter embodied in this thesis for the award of any other degree.

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This is to certify that the above statement made by the candidate is correct and true to the best of my knowledge

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Date: Jessica

Place:

Dedication

Every challenging work needs self-efforts as well as guidance of elders

especially those who were very close to our heart.

My humble effort I dedicate to my sweet and loving

Father & Mother,

Whose affection, love, encouragement and prays of day and night make me

able to get such success and honor,

Along with all hard working and respected

Teachers

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ABBREVIATIONS

S.No.	Abbreviation	Full form
1.	µg	Micro gram
2.	µl	Micro litre
3.	µM	Micro molar
4.	ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
5.	ATR	Attenuated total reflection
6.	ALP	Alkaline phosphatase
7.	CAGR	Compound annual growth rate
8.	CMA	Chaperone-mediated autophagy
9.	BLAST	Basic local alignment search tool
10.	DA	Dopaminergic
11.	DNA	Deoxyribonucleic acid
12.	dNTP	Deoxynucleotide triphosphate
13.	DPPH	1,1-diphenyl-2-picrylhydrazyl
14.	EA	Ethyl acetate
15.	EDTA	Ethylene diamine tetra acetic acid
16.	ESI	Electrospray ionization
17.	Et Br	Ethidium bromide
18.	FC	Folin-ciocalteu
19.	FTIR	Fourier-transform infrared spectroscopy
20.	FMA	Fusarium minimal agar
21.	FRS	Free radical scavenging
22.	HPLC	High performance liquid chromatography
23.	Hr	Hours

24.	ITS	Internal transcribed spacer
25.	IC_{50}	Inhibitory concentration
26.	KW	Kilowatt
27.	L	Litre
28.	LC-MS	Liquid chromatography–mass spectrometry
29.	MEA	Malt extract agar
30.	mg	Milli gram
31.	MHA	Muller hinton agar
32.	MHB	Muller hinton broth
33.	MAO-B	Monoamine oxidase B
34.	min	Minutes
35.	ml	Milli litre
36.	m/z	Mass-to-charge ratio
37.	mm	Millimetre
38.	miRNA	MicroRNA
39.	mAU	milli-Absorbance Units
40.	NAC	Non-Abeta component
41.	ng	Nano gram
42.	O.D	Optical density
43.	PBS	Phosphate Buffered Saline
44.	PD	Parkinson's disease
45.	PCR	Polymerase chain reaction
46.	PDA	Potato dextrose agar
47.	PDB	Potato dextrose broth
48.	pH	Potential of hydrogen
49.	PLA	Pine leaf agar
50.	RNA	Ribo nucleic acid
51.	RNAi	RNA interference
52.	rpm	Revolutions per minute

53.	Rf	Retardation factor
54.	SD	Standard deviation
55.	SDA	Soubard's dextrose agar
56.	SN	Substantia nigra
57.	SNA	Synthetic nutrient deficient agar
58.	Sp.	Species
59.	STD	Standard
60.	TE	Tris EDTA
61.	TAE	Tris acetate EDTA
62.	TEAC	Trolox equivalent antioxidant capacity
63.	TFC	Total flavonoid content
64.	TLC	Thin-layer chromatography
65.	TPC	Total phenolic content
66.	US	United States
67.	UV	Ultra violet
68.	UPLC	Ultra-Performance Liquid Chromatography
69.	WA	Water agar

EXECUTIVE SUMMARY

Piceatannol or trans-3,3',4,5'tetrahydroxystilbene is a natural stilbene, type of polyphenolic flavonoid whose structure is similar (except producing an extra hydroxyl group) to resveratrol. It is one of the important dietary stilbenes commonly found in red wine, berries, peanuts, grapes, and white tea. Piceatannol having high demands due to its multifarious application in neurodegenerative disorder and cardiovascular disorders.

Endophytic fungi are microorganisms which colonize inside plant tissue without causing apparent harm to the host. Besides upon colonization of host plant endophytes synthesize an array of secondary metabolite which may defend the host plant against survival and stress conditions and in turn the host plant supply nutrients and habitat for endophytic fungi. Therefore in addition to interaction of endophytes with the host plant, endophyte acquires the property of their host plant and start producing analogue bioactive compounds which some have medicinal value.

The current study reports the exploration of endophytic fungi isolated from *Vitis vinifera* for their potential to produce piceatannol. In the present study culture filtrate of endophytic fungi obtained from potato dextrose broth were screened for Biochemical assays namely: Liebermann, Chromogenic reaction and Acetic anhydride test were used for the identification of phenolic compounds, where out of 50 endophytic isolates, only 21 isolates were found to be positive among which 6 isolates were observed to show 80-90% activity and subjected to mass production. These were subsequently extracted with EA solvent and again tested for presence of piceatannol. Out of 6 isolates the EA extract of #10(B)VVLPM were found to be potent producer of piceatannol. Using TLC #10(B)VVLPM was separated to 4 bands by using solvent system, chloroform: dichloromethane in 1:1 ratio. In TLC fungal piceatannol visualized as violet color spot with symmetrical Rf 0.65 as standard. The concentration of fungal piceatannol was determined by HPLC using C18 reverse phase discovery column which exhibited symmetrical peak at RT of 6.5 min whereas the concentration of crude fungal piceatannol #10(B)VVLPM, was found to be 7.56 µg/ml. In order to confirm tentative identification, functional groups and to obtained

more quantitative data FTIR and LC-MS analysis was performed. The m/z of 245.09 of #10(B)VVLPM was obtained which was similar to m/z of standard piceatannol. The fungal piceatannol producing endophytic fungi #10(B)VVLPM in our study showing strong antioxidant potential. The EA extract fungal piceatannol in DPPH assay exhibit very good scavenging activity as compared to standard piceatannol. In case of TEAC, TPC and TFC assay also same trend as in case of DPPH was obtained.

The potent piceatannol producing endophytic isolates #10(B)VVLPM, was tentatively identified as *Fusarium* sp. The genomic DNA of the selected isolate was extracted, amplified and further send for sequencing for molecular identification.

Keywords: Endophytic, Fungi, Piceatannol, *Fusarium* sp, Grapes, α-synuclein, Parkinson's disease

1.0. INTRODUCTION

Parkinson's disease (PD) is an age-related neurodegenerative disorder. It is the second most encountered disorder after Alzheimer's disease (Kalia and Lang, 2016). It affects approximately 1–2% of the global community over the age of 65. Lewy bodies (Intracytoplasmic aggregates) are one of the important indicators of PD and some other neurodegenerative disorders. It present inside the cytoplasm of nerve cells. They are mostly composed of α-synuclein fibrils (Shahpiri et al., 2016; Caruana et al., 2016).

α-synuclein is a 140 residue amino acid protein highly expressed in brain. Deposits of α-synuclein fibrils are the key player in the etiology and pathogenesis of PD. However, the soluble α-synuclein oligomers are the most toxic species. Basically, these species can induce toxicity by disrupting the cellular membranes by forming structures with pore-like morphologies (Temsamani et al., 2016; Bensalem et al., 2015).

L-dihydroxy-phenyl alanine (L-dopa or Levodopa) is the center for attraction for Parkinson's pharmacophore therapy, but still these drugs have many side effects, such as dyskinesia, motor fluctuations, and other non-motor complications (Chao et al., 2012; Bensalem et al., 2015). Owing to these side effects many researchers are aiming an alternative component which can target a single molecule or pathway, these include conventional drugs such as natural products to target multiple pathway. Thus a possible strategy of delaying or preventing PD is by exploration of natural small molecule which has favorable toxicological profile as well as cost effective that can inhibit the α-synuclein aggregation (Lashuel et al., 2013; Xu et al., 2002; Rocha et al., 2018).

The ability of plants to yield new therapeutic agents has motivated extensive investigation in search for novel natural product which can inhibit the α-synuclein aggregation. Among them, dietary polyphenols have gained tremendous attention because of their low toxicity and potential in human health (Nagao et al., 1999; Wang et al., 2015). Several studies have indicated that dietary polyphenols viz. resveratrol, piceatannol and ampelopsin A, have the ability to destabilize the α-

synuclein by forming soluble, non-cytotoxic, oligomeric complexes with the α-Synuclein protein (Meng et al., 2010; Ahsan et al., 2015; Temsamani et al., 2016; Caruana et al., 2016). Piceatannol or trans-3,3',4,5'tetrahydroxystilbene is a natural stilbene, type of polyphenolic flavonoid whose structure is similar (except producing an extra hydroxyl group) to resveratrol (Maruki-Uchida et al., 2018; Bavaresco et al., 2002). It is one of the important dietary stilbenes commonly found in red wine, berries, peanuts, grapes, and white tea (Dhanapal et al., 2018; Kil et al., 2017). The report presented by Zion market research (Global news wire Newyork, NY, May 14, 2018) on global Parkinson's disease therapeutics market was valued at approximately 2.36 US billion dollars and expected to generate revenue of around 4.31 US billion dollars by 2023 with growing CAGR of 10.6% between 2013-2017. With increasing demand of piceatannol also finding their application in nutraceuticals as well as in cosmeceutical market.

Use of microbial fermentation to produce valuable compounds on large scale appears as a plausible alternative which can make the process efficient, cost-effective as well as easy to manage (Tassoni et al., 2005). Hence scientists and researchers are exploring techniques which can mimic the phenyl propanoid pathway in microorganisms (Strobel et al., 2003; Suryanarayanan et al., 2009).

In last few decades endophytic fungi are renowned as natural ware house of novel bioactive compounds possessing antimicrobial, anti-cancer, neuroprotective and cardioprotective activities (Strobel et al., 2003; Suryanarayanan et al., 2009). Endophytic fungi are able to produce identical analog compound as their host. There are few reports where endophytic fungi are capable to produce stilbene compounds like resveratrol (Dwibedi et al., 2018; Liu et al., 2016); however there exist no reports on endophytic fungi able to produce piceatannol.

Hence the aim of present thesis was to screen the endophytic fungi of *Vitis vinifera* which are capable to produce piceatannol through microbial fermentation and their application in destabilizing the α-synuclein aggregation as well as anti-oxidant and anti–microbial activity.

2.0. REVIEW OF LITERATURE

2.1. Parkinson's Disease and Its Symptoms

Parkinson's disease (PD) is an age-related neurodegenerative disorder. It is the second most encountered disorder after Alzheimer's disease (Kalia and Lang, 2016). PD affects approximately 1–2% of the general population over the age of 65. It was first described by James Parkinson in 1817 (Parkinson et al., 1817). It is only 10% familial and 90% sporadic disease (Olanow et al., 2013). PD includes motor symptoms (tremor, rigidity, and unbalancing) and non-motor symptoms (hyposmia, constipation, depression, and sleep disorder), due to decrease in dopamine levels in synaptic terminals. The loss of dopaminergic neurons in the substantia nigra leads to the decrease of dopamine levels (Fig.2.1) (Cheng et al., 2010). The affected nerve cells contain Lewy bodies (intracytoplasmic inclusions). Lewy bodies are the important indicators of PD and few other similar diseases. Lewy bodies are mainly made up of α-synuclein fibrils (Spillantini et al., 1998).

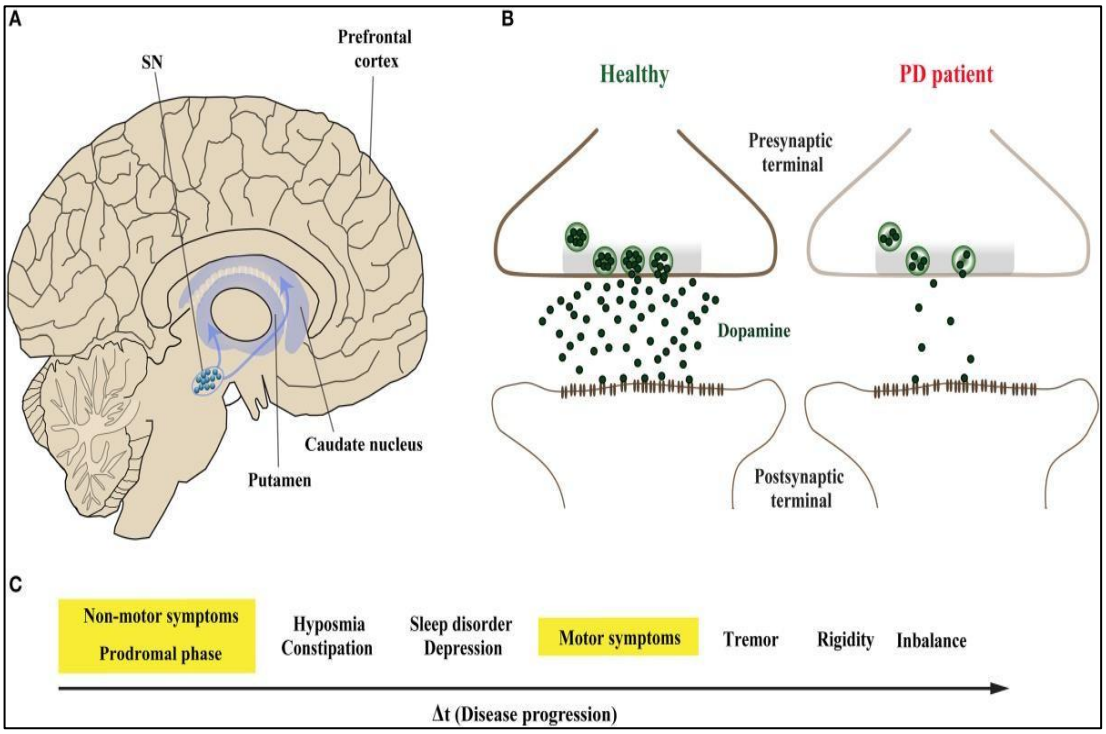

Fig.2.1: A) Shows the dopaminergic (DA) neurons in the substantia nigra (SN), B) Comparison between healthy controls (left) and striatal neurons (right), loss in dopaminergic neurons results in the loss of dopamine secretion (neurotransmitter) on synaptic terminals (right), C) The resulting motor symptoms and non-motors symptoms.
(Source:https://www.frontiersin.org/files/Articles/338212/fnins-12-00080-HTML/image_m/fnins-12-00080-g001.jpg)

2.2. α-Synuclein

α-Synuclein is a type of protein highly expressed in the brain and it is mainly found in the presynaptic terminal of the neurons (nerve cell), the small amount of α-Synuclein is also found in red blood cells, heart, muscles, and other tissues (Marques et al., 2012). α-Synuclein is made of 140 amino acids encoded by SNCA gene, these amino acids divided into three different regions: Amphipathic region (a positively charged lysine-rich N-terminal plays an important role in regulating its interactions with membranes), NAC domain (a central hydrophobic region which is highly capable to aggregate (Giasson et al., 2001)), and acidic tail (a highly acidic C-terminal domain involved in interactions with small molecules, and regulating nuclear localization (Bayer et al., 1999; Guardia-Laguarta et al., 2014)) (Fig.2.2).

