

# Screening and Purification of Resveratrol from endophytic fungi

A  
Thesis submitted  
in partial fulfilment of the requirement of the degree  
of  
**Master of Technology**  
In  
**Biotechnology**



Submitted By  
**Anugya Srivastava**  
Roll no. 601304003

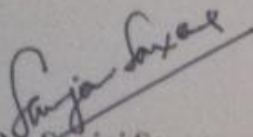
Under the supervision of  
**Dr. Sanjai Saxena**  
Associate Professor  
Department of Biotechnology  
Thapar University

DEPARTMENT OF BIOTECHNOLOGY  
Thapar University, Patiala, Punjab, 147004

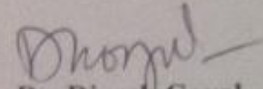
**July 2015**

*Certificate*

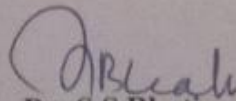
This is to certify that the thesis entitled "Screening and purification of Resveratrol from endophytic fungi" being submitted by Ms Anugya Srivastava (Roll No-601304003) in partial fulfillment of the requirements for the award of degree of Master of Technology in Biotechnology, Thapar University, Patiala 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.

  
Dr. Sanjai Saxena

Associate Professor/Supervisor  
DBT, Thapar University

  
Dr. Dinesh Goyal

Professor & Head  
DBT, Thapar University

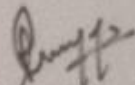
  
Dr. S.S Bhatia

Dean, Academic Affairs  
Thapar University, Patiala

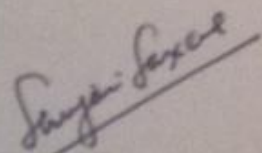
*Candidate's Declaration*

I hereby declare that the work being presented in the thesis entitled "Screening and purification of Resveratrol from endophytic fungi" in partial fulfillment of the requirements for the award of degree of Master of technology, Department of Biotechnology, Thapar University, Patiala is my own laboratory work during the period of **July 2014 to June 2015**, under the conception and supervision of Dr. Sanjai Saxena, Associate professor, Department of Biotechnology, Thapar University, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree.

Patiala  
Date: July 10<sup>th</sup>, 2015

  
Anugya Srivastava  
Roll No. 601304003

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

  
**Dr. Sanjai Saxena**  
Associate Professor / Supervisor

**Dr. Dinesh Goyal**  
Professor & Head

Department of Biotechnology  
Thapar University, Patiala 147004

## ACKNOWLEDGEMENT

Although writing a few words on a piece of paper is not a proper way of acknowledging those who have helped me in the completion of this project, yet the words coming from my heart and soul needs no mode of communication.

I got an excellent opportunity to carry out my project work under the encouraging guidance of my project advisor **Dr. Sanjai Saxena, Associate Professor, Department of Biotechnology, Thapar University, Patiala**. It is a great occasion for me to express my deep sentiments, legitimate regards and gratitude towards him. He took a lot of pain to supervise this project work, whose expert guidance, advice, suggestion, explicit decision and deep personal interest. Speech fails me and my fumble for words of gratitude for my respected advisor.

My sincere thanks to **Dr. Dinesh Goyal, Professor and Head, Department of Biotechnology, Thapar University, Patiala** for providing me an opportunity to do this project work. My gratitude is due to all faculty members for their help, inspiration and moral support, during the course of my study.

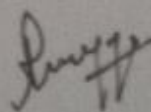
I thank **Mr. Vineet Meshram, Ms. Mahiti Gupta, Ms Neha Kapoor and Mr Vagish Dwibedi** whose scientific acumen, meticulous guidance, unending enthusiasm and constant encouragement led me to complete this painstaking venture.

My whole hearted thanks to **Mr. Ram Naval** and all other lab attendants of Department as well as TIFAC-CORE for their valuable help and immense support.

I thank the almighty whose blessings have enabled me to accomplish my dissertation work successfully. I express my deep gratitude to my parents for their love and support.

Date: 13<sup>th</sup> July, 15

Place: Patiala

  
Anugya Srivastava

*This thesis is dedicated to my seniors*

*For their endless love, support and encouragement*

## CONTENTS

---

---

<b>S.No</b>	<b>Contents</b>	<b>Page no.</b>
1.	Executive summary	
2.	Introduction	1-3
3.	Review of literature	4-12
4.	Aim of study	13
5.	Materials and methods	14-20
6.	Results & discussion	21-37
7.	Conclusion	38
8.	Bibliography	39-48

## List of Tables

---

---

<b>S.No</b>	<b>Table</b>	<b>Page no.</b>
1.	Sources of Resveratrol	3
2.	Vital active compound from endophytic fungus	9
3.	Different reagent used during PCR reaction	4
4.	Endophytic isolates used for screening of Resveratrol production	22-25
5.	The biomass production of different cultures under study	26
6.	Weight of bioactive residue of different culture under study	28
7.	Different mobile phase used for TLC of ethyl acetate of #5VVSTL	28-29
8.	Peak area and retention time of different concentrations of Resveratrol	30
9.	BLAST analysis of final sequences of #5VVSTL	36

---

## List of Figures

S.No	Figures	Page no.
1.	Structure of resveratrol	1
2.	Pure culture of different endophytic fungi on PDA plates	21
3.	Biochemical assay for screening of Resveratrol	27
4.	Violet colored ethyl acetate extract of #5VVSTL	27
5.	TLC of purified and standard Resveratrol	29
6.	Data of peak (mAU) and concentration (mg/mL) of standard Resveratrol	38
7.	HPLC graph of different concentration of standard Resveratrol	38
8.	HPLC spectra of (a) standard Resveratrol (b) crude ethyl acetate extract of #5VVSTL (c) Purified fungal Resveratrol from #5VVSTL (d) combined spectra of purified Resveratrol along with standard Resveratrol	31
9.	Colony morphology of <i>Quambalaria cyanescens</i> (#5VVSTL) over different media	32
10.	Microscopic features of <i>Quambalaria cyanescens</i> (#5VVSTL) over different medium	33
11.	a) Genomic DNA isolation of #5VVSTL b) PCR amplicons of ITS region of #5VVSTL	35
12.	Maximum parsimony phylogenetic tree based on ITS region sequence.	37

## Abbreviations

---

---

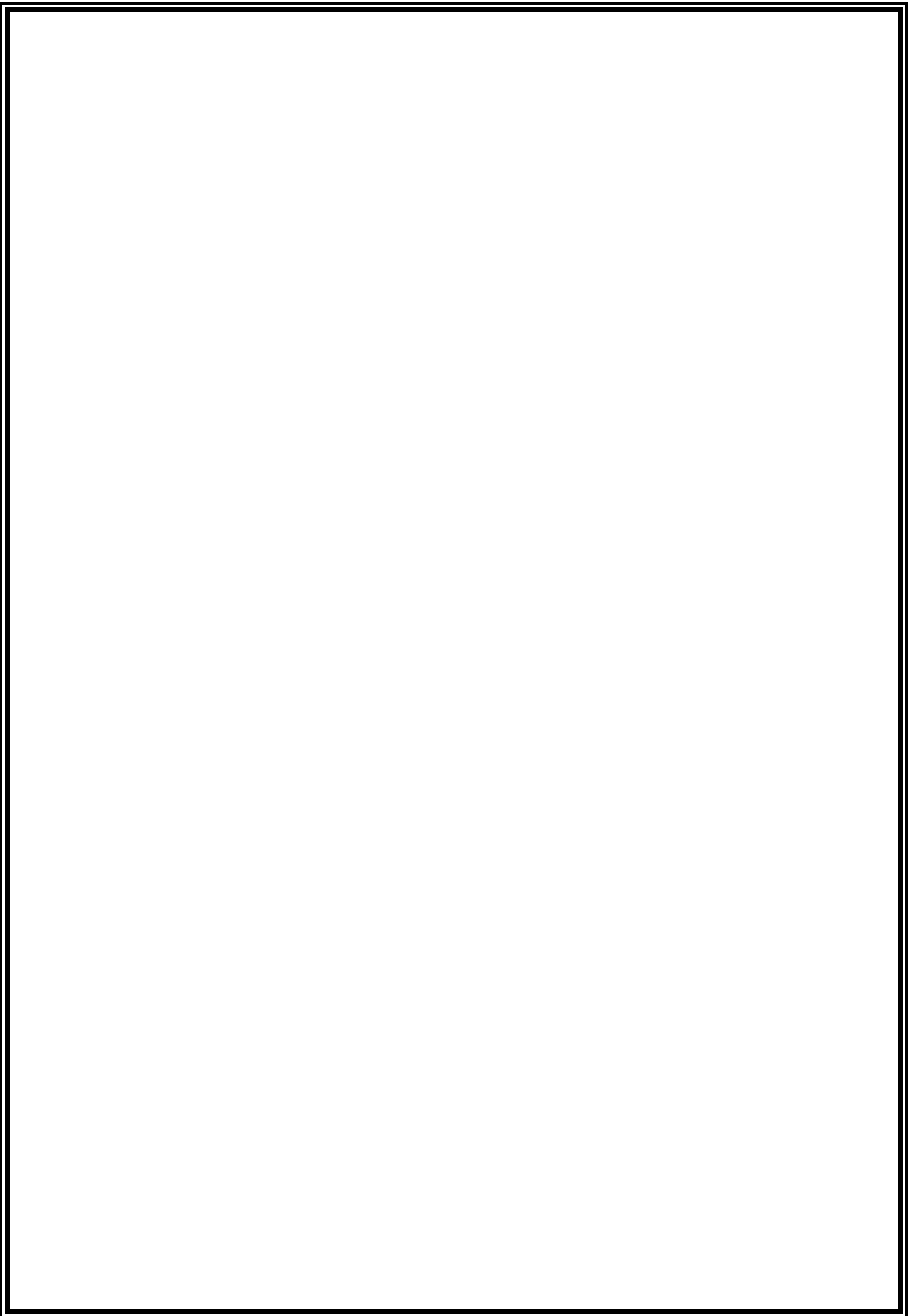
S.No	Abbreviation	Full form
1.	ACN	Acetonitrile
2.	AD	Alzheimer's dementia
3.	COX	Cyclooxygenase
4.	DNA	Deoxyribonucleic acid
5.	dNTP	Deoxynucleotide triphosphate
6.	EA	Ethyl acetate
7.	EDTA	Ethylene diamine tetra acetic acid
8.	ITS	Internal transcribed spacer
9.	L	Litre
10.	M	Molar
11.	mAU	Milli-absorbance unit
12.	mM	Milli-molar
13.	mg	Milli-gram
14.	mL	Milli-litre
15.	mm	Milli-metre
16.	nm	Nanometer
17.	NO	Nitric oxide
18.	PCR	Polymerase Chain Reaction
19.	ppm	Parts per million

---

---

20.	µg	Microgram
21.	µL	Microlitre
22.	R <sub>f</sub>	Retention factor
23.	rpm	Rotation per minute
24.	CDA	Czepak Dox agar
25.	CDB	Czepak Dox broth
26.	CMA	Corn Meal Agar
27.	GLA	Grape leaves agar
28.	HPLC	High performance liquid chromatography
29.	MEA	Malt Extract Agar
30.	PDA	Potato Dextrose Agar
31.	RESV	Resveratrol
32.	SIRT 1	Sirtuin 1
33.	SNA	Synthetischer nährstoffarmer agar
34.	TAE	Tris acetate EDTA
35.	TE	Tris EDTA
36.	TFA	Tri-fluro acetic acid
37.	TLC	Thin layer chromatography
38.	WA	Water Agar

---



## Executive Summary

Resveratrol is a phenolic compound naturally produced during pathogenic attack by plants defense system. It is being exploited as potent antioxidant, anti-cancer, anti-inflammatory and anti-aging agent. There exists an urgent need for exploration of alternative natural sources of resveratrol production which can contribute in meeting the increasing demand of this valuable drug and preserve the biodiversity of nature also.

Endophytic fungi exist within the tissues of host plants in symbiotically fashion without exerting any negative effects to the host plant. The endophytic fungi are believed to be “Gold mines” of novel pharmacologically important bioactive compounds. The present study reports the exploration of endophytic fungi isolated from various medicinally important plants viz. *Aegle marmelos*, *Cinnamomum malabaricum*, *C. camphora*, *Vitis vinifera*, *Catharanthus roseus* and *Raulwofia serpentina* for their potential to produce resveratrol. In the present study culture filtrates of endophytic fungi obtained from Czapek Dox broth were screened for their potential to produce resveratrol extracellularly. Libermann and Acetic anhydride tests were used for confirming the presence of resveratrol initially. After the preliminary screening 9 endophytic isolates were further subjected for mass production of secondary metabolites on CDB. These were subsequently extracted with solvents and tested for their presence of resveratrol. Out of selected 9 cultures, the crude EA extract of #5VVSTL and #16CRLPAL were found to be potent producers of Resveratrol. The resveratrol content in ethyl acetate extract of #5VVSTL was found to be maximum. Hence, Ethyl acetate extract of #5VVSTL was separated into 7 fractions by using Chloroform:Ethyl acetate:Formic acid in ratio 10:2.4:0.4 as solvent system. The fungal resveratrol was purified by scraping off TLC plates 40 mg of resveratrol was obtained by this method. The purity and concentration of TLC Purified Resveratrol was then determined by

using HPLC using C18 reverse phase discovery column the mobile phase combined of H<sub>3</sub>PO<sub>4</sub> (0.1%): Acetonitrile (10-70%). The purified fungal Resveratrol and standard resveratrol exhibited same retention time of 35.2 min and visualized at violet color spot on TLC plate with R<sub>f</sub> value of 0.63.

The potent resrveratrol producing endophytic isolate, #5VVSTL was identified as *Quambalaria cyanescens* by using morphological and molecular taxonomic techniques.

# *Chapter 1*

---

## *Introduction*

## 1. Introduction

The French people despite their dietary habits do not suffer from cardiovascular problems which are predominantly associated with their habit of daily consumption of red wine which they believe keeps the doctor at bay. This is referred as the “French Paradox” which is attributed to a molecule “Resveratrol” from red wine. Natural products play a major role as active substances, model molecules for the discovery and validation of drug targets. In fact, about 50% of the drugs introduced to the market during the last 20 years are derived directly or indirectly from small biogenic molecules. Plants and fungi are the chief source of natural compounds used for medicine, in which medicinal plants. (Cai et al., 2004; Newman and Cragg, 2007).

Resveratrol (3,5,4'-trihydroxy-trans-stilbene), a stilbenoid, consisting of two aromatic ring which are attached by methylene bridge produced naturally by 72 different plant species when under attack by pathogens. It is found in the roots of the Japanese Knotweed and also in skin of red grapes. First isolated from the poisonous, but medicinal, *Veratrum album*, variety grandiflorum (Takaoka, 1939). The name resveratrol was derived from its source since it is resorcinol derivative from a *Veratrum* species (Lancon et al., 2007). Resveratrol exists as cis and trans isomer. *Trans*-resveratrol in the powder form was found to be stable under "accelerated stability" conditions of 75% humidity and 40 °C in the presence of air. Predominantly the beneficial resveratrol is trans- resveratrol form. Resveratrol content also was stable in the skins of grapes and pomace taken after fermentation and stored for a long period. The grapevine is attacked with the fungus known as *Botrytis cinerea* the resveratrol is produced as a self-defence mechanism to protect itself from the infection.

A very well known Ayurvedic preparation is Darakchasava which is prepared from grape vine (*Vitis vinifera*) (Paul et al., 1991). Darakchasava has been prescribed as an ayurvedic medicine for use as a cardiotoxic and treatment of diseases like worm infection (Parker et al., 2005), epistaxis (Korneich et al., 2012) and heart diseases (Petrouski et al., 2011). Resveratrol is a major component of Darakchasava (Soleas et al., 1997). Biologically resveratrol is synthesized in the skin of grapes and reaches peak production when the grape reaches maturity (Wang et al., 2013). Stilbene synthase is the terminal enzyme required for the biosynthesis of resveratrol which is activated in the presence of exogenous stress factors,

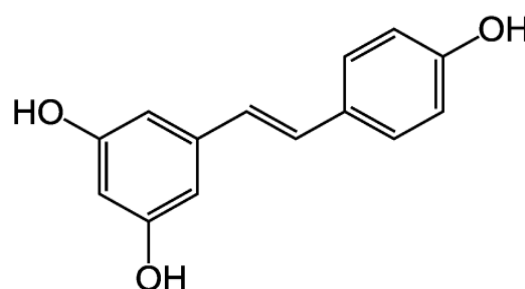


Figure 1 structure of Resveratrol

such as injury, UV irradiation and chemical signal from pathogenic fungi (Adrian et al., 2000, Cantos et al., 2000). The resveratrol was first mentioned by Michio Takaoka in 1940, where he has isolated it from the medicinal plant Japanese knotweed (*Polygonum cuspidatum*).

Peanuts (*Arachis hypogaea* Linn.), a member of the Papilionaceae family, have been discovered as potent source of resveratrol production (Chen et al., 2002). Many studies in Korea have showed that many food contents such as peanut butter and roasted peanuts have the high content of resveratrol (Burns et al., 2002).

### **Medicinal properties of resveratrol**

Resveratrol has been the subject of intensive investigation in recent years and is reported to be an extremely potent antioxidant, a modulator of genetic expression via signal transduction, an inhibitor of inflammatory mediators, and to have other actions including phyto-hormonal effects. This combination of biological properties and cosmetic effects makes resveratrol a unique candidate anti-aging agent.

Numerous studies have been found that resveratrol has many health benefit properties such as anti-oxidative, anti-inflammatory, anti-cancer, chemopreventive abilities etc (Behr et al. 2009; Fremont et al., 1999). The most interesting activity of resveratrol is that it has potential to slow down the aging process and prolong the life span through the synergetic action with flurosphore- signaling molecule (Bhat et al., 2001).

It has been reported to exhibit a wide range of pharmacological properties and is believed to play an important role in the prevention of human cardiovascular disease (Gusman et al., 2001) as it is responsible for increasing the nitric oxide production and down-regulating vasoactive peptides (known as endo-thelins). It also induces the anti-inflammatory effects via cyclooxygenase (COX) inhibition and down-regulation of eicosanoid synthesis (Pace-Asciak et al., 1995; Szmítko et al, 2005).

Resveratrol shows the protection of brain against the effects of traumatic injury by decreasing cerebral ischemic injury in a rat model (Wang et al., 2002). This study documented the ability of resveratrol to traverse the blood–brain barrier. It also binds to the estrogen receptor and evidence suggests that regular wine consumption attenuates perimenopausal symptoms and protects against osteoporosis.

Resveratrol has an ability to delay the onset of neurodegradation due to the formation of  $\beta$ -amyloid plaque formation and oxidative stress. Alzheimer's dementia (AD) leads to progressive loss in the memory but the etiology of AD is completely unknown but formation of  $\beta$ -amyloid plaques have been found to be highly neurotoxic which occurs in AD.

Oxidative stress serves as a precursor for the amyloid precursor protein formation leading to AD (Karuppagounder et al., 2009).

### **Why Endophytic fungi be explored as a source of Resveratrol?**

Plant endophytic fungi is novel and abundant microorganism resource, possessing the unique ability to manufacture the analogous compounds originated from their host plants, as well as other bioactive compounds (Strobel et al., 2004). In the past two decades researchers mainly focused on the investigation of endophytic fungal diversity, relationships between endophytic fungi and their host plants, seeking for natural bioactive compounds originated from the endophytic fungi, and improving the productivity of some potential candidates by taking advantage of genetic engineering, microbial fermentation projects and other measures (Strobel et al., 2004).