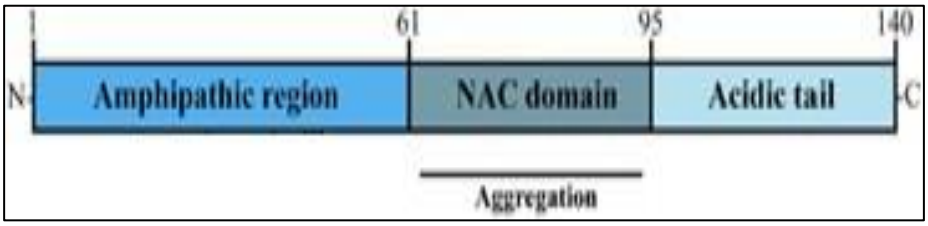

Fig.2.2: Shows the α-Synuclein protein domain structure.
(Source:https://www.frontiersin.org/files/Articles/338212/fnins-12-00080-HTML/image_m/fnins-12-00080-g002.jpg)

The normal function of native α-synuclein has remained unclear; many controversial reports are present, which predict that the α-synuclein has a significant role in the regulation of vesicle exocytosis and in the transmission of synaptic vesicles (Chandra et al., 2004). α-synuclein also work as a neuroprotectant due to its ability to bind to vesicles and membranes (Chandra et al., 2005).

2.3. α-Synuclein genetic mutations and modifications

Genetic modifications and mutations are responsible for α-Synuclein misfolding and aggregation in both familial and sporadic PD. Six missense mutations: p.A30P, p.E46K, p.A53T p.G51D, p.H50Q, p.A53E in α-Synuclein encoding SNCA gene on chromosome 4q21-q23 is responsible for familial PD (Pasanen et al., 2014; Lesage et al., 2013). These mutations in familial PD are very rare, only a few families were identified for each mutation. While the caspase-1 cleavage on ASP121 site induces sporadic PD, studies have shown that inflammasome is responsible for activating the

caspase-1 enzyme which farther leads to cut off ASP121 to 140 amino acids. Head trauma, brain infections, mitochondrial oxidative stress (rotenone etc), high cholesterol and lysosomal storage diseases are the risk factors which are responsible for activation of the inflammasome (Codolo et al., 2013; Wang et al., 2016).

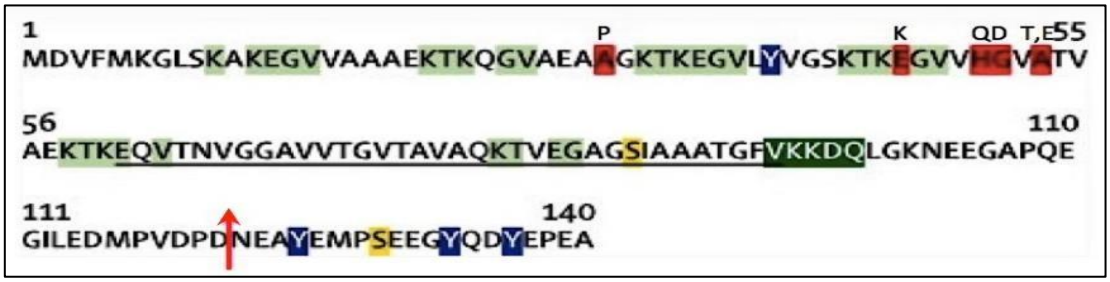

Fig.2.3: Amino acid structure of human α-synuclein (Dehay et al., 2015). Clinical Mutations (p.A30P, p.A53T, p.H50Q, p.E46K, p.A53E, p.G51D) are in red. Amphipathic N-terminal region marked in grey. Phosphorylation sites are colored in yellow. Central hydrophobic NAC region is underlined. Red arrow is caspase-1 cleavage site (ASP121). Nitration sites are colored in blue. CMA recognition sites are marked in green Neuropathological reports on autopsies of PD patients with p.A30P, p.A53T, p.G51D and p.E46K mutations and sporadic PD patient with caspase-1 cleavage on ASP121 described the level of dopamine due to the synucleinopathy (Lesage et al., 2013).

2.4. Mechanism of α-Synuclein aggregation and propagation

SNCA gene mutations and modifications enable the protein to change its structure upon interacting with different other biological components for e.g.: specific ligands, lipids, proteins or biological membranes (Ullman et al., 2011).

The confirmation unfolded α-Synuclein monomer, which furthur interact to form oligomers and ultimately enhance formation of small amyliod fibrils, finally inducing the formation of fibrils. The deposition of these fibrils further leads to formation of Lewy bodies (intracellular inclusions) (Dehay et al., 2015) (Fig.2.4).

In diseased conditions, α-synuclein oligomer can be secreted out of neurons by using different secretary mechanisms (Jang et al., 2010; Alvarez-Erviti et al., 2011). This further helps the toxic oligomers to penetrate into other neurons. Endocytosis, direct penetration, transynaptic dissemination, and membrane-receptor mediated access are the different mechanisms used for the transfer of toxic α-synuclein oligomers into other cells (Lee et al., 2008). After the successful penetration of toxic α-synuclein into other neuron it can further seed the toxic α-synuclein aggregation (Conway et al., 2000).

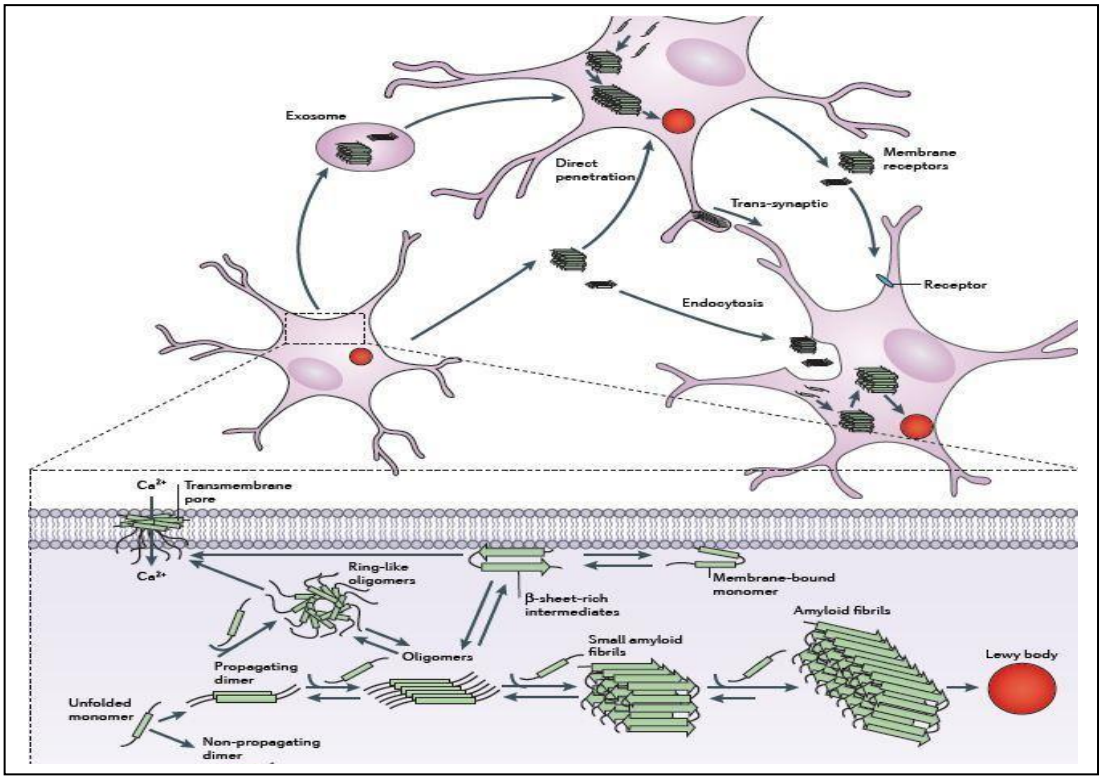

Fig.2.4: α-synuclein fibril aggregate formation from the monomeric form and transfer of toxic α-synuclein aggregate between nerve cells.
(Source:https://scienceofpd.files.wordpress.com/2015/12/mechanism-of-syunuclein-propagation- and-fibrillization.jpg)

2.5. Toxicity of α-synuclein

Studies have shown that conversion of α-synuclein monomer to fibrils contribute to α-synuclein toxicity, which plays an important role in neurodegeneration (Azeredo da Silveira et al., 2008). It was seen that different intermediates on the pathway to lewy body formation and mechanisms are involved in α-synuclein toxicity. α-synuclein oligomers are the most toxic species comparison to others (Winner et al., 2011). It plays an important role in the toxicity of neurons and glial cells by triggering lysosomal leakage, disrupting mitochondria or damaging microtubules (Dehay et al., 2015). Recently researchers found that toxic oligomers interrupt the synaptic proteins axonal transportation which results in synapses dysfunction and probably neurodegeneration (Scott et al., 2010). Moreover, the protofibrils can also alter the membrane and permeabilize nerve cells which results in calcium influx from the extracellular to intracellular space, finally results in cell death (Danzer et al., 2007). All these studies indicate that α-synuclein oligomers and fibrils are the key player of α-synuclein toxicity.

2.6. Strategies to resist α-synuclein toxicity

The aggregation of α-synuclein plays a major role in the pathology of PD. There are many different targets are available to prevent the α-synuclein toxicity. Fundamentally, out of which two main targets are: (1) Increasing protein clearance and (2) Targeting α-synuclein aggregation (Schapira et al., 2014).

2.6.1 Increasing protein clearance

The first move against α-synuclein toxicity is to decrease its expression by using two different strategies written below.

(i) Reducing the synthesis

Gene silencing approach can be used to reduce the α-synuclein synthesis. Silencing of SNCA gene using small hairpin RNA was reported in rodent models which results in degeneration and inflammation activation while no results are reported in monkey and squirrel (McCormack et al., 2010). Toxic RNA silencing is another possible approach which may result in endogenous RNAi saturation further leads to miRNA interference (Brundin et al., 2017).

(ii) Increasing the clearance

Another therapeutic possibility is to enhance the clearance process of α-synuclein aggregations by increasing the lysosomal or proteasomal activity of the nerve cell. ALP Mediated α-synuclein degradation mechanism is used under the pathological conditions and macroautophagy is activated by CMA (α-synuclein-mediated impairment) (Xilouri et al., 2009).

2.6.2. Targeting α-synuclein aggregation

α-synuclein aggregation inhibition is an attractive target for new drug development. These days' researchers are focusing more on α-synuclein disaggregation pathway which may protect the nerve cell from α-synuclein toxicity in *in-vitro* by decreasing the presence of oligomers (Auluck et al., 2002). Since oligomers are the most toxic species of α-synuclein, forming stable fibrils is another interesting strategy to prevent cell death. Strategy to identify α-synuclein aggregation inhibiting compound may also be used (Bieschke et al., 2010).

2.6.3. Additional strategies

The spreading property of α-synuclein explores the new targets, by better understanding of spreading mechanism we can develop new therapeutic strategies. Secretion and uptake of aggregated α-synuclein are two main components involved in α-synuclein spreading. Exosome- mediated transfer, endocytosis, receptor-dependent uptake, trans-synaptic dissemination and direct membrane penetration are the different pathways involved in cell to cell transfer of α- synuclein (Lashuel et al., 2013; Lee et al., 2006).

Our present study aims to produce piceatannol from endophytic fungi which can be used as a novel inhibitor and disruptor of α-synuclein.

Table.2.1: List of the treatments that prevent/delay PD.

Involvement	Drug	Efficacy conclusions	Safety	Implications for clinical practice	References
MAO-B inhibitors	Selegiline	Insufficient evidence	Acceptable risk with specialized Monitoring	Investigational	Rascol et al., 2016
	Rasagiline	Insufficient evidence		Investigational	
Levodopa/peripheral decarboxylase inhibitor	Standard IR Formulation	Insufficient evidence		Investigational	PD Med Collaborative Group, 2014; Pahwa et al., 2014
Supplements	Coenzyme Q10	Nonefficacious		Not useful	Storch et al., 2007; Beal et al., 2014
	Creatine	Nonefficacious		Not useful	Kieburtz et al., 2015
	Vitamin D	Insufficient evidence		Investigational	Suzuki et al., 2013

Dopamine agonists	Ropinirole	Insufficient evidence		Investigational	Schapira et al., 2013; Schapira et al., 2014
	Pramipexole	Nonefficacious		Not useful	
	Pergolide	Unlikely efficacious		Not useful	

(Note: MAO-B, monoamine oxidase B)

2.7. Natural products to inhibit α-synuclein aggregation

L-dihydroxy-phenyl alanine (L-dopa or Levodopa) is the center of attraction for Parkinson's pharmacophore therapy, but still all these drugs have many side effects, such as dyskinesia, motor fluctuations, and other non-motor complications (Chao et al., 2012; Bensalem et al., 2015). Because of these side effects many researchers are looking for an alternative compound which can target a single molecule or pathway, these include conventional drugs such as natural products to target multiple pathways (Lashuel et al., 2013; Xu et al., 2002; Rocha et al., 2018).

Among them, dietary polyphenols have gained tremendous attention because of their low toxicity and potential in human health (Nagao et al., 1999; Wang et al., 2015). Studies have been indicated that natural phenols/polyphenols tend to destabilize the α-synuclein by forming soluble, non-cytotoxic, oligomeric complexes with the α-Synuclein protein. These promising agents include resveratrol, piceatannol, and ampelopsin A. The study shown that piceatannol have highest inhibitory activity against α-synuclein aggregation, while other stilbenes were less active (Temsamani et al., 2016). The mechanisms of action of these phenols against α- synuclein aggregation are still unknown.