Endophytic fungi are prolific producers of bioactive novel compounds that have commercial value. An array of natural products has been characterized from endophytes, which include anti-cancerous, anti-oxidants, anti-fungal, anti-bacterial, anti-viral, anti-insecticidal and immunosuppressant. World's first billion-dollar anticancer drug, paclitaxel (Taxol) is produced by *Pestalotiopsis microspora* which is a colonizer of Himalayan yew tree *Taxus wallichiana*. (Strobel and Daisy, 2003).

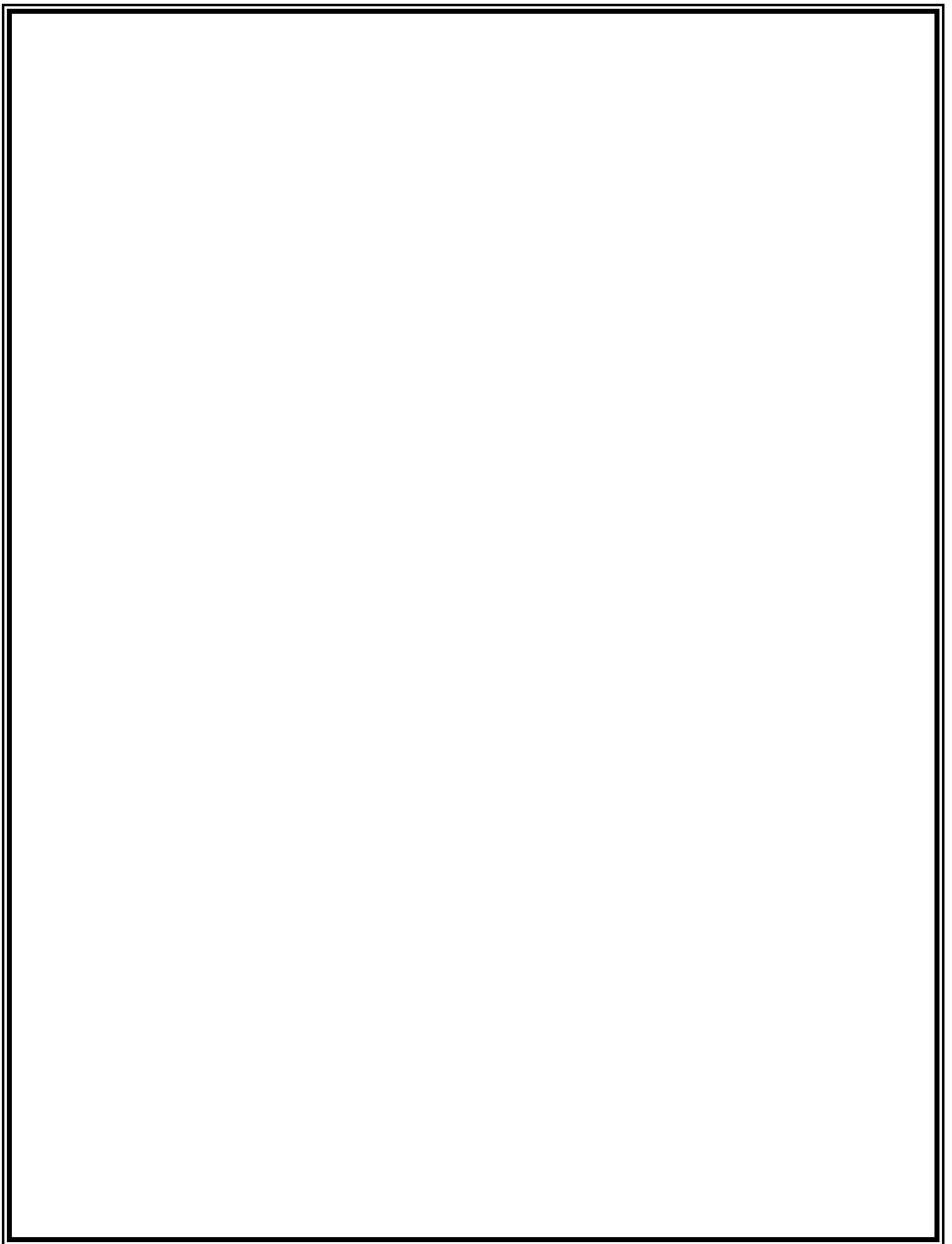
Resveratrol as a potent drug molecule for the cure of various diseases is supposed to be produced by the plant sources or through the gene transfer method but both the ways of resveratrol production is cumbersome and expensive process. As endophytic fungi reside inside the host plant it mimics all the property of the host so it could be a potent source for production of resveratrol for commercial use. Therefore endophytes can be explored for novel biological active secondary metabolites.



## *Chapter 2*

---

### *Review of Literature*



## **2. Review Literature**

Resveratrol (RSV) or trans-3, 4, 5-trihydroxystilbene was originally identified as a phytoalexin that in nature protects plants against infection against fungi especially *Botrytis cinerea* (Langcake and Pryce, 1976). Resveratrol was identified as a phytochemical since 1940's when it was first isolated from white hellebore (*Veratrum grandiflorum O. Loes*) and later from the medicinal plant *Polygonum cuspidatum*. Resveratrol is found in at least 72 plant species (Dercks and Creasy, 1989) and is formed via a condensation reaction between 3 molecules of malonyl CoA and 1 molecule of 4-coumaroyl CoA (Soleas et al., 1997). Both of these plants find use in the Chinese as well as Japanese medicine. Resveratrol was initially identified as an inhibitor of enzymes involved in Arachidonate metabolism in the leukocytes and of partially purified kinases. Resveratrol is also present in peanuts, soys and other plant product. In spite of their dietary habits the French population has a lower incidence of cardiovascular diseases –i.e. the French paradox has been attributed to uptake of resveratrol by red wine consumption which is a long standing component of their diet (Fremont, 2000). Thus this paradox has been the primary driver for impetus in research on resveratrol. With the increasing researches the market value and demand of resveratrol is increasing globally. In order to meet the market requirement the resveratrol has to be produced through cheaper and easy method.

### **2.1. Medicinal value of Resveratrol**

Incidentally the first use of grape extracts as medicine has been dated 2000 years ago as “darakchasava”, a well-known Indian herbal preparation of which the main ingredient is *Vitis vinifera* (Paul et al., 1999). This Ayurvedic medicine was prescribed as a cardi tonic. Today resveratrol has wide ranging biological activities and consequently many different targets and mechanisms of action. Resveratrol can prevent or slow the progression of several diseases, including cardiovascular disease (Bradamante et al., 2004), carcinogenic and neurodegenerative (Jang and Surh, 2001), prevent many aging processes and increase longevity as well (Baur et.al, 2006). Resveratrol also has anti-inflammatory (Langcake et al., 1996), antioxidant and antimicrobial properties (Docherty et al., 2007).

#### **2.1.1 Anti- cancer and chemopreventive activity**

Resveratrol possesses cancer chemo-preventive and cytostatic properties via initiation,

promotion and progression of carcinogenesis (Jang et al., 1997). Wolter et al., (2001) reported that resveratrol inhibited growth and proliferation of Caco-2 cells through apoptosis. The resveratrol has the potency to inhibit the COX enzyme which primarily catalyzes the conversion of arachidonic acid into prostaglandins, a pro-inflammatory substance which can stimulate tumor cell growth, suppress immune surveillance (Plescia et al., 1975; Goodwin, 1984) and can activate carcinogens to forms that damage genetic material (Zenser et al., 1983). It work as a chemopreventive agent also by producing its effect on cell apoptosis and anti-proliferation (Aluyen et al., 2012).It induces apoptosis by inhibit (ERK-1/2) gene suppressing the levels of estrogen and insulin growth factor -1 (IGF-1) receptor (Sheth et al., 2012) and can also induced anti-angiogenesis by suppression of PKG signaling and decreased expression of the cell-survival proteins c-IAP1, c-IAP2 and XIAP (Wong & Fiscus, 2015).

### **2.1.2 Neuroprotective activity**

Resveratrol has been reported to be effective in neuroprotective activity which is attributed to its anti-oxidant properties. It has been found effective against ischemic brain injury and in kainic acid induced brain seizures or neuronal damage in rodents (Wang et al., 2002; Wang et al., 2004). Resveratrol has an ability to delay the onset of neurodegradation due to the formation of  $\beta$ -amyloid plaque formation and oxidative stress (Karuppagounder et al., 2009). Resveratrol has also been tested for its beneficial effects in 6-hydroxydopamine induced Parkinson's rat model (Sun et al., 2010). In case of alzheimer's disease (AD) resveratrol inhibits activity on  $\beta$ -secretase, the generation of the reactive oxygen intermediates, and the aggregation of  $\beta$ A peptide. The derivatives of resveratrol such as the dimers scirpusin A and  $\epsilon$ -viniferin glucoside, could also be considered as a potent preventive and therapeutic agents for the cure of AD (Richard et al., 2011).The property of resveratrol to resveratrol ameliorate *dnj-14* mutant phenotypes can increase the lifespan which induces a SIRT- independent *Caenorhabditis elegans* worm neuroprotective mechanism leading it to be a potent drug for the cure of a human neurodegenerative disorder; Adult onset neuronal lipofuscinosis (ANCL) which characterizes the progressive neuronal dysfunction and premature death (Kashyap et al., 2014).

### **2.1.3 Cardioprotective activity**

Cardiovascular diseases are the leading causes of mortality throughout the world. The common

risk factors for cardiovascular diseases are hypertension, obesity, dyslipidemia, insulin resistance, glucose intolerance and increased oxidative stress. A significant observation was the South French population despite high fat consumption in their diet and heavy use of tobacco products had a very low incidence of cardiovascular disease. This is referred to as French Paradox and may be associated with the dietary consumption of red wine which is a dietary source of resveratrol and may be associated with the positive cardiovascular effects of moderate wine consumption (Constant, 1997). It has been found that resveratrol inhibits LDL (low density lipoprotein) oxidation which is the key event in the initiation of atherosclerosis (Belguendouz et al., 1997; Zou et al., 1999; Zou et al., 2000).

The interaction of resveratrol with multiple molecular targets and many intracellular pathways such as action on sirtuins through multiple transcription factor induces autophagy which is another form of stress adaptation it is a first line of protection against oxidative stress leading to cardiovascular diseases (Petrovski et al., 2011).

#### **2.1.4 Anti-ageing activity of Resveratrol**

Anti-ageing interventions essentially refer to both cosmetic maintenance of age through appropriate healthful appearance as well as stemming up the development of degenerative diseases optimizing in the brain as well as in other tissues to prolong life. A family of enzymes called Sirtuins specifically Sirtuin 1 (SIRT1) possesses anti-ageing action. Resveratrol has been found to activate SIRT1 and also exhibits the anti-oxidant effects.

Thus SIRT1 activation leads to modulation of a variety of proteins including peroxisome proliferator activated receptor co-activator-1  $\alpha$ , protein kinase B, and NF $\kappa$ B. SIRT belongs to histone deacetylases. During *in vitro* screening of SIRT1 activators, Resveratrol was identified as the most potent of all the 18 inducers who activity was tested during the assay (Baur and Sinclair, 2006; Balan et al., 2008). Resveratrol extends the life span in *Saccharomyces cerevisiae*, *Caenorhabditis elegans* (Tissenbaum and Guarente, 2001) and *Drosophila melanogaster* only of the genes of SIRT2 are present in these organisms. Resveratrol has shown to extend the lifespan of short lived fish by 59% concomitant with maintenance of learning and motor function (Valenzano and Cellerino, 2006; Terzibasi et al., 2007). Resveratrol has also been found to be an activator of human SIRT1 (Borra et al., 2005). The selective knock-out or over-expression of SIRT1 in human mesenchymal stem cells leads to acceleration or delay of senescence, resveratrol transiently promotes proliferation of mesenchymal stem cell without

affecting their lifespan (Yuan et al., 2012) which prolongs the lifespan of cell leading to an anti-aging effect.

### **2.1.5 Anti-microbial activity of Resveratrol**

Phytoalexins are low molecular weight compounds which exhibit biological activity against a range of plant as well as human pathogens (Jeandt et al., 2002; Mahady, 2005). Resveratrol was initially identified as a phytoalexin in *Vitis vinifera* (grapes) which has been subjected to intensive investigation because of multiple implications on human health. as resveratrol possesses anti-oxidant properties it is expected that it may also possess antimicrobial effects and may be used to prevent microbial infections. Resveratrol has been previously reported to possess anti-viral activity. Resveratrol has been found to inhibit the growth of *Helicobacter pylori* which is responsible for duodenal ulcer disease, dyspepsia, peptic ulcer and gastric B cell lymphoma (Mahady and Pendland, 2000). It is found that its activity is more towards gram-negative bacteria than gram-positive bacteria (Alam et al., 2013).

### **2.1.6 Anti – diabetic agent**

Diabetes mellitus is a modern day epidemic which is characterized by high blood sugar level which may be due to abnormal insulin secretion or insulin receptor or post receptor events affecting the overall metabolism of carbohydrates, fats and proteins. Resveratrol has been found to improve insulin action in different animal models (Sharma et al., 2011). The anti-hyperglycemic and anti-oxidant activity of resveratrol has been assessed by its modulatory effects on the activities of carbohydrate metabolizing enzymes in the kidney and in the hepatic tissues of streptozotocin-nicotinamide induced diabetic rats (Palsamy and Subramanian, 2009; Movahed et al., 2013).

## **2.2 Sources of resveratrol**

All of the families found to contain resveratrol belong to the spermatophytes division- *Vitaceae*, *Myrtaceae*, *Dipterocarpaceae*, *Cyperaceae*, *Gnetaceae*, *Leguminosae*, *Pinaceae*,

*Moraceae*, *Fagaceae*, *Liliaceae* (Langcake and Pryce, 1976). Resveratrol has most often been reported in non-edible plants: vine, eucalyptus, spruce, and the tropical deciduous tree *Bauhinia racemosa*, *Pterolobium hexapetallum* (Cassady et al., 2000; Soleas et al., 1997). Foods known to contain resveratrol are limited to grapes, wine, grape juice, cranberries, cranberry juice (0.278 ppm, Wang et al., 2002), peanuts, and peanut products (0.03–0.147 ppm in roasted peanuts: Sanders et al., 2000 and Sobolev and Cole, 1999;  $5.14 \pm 2.85$  ppm in boiled peanuts: Sobolev & Cole, 1999; and 0.27–0.75 ppm in peanut butter: Ibern-Gómez et al., 2000 and Sobolev and Cole, 1999). Recently, Callemien et al., (2003) detected *trans*-resveratrol, *trans*-piceid, and *cis*-piceid in hop, this suggesting that stilbenes might also be found in beer. (Vastano et al., 2000) have shown that the roots of the weed *Polygonum cuspidatum* constitute one of the richest sources of resveratrol (2960–3770 ppm).

Source	Percentage of Resveratrol	Reference
<b>Plants</b>		
<i>Polygonum cuspidatum</i>	73.8%	Wang et al., 2013
<i>Arachis hypogea</i>	0.09 to 0.30 µg/gm	Lee et al., 2004
<i>Vitis rotundifolia</i> (muscadine berries)	In seed- 30.1 % In skin- 23.4 %	Ector et al., 1996
<b>Food Products</b>		
Roasted peanut	0.27 µg/gm- traces	Lee et al., 2003
Peanut butter	0.27 to 0.70 µg/gm	Lee et al., 2003
<b>Fungi</b>		
<i>Alternaria</i> sp from <i>Vitis vinifera</i>	6-123 µg/L	Shi et al., 2012

Table no. 1 Sources of Resveratrol

### 2.3. Endophytes: Bioresource to novel metabolites

Endophytes are microbial entities that live within living tissues of plants. In most cases their interaction with the host plant is symbiotic and probably mutualistic. Fungal endophytes comprise a diverse group of fungi that inhabit living plant tissues without inducing disease symptoms (Petrini, 1991). Endophytes residing in tropical plant species benefit the host plant by

providing protection from insect attack (Arnold and Lewis, 2005). Endophytic fungi have been found in most plant species including terrestrial plants from different parts like fruits, stems or bark, roots, leaves, tubers, buds, xylem and rachis of grasses, palms, banana, mangroves and halophytes.

The types of plants having this association are quite diverse, which include mosses, liverworts, pteridophytes, gymnosperms and angiosperms. It is now widely accepted that endophyte-free plants are few, and this is especially true for shrubs and trees (Gennaro et al., 2003). An array of natural products has been characterized from endophytes, which include anti-cancerous, anti-oxidants, anti-fungal, anti-bacterial, anti-viral, anti-insecticidal and immunosuppressants (Strobel et al., 2003).

COMPOUND	SOURCE ORGANISM	ACTIVITY	REFERENCES
Paclitaxol	<i>Taxus andreanae</i>	Prostate, ovarian, breast and lung cancers	Slichenmyer & Von Hoff, 1991
Podophyllotoxin	<i>Diphylleia sinensis</i>	Anticancer, antiviral, antioxidant	Puri et al., 2006
Camptothecin	<i>Entrophospora infrequens</i>	Antineoplastic agents	Kaufmann, 1989
Ambuic acid	<i>Pestalotiopsis microspora</i>	Antifungal agent	Li et al., 2001
BrefeldinA	<i>Phoma medicaginis</i>	Antibacterial	Wang et al., 2007

Table no.2 Vital active compound from endophytic fungus.

#### 2.4. Production of Resveratrol

The main producer of trans-Resveratrol from root extracts is China using an extraction process. The level of trans-resveratrol purity in these extracts varies between 49%-99% (Changsha Nutramax Inc. 2009). Plant cell suspension cultures of *Polygonum* species, *Vitis vinifera* and *Arachis hypogea* have been attempted for trans-resveratrol production under controlled conditions but production higher than native state have not been achieved by untreated plant cell cultures. The additional challenge posed by plant cell cultures is instability in the production of

secondary metabolites during long term cultivation (Kiselev and Bulgakov, 2009; Kiselev et al., 2009). Plant cell and tissue culture offer alternatives for the production of resveratrol. The production of trans-resveratrol in plant cell cultures e.g., cell cultures of *Vitis* sp, *Arachis hypogea*, has been analyzed by several groups (Morales et al., 1998; Chen et al., 2007; Medina-Bolivar et al., 2007; Kiselev et al., 2007). Microorganisms have also been genetically manipulated in order to optimize the production of resveratrol through the fermentation route. Transgenic yeast *Saccharomyces cerevisiae* (CEN.PK113-3b strain) has been developed and is being tested for the production of trans-resveratrol (Beekwilder et al., 2006).

The recent method used for resveratrol production is through recombinant gene expression system in *E.coli* by screening genes of biosynthetic pathway and combinations of 4CL and STS genes. This includes the gene expression system, strain background and Malonyl-CoA availability (Lim et al., 2011)

## **2.5 Screening for resveratrol**

### **2.5.1 Biochemical Tests:**

Phenolic compounds are not only widely employed in industry but are distributed in liberal variety throughout the animal and vegetable kingdoms. Many are found by the biologists to occur naturally as products of metabolism; several are used as medicine or antiseptics. Since quantities available were often small, the hydroxyl groups remain in the final product the acidic properties of this group determine the conditions under which color appears or changes of color take place (Jumaily et al., 2014).

The Liebermann reagent named after Hungarian chemist Leo-Liebermann (1852-1926) is been used as a simple spot-test to presumptively identify organic compound with free para position. It is composed of a mixture of potassium nitrite and concentrated sulfuric acid is used to screen out the cultures likely to produce resveratrol as it has the free para position in the structure. After that the confirmatory test of using acetic anhydride in place of potassium nitrite enhances the possibility of presence of resveratrol in the culture broth (Jumaily et al., 2014).