2.8. Piceatannol

Piceatannol or trans-3,3',4,5'tetrahydroxy is a naturally occurring stilbene (Fig.2.5), a type of polyphenolic flavonoid whose structure is similar (except producing an extra hydroxyl group) to resveratrol (Maruki-Uchida et al.,2018; bavaresco et al., 2002). It is one of the important dietary stilbene which is normally present in berries, red wine, white tea, peanuts, and grapes (Dhanapal et al., 2018; Kil et al., 2017). Piceatannol is

less studied than resveratrol although it shows a variety of physiological activities of therapeutic benefit (Heo et al., 2017; Piotrowska et al., 2012). This makes piceatannol a highly demanded therapeutic compound.

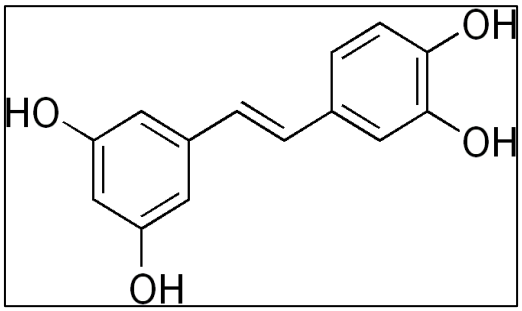

Fig.2.5: Chemical structure of piceatannol.
(Source:https://upload.wikimedia.org/wikipedia/commons/8/8e/Piceatannol.png)

Piceatannol is first isolated from the *Euphorbia lagascae* seeds as an antileukemic factor (Ku et al., 2005). It has been found in several plants, *Vitis vinifera* (grapes), *Polygonum cuspidatum* (Japanese knotweed), *Arachis hypogaea* (groundnuts), and *Vaccinium* (cranberries) but normally in very small quantity (Heo et al., 2017) (Piotrowska et al., 2012). Passion fruit seed is known as a richest source of piceatannol. It is produced being phytoalexins against the respond to the fungal attack or another environmental stress inside some plants. Unlike resveratrol, piceatannol is considerably less accessible because of the low levels exhibit in plants (Ku et al., 2005).

The report presented by Zion market research (Global news wire Newyork, NY, May 14, 2018) on global Parkinson's disease therapeutics market was valued at approximately 2.36 US billion dollars and expected to generate revenue of around 4.31 US billion dollars by 2023 with growing CGAR of 10.6% between 2017-2013. With increasing demand of piceatannol, it is also finding their application in nutraceuticals as well as in cosmeceutical market, which made piceatannol an extremely required compound (Heo et al., 2017). The yield of piceatannol from the plant is very low and time-consuming, which contribute to its higher prices. However, this method can't meet the increasing demand of piceatannol in the market. Hence, there is an urgent need for exploration and development of a new approach for commercial production of piceatannol (Ku et al., 2005).

2.9. Microbial fermentation

Microbial fermentation has been used for the manufacturing of many different valuable products, for example, organic acids, antibiotics, amino acids, and vitamins commercially. The benefit of utilizing these microbes for large-scale production is the efficiency and cost- effectiveness, as well as easy to manage (Heo et al., 2017; Tassoni A et al., 2005). Therefore, researchers are investigating techniques of mimicking the piceatannol biosynthetic pathways in microorganisms or optimizing plant cell cultures (Ku et al., 2005; Heo et al., 2017; Strobel G et al., 2003; Suryanarayanan T S et al., 2009). The different encouraging approach is an investigation of the microorganisms which may mimic the production of piceatannol below free-fermenting situation. It has been already proven that endophytic fungi have the capacity to producing some functional compounds as their host (Strobel G et al., 2003) (Suryanarayanan T S et al., 2009). There are few reports where endophytic fungi are capable to produce stilbene compounds like resveratrol (Dwibedi et al., 2018; Liu et al., 2016), however there exist no reports on endophytic fungi able to produce piceatannol.

2.10. Endophytic fungi

The endophytic fungi reside within the plant tissue without showing the symptoms of their existence and are present virtually in all parts of the plant. These obtain nutrients from the host plant and contribute positively to the host fitness and are thus known as mutual symbionts (Sadrati etal., 2013). It is said to be an endophyte if it lives within a plant for at least part of its life cycle without causing any apparent disease. The endophytic fungi have gained the attention of scientists to explore them as the potential producers of novel and bioactive compounds (Strobel et al., 2003). Over past two decades, different valuable bioactive compounds possessing insecticidal, antimicrobial, cytotoxic and anti-cancer activities have been discovered from endophytic fungi. These bioactive compounds can be classified mainly as steroids, alkaloids, terpenoids, isocoumarins, quinones, phenylpropanoids, lignans, lactones and phenols (Zhang et al., 2006; Xu et al., 2008). It was hypothesized that endophytic fungus belongs to *Vitis vinifera* are capable of producing piceatannol in free fermentative condition.

2.11. *Vitis vinifera*

Grapes (*Vitis vinifera*) are known as world's largest non-climatic fruit crop. The annual production of *Vitis vinifera* is approximately 58 million metric tonnes (Book, 1997). It is the richest source of phenolic compounds such as stilbene, flavonoid and phenolic acid; these polyphenoles are present in every part of *Vitis vinifera*: skin, flesh, stem, leaf and seeds (Xia et al., 2010). The types, actions and amount of phenolic compound present in grapes are dependent on the environmental factors (Ojeda et al., 2002). Researchers found that grape polyphenoles helps to prevent inflammatory and cardiovascular diseases. It also helps to prevent or slow down the cell damage caused by oxidative stress and decreasing the oxidation of low density lipoprotein cholesterol through their antioxidant effects (Stein et al., 1999; Sovak et al., 2001). Studies investigation showed that the French people tend to have less heart disease than others due to their regular consumption of wine also known as "French paradox" (Renaud et al., 1992). *Vitis vinefera* a rich source of polyphenols can therefore be utilized as a great therapeutic agent.

3.0. AIM OF THE STUDY

- Screening of endophytic fungi for the production of piceatannol.
- Qualitative and quantitative analysis of piceatannol.
- Identification of selected endophytic fungi.

4.0. MATERIAL AND METHOD

4.1. Procurement, maintenance and preservation of endophytic fungi

50 cultures were obtained from the repository of endophytes maintained by Prof. Sanjai Saxena, Thapar Institute of Engineering and Technology, Patiala. The procured cultures were inoculated on Potato Dextrose Agar (PDA; Hi-Media, India) plates and incubated at 28˚C and constantly observed for the uniform fungal growth. The fungal hyphae appearing out of the inoculated sample was taken by inoculation loop and sub-cultured on to new PDA (Potato dextrose agar) plate. These were then aseptically transferred to PDA slants containing 10% glycerol for long-term storage.

4.2. Production of culture filtrates

The 5 mm mycelial disc of 7-8 days old endophytic fungi was inoculated in 50 ml of pre- sterilized Potato Dextrose Broth (PDB) medium in 250 ml Erlenmeyer flasks. The flasks were incubated in incubator shaker at 28±2˚C, 120 rpm for 7-8 days. After incubation was over the fungal biomass was separated by using Whatman filter paper 4 followed by centrifugation at 10,000 rpm for 10 min. The supernatant was passed through 0.2-µm nitrocellulose membrane to obtain cell-free filtrate/spent broth (Rodrigues et al., 2000; Vicente et al., 2001).

4.3. Screening of piceatannol producing endophytic fungi

The endophytic fungi culture filtrates were screened for piceatannol production using three biochemical assays namely (i) Liebermann test (ii) Acetic anhydride test and (iii) Chromogenic test. Piceatannol is a kind of polyphenol belonging to a flavonoid family owing a stilbene kind structure and chemically holds a free para position like phenols and stilbene which produces green/blue-violet color via adding of sodium nitrite and greenish-brown precipitate after reaction with acetic anhydride (Shi et al., 2012).

4.3.1. Liebermann test

This test is known to detect the presence of free para position in phenolic compounds. Briefly, culture filtrate and concentrated sulfuric acid (Sigma Aldrich,

USA; 339741) were mixed in equal ratios followed by addition of two to three drops of 1% sodium nitrate (Hi-Media, India; GRM1722). The appearance of a green/blue-violet color indicates the presence of phenolic compound (Al-Jumaily et al., 2014). Piceatannol served as the positive control, while uninoculated PDB and methanol (Hi-Media; AS061) were used as negative control.

4.3.2. Acetic anhydride test

This test detects the free –OH group in the phenolic compounds. Briefly, the reaction was initiated by the addition of 100 µl of acetic anhydride (Sigma Aldrich; 320102) and 500 µl of concentrated sulfuric acid to 100 µl of test compound and then observed for appearance of greenish brown precipitate formation which indicated the presence of free –OH phenolic compounds in the test sample (Al-Jumaily et al., 2014). Piceatannol (1 mg/ml) served as the positive control, while un-inoculated PDB and methanol were used as negative control.

4.3.3. Chromogenic reaction

This test is known to detect the presence of phenolic compounds by using ferric chloride- potassium ferricyanide color reaction, the reaction was begun via the addition of 2 ml of MeOH and 3-4 drops of chromogenic agent (0.1% $FeCl_3$: 0.1% $K_3[Fe(CN)_6]$ = 1:1 (v/v)) into 2 ml of test sample and then observed for appearance of blue color indicating the presence of phenolic compounds in the test sample (Al-Jumaily et al., 2014). Piceatannol (1 mg/ml) served as the positive control, while un-inoculated PDB and methanol were used as negative control.

4.4. Solvent extraction

The cultures exhibiting positive Liebermann, Acetic anhydride and Chromogenic tests were subjected to Liquid-liquid extraction using ethyl acetate. The culture filtrate and ethyl acetate were taken up in a ratio of 1:3 and extracted three times. The organic layers was pooled and dehydrated by the addition of anhydrous sodium sulfate. Subsequently, the solvent fraction was evaporated in a rotary evaporator (DLAB RE 100-Pro, China) at 30 °C to dryness to get the crude fraction (Kjer et al., 2010). The fraction so obtained was weighed, reconstituted in methanol, and again screened for the presence of phenolics/piceatannol by Liebermann, Acetic anhydride

and Chromogenic assays as described previously. Crude fractions which exhibited positive Liebermann test and Acetic acid tests were subjected to TLC separation to confirm the presence of piceatannol.

4.5. TLC analysis

The crude bioactive fraction of the cultures exhibiting piceatannol production was fractionated by subjecting to preparative thin layer chromatography (TLC). The TLC plate of 0.5 mm thickness was prepared by coating silica gel on to 20 x 15 x 5 mm clean glass plates and was activated by incubating at 100ºC for 2 h prior to use. The sample was spotted 1 cm above the edge of activated TLC plate with the help of capillary tube. Simultaneously, the TLC plate was immersed in TLC chamber. The different solvent systems (Binary and tertiary) consisting of the mixture of solvents of different polarities and ratios were used for separation. The TLC plate was kept inside the pre-immersed TLC chamber in such a manner that sample spot is above the solvent level. After the solvent rise up to the 75% of the TLC plate surface, the plate was taken out and left to air dry. The chromatogram was visualized by TLC plate under UV light. Piceatannol was used as a standard for the comparison of retention factor (Rf) value. (Rf) value of individual band was taken via the ratio of distance traveled by solute to that of solvent (Park et al., 2013; Babu et al., 2005).

$$\mathrm{Rf} = \frac{\text{Distance moved by solute}}{\text{Distance moved by solvent}}$$

4.6. High-performance liquid chromatography

The concentration of selected crude isolate was determined by HPLC (Perkin Elmer-200 series pump). 50 µg of the crude isolate was dissolved in methanol and injected into HPLC column. Isocratic mode of elution of orthophosphoric acid (0.1%, pH 2.3; Sigma Aldrich) and acetonitrile, 45%-55% ratio was used as mobile phase with a flow rate of 1 ml/min. To determine the concentration of fungal Piceatannol, different dilutions of standard Piceatannol (Stock- 1 mg/ml, Sigma Aldrich) ranging from 0.025 - 0.6 mg/ml were prepared in HPLC grade Methanol. 20 µl of each dilution was injected into C18 (5 µm) reverse phase Discovery column (Sigma Aldrich) with 4.6 mm Internal Diameter x 150 mm Length. The data of the peak area vs. concentration

of the standard piceatannol obtained were used to estimate the quantity of fungal piceatannol in crude ethyl acetate fraction (Dwibedi et al., 2018; Shi et al., 2012).

4.7. LC-MS analysis

The extracts (Stock-1mg/ml) in methanol (Merck, HPLC grade) were analyzed in LC-MS system (UPLC-WATERS, MASS-XEVO G2XS QTOF-WATES) equipped with an electrospray ionization source (ESI). For detection of piceatannol, the negative ion mode (m/z- H^-) was used. Mass scan range was from 100 to 700 m/z. 1.0µl of each sample was injected into Acquity BEH C18 1.7µ (2.1*50mm) column. Isocratic mode of elution of orthophosphoric acid (0.1%, pH 2.3; Sigma Aldrich) and acetonitrile, 45%-55% ratio was used as mobile phase with a flow rate of 1 ml/min and the column temperature was maintained at 35˚C with mass spectroscopic conditions of Capillary voltage-3kV, cone voltage-30, source temperature-120°C, desolvation temperature-400˚C, Cone gas-60L/hr, desolvation gas-800L/hr (Bavaresco et al., 2002).

4.8. FT-IR analysis:

The FTIR absorption spectroscopy of ethyl acetate extract (1 mg/ml in methanol) was performed by using instrument FTIR (AGILENT CARY-600 Series). All spectra are taken via the ATR method. The temperature of the instrument was maintained below 25˚C. Firstly, the FTIR of solvent i.e., methanol was done to obtain the baseline for the sample spectrum followed by both the standard and sample to be analyzed (Jiang et al., 2008).

4.9. Identification of Endophytic Fungi

The selected isolate showing piceatannol production was identified by classical and molecular taxonomy

4.9.1. Morphotaxonomy of Piceatannol-Producing Endophytic Fungus

For morphotaxonomic studies, the endophytic fungus producing piceatannol has inoculated on different nutrient media viz. Potato dextrose agar (PDA (5.4pH)), Pine leaf agar (PLA), Potato dextrose agar (PDA (7.0pH)), and Water agar (WA), Synthetic nutrient-deficient agar (SNA), Fusarium minimal agar (FMA) for 10 days at 26 ± 2°C

including 12 h of photoperiod. Growth rate and features, including its microscopic characteristics like hypha features, conidia formation, and another cellular bodies such as fruiting bodies (asexual or sexual spores) or structures, were critically observed under a Nikon stereo zoom microscope. All the micrometric observations were carried out using the Nikon NIS element software by taking at least 30-40 observations per structure (Shi et al., 2012; Wang et al., 2016).

4.9.2. Molecular identification of endophytic fungi

4.9.2.1. DNA isolation

The fungal genomic DNA was isolated from 5-7 days old culture grown on PDA plate using Wizard® Genomic DNA purification kit (Promega, USA). The 3-4 mycelial plugs of 5 mm diameter were crushed in liquid nitrogen using sterile pestle and mortar. 1 ml of cell lysis solution was added followed by addition of 660- 750 µl of Nuclei lysis buffer and was again crushed. The contents were shifted to a 2.0 ml microcentrifuge tube and vortexed followed by incubation at 65°C in water bath for 15 min. After the incubation is over, the microcentrifuge tubes were centrifuged for 5 min at 12,000 rpm to eliminate cell debris. Further, 5 µl of RNase was added to each tube and incubated for 15 min at 37°C followed by addition of 200 µl of protein precipitation solution. After this, the micro centrifuge tubes were centrifuged for 3 minutes at 12,000 rpm to eliminate protein contamination. The aqueous phase containing DNA was transferred and to it chilled isopropanol was added and centrifuged for 3 min at 13,000 rpm. The pellet of DNA was rinsed using 70% ethanol and centrifuged for 1 min at 13,000 rpm. The pellet was air-dried and suspended in 50 µl of DNA dehydration buffer (Tris EDTA buffer (pH 8)). The qualitative estimation of the DNA isolated was done by agarose gel electrophoresis (Dwibedi et al., 2018; White et al., 1990).

4.9.2.2. Agarose gel electrophoresis

1X TAE (Tris Acetate EDTA) buffer was used to develop 0.8% agarose gel including 0.5 µg/ml of ethidium bromide (EtBr) and then the gel was casted in the electrophoretic apparatus. The gel was left to harden and the comb was carefully taken out. The running buffer (1X TAE) was poured in the electrophoretic tank. The

DNA samples and the 6X loading dye were loaded into wells and allowed to run at 80V for 1 hr. The DNA fragments were visualized under UV trans-illuminator. Gel imaging was done using UV light in Bio-Rad Gel documentation system using Quantity-1-D analysis software. Quantitative estimation of the genomic DNA was done by spectrophotometric analysis of the sample. The absorbance of the individual sample was obtained at 260 nm and 280 nm to confirm the concentration and purity of the DNA. 1 OD is equivalent to 50µg/ml DNA sample. The concentration of the DNA sample was determined by applying following formula:

Concentration (µg/ml) = O.D 260nm x 50µg/ml x Dilution factor

The purity of the DNA sample was obtained by using the ratio of absorbance at 260 nm and at 280 nm. If the ratio is less than 1.6, then there is RNA contamination, if the ratio lies between 1.6-1.8, DNA sample is in purified form. If the ratio is higher than 1.8, the DNA might be contaminated with protein (Dalawai et al., 2017).

4.9.2.3. PCR amplification

Amplification of ITS1-5.8S-ITS2 rDNA sequence was done using universal primer pair i.e. ITS 1 and ITS 4, synthesized by Integrated DNA Technologies (IDT), USA, in a Thermocycler (My Cycler, Bio-Rad Laboratories, Inc.). The amplification reaction was carried out by using the primers ITS1 (5′ TCC GTA GGT GAA CCT GCG G 3′) AND ITS 4 (5′ TCC TCC GCT TAT TGA TAT GC 3′) (White et al., 1990). Amplification was achieved in the 25µl reaction mixture including 25 ng of fungal DNA, 0.8 µM of both ITS1 and ITS4 primers, 2.5mM of dNTP (Bangalore GeNei), 1.5 U of Taq DNA Polymerase (Bangalore GeNei), 1.5 mM MgCl2 (Bangalore GeNei) into 10 X Taq buffer (Bangalore GeNei). The Thermal cycling conditions include primary denaturation for 5 minutes at 96ºC supported by 30 cycles of 95ºC for 1 minutes, 58ºC for 1.30 minutes, 72ºC for 1 minutes supported by the final extension at 72ºC for 5 minutes. The PCR amplicons were examined by utilizing gel electrophoresis i.e. 1.5 % agarose gel at 80 V for 1 hr. Gel imaging was done using UV light inside Bio-Rad Gel Documentation System. An estimated 550- 600bp PCR amplicon was purified by using the Wizard® SV Gel and PCR clean-up system kit (Promega, USA)

(Dwibedi et al., 2018; Shearin et al., 2018). The purified amplicon was sent for sequencing to Genei Laboratories, Bangalore.

Table 4.1: Different reagents used during PCR reaction

S.no	Reagents	Stock Concentration	Quantity	Final concentration /25µl
1.	Autoclaved double distilled Water	_	15µl	_
2.	Taq buffer	10 X	2.5µl	1X
3.	dNTPs	2.5mM	2.0µl	0.2mM
4.	Forward Primer (ITS1)	10µM	2.0µl	0.8 µM
5.	Reverse Primer (ITS4)	10µM	2.0µl	0.8 µM
6.	Taq DNA Polymerase	3U/µl	1.0 µl	2.5 U
7.	Template DNA	25 ng/µl	1 µl	25ng

4.9.2.4. Sequence assembly and Phylogenetic identification

The received chromatograms were manually fixed and the conclusive sequence was submitted in the GenBank. The homology of the isolate was proved via subjecting the final sequence to sequence similarity search using BLAST. The final sequence along 15 reference taxa was aligned by utilizing CLUSTAL W in MEGA 5.0 (Tamura et al., 2011). An evolutionary relationship was understood by using Maximum Parsimony method. The Close-Neighbor- Interchange algorithm was utilized to achieve Maximum Parsimony tree including search level 3 in which the primary trees were obtained with the arbitrary interest of sequences (20 replicates). The tree was represented to scale; with branch distances evaluated using the average pathway method (Nei and Kumar., 2000) and are in the units of the number of variations over the complete sequence. Gaps were considered as missing data. 1000 bootstrap replicates were taken to infer evolutionarily relationship (Felsenstein., 1985).

4.10. Biological activity

4.10.1. DPPH scavenging assay

The antioxidant activity was calculated using 2, 2-diphenyl-1-picrylhydrazyl scavenging assay (Kitts et al., 2000). The extracts were diluted to concentrations ranging from 10-50 µg/ml and same concentration range was used for piceatannol used as a standard.1 ml of freshly prepared 100 µM DPPH solution was mixed with 50 µl of the fungal extract. Pure methanol and working 100 µM DPPH were taken as blank and control respectively. The reaction mixture was incubated at room temperature in dark for 30 minutes. Piceatannol (10-50 µg/ml) was used as standard. ELISA reader was used to measuring the absorbance at 517 nm. The antioxidant activity was represented as µg of piceatannol per mg of extract. Percentage free radical scavenging (%FRS) at different concentration was calculated using the formula:

$$\%\text{FRS} = \frac{\text{Absorbance (Control)} - \text{Absorbance(Sample)}}{\text{Absorbance(Control)}} \text{x}100$$

A linear regression was plotted between the different concentrations and their respective %FRS which was then used to calculate the IC_{50} (Popli et al., 2018).

4.10.2. Trolox equivalent antioxidant capacity assay (TEAC)

TEAC assay uses the capacity of an antioxidant to scavenge ABTS to determine the level of its activity (Re et al., 1999). The ABTS free radical was produced by mixing 7mM ABTS (2, 2'- azino-bis-3ethylbenzothiazoline 6-sulphonic acid) prepared into 0.1 M PBS of pH-7.4 with 2.45 mM potassium persulphate in the same volume followed by 16-hour incubation at room temperature in dark. This ABTS free radical was diluted in Phosphate-buffered saline (PBS) to an absorbance of 0.9-1.0. Then 10µl of the extract was added to 1ml of working ABTS solution and the reaction mixture was incubated for 6 minutes. The decrease in absorbance was noted at 734nm. Phosphate buffer saline was taken as blank and ABTS was utilized as the control. Piceatannol (50-250 µg/ml) was used as the standard. Free radical scavenging (%FRS) at different concentration was calculated using the formula:

$$\%\text{FRS} = \frac{\text{Absorbance (Control)} - \text{Absorbance(Sample)}}{\text{Absorbance(Control)}} \text{x}100$$

A linear regression was plotted between the different concentrations and their respective %FRS which was then used to calculate the IC50

4.10.3. Total phenolic content assay (TPC)

Folin-Ciocalteu (FC) reagent (Sriplang et al., 2007) was used to determine the total phenolic content. To 150 µl of 1mg/ml extract, 2.25 ml of deionized water and 150 µl of FC reagent were added. The reaction mixture was incubated at room temperature for 10 minutes before adding 300 µl of 5% (w/v) sodium carbonate and incubated it for another hour at room temperature. Piceatannol (10-50 µg/ml) was used as standard and absorbance was noted at 760 nm. A linear regression was plotted between the concentrations of standard and their absorbance using which the concentration of the sample was calculated. The total phenolic content was represented as µg of piceatannol equivalent per mg of extract.

4.10.4. Total flavonoid content assay (TFC)

The TFC was discovered by Luximon Ramma et al., 2002. 800 µl of deionized water and 60 µl of 5% (w/v) sodium nitrate were added to 200 µl of 1mg/ml extract. The reaction mixture was incubated at room temperature for 5 minutes. Then 60 µl of 10% (w/v) aluminum chloride and 400 µl of 1N sodium hydroxide were added to the reaction mixture. The complete volume was brought to 2ml using deionized water. Absorbance was noted at 510 nm and piceatannol (10-50 µg/ml) was used as a standard. A linear regression was plotted between the concentrations of standard and their absorbance using which the concentration of the sample was calculated. The TFC was expressed as µg of piceatannol per mg of extract.

4.11. DNA nicking Assay

The ability of fungal ethyl acetate extract to protect lambda DNA from oxidative damage produced by Fenton's reagent was estimated by DNA nicking assay. The reaction was performed in an Eppendorf tube containing 15 μL of lambda DNA (0.5 μg) with and without fungal extract (2μl of 1mg/ml) in Fenton reagent (3 μl of 2mM Feso4, 3 μl of 30% H_2O_2 in Tris buffer 10 mM) and the final reaction volume of

mixture was brought up to 30μl using TE buffer also used as a control. The reaction mixtures were allowed to incubate for 30 min at 37˚C. After that 1 μl of 0.5 mM EDTA (pH 8.0) was added to stop the reaction. The relative difference between oxidized and native DNA was analyzed by using gel electrophoresis. 2 μl bromophenol blue dye was added in 8 μl of the reaction mixture. 10 μL reaction mixtures were loaded in 1% agarose gel prepared in Tris-acetate-EDTA buffer (pH-8.5) followed by ethidium bromide staining. The gel was run at 80 V for 1 hour at room temperature and after that band intensity was documented using XR+ molecular imager gel documentation system (Bio-RAD, USA) (Danagoudar et al., 2017; Golla et al., 2014).

4.12. *Invitro* antimicrobial assay

The *invitro* antimicrobial assay was performed using agar well diffusion method. The endophytic fungi were screened for its antimicrobial potential against a panel of test microorganisms comprising of, 3 gram positive bacteria viz. MTCC 96 (*Staphylococcus aureus*), NCTC 6571 (*Staphylococcus aureus*), MTCC 441 (*Bacillus subtilis*) and 1 gram-negative bacteria MTCC 647 (*Pseudomonas aeruginosa*), other 2 are fungi ATCC 227 (*Candida albican*) and MTCC 3011(*Candida albican*). Test organisms were obtained from a pre-existing depository maintained by Dr. Sanjai Saxena, Professor, Thapar Institute of Engineering and Technology, Patiala. The cultures were revived by inoculating them in Muller Hinton Broth (MHB; Hi-Media, India) one day before performing the assay. The test microorganisms were diluted in 0.9% w/v saline and visually adjusted with 0.5 McFarland solutions to achieve 106 CFU/ml. The test organism was then spread on the Muller Hinton Agar (MHA, Hi-Media, India) for anti-bacterial assay and Saboraud's Dextrose Agar (SDA) for anti-candidal activity with the help of cotton swab. Subsequently, 5mm wells were scooped out with sterile cork-borer on pre-made MHA plates and 30 µl of the sample was poured in each well. The inoculated plates were incubated at 37˚C for 16-24 hr. Streptomycin (1mg/ml; Hi-Media, India) was used as the positive control and the un-inoculated PDB as the negative control. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organisms. All the tests were performed in triplicate and the results expressed as mean ± SD (Baris et al., 2006).

5.0. RESULTS

5.1. Sub-culturing of endophytic isolates

In the present study a total of 50 endophytic fungi were procured from existing repository of Dr. Sanjai saxena, DBT, TIET Patiala, and screened for piceatannol production. All of the isolates of endophytic fungi were preserved in PDA-glycerol slants for long-term preservation. The endophytic fungi isolates in this investigation were isolated from Vitaceae (*Vitis vinifera*), which is a medicinally valuable plant, obtained from the biodiversity hotspots of India. During the centuries, humans have constantly relied on the plants for the treatment of numerous disorders such as diabetes, cancer etc. During the earlier two decades, several important unique bioactive compounds including anti-aging, anti-microbial, and anticancer characteristics have been discovered from the endophytic fungi. Throughout the prolonged duration of co-evolution, a symbiotic relationship was formed within endophyte and its host plant. Few of the endophytes have the capability to mimic the character of the host plants and are able to produce the identical or related bioactive compounds as those obtained from their host plants. Therefore the existing investigation involves the exploitation of endophytic fungi isolated from the therapeutic plant for the production of novel bioactive compounds. All of the 50 endophytic fungi were isolated from different parts of *Vitis vinifera***-** 45 were isolated from leaf, 5 were from the stem.