#### **2.5.2.1. Thin Layer Chromatography (T.L.C)**

TLC is simple, quick, and inexpensive procedure that gives a quick resolution of the components

present in a mixture. TLC is also used to support the identity of a compound in a mixture when the  $R_f$  of a compound is compared with the  $R_f$  of a known compound (preferably both run on the same TLC plate). According to the principle of TLC the components will differ in solubility and in the strength of their adsorption to the adsorbent and will separate accordingly. TLC method was used by several researchers to identify resveratrol (Babu et al., 2005; Malele, 2014 and Park and Boo, 2013). Babu et al., (2005) has performed thin layer chromatography using solvent Chloroform: ethyl acetate: formic acid (2.5:1:0.1v/v/v) and reported the presence of resveratrol from *Polygonum cuspidatum* and similarly Malele R.S., (2014) has also used this solvent system to report the presence of resveratrol in grape berry skin.

The presence of resveratrol in *Vitis vinifera* is also been reported using TLC in solvent system of Chloroform: methanol: Acetic acid (90:10:1 v/v/v) by Park and Boo, (2013).

#### **2.5.2.1 High Performance Liquid Chromatography (HPLC)**

HPLC is a separation technique that involves the placement (injection) of a small volume of liquid sample into a tube packed with porous particles i.e. column (stationary phase) where individual components of the sample are transported along the packed tube (column) by a liquid moved by gravity. The components of the sample are separated from one another by the column packing that involves various chemical and/or physical interactions between their molecules and the packing particles.

HPLC was used by several researchers to identify and purify resveratrol (Shi et al., 2012; Wang et al., 2013 and Sanders et al., 2000). Shi et al., 2012 has performed HPLC using Acetonitrile: Water as mobile phase in the gradient mode with C18 column as stationary phase. The flow rate was taken as 1mL/min to identify the production of resveratrol by an *alternaria* sp. Similarly Babu et al., (2005) has also used Acetonitrile: Water (60:40 v/v) but in isocratic mode with the flow rate of 0.6 mL/min at wavelength of 280nm to report the presence of resveratrol.

Wang et al., (2013) has reported the presence of resveratrol from *Polygonum cuspidatum* with mobile phase comprising of Acetic acid: Acetonitrile: Water (2:20: 80 v/v/v) at the wavelength of 290nm with the flow rate of 1mL/min. Sanders et al, 2000 have eluted resveratrol with the linear gradient of Acetonitrile and 0.1% TFA at 308nm from Peanuts. The isocratic mobile phase of Acetonitrile: Water (40:60 v/v) with the flow rate of 0.3mL/min at wavelength

of 306nm was used to identify the presence of resveratrol from Korean Peanut Butter and the content found was too high (Lee et al., in 2004).

## **2.6. Identification of Endophytic fungi**

### **2.6.1 Morphological identification:**

Morphological taxonomy has to be carried by microscopic study of the fungal structures such as mycelia, fruting bodies, spores are very important features as fungal taxonomy is traditionally based on comparative morphological features (Zhang et al., 2008). Various optimization of growth conditions have been used in case of the fungi which are non-sporulating in the culture as , they cannot be identified by the conventional techniques otherwise so different media such as Potato dextrose agar (PDA), Water agar (WA),Malt Extract Agar (MEA), Corn meal agar (CMA) have been used in which the fungi sporulate. (Guo et al., 2000).

### **2.6.2 Molecular technique:**

In contrast the molecular techniques exhibit more sensitivity and specificity for identifying microorganisms and can be used to classify the microbes on the diverse hierarchical taxonomic levels (Sette et al., 2006). Most of the endophytic fungi are being identified and detected by the comparative analysis of ribosomal DNA sequences especially ITS region. The arbuscular mycorrhizal fungi is identified using the ITS (internal transcribed site) region. The 5.8S ribosomal gene flanking region ITS 1 and ITS 4 have been used to identify the resveratrol producing fungi (Shi et al., 2012).

## *Chapter 3*

---

### *Aim of the study*

### **3. Aim of the study**

1. Isolation of endophytic fungi from the plant *Vitis Vinifera*
2. Screening and identification of resveratrol producing endophytic fungi
3. Purification of resveratrol produced from endophytic fungi.

# *Chapter 4*

---

## *Materials & Methods*

## **4. Material and Method**

### **4.1 Plant Sample collection and Isolation of endophytic fungi**

Healthy plant parts of *Vitis vinifera* were collected from the Rajendra nagar of Lucknow, Uttar Pradesh in the month of September 2014 and were transferred to sterile zip-lock plastic bags and stored at 4<sup>0</sup>C until isolation. Briefly describing, the collected plant sample (stems and leaves) were surface sterilized by immersing them in 0.1% sodium hypochlorite solution for 2-3 min followed by 70% ethanol for 1 min and then subsequent washing with 30% ethanol for 45 sec. The sterilized sample was cut into 1-2 mm pieces with the help of sterile blade and was inoculated onto Potato Dextrose Agar (PDA) plate supplemented with chloramphenicol (100 mg/ml). The inoculated plates were incubated at 28°C to promote the growth of endophytes and were regularly monitored for the profuse fungal growth. The fungal hyphae emerging out of the inoculated sample was picked up by inoculation loop and, subcultured on to fresh PDA plate to get pure culture. The purified endophytic isolates were transferred aseptically to PDA slants containing 20% glycerol for long term preservation.

### **4.2 Procurement, Maintenance and Preservation of endophytic fungi**

63 cultures were procured from the pre-existing repository of endophytic cultures in Dr. Sanjai Saxena, Thapar University, Patiala Laboratory. These cultures were maintained and preserved as described above.

### **4.3 Production of culture filtrates**

All the endophytic cultures for subjected to culture filtrate production by inoculating 5 mm mycelial disc of 7-10 day old fungal culture to 50 ml pre-sterilized Czepak Dox Broth (CDB) medium in 250ml Erlenmeyer flasks. The flasks were incubated in incubator shaker at 26°C, 120 rpm for 7 days. After the incubation is over, spent broth was separated from mycelia by filtration through Whatman filter paper 4 followed by centrifugation at 12,000 rpm for 10 min to get cell free filtrate (Rodrigues et al., 2000, Vicente et al., 2001).

### **4.4 Screening of Resveratrol producing endophytic fungi**

The culture filtrates of endophytic fungi were then screened for Resveratrol production by using two biochemical assays for the detection of phenolic compounds.

#### **4.4.1 Libermann Test (Jumaily et al., 2014)**

This assay is used to detect phenolic compounds with free para position. To the 2ml of culture filtrate of each endophytic fungi, 1 ml of conc  $H_2SO_4$  followed by few drops of  $NaNO_2$  (1mg/ml) was added. The appearance of green/blue-violet colour indicates the presence of phenolic compound. Resveratrol (Sigma-Aldrich) and Un-inoculated Czepak Dox broth was used as positive and negative control respectively.

#### **4.4.2. Acetic Anhydride test (Jumaily et al., 2014)**

This test is based upon the detection of phenolic compound with free  $-OH$  group. To 1 ml of culture filtrate, 100 $\mu$ L of acetic anhydride and 500 $\mu$ L of conc.  $H_2SO_4$  was added. The reaction mixture was then observed for the appearance of greenish brown precipitate indicates the presence of phenolic compounds in the test sample. Resveratrol (Sigma-Aldrich) and Un-inoculated Czepak Dox broth was used as positive and negative control respectively.

#### **4.5 Solvent Extraction**

The cultures exhibiting resveratrol production was then subjected to liquid-liquid extraction for the probable isolation of bioactive residue. The culture filtrate was extracted with ethyl acetate in the ratio of 1:3. The organic layer was pooled out and the extraction procedure was repeated thrice. The organic layer was dried with addition of anhydrous sodium sulphate and evaporated in rotatory evaporator at 30°C to dryness to get crude bioactive fraction. The fraction so obtained was weighed, reconstituted in methanol and again screened for the presence of resveratrol by biochemical assays used above.

#### **4.6 TLC analysis**

The crude bioactive fraction of the cultures exhibiting resveratrol production was fractionated by subjecting to preparative thin layer chromatography (TLC). The TLC plate of 0.5mm thickness was prepared by coating silica gel (Merck) on to 20 x 15 x 5 mm clean glass plates and was activated by incubating at 100°C for 2h prior to use. The sample was spotted on to activated TLC plate just 1 cm above the edge of plate with the help of capillary. Simultaneously, the TLC chamber was saturated with different solvent systems (Binary and tertiary) consisting of mixture of solvents of different polarities and ratios for 30 min. The TLC plate was kept in saturated TLC

chamber in such a way that the sample spot is above the solvent level. When the solvent front reaches up to the desired level, the TLC plate was taken out and allowed to air dry. The Chromatogram was developed by visualizing the TLC plate under UV light. Resveratrol was used as standard for the comparison of  $R_f$  value. Retention factor ( $R_f$ ) value of each band was obtained as the ratio of distance moved by solute to that of solvent.

$$\text{Retention Factor } (R_f) = \frac{\text{Distance moved by solute}}{\text{Distance moved by solvent}}$$

#### **4.7 High Performance Liquid Chromatography**

The concentration of crude bioactive fraction of selected positive isolate was determined by HPLC (Perkin Elmer- 200 series pump). 50 $\mu$ g of crude bioactive fraction was dissolved in methanol and injected into the HPLC column. Gradient elution of orthophosphoric acid (0.1%) and acetonitrile ranging from 10% to 70% was used as mobile phase with a flow rate of 1 ml/min. To determine the concentration of fungal Resveratrol, different dilutions of standard Resveratrol (Stock- 1 mg/ml, Sigma Aldrich) ranging from 0.1 - 1.0 mg/ml were prepared in HPLC grade Methanol. 20 $\mu$ l of each dilution was injected into C18 (5  $\mu$ m) 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 Resveratrol obtained were used to estimate the quantity of fungal resveratrol in crude EA fraction.