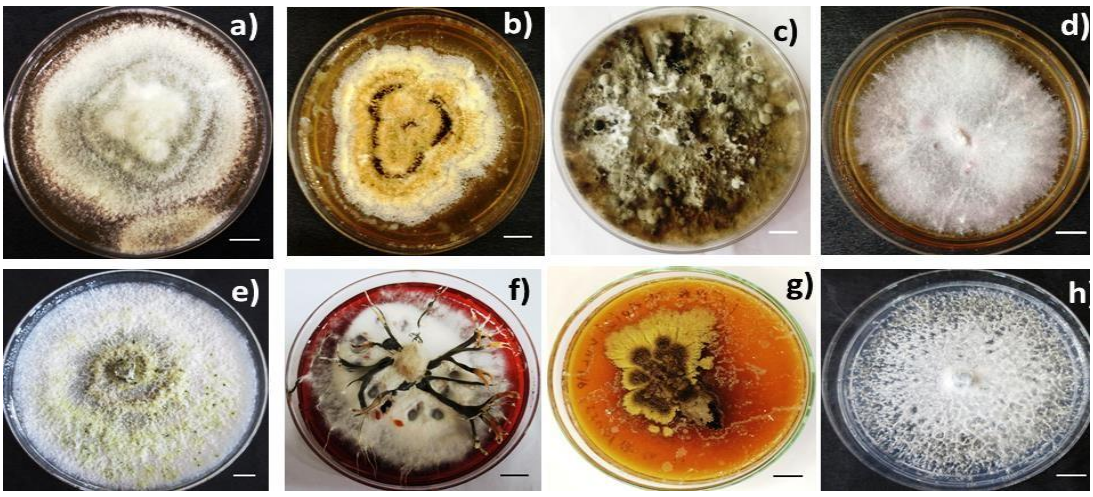

Fig. 5.1: Pure cultures of different endophytic fungi procured from lab a) *Fusarium* sp.; b) Unidentified.; c) *Nigrospora* sp.; d) *Alternaria* sp.; e) Unidentified.; f) *Xylaria* sp.; g) *Xylaria* sp.; h) *Fusarium* sp (Bar : 10mm).

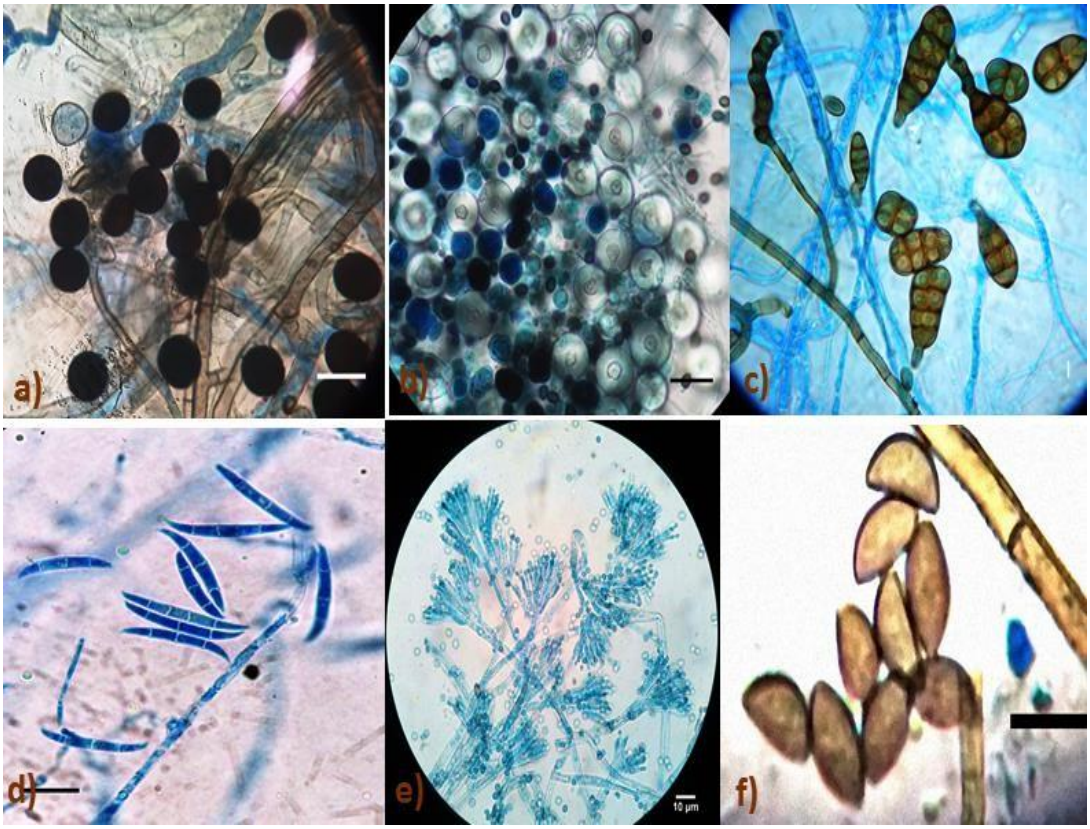

Fig.5.2: Microscopic feature of endophytic fungi screened during the study a) *Nigrospora* sp.; b) *Aspergillus* sp.; c) *Alternaraia* sp.; d) *Fusarium* sp.; e) *Penicillium* sp.; f) *Arcopilus* sp. (Bar : 10µm).

Table 5.1: List of some endophytic fungi screened during the study.

Culture Code	Plant Part	Location	Culture identification (Tentative identification)	Biochemical screening		
				Acetic Anhydride Test	Chromogenic reaction	Liebermann Test
#12VVLPM	Leaf	Pune, Maharashtra	*Arcopilus sp.*	++	+++	+++
#10(B)VVLPM	Leaf		*Fusarium sp.*	+++	+++	+++
#16(B)VVLPM	Leaf		*Fusarium sp.*	+	++	+
#18VVLPM	Leaf		*Botryosphaeria sp.*	-	-	-
# 19(P) VVLPM	Leaf		*Epicoccum sp.*	-	-	-
# 20(b) VVLPM	Leaf		*Botryosphaeria sp.*	++	+++	++
# 27VVLPM	Leaf		*Pestalotiopsis sp.*	-	-	-
#15(P)VVLPM	Leaf		*Xylaria_sp.*	-	-	-
#21VVLPM	Leaf		*Aspergillus sp.*	++	++	++
#18(P)VVLPM	Leaf		*Aurobasidium sp.*	-	-	-
#42VVLPM	Leaf		*Fusarium sp.*	++	+++	++
#36(P)VVLPM	Leaf		*Aspergillus sp.*	++	+++	+
#10(P)VVLPM	Leaf		*Pheoacremonium_sp.*	-	-	-
#19VVLPM	Leaf		*Fusarium sp.*	-	-	-
#22(P)VVLPM	Leaf		*Xylaria sp.*	++	+++	+++
#19VVSPM	Stem		*Alternaria sp.*	-	-	-
#17(B)VVLPM	Leaf		Unidentified	-	-	-
#11VVSPM	Stem		*Aspergillus sp.*	-	-	-
#10VVLPM	Leaf		*Lasiodiplodia sp.*	-	-	-
#31VVLPM	Leaf		Unidentified	-	-	-
#29VVLPM	Leaf		*Pestalotiopsis sp.*	-	-	-
#27(C)VVLPM	Leaf		*Pheoacremonium_sp.*	-	-	-
#1VVLPM	Leaf		Unidentified	+	++	++
#12(D)VVLPM	Leaf		*Aspergillus sp.*	+	++	++
#3VVLPM	Leaf		*Nigrospora sp.*	-	-	-
#22VVLPM	Leaf		Unidentified	-	-	-

#35VVLPM	Leaf		*Aspergillus sp.*	+	++	++
#101(C)VVLPM	Leaf		*Fusarium sp.*	-	-	-
#8(B)VVLPM	Leaf		*Nigrospora sp.*	-	-	-
#139(P)VVLPM	Leaf		*Pestalotiopsis sp.*	+++	+++	++
#59(P)VVLPM	Leaf		*Aspergillus sp.*	++	++	+
#107(P)VVLPM	Leaf		*Alternaria sp.*	+	++	++
#30(B)VVLPM	Leaf		*Pheoacremonium_sp.*	++	++	++
#176VVSSWN	Stem	Nashik, Maharashtra	*Epicoccocum_sp.*	-	-	-
#591VVLSWN	Leaf		*Botryosphaeria_sp.*	++	+	++
#4(P)VVLNM	Leaf		*Fusarium sp.*	++	++	+
#83VVLNM	Leaf		*Aspergillus sp.*	-	-	-
#109VVLNM	Leaf		*Alternaria sp.*	+++	+++	++
#105VVLNM	Leaf		*Aspergillus sp.*	-	-	-
#671VVLSWN	Leaf		*Botryosphaeria_sp.*	-	-	-
#10(B)VVLNM	Leaf		*Fusarium sp.*	-	-	-
#10(A)VVLNM	Leaf		*Phomosis* sp.	-	-	-
#50(D)VVLLK	Leaf	Alambagh, Lucknow	*Fusarium sp.*	+	++	++
#14VVRLLK	Leaf		Unidentified	-	-	-
#23(D)VVLLK	Leaf		Unidentified	-	-	-
#1(D)VVLLK	Leaf		Unidentified	-	-	-
#9(B)VVLLK	Leaf		*Fusarium sp.*	-	-	-
# 1VVRLLK	Leaf		Unidentified	++	+	+
# 1VVRSTL	Stem		*Lasiodiplodia sp.*	-	-	-
# 8VVGSTL	Stem		Unidentified	+++	+++	+++

Note: (+)moderate (50-65%); (++) good (80-90%); (+++) very high (>90%) and (-) no phenolic content.

5.2. Preservation of endophytic fungi

The isolated endophytes were aseptically kept in the PDA slants and vials (Fig.5.3) including 10% glycerol for storing them for a long time as it is not feasible to save cultures in plates for a long-term duration.

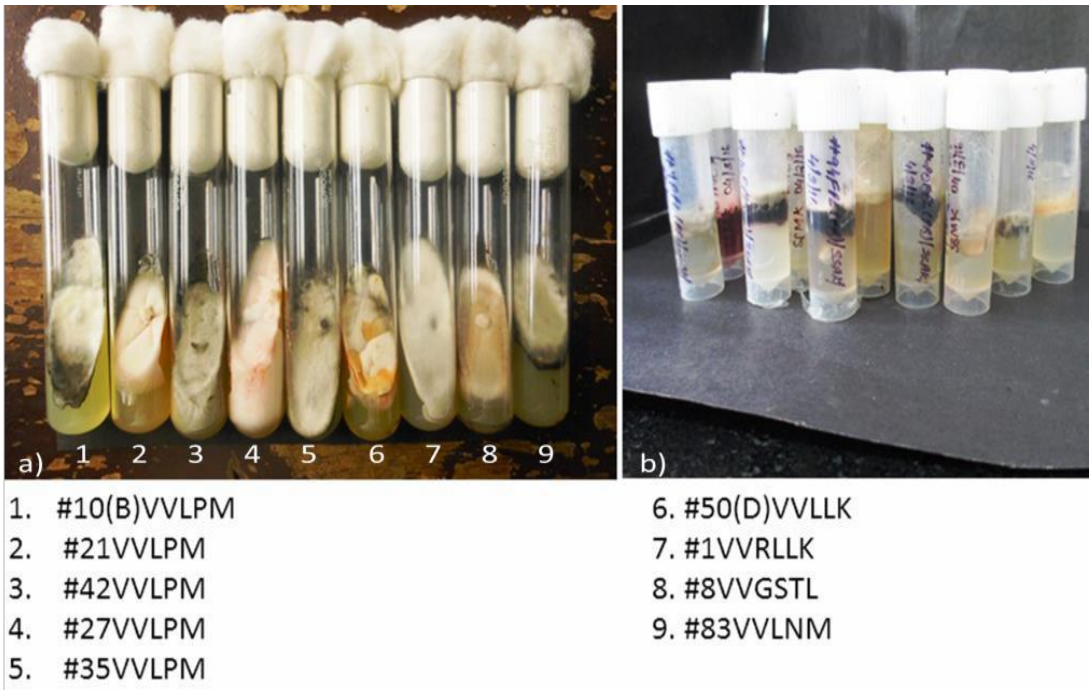

Fig. 5.3: Endophytic isolates preserved for long term storage by different methods, a) PDA slants b) PDA vials.

5.3. Production of culture filtrate

The isolates following existing research were constrained to secondary metabolites production in the PDB medium. Biomass production was recognized as a guideline to estimate the growth rate of fungal cultures. #12VVLPM culture was exhibiting the highest and #139(P)VVLPM giving the lowest biomass production. Biomass production of some of the fungal cultures ranking from lowest to highest is recorded in table 5.2.

Table 5.2: Biomass production of different culture under study.

Culture Code	Yield of crude compound (mg)
#10(B)VVLPM	2.0
#12VVLPM,	9.0
#22(P)VVLPM	5.0
#8VVGSTL	16.0
#139(P)VVLPM	20.0
#109VVLNM	2.0

5.4. Biochemical screening assays of piceatannol producing endophytic fungi

Three biochemical assays for the identification of phenolic compounds namely: Liebermann, Chromogenic reaction, and Acetic anhydride tests were done for probable identification of piceatannol in extracts of each endophytic fungi. Out of 50 endophytic fungi only 21 isolates were found to be positive in preliminary screening assay and out of 21 isolates, 6 isolates namely #12VVLPM, #10(B)VVLPM, #22(P)VVLPM, #139(P)VVLPM, #109VVLNM and #8VVGSTL were found potent piceatannol producers. In Liebermann, Chromogenic reaction and Acetic anhydride test, the creation of the red precipitate, blue, and dark greenish brown coloration respectively verify the presence of piceatannol (Figure 5.4). In all 3 tests, #12VVLPM, #10(B)VVLPM, #22(P)VVLPM, #139(P)VVLPM, #109VVLNM and #8VVGSTL were strong piceatannol producers. However, #10(B)VVLPM showed the highest content of phenolic content among all 6 showing greater than 90% activity hence and therefore could be recognized as a possible yielder of fungal piceatannol (Table 5.1). Accordingly, 8 cultures were further subjected to liquid-liquid extraction.

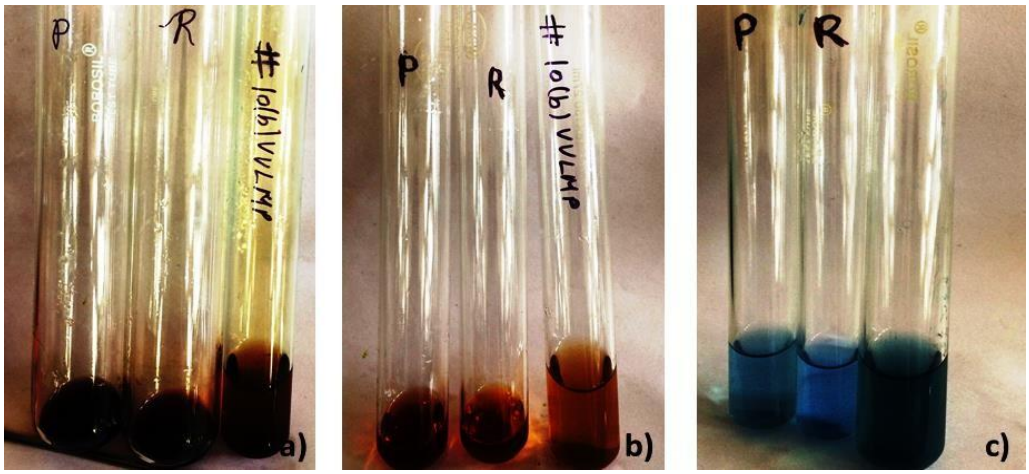

Fig. 5.4: Biochemical assays for screening piceatannol a) Liebermann Test b) Acetic Anhydride Test c) Chromogenic reaction. Positive Control- piceatannol, Test- ethyl acetate fraction of #10(B)VVLPM.

5.5. Solvent extraction

The liquid-liquid extraction of cell-free filtrates of 6 cultures namely: #10(B)VVLPM, #12VVLPM, #22(P)VVLPM, #139(P)VVLPM, #8VVGSTL and #109VVLNM was carried out using ethyl acetate. The bioactive residue reconstituted in methanol. The highest yield of the bioactive residue collected from #139(P)VVLPM (20.0 mg) and the lowest yield in the case of #10(B)VVLPM and #109VVLNM (2.0 mg) (Table 5.3). Ethyl acetate

extracts of these fungal cultures were subjected to biochemical screening assays. #10(B)VVLPM, #12VVLPM, #22(P)VVLPM, #139(P)VVLPM, #8VVGSTL and #109VVLNM were observed to be the potent piceatannol producers. As in biochemical screening #10(B)VVLPM exhibited activity greater than 90% , so it was chosen for further investigation.

Table 5.3: Yield of bioactive residue of different cultures under study.

Culture Code	Yield of crude compound (mg)
#10(B)VVLPM	2.0
#12VVLPM,	9.0
#22(P)VVLPM	5.0
#8VVGSTL	16.0
#139(P)VVLPM	20.0
#109VVLNM	2.0

5.6. Thin layer chromatography (TLC)

The crude ethyl acetate fraction of #10(B)VVLPM was separated to various combination of solvents (Table 5.4). To achieve good sepration of crude ethyl acetate extract of #10(B)VVLPM was seen in dichloromethane : chloroform in the proportion of 5: 5 which gave 4 different bands. The Rf value of Band 1 to Band 4 was 0.125, 0.25, 0.325 and 0.650 respectively. The standard piceatannol showed Rf value of 0.650 which was similar as that of Rf value of Band 4 of crude ethyl acetate extract of #10(B)VVLPM.

Table 5.4: Different mobile phase used for TLC of ethyl acetate extract of #10(B)VVLPM.

S.No.	Solvent System	Ratio Used (V/V/V)	Result
1	Hexane : chloroform	9:1	No separation
2	Hexane : chloroform	7:3	No separation
3	Hexane : chloroform	5:5	No separation
4	Hexane : chloroform	3:7	No separation
5	Hexane : chloroform	1:9	No separation
6	Hexane : ethyl acetate	1:9	No separation
7	Hexane : ethyl acetate	3:7	No separation
8	Hexane : ethyl acetate	5:5	No separation
9	Hexane : ethyl acetate	7:3	No separation
10	dichloromethane : chloroform	9:1	No separation
11	dichloromethane : chloroform	8:2	No separation
12	dichloromethane : chloroform	7:3	No separation
13	dichloromethane : chloroform	6:4	No separation
14	**dichloromethane : chloroform**	**5:5**	**4 bands**

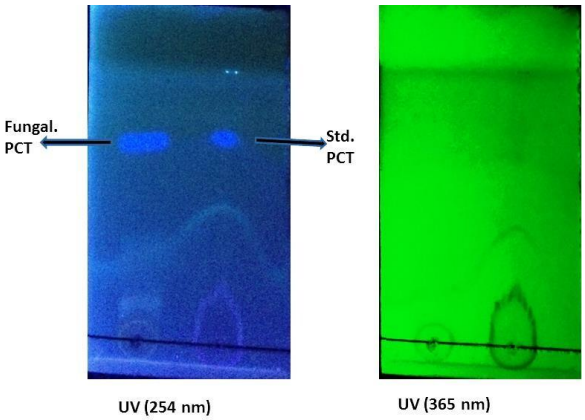

Fig.5.5: TLC of Fungal and standard piceatannol under the UV at 254 and 365 nm

5.7. Confirmation and quantification of fungal piceatannol (#10(B)VVLPM) through liquid chromatography

Liquid chromatography is a very common method for qualitative and quantitative analysis, Confirmation of fungal piceatannol was confirmed by HPLC analysis, which exhibited a symmetrical peak at retention time of 6.5 min. which is almost identical to standard piceatannol. The concentration of crude extract of #10(B)VVLPM was found to be 7.56µg/ml ascertained from the data of peak area vs. concentration of standard piceatannol (y=5E+07x – 2E+06; R^2=0.9986) (Fig.5.6 and 5.7) (Table 5.5).

Table 5.5: Peak area of different concentration of Piceatannol

Concentration (mg/ml)	Peak area (mAU)
0.025	53928.24
0.050	153084.21
0.100	3238206.52
0.200	8429410.14
0.300	13306900.98
0.500	22360449.36
0.600	27749124.55

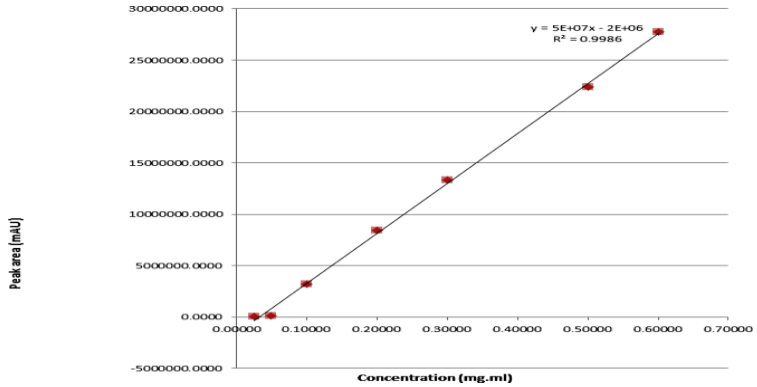

Fig.5.6: DATA of peak area (mAU) and concentration (mg /ml) of Piceatannol

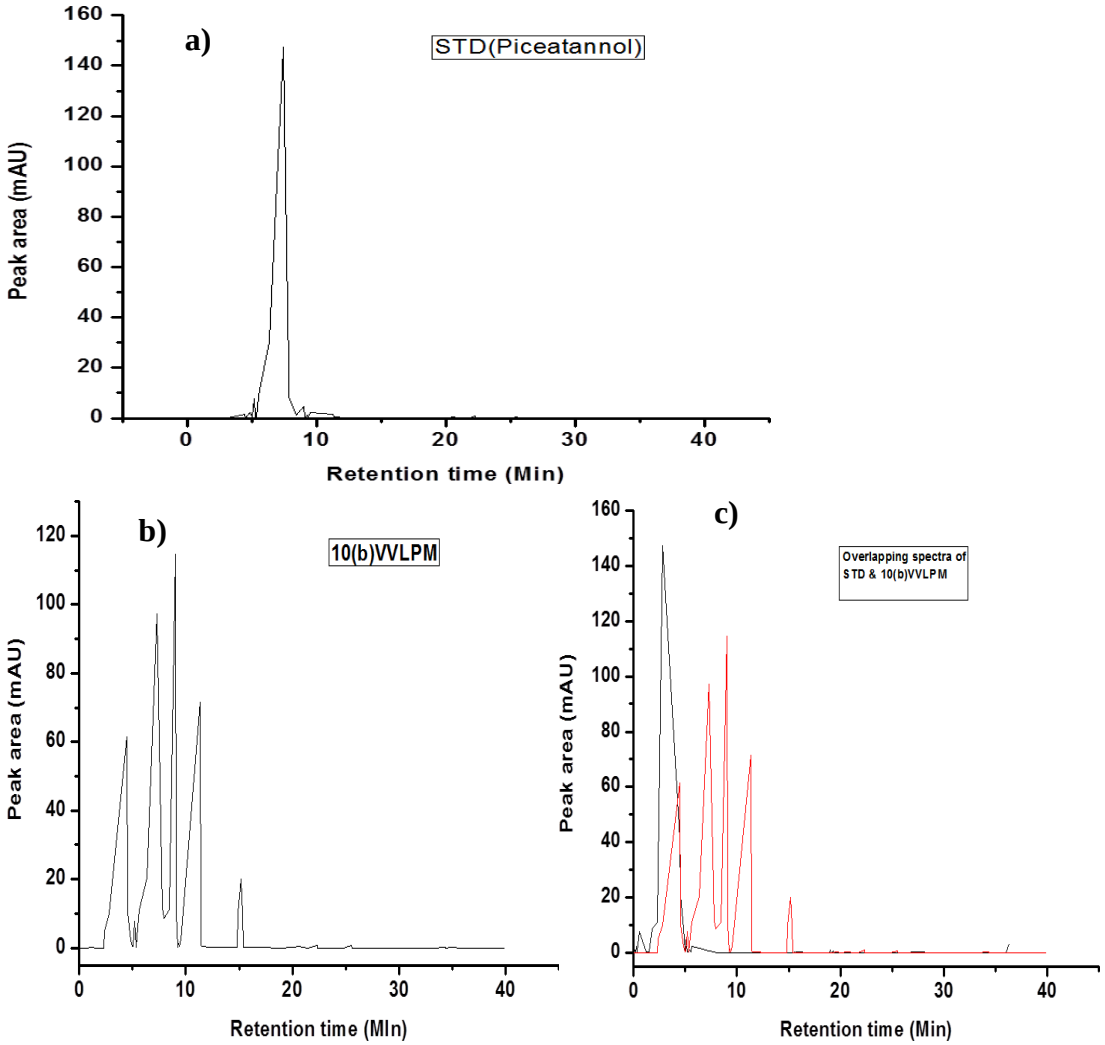

Fig.5.7: HPLC spectra of a) STD piceatannol, b) Crude residue of #10(B)VVLPM, c) Overlapping spectra of STD piceatannol and crude fungal piceatannol (#10(B)VVLPM).

5.8. FTIR analysis

In order to confirm whether the obtained extract consists of piceatannol or not Fourier Transform Infrared Spectroscopy was done for the sample as well as standard piceatannol. So if the sample to be characterized consists of the characteristic transmittance peaks of the standard (piceatannol), implies both have same functional groups thus indicating the presence of standard. The peaks at wavenumber of ~3300 cm^{-1} and ~2330 cm^{-1} in both of the samples attribute to the presence of hydroxyl group (OH). Piceatannol consists of aromatic ring so the presence of characteristic peak of C=C aromatic stretching which lies in region 1550-1600 cm^{-1} also confirms the presence of piceatannol in the sample .

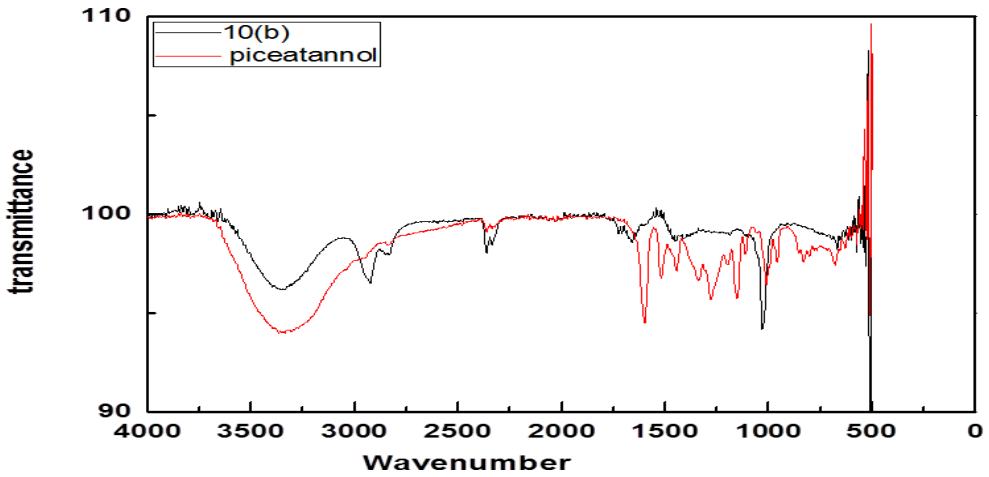

Fig.5.8: FTIR absorption spectra of #10(B)VVLPM and piceatannol.

5.9.LC-MS Analysis

In order to confirm the tentative identification of piceatannol and to obtain more accurate quantitative data the LC-MS analysis was performed. The LC-MS analysis of the sample (#10(B) VVLPM) was carried out in order to determine the presence of piceatannol which exhibited higher piceatannol content as well as antioxidant activity. The m/z of 245.09 of STD piceatannol was obtained. Symmetrical m/z of 245.09 was obtained in sample (#10(B) VVLPM) similar to that of standard piceatannol (Fig.5.9).