#### **4.8 Purification of fungal resveratrol**

For the isolation of pure resveratrol, band of interest was scrapped off from the TLC plate and the fraction so collected was reconstituted in Methanol followed by centrifugation at 8000 rpm for 15 min to remove excess silica. After that the fraction collected was passed through the Whatman filter-paper no.1 and was completely evaporated in rotatory evaporator at 30°C to get purified fraction. The purified fraction was dissolved in methanol and stored at -20° C.

#### **4.9 Identification of Endophytic Fungi**

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

### **4.9.1 Morphotaxonomy**

The isolate found to be potential producer of resveratrol was examined under the microscope for the identification on the basis of their morphological and microscopic characters. The culture was grown over different media namely Potato dextrose Agar (PDA), Corn meal agar (CMA), Water agar (WA), Grape leaf agar (GLA), synthetic nutrient deficient Agar (SNA), Malt extract agar (MEM), Czepak Dox Agar (CDA) for 4-5 weeks at 26° C. Morphological characteristics such as colony color, appearance, growth rate was observed carefully and noted. For microscopic features, a drop of water was put on clean glass slide, upon which the mycelial mass was placed and teased properly with the help of fine tip needle. It was then stained with Lactophenol cotton blue (Hi Media). The slide was covered with 18 x 10 mm cover slip avoiding the formation of air bubble and mounted with DPX. The slide was observed at 10X, 40X and 100X using Nikon binocular microscope.

### **4.9.2 Molecular Identification of endophytic fungi**

#### **4.9.2.1 DNA Isolation**

The fungal genomic DNA was isolated from 5-7 day old culture grown on PDA plate using Wizard<sup>®</sup> Genomic DNA purification kit (Promega, USA). 2-3 mycelial plug of 5 mm diameter was crushed into very fine powder in sterile pestle and mortar by using liquid nitrogen. 660-750µl of the Nuclei lysis buffer was added and it was again crushed. The contents were transferred to a 1.5 ml micro centrifuge tube and vortexed followed by incubation at 65°C in water bath for 15 min. After the incubation, the micro centrifuge tubes were centrifuged at 12,000 rpm for 5 min to remove cell debris. Further 5µl of RNase was added to each tube and incubated at 37°C for 15 min followed by addition of 200 µl of protein precipitation solution. After this, the micro centrifuge tubes were centrifuged at 12,000 rpm for 3 min to remove contaminating proteins. The aqueous phase containing DNA was transferred to iso-propanol containing micro-centrifuge tube and centrifuged at 13,000 rpm for 3 min. The DNA pellet was rinsed with 70% ethanol followed by centrifugation at 13,000 rpm for 1 min. Then pellet was air dried and dissolved in 50µl of DNA dehydration buffer (Tris EDTA buffer (pH 8)). The qualitative estimation of the DNA isolated was done by agarose gel electrophoresis.

#### 4.9.2.2 Agarose gel electrophoresis

0.8% agarose gel containing 0.5µg/ml of ethidium bromide (EtBr) was prepared in 1X TAE (Tris Acetate EDTA) buffer and then gel was casted in the electrophoretic apparatus. The gel was allowed to solidify and the comb was carefully removed. The running buffer (1X TAE) was poured into the electrophoretic tank. The DNA samples mixed with the 6X loading dye were loaded into wells and allowed to run at 50V for 1h. The DNA fragments were visualized under UV transilluminator. Gel imaging was performed under 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 sample was taken at 260 nm and 280 nm to determine the concentration and purity of the sample. 1 OD is equivalent to 50µg/ml DNA sample. The concentration of the DNA sample was calculated using following formula.

$$\text{Concentration } (\mu\text{g/ml}) = \text{O.D}_{260\text{nm}} \times 50\mu\text{g/ml} \times \text{Dilution factor}$$

The purity of the DNA sample was determined by taking the ratio of absorbance at 260nm and at 280nm. If the ratio is less than 1.6, then there is RNA contamination, if the ratio lies between 1.6-1.8, then DNA sample is pure. If the ratio is more than 1.8, the DNA might be contaminated with protein.

#### 4.9.2.3 PCR amplification

ITS1-5.8S-ITS2 rDNA sequence was amplified 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.). 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 performed in 25µl reaction mixture containing 25ng of extracted fungal DNA, 0.8 µM of each primer (ITS1 and ITS4), 2.5mM of dNTP (Bangalore GeNei), 1.5 mM MgCl<sub>2</sub> (Bangalore GeNei), 1.5 U of Taq DNA Polymerase (Bangalore GeNei) in 10 X Taq buffer (Bangalore GeNei). The Thermal cycling conditions consisted of initial denaturation at 96°C for 5 min followed by 39 cycles of 95°C for 1 min, 58°C for 1.30 min, 72°C for 1 min followed by final extension at 72°C for 5 min. The PCR amplicons were examined using gel electrophoresis in a 1.5 % agarose gel at 40V for 1.30 hr. Gel imaging was performed under UV light in Bio- Rad Gel documentation System.

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

Table 3 Different reagents used during PCR reaction

An approximate 550- 600bp PCR amplicon was purified by using the Wizard<sup>®</sup> SV Gel and PCR clean up system kit (Promega, USA). The purified amplicon was sent for direct sequencing to Xcelris Labs Ltd., Ahmadabad, Gujarat.

#### 4.9.2.4 Sequence assembly and Phylogenetic identification

The obtained chromatograms were manually edited and final sequence was submitted in the GenBank. The homology of isolate was established by subjecting the final sequence to sequence similarity search using BLAST. The final sequence along with 15 reference taxa was aligned using CLUSTAL W in MEGA 5.0 (Tamura et al., 2011). The evolutionary relationship was inferred using the Maximum Parsimony method. The Maximum Parsimony tree was obtained by using the Close-Neighbor-Interchange algorithm (Nei and Kumar, 2000) with search level 3 in which the initial trees were obtained with the random addition of sequences (20 replicates). The tree was drawn to scale, with branch lengths calculated using the average pathway method (Nei and Kumar, 2000) and are in the units of the number of changes over the whole sequence. Gaps

and missing data were eliminated. 1000 bootstrap replicates were taken to infer evolutionary relationship (Felsenstein, 1985).

# *Chapter 5*

---

## *Results & Discussion*

## 5. Results

### 5.1 Sub-culturing of endophytic isolates

In the present study, 72 Endophytic fungi were sub cultured regularly on PDA plates and maintained at 28°C. All the isolates were preserved on to PDA-glycerol slants for long term storage. The isolates in the present study were isolated from medicinally important plants belonging to families Rutaceae (*Aegle marmelos*), Lauraceae (*Cinnamomum malabaricum*, *Cinnamomum zeylanicum*, *Cinnamomum camphora*), Apocynaceae (*Catharanthus roseus*, *Raulwofia serpentina*) and Vitaceae (*Vitis vinifera*) collected from the biodiversity hot spots of India. Throughout the ages, human beings have always relied upon the plants for the medication of various diseases such as Malaria, fever, warts, cancer, diabetes etc. In the past two decades, many valuable novel bioactive compounds with antimicrobial, anti-aging, cytotoxic and anticancer potential have been successfully discovered from the endophytic fungi. During the long period of co-evolution, a responsive relationship was created between endophyte and its host plant. Some of the endophytes have the ability to mimic the property of host plants and are capable of producing the same or similar bioactive compounds as those derived from their host plants. Hence the present study undertakes the exploitation of endophytic fungi isolated from medicinal plants for the production of novel bioactive compounds.

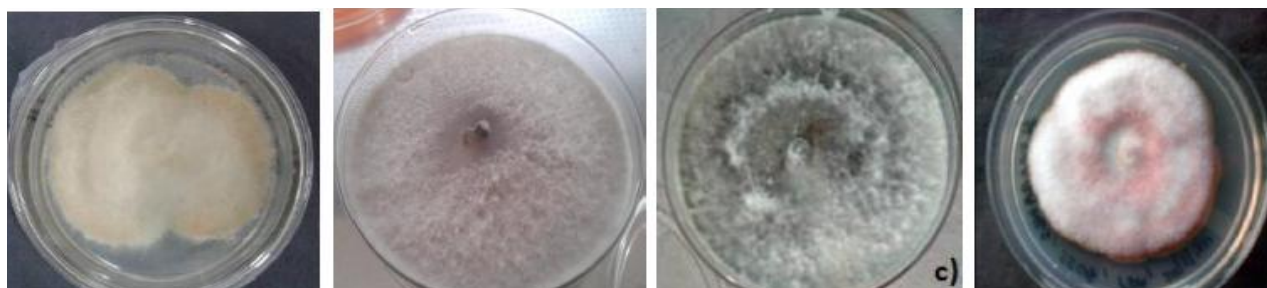


Figure 2 Pure cultures of different endophytic fungi on PDA plates

Out of 72 endophytic fungi (Figure 2), 2 were isolated from leaves of *Aegle marmelos*, 48 endophytic fungi were isolated from *Cinnamomum malabaricum*- 11 were from bark, 12 from leaf, 14 from stem, 11 from stem internal tissue, 4 endophytic fungi were isolated from stem internal tissue and one from stem of *Cinnamomum camphora*, 9 were isolated from *Vitis vinifera*- 8 were isolated from leaf and 1 from stem, 14 endophytic fungi were isolated from *Catharanthus roseus* - 12 were from stem and 2 from leaf, 1 endophyte was isolated from bark of *Cinnamomum zeylanicum* as well as from stem of *Raulwofia serpentina*.

S.no	Culture Code	Host Plant	Identification	Plant Part	Place of Collection	Biochemical Tests	
						Acetic	Libermann
						Anhydride Test	Test
1	#6AMLWLS	<i>Aegle marmelos</i>	<i>Fusarium incarnatum</i>	Leaves	Wayanad wildlife Sanctuary, Karnataka	-	-
2	#16AMLWIS	<i>Aegle marmelos</i>	<i>Muscodor kashayum</i>	Leaves	Wayanad wildlife Sanctuary, Karnataka	++	-
3	#1CCSTITD	<i>Cinnamomum camphora</i>	<i>M. darjeelingensis</i>	Stem	Tiger Hills, Darjeeling, W.B	-	-
4	#6CCSTITD	<i>Cinnamomum camphora</i>	<i>M. ghoomensis</i>	Stem internal tissue	Tiger Hills, Darjeeling, W.B	-	-
5	#6(b)CCSTITD	<i>Cinnamomum camphora</i>	<i>M. indica</i>	Stem internal tissue	Tiger Hills, Darjeeling, W.B	-	-
6	#36CCSTITD	<i>Cinnamomum camphora</i>	Unidentified	Stem internal tissue	Tiger Hills, Darjeeling, W.B	-	-
7	#1639CCSTITD	<i>Cinnamomum camphora</i>	<i>M. camphora</i>	Stem internal tissue	Tiger Hills, Darjeeling, W.B	-	-
8	#4CMBABRT	<i>Cinnamomum malabaricum</i>	<i>Bionectria sp.</i>	Bark	BRT Wildlife Sanctuary, Karnataka	-	-
9	#12CMBABRT	<i>Cinnamomum malabaricum</i>	<i>Bionectria sp.</i>	Bark	BRT Wildlife Sanctuary, Karnataka	-	-
10	#18CMBABRT	<i>Cinnamomum malabaricum</i>	<i>Clonostachys</i>	Bark	BRT Wildlife Sanctuary, Karnataka	-	-
11	#2CMBANEY	<i>Cinnamomum malabaricum</i>	<i>Alternaria sp.</i>	Bark	Neyyar, Kerala	++	+++
12	#12CMBANEY	<i>Cinnamomum malabaricum</i>	<i>Alternaria sp.</i>	Bark	Neyyar, Kerala	-	-
13	#18CMBANEY	<i>Cinnamomum malabaricum</i>	<i>Botryosphaeria sp.</i>	Bark	Neyyar, Kerala	+	-
14	#20CMBANEY	<i>Cinnamomum malabaricum</i>	<i>Lasiodiplodia sp.</i>	Bark	Neyyar, Kerala	-	-
15	#21CMBANEY	<i>Cinnamomum malabaricum</i>	<i>Fusarium sp.</i>	Bark	Neyyar, Kerala	-	-
16	#22CMBANEY	<i>Cinnamomum malabaricum</i>	<i>Fusarium sp.</i>	Bark	Neyyar, Kerala	+++	++
17	#4CMLBRT	<i>Cinnamomum malabaricum</i>	<i>Alternaria sp.</i>	Leaves	BRT Wildlife Sanctuary, Karnataka	-	-
18	#27CMLBRT	<i>Cinnamomum malabaricum</i>	<i>Aspergillus sp.</i>	Leaves	BRT Wildlife Sanctuary, Karnataka	-	-
19	#40CMLBRT	<i>Cinnamomum malabaricum</i>	<i>Alternaria sp.</i>	Leaves	BRT Wildlife Sanctuary, Karnataka	-	-

20	#1CMLNEY	<i>Cinnamomum malabaricum</i>	Unidentified	Leaves	Neyyar, Kerala	-	-
21	#2CMLNEY	<i>Cinnamomum malabaricum</i>	Unidentified	Leaves	Neyyar, Kerala	-	-
22	#17CMLNEY	<i>Cinnamomum malabaricum</i>	<i>Pestalotiopsis</i> sp.	Leaves	Neyyar, Kerala	+++	++
23	#18CMLNEY	<i>Cinnamomum malabaricum</i>	Unidentified	Leaves	Neyyar, Kerala	-	-
24	#23CMLNEY	<i>Cinnamomum malabaricum</i>	Unidentified	Leaves	Neyyar, Kerala	-	-
25	#29CMLNEY	<i>Cinnamomum malabaricum</i>	<i>Bionectria</i> sp.	Leaves	Neyyar, Kerala	-	-
26	#37CMLNEY	<i>Cinnamomum malabaricum</i>	<i>Bionectria</i> sp.	Leaves	Neyyar, Kerala	-	-
27	#31CMLPITNEY	<i>Cinnamomum malabaricum</i>	Unidentified	Leaves	Neyyar, Kerala	-	-
28	#35CMLPLM	<i>Cinnamomum malabaricum</i>	Unidentified	Leaves	Palampur	-	-
29	#43CMSTITBRT	<i>Cinnamomum malabaricum</i>	Unidentified	Stem internal tissue	BRT Wildlife Sanctuary, Karnataka	-	-
30	#6610CMSTITBRT	<i>Cinnamomum malabaricum</i>	<i>M. strobilii</i>	Stem internal tissue	BRT Wildlife Sanctuary, Karnataka	-	-
31	#4CMSTITNEY	<i>Cinnamomum malabaricum</i>	<i>Arthrinium</i> sp.	Stem internal tissue	Neyyar, Kerala	-	-
32	#21CMSTITNEY	<i>Cinnamomum malabaricum</i>	<i>Arthrinium</i> sp.	Stem internal tissue	Neyyar, Kerala	-	-
33	#54CMSTITNEY	<i>Cinnamomum malabaricum</i>	<i>Pestalotiopsis</i> sp.	Stem internal tissue	Neyyar, Kerala	-	-
34	#1622CMSTITNEY	<i>Cinnamomum malabaricum</i>	<i>Curvularia</i> sp.	Stem internal tissue	Neyyar, Kerala	++	++
35	# #1CMSTNEY	<i>Cinnamomum malabaricum</i>	<i>Arthrinium</i> sp.	Stem	Neyyar, Kerala	-	-
36	#11CMSTNEY	<i>Cinnamomum malabaricum</i>	<i>Pestalotiopsis</i> sp.	Stem	Neyyar, Kerala	-	-
37	#13CMSTNEY	<i>Cinnamomum malabaricum</i>	<i>Pestalotiopsis</i> sp.	Stem	Neyyar, Kerala	-	-
38	#26CMSTNEY	<i>Cinnamomum malabaricum</i>	Unidentified	Stem	Neyyar, Kerala	-	-
39	#35CMSTNEY	<i>Cinnamomum malabaricum</i>	Unidentified	Stem	Neyyar, Kerala	-	-
40	#36CMSTNEY	<i>Cinnamomum malabaricum</i>	Unidentified	Stem	Neyyar, Kerala	-	-
41	#43CMSTNEY	<i>Cinnamomum malabaricum</i>	Unidentified	Stem	Neyyar, Kerala	-	-

42	#44CMSTNEY	<i>Cinnamomum malabaricum</i>	<i>Arthrinium sp.</i>	Stem	Neyyar, Kerala	-	-
43	#45CMSTNEY	<i>Cinnamomum malabaricum</i>	Unidentified	Stem	Neyyar, Kerala	-	-
44	#49CMSTNEY	<i>Cinnamomum malabaricum</i>	Unidentified	Stem	Neyyar, Kerala	-	-
45	#55CMSTNEY	<i>Cinnamomum malabaricum</i>	Unidentified	Stem	Neyyar, Kerala	+++	+++
46	#59CMSTNEY	<i>Cinnamomum malabaricum</i>	Unidentified	Stem	Neyyar, Kerala	-	-
47	#64CMSTNEY	<i>Cinnamomum malabaricum</i>	Unidentified	Stem	Neyyar, Kerala	-	-
48	#28CZBAG	<i>Cinnamomum zeylanicum</i>	<i>Curvularia sp.</i>	Bark	Guwahati, Assam	++	+++
49	#16CRLPAL	<i>Catharanthus roseus</i>	<i>Fusarium sp.</i>	Leaves	Palampur	+++	+++
50	#37CRLPAL	<i>Catharanthus roseus</i>	<i>Schizophyllum sp</i>	Leaves	Palampur	-	-
51	#2CRSTBRT	<i>Catharanthus roseus</i>	Unidentified	Stem	BRT Wildlife Sanctuary, Karnataka	-	-
52	#5CRSTBRT	<i>Catharanthus roseus</i>	Unidentified	Stem	BRT Wildlife Sanctuary, Karnataka	-	-
53	#6CRSTBRT	<i>Catharanthus roseus</i>	Unidentified	Stem	BRT Wildlife Sanctuary, Karnataka	-	-
54	#15CRSTBRT	<i>Catharanthus roseus</i>	Unidentified	Stem	BRT Wildlife Sanctuary, Karnataka	-	-
55	#17CRSTBRT	<i>Catharanthus roseus</i>	<i>Fusarium sp.</i>	Stem	BRT Wildlife Sanctuary, Karnataka	-	-
56	#17(b)CRSTBRT	<i>Catharanthus roseus</i>	Unidentified	Stem	BRT Wildlife Sanctuary, Karnataka	-	-
57	#26CRSTBRT	<i>Catharanthus roseus</i>	<i>Alternaria sp.</i>	Stem	BRT Wildlife Sanctuary, Karnataka	-	-
58	#29CRSTBRT	<i>Catharanthus roseus</i>	<i>Xylaria sp.</i>	Stem	BRT Wildlife Sanctuary, Karnataka	-	-
59	#37CRSTBRT	<i>Catharanthus roseus</i>	<i>Xylaria sp.</i>	Stem	BRT Wildlife Sanctuary, Karnataka	-	-
60	#42CRSTBRT	<i>Catharanthus roseus</i>	<i>Fusarium sp.</i>	Stem	BRT Wildlife Sanctuary, Karnataka	-	-
61	#43CRSTBRT	<i>Catharanthus roseus</i>	Unidentified	Stem	BRT Wildlife Sanctuary, Karnataka	-	-
62	#4RSLBRT	<i>Rauwolfia serpentina</i>	<i>Fusarium sp.</i>	Leaves	BRT Wildlife Sanctuary, Karnataka	-	-
63	#22CRSTNEY	<i>Catharanthus roseus</i>	<i>Xylaria sp.</i>	Stem	Neyyar, Kerala	+++	++
64	#1VVLL	<i>Vitis vinifera</i>	Unidentified	Leaves	Rajendra nager, Lucknow	++	+