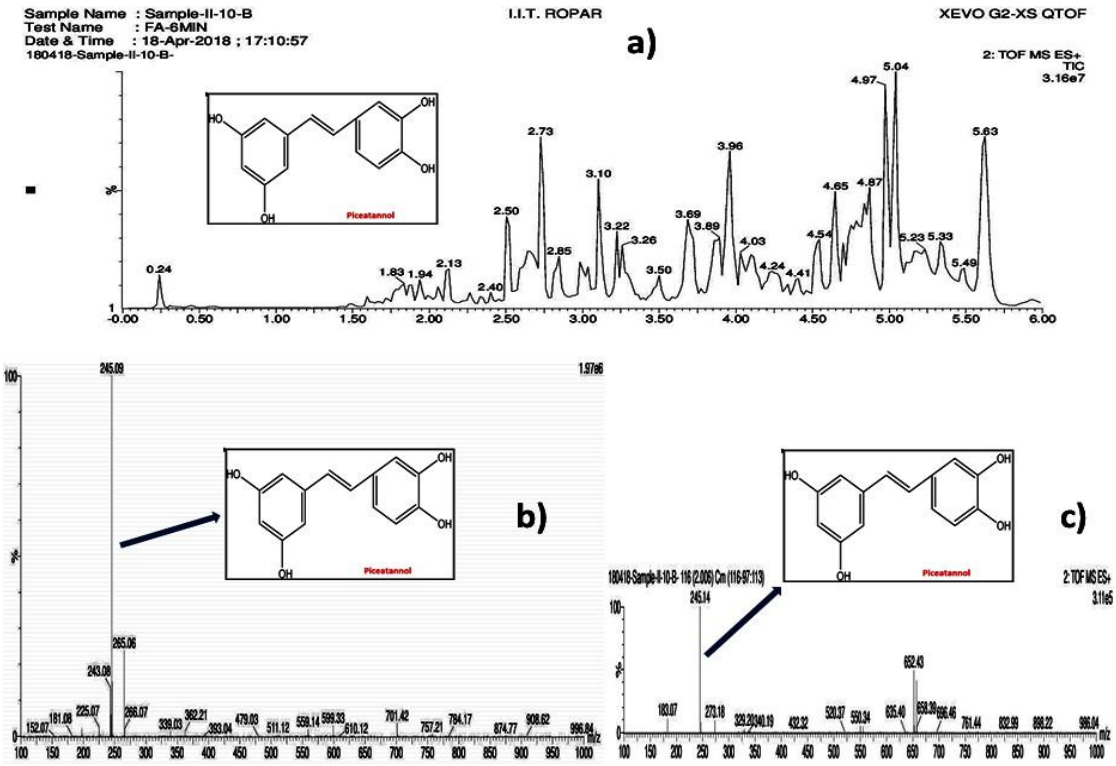

Fig.5.9: Liquid chromatography, mass spectrometry assay of a) LC chromatogram #10(B)VVLPM b) Standard piceatannol c) #10(B)VVLPM (Fungal piceatannol).

5.10. Identification of the selected piceatannol producing endophytic fungus

The potential piceatannol producing endophytic fungus #10(B)VVLPM was identified using morphotaxonomic and molecular taxonomic methods.

5.10.1. Morphotaxomony

The endophytic fungus #10(B) VVLPM produced white, fast growing (90± 0), floccose aerial mycelium on PDA and FMA media after 10 days of incubation with 12 h photoperiod (Fig.5.10). Fungus start growing with white in color and later becomes tan color with smooth margin over PDA and dark brown over FMA (Fig.5.10). On SNA medium fungus was off-white in color initially with wooly appearance and later become Ivory color with smooth margin (Fig. 5.10). On PLA medium it was brown in color and ivory color in Water Agar (WA) (Fig.5.10 & Table 5.6). Over Pine Leaf Agar (PLA) and Water Agar (WA) the margins were smooth and flat respectively.

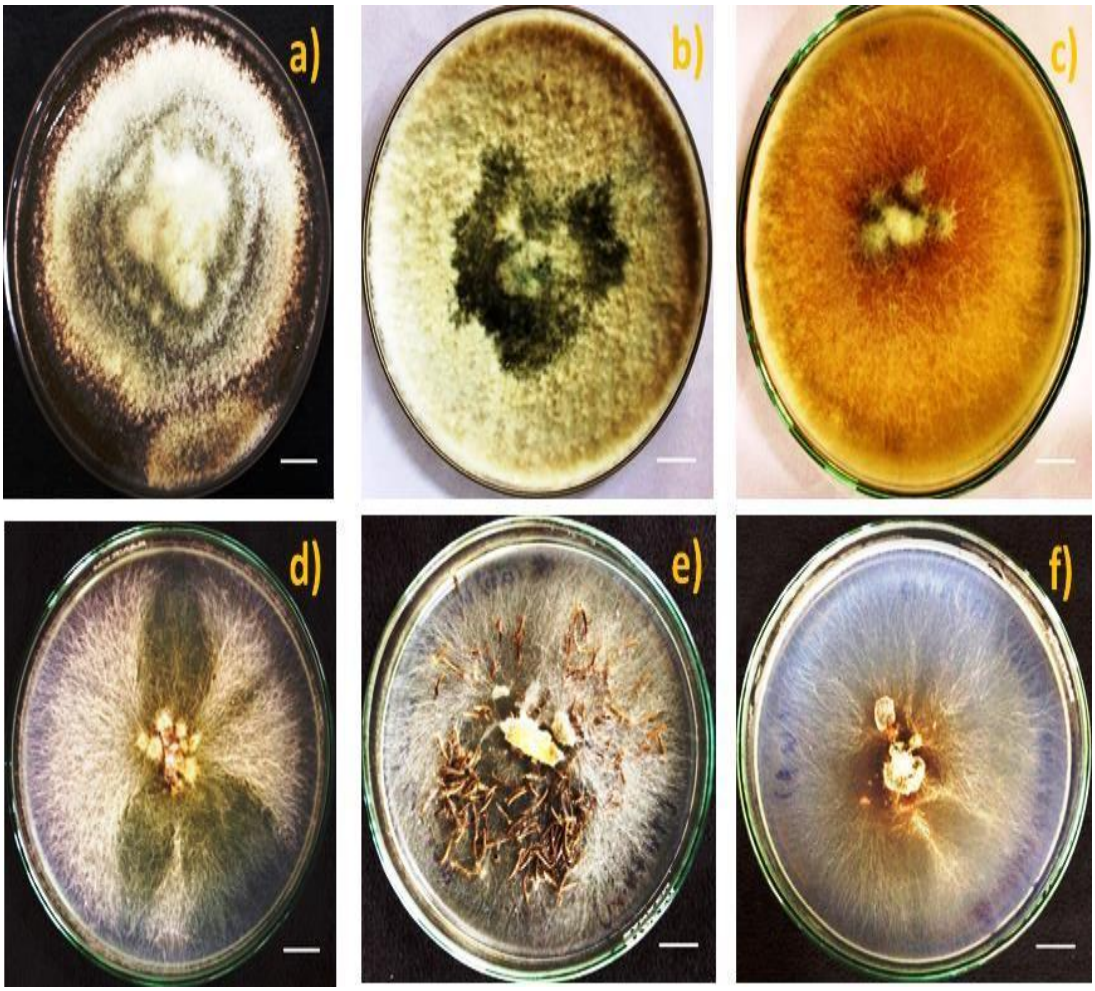

Fig. 5.10 : Morphological feature of #10(B)VVLPM; colony morphology on a)PDA (5.4 pH) medium; b) PDA (7.0 pH) medium; c) FMA medium; d) SNA medium; e) PLA medium; f) WA medium (Bar : 10mm).

Table 5.6: Colony morphology of #10(B)VVLPM on different medium after 20 days, 28˚C, 12h dark

Medium	Colony color		Colony diameter* (mm)	Margin	Pigment	Odour
	Front	Back				
PDA	Dark brown	Pale Brown	90 ± 0	Circular, Smooth	Pale brown	Fruity smell
FMA	Yellowish brown	Pale Brown	90 ± 0	Circular, Smooth	Yellowish Brown	Fruity smell
PLA	Whitish brown	Dark Brown	49 ± 2	Flat	No pigment	No odour
SNA	Off-white	Whitish Pink	55 ± 3	Smooth	No pigment	No odour
WA	Off-white	Ivory	42 ± 2	Flat	No pigment	No odour

(*Data represented as mean ± SD)

5.10.2 Molecular identification

5.10.2.1 Genomic DNA isolation and PCR amplification

The genomic DNA isolation of the potent piceatannol producing endophytic fungi, #10(B)VVLPM, was done and the size of the genomic DNA was found to be approximately more than 10 kb (Fig. 5.11). The concentration of DNA was estimated by taking the absorbance at 260 nm and the amount was 25 ng/µl by using formula concentration (µg/ml) = O.D 260nm x 50 µg/ml x dilution factor The PCR amplicon was resolved on 1.5% agarose gel in order to check the size on the basis of the mobility and comparison with the 100bp ladder. The size of the amplicon was found to be approximately 550 bp to 600 bp (Fig.5.11). This size can be easily compared to the ITS region, which was amplified in order to characterize the fungi at molecular level.

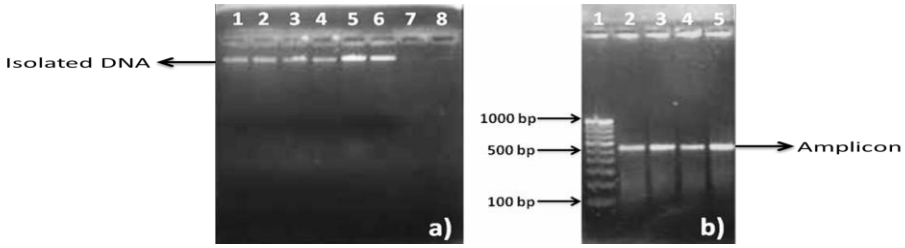

Fig.5.11: a) Genomic DNA isolation of #10(B)VVLPM, Lane 1-6: DNA sample of #10(B)VVLPM. b) PCR amplicon of ITS region of #10(B)VVLPM, Lane 10: 100bp ladder, Lane 2-5: ITS amplicon of #10(B)VVLPM

5.11. Biological activity

5.11.1. *In-vitro* antioxidant assay

The crude ethyl acetate fraction of #10(B)VVLPM was estimated for its capability of antioxidant activity using the DPPH free radical scavenging system, Trolox equivalent antioxidant capacity assay (TEAC), Total phenolic content assay (TPC), and Total flavonoid content assay (TFC).

5.11.2. DPPH scavenging assay

The crude fungal piceatannol in the DPPH assay exhibited very good scavenging activity (IC_{50}=117.021±13.38) as compared to standard piceatannol which showed (IC_{50}=129.01±18.71), there was a notable change in color from purple to yellow during scavenging reaction (Fig.5.12).

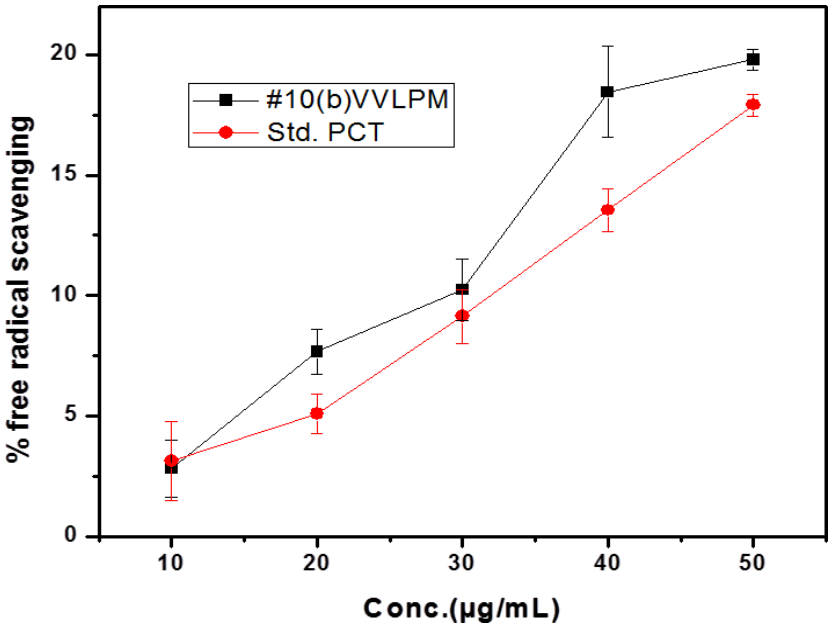

Fig.5.12: DPPH scavenging activity of #10(B)VVLPM compared with standard piceatannol.

5.11.3. TEAC assay

The results illustrated that #10(B)VVLPM has a stronger activity to scavenge the free radicals. An activity of the raw ethyl acetate fraction #10(B)VVLPM, was obtained 109.39±5.73 IC50*(µg/ml). Which is similar to the piceatannol (Fig.5.13) used as the standard, which showed scavenging activity of 112.35±4.54 IC50*(µg/ml).

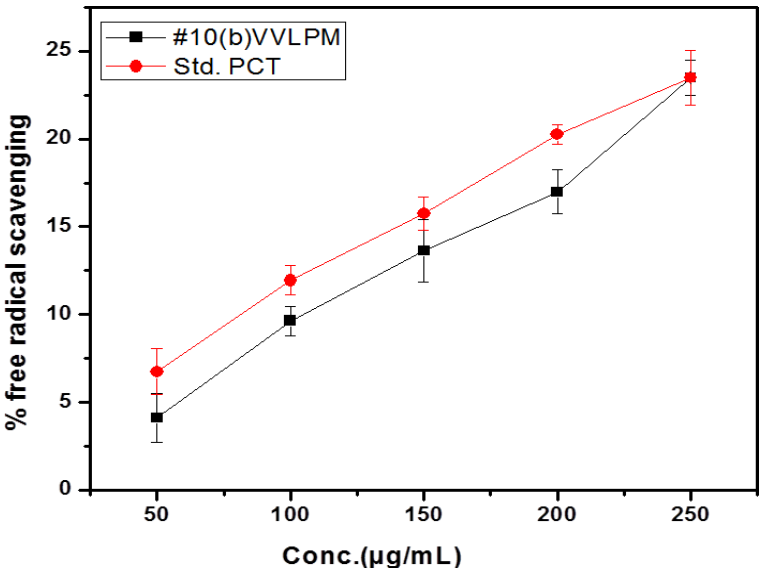

Fig.5.13: TEAC scavenging activity of #10(B)VVLPM compared with standard piceatannol.