65	#1(b)VVLL	<i>Vitis vinifera</i>	Unidentified	Leaves	Rajendra nager, Lucknow	-	-
66	#3VVLL	<i>Vitis vinifera</i>	Unidentified	Leaves	Rajendra nager, Lucknow	-	-
67	#4VVLL	<i>Vitis vinifera</i>	Unidentified	Leaves	Rajendra nager, Lucknow	-	-
68	#8VVLL	<i>Vitis vinifera</i>	Unidentified	Leaves	Rajendra nager, Lucknow	-	-
69	#9VVLL	<i>Vitis vinifera</i>	Unidentified	Leaves	Rajendra nager, Lucknow	++	++
70	#13VVLL	<i>Vitis vinifera</i>	Unidentified	Leaves	Rajendra nager, Lucknow	-	-
71	#35VVLL	<i>Vitis vinifera</i>	Unidentified	Leaves	Rajendra nager, Lucknow	-	-
72	#5VVSTL	<i>Vitis vinifera</i>	<i>Quambalaria sp.</i>	Stem	Rajendra nager, Lucknow	+++	+++

[-] no pigmentation, (+) average (70-80%), (++) good (80-90%), (+++) very good (>90%)

Table No. 4 Endophytic isolates used for Resveratrol production

## 5.2 Production of culture Filtrate

The isolates under present investigation were subjected to secondary metabolites production in CDB medium. Biomass production was considered as a parameter to infer the growth rate of fungal cultures. #8VVLL was showing the highest and #1CCSTITD showing the lowest biomass production. Biomass production of some of fungal cultures ranging from highest to lowest is listed in Table no.5. The pH of culture filtrates of most of the isolates were found to be static except that of #16AMLWLS, #1CCSTITD, #2CCSTITD and #6CCSTID whose pH value drops down to 6.7.

Culture Code	Weight of biomass (in gram)
# 1CMLNEY	2.30
# 3VVLL	3.14
# 43CMSTITBRT	1.93
# 8VVLL	3.75
# 20CMBANEY	1.81
# 5VVSTL	0.82
# 6610CMSTITBRT	0.78
# 1CCSTITD	0.56

Table no.5 Biomass production of different cultures under study

## 5.3 Biochemical screening assays of Resveratrol producing endophytic fungi

Two biochemical tests for the detection of phenolic compounds viz. Liebermann and Acetic anhydride tests were used for probable detection of Resveratrol in ethyl acetate extracts of all endophytic fungi. Only 9 isolates viz. #16CRLPAL, #5VVSTL, # 54CMSTITNEY, #2CMBANEY, #17CMLNEY, #28CZBAG, #55CMSTNEY, #35CMSTNEY were found to be positive for resveratrol production. In Liebermann and Acetic anhydride test, formation of Red precipitate and dark greenish brown coloration respectively confirms the presence of Resveratrol (Figure 3). In both the tests, #5VVSTL and #16CRLPAL were potent Resveratrol producers followed by #54CMSTITNEY, #17CMLNEY, #55CMSTNEY, #35CMSTNEY which were

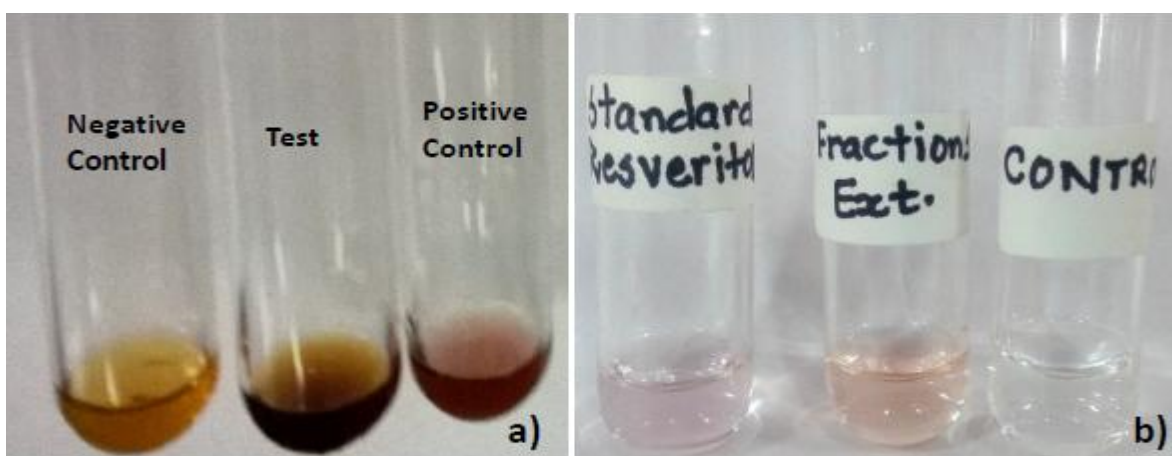


Figure 3. Biochemical assays for screening Resveratrol a) Liebermann Test b) Acetic Anhydride test. Negative control- un-inoculated CDB medium, Positive Control- Resveratrol, Test- ethyl acetate fraction of #5VVSTL.

found to exhibit 80 -90 % activity and two cultures viz. #2CMBANEY, #28CZBAG were showing 70-80% activity. Hence, these nine cultures were further subjected to liquid-liquid extraction.

#### 5.4 Solvent extraction

The cell free filtrates of #5VVSTL and #16CRLPAL were subjected to liquid-liquid extraction by ethyl acetate. The bioactive residue was reconstituted in Methanol. The maximum yield of the bioactive residue was obtained from #5VVSTL (280 mg) and was found to be lowest in the case of #16CRLPAL (110 mg) (Table 6). Ethyl acetate extracts of these selected fungal cultures were again subjected to biochemical screening assays. #5VVSTL and #16CRLPAL were found to be most potent Resveratrol producer. #5VVSTL produced deep violet colored compound similar to that of color of red wine (Figure 4). As the yield of #5VVSTL was found to be greater than #16CRLPAL so it was selected for further analysis.



Figure 4 violet colored ethyl acetate extract of #5VVSTL

<b>Culture Code</b>	<b>Yield of crude compound (mg)</b>
5VVSTL	280.01
16CRLPAL	110.10
54(B)CMSTITNEY	180.40
96CMSTITNEY	150.00
2CMBANEY	120.10
17CMLNEY	180.20
28CZBAG	113.31
55CMSTNEY	150.20
35CMSTNEY	180.10

Table 6 Yield of bioactive residue of different cultures under study

### 5.5 Thin Layer Chromatography (TLC)

The crude ethyl acetate fraction of #5VVSTL was subjected to different combinations of solvents (Table 7) to achieve good separation. The best separation of crude EA extract of #5VVSTL was observed in Chloroform: ethyl acetate: formic acid in the ratio of 10: 2.7: 0.4 which resolved the extract into 7 different bands. The  $R_f$  value of Band 1 to Band 7 was 0.06, 0.14, 0.27, 0.51, 0.63, 0.78 and 0.96 respectively. The standard Resveratrol exhibited  $R_f$  value of 0.63 which was same as that of  $R_f$  value of Band 5 of crude EA extract of #5VVSTL.

<b>S.No.</b>	<b>Solvent System</b>	<b>Ratio Used (V/V/V)</b>	<b>Result</b>
1	n- hexane : ethyl acetate : formic acid	2.5 : 2.375 : 0.25	No separation
2	n- hexane : ethyl acetate : formic acid	2 : 1.9 : 0.1	No separation
3	n- hexane : methanol : formic acid	2.5 : 2 : 0.025	No separation
4	Chloroform : methanol : acetic acid	3 : 0.33 : 0.03	No separation
5	ethyl acetate : methanol : formic acid	2.5 : 2 : 0.02	No separation
6	Benzene : Methanol	3 : 0.33	No separation

7	Methanol : Hexane	2 : 2	No separation
8	Chloroform : ethyl acetate : water	5 : 4 : 1	No separation
9	n- hexane : ethyl acetate : water	4 : 4 : 1	No separation
10	Chloroform : ethyl acetate : formic acid	10 : 4 : 0.4	Single Band
11	Chloroform : ethyl acetate : formic acid	10 : 2 : 0.4	Single Band
12	<b>Chloroform : ethyl acetate : formic acid</b>	<b>10 : 2.7 : 0.4</b>	<b>7 bands</b>

Table no.7 Different mobile phase used for TLC of ethyl acetate extract of #5VVSTL

### 5.6 Purification of Resveratrol by TLC

The band with  $R_f$  value same as that of Standard Resveratrol was scrapped off from TLC plate and was suspended in Methanol. The yield of the purified fraction was 40 mg. It was again subjected to preparative TLC along with standard Resveratrol in optimized solvent system. The Purified and standard Resveratrol were visualized under UV light as violet colored single spot on TLC at same  $R_f$  of 0.63 (Figure 5)

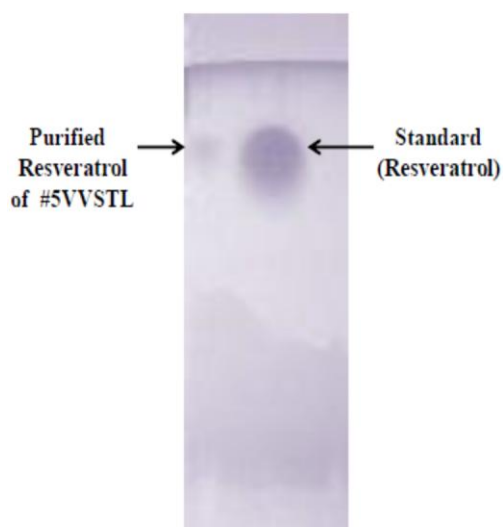