5.11.4. Total phenolic content assay (TPC)

The total phenolic content of #10(B)VVLPM was calculated from the equation (y = 0.0016x + 0.0508, R^2 = 0.9943) and the crude extract of #10(B)VVLPM showed a concentration of 23.56±1.09 (µg piceatannol equivalent /mg of sample) (Table 5.7).

5.11.5. Total flavonoid content assay (TFC)

The flavonoid content of #10(B)VVLPM was calculated from the equation (y = 0.0016x + 0.0508, R^2 = 0.9943) and the crude extract of #10(B)VVLPM exhibited a concentration of 265.0±8.29 (µg piceatannol equivalent/ mg of sample) (Table 5.7).

Table 5.7: DPPH free radical scavenging activity, Trolox equivalent antioxidant capacity (TEAC) assay, TPC, and TFC of #10(B)VVLPM.

S.No.	Sample	IC50* (µg/ml)		TPC	TFC
		DPPH	TEAC	Conc. (µg Piceatannol equivalent/ mg of sample)*	Conc. (µg Piceatannol equivalent/ mg of sample)*
1	Standard Piceatannol	129.01±18.71	112.35±4.54	-	-
2	#10(B)VVLPM	117.02±13.38	109.39±5.73	23.56±1.09	265.0±8.29

(*Data presented are mean ± standard deviation of three replicates)

5.11.6. Lambda DNA nicking assay

The assay was carried out to understand the possible role of fungal piceatannol (#10(B)VVLPM) on prevention of damage to DNA by free radical. Fungal extracts of

our results indicates that fungal extracts of #10(B)VVLPM exhibited good antioxidant activity and have efficiently able to decrease the oxidative stress on Lambda DNA (Fig.5.14).

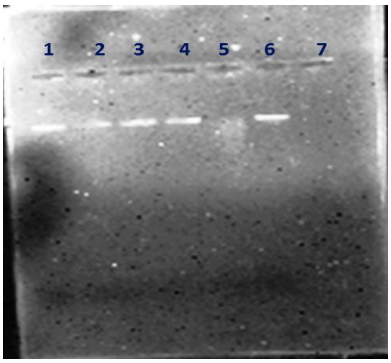

Fig.5.14: Lambda DNA reactions in Lane 1-4: Fenton reagent+ Lambda DNA + #10(B)VVLPM + 0.5mM EDTA, Lane 5: Fenton reagent+ Lambda DNA + 0.5M EDTA, Lane 6: Fenton reagent+ Lambda DNA+ Piceatannol + 0.5mM EDTA, Lane 7: Blank

5.11.7. *In-vitro* Antimicrobial Assay

The antimicrobial activity of the #10(B)VVLPM culture was successfully carried out, which gave excellent antimicrobial activity. The zone of inhibition of the culture was measured against different microorganisms namely: *Pseudomonas aeruginosa* (MTCC647), *Bacillus subtilis* (MTCC441), *Candida albican* (ATCC227; (MTCC3011), *Staphylococcus aureus* (NCTC6571; MTCC96). The maximum zone obtained was 14.66mm against MTCC441 and the lowest zone of inhibition received was 8.33mm against MTCC647. The culture antimicrobial activity is demonstrated in (Table 5.8).

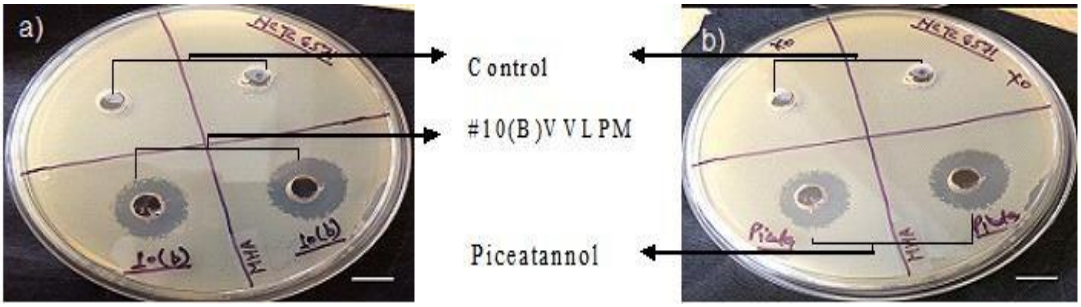

Fig.5.15: Plates depicting antimicrobial assay of #10(B)VVLPM and piceatannol against NCTC6571.

Table 5.8: List of cultures which possess antimicrobial activity against different microbes.

SAMPLE	ATCC227	NCTC6571	MTCC3017	MTCC441	MTCC647	MTCC 96
Piceatannol	12.00 ± 1.00	10.33 ± 0.58	11.00 ± 1.00	15.67 ± 0.58	10.33 ± 0.58	11.67 ± 0.47
#10(B)VVLPM	11.67 ± 0.58	9.67 ± 1.15	11.33 ± 0.58	14.67 ± 0.58	8.33 ± 0.58	11.67 ± 0.47
Positive control	15.67 ± 0.58	15.33 ± 0.58	16.67 ± 0.58	17.00 ± 1.00	16.33 ± 0.58	15.33 ± 0.47
Negative control	5.00 ± 0.00	5.00 ± 0.00	5.00 ± 0.00	5.00 ± 0.00	5.00 ± 0.00	5.00 ± 0.00

*Note: - Data represented are mean ± standard deviation.

6.0. DISCUSSION

Parkinson disease (PD) is the second most encountered neurodegenerative disorder which results in a series of motor (bradykinesia, rigidity) and non-motor symptoms (sleep disturbances, constipation etc) (Obeso et al., 2010). Biochemical basis of PD is the loss or inhibition of dopamine activity in corpus striatum whereas major hallmark of PD is the presence of lewy bodies (Fearnley et al., 1991).

Lewy bodies are mainly constituted of α-synuclein fibrils which are small soluble proteins, located on human chromosome 4q 21.3-22 (Spillantini et al., 1997). The physiological pathway for aggregation of α-synuclein is first unfolding of the tetramer to kinetically trapped monomer and then re-association to a disordered aggregate and eventually fibrillar lewy bodies. Various polyphenol compounds are studied for the purpose due to their high anti-oxidant properties and shows dose dependent inhibition of α-synuclein aggregation and fibrillation (Caruana et al., 2011).

Polyphenols are major natural secondary metabolites structurally diverse and essential for a variety of functions. Piceatannol or Trans-3,3',4,5' tetrahydroxystilbene is a natural stilbene, a type of polyphenolic flavonoid (Uchida et al., 2018; L.bavaresco et al., 2002). Piceatannol shows various physiological activities with therapeutic benefits and researchers confirmed that piceatannol inhibit/disrupt the aggregation of α-synuclein responsible for causing Parkinson disease (Temsamani et al., 2016). It is one of the important dietary stilbenes commonly found in red wine, berries, grapes, and white tea along with various plants like grapes, groundnuts, cranberries and passion fruit. The yield of piceatannol from the plant is very low and time-consuming, which contribute to its higher prices (Dhanapal et al., 2018; kil et al., 2017). However, plant based method can't meet the increasing demand of piceatannol in the market. Hence, different encouraging approaches are in investigation of the endophytic fungi which may mimic the production of piceatannol in free-fermenting situation.

The endophytes are the micro-symbionts which reside within plant without causing any harm to plant. Endophytic fungi are considered to be ware houses of plethora of

bioactive compounds which exhibits antimicrobial, antifungal and immunosuppressive activities. It has been already proven that endophytic fungi have the capacity to producing same functional compounds as their host (Strobel et al., 2003; Suryanarayanan et al., 2009).

In the current research, the study was performed for the production of piceatannol through free fermentation conditions by using different endophytic fungi previously isolated from different climatic regions. Biochemical assays namely: Liebermann, Chromogenic reaction and Acetic anhydride test were used for the identification of phenolic compounds, where out of 50 endophytic fungi culture filtrates, only 21 isolates were found to be positive among which 6 isolates were observed to show 80-90% activity whether in previous studies reported by shi et al in 2012, 21 resveratrol producing cultures out of 65 were found whereas Dwibedi et al in 2018 found 29 strains out of 53 in preliminary screening.

Out of six isolates which is found to be potent producer of piceatannol, #10(B)VVLPM was showing maximum activity (>90%) and thus chosen for the study. TLC is a method often used for separation as well as identification of bioactive compounds. In TLC light fungal piceatannol visualized as violet color spot with symmetrical Rf 0.65 as standard. Therefore the strain #10(B)VVLPM was chosen as a target strain for the production of piceatannol.

Now days HPLC is the most common method for qualitative as well as quantitative analysis for the bioactive compounds, under the same condition and same chromatographic system each compound has fix retention time. Thus unknown compounds and standard compound can be identified preliminary by same compounds owe to the similar retention time under same chromatographic condition (Shi et al 2012; Bavaresco et al 2002).

In the present study the concentration of fungal piceatannol was determined by HPLC using C-18 reverse phase discovery column which exhibited symmetrical peak at Rf of 6.5 min whereas the concentration of crude fungal piceatannol #10(B) VVLPM, was found to be 7.56 µg/ml. The isolate #10(B) VVLPM became the principle focus of the study, since it produced highest level of piceatannol amongst the six

isolates. Piceatannol production by #10(B)VVLPM was stable over several sub culturing and it did not decreased as those found in case of *Alternaria* sp. MG2 and *Botryosphaeria* sp. YG3 (Shi et al 2012; Liu et al 2016). Further analysis was made and results were confirmed through FTIR and LCMS. The m/z of 245.09 of fungal piceatannol obtained from #10(B) VVLPM was obtained which was similar to m/z of standard piceatannol.

The fungal piceatannol producing endophytic fungi #10(B) VVLPM in our study showing strong antioxidant potential. The EA extract of #10(B) VVLPM exhibited a very good scavenging activity as compared to standard piceatannol. The two piceatannol derivatives produced by endophytic *Alternaria* sp. also displayed moderate antioxidant activity by DPPH scavenging (Wang et al 2015). Shrikanta et al (2015) also observed antioxidant property which was attributed to the presence of piceatannol in grapes, jack fruit and jamun. Apart from DPPH scavenging potential, piceatannol also exhibited hydrogen peroxide and superoxide anion radical scavenging activities (Armijos et al 2018). Further, the fungal piceatannol in the present study also displayed strong antimicrobial activity against a spectrum of drug resistant *Staphylococcus aureus*. Yim et al in 2010 demonstrated that piceatannol possessed antimicrobial properties against a battery of Gram positive and Gram negative bacteria. The piceatannol was found to exhibit antimicrobial activity against surrogate, methicillin sensitive and methicillin resistant *S. aureus*. It is well known the DNA protective activity of polyphenols in our result protective effect of #10(B)VVLPM lambda DNA showing efficient decrease in oxidative stress by using this assay similar to Golla et al 2014.

The biological properties of the fungal piceatannol produced by *Fusarium sp.* might also be attributed to the inherent property of the fungus to produce various bioactive metabolites which can have therapeutic implications.

Hence the aim of our current study focus on screening of endophytic fungi from *Vitis vinifera* for production of piceatannol through microbial fermentation so that we can produce piceatannol on industrial level in bulk by easy and cost effective manner for their application to treat various neurodegenerative disorder like Parkinson's and Alzheimer disease.

7.0. CONCLUSION

In the current study we conclude that the *Fuserium* sp. (#10(B)VVLPM) is a potent producer of piceatannol. The results were confirmed through TLC, HPLC, FT-IR, and LC-MS analysis. The antioxidant and antimicrobial potential of the isolate #10(B)VVLPM, was also determined. The isolate showing strong scavenging activity of DPPH (IC50=117.02±13.38) and TEAC (IC50=109.35±53) as compared to standard piceatannol.

Thus further work on optimization and purification of the compound is warranted to meet the industrial scale production of piceatannol.

8.0. REFERENCES

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APPENDIX

Media

1. Potato dextrose agar

Potato dextrose agar – 39gm

Distilled water – 1L

Final pH (at 26°C)- 5.6±0.2

Autoclave at 121ºC for 15 min

2. Malt extract agar

Malt extract – 30gm

Mycological peptone – 5gm

Agar – 15gm

Distilled water – 1L

Final pH - 7.6 ± 0.2 at 37°C

Autoclave at 121ºC for 15 min

3. Synthetic nutrient deficient agar

Glucose – 0.2gm

Sucrose – 0.2gm

Pot. dihydrogen phosphate – 1gm

Potassium nitrate – 1gm

Magnesium sulphate – 0.25gm

Potassium chloride – 0.5gm

Agar – 15gm

Distilled water – 1L

Final pH – 5.4 ± 0.2 at 26°C

Autoclave at 121ºC for 15 min

4. Water agar

Agar – 15gm

Distilled water – 1L

Autoclave at 121ºC for 15 min

5. Pine leaf agar

Pine leaves

Agar – 15gm

Distilled water – 1L

Autoclave at 121ºC for 15 min

6. Fusarium minimal agar

Distilled water – 1L

Sucrose – 30g

$MgSO_4 \cdot 7H_2O$ – 0.5g

KC1 – 0.5g

$FeSO4 \cdot 7H_2O$ – 10mg

$NaNO_3$ – 2g

Trace elements* – 2ml

Agar – 20g

Autoclave at 121ºC for 15 min

Buffers

1. 50XTAE

Tris base – 242g

Glacial acetic acid – 57.1ml

0.5M EDTA – 10ml

Distilled water – 1L

2. 1X TE Tris-HCl (pH 8.0)

10 mM EDTA - 0.1 mM

Distilled water - 100ml

Jessica (601604004)

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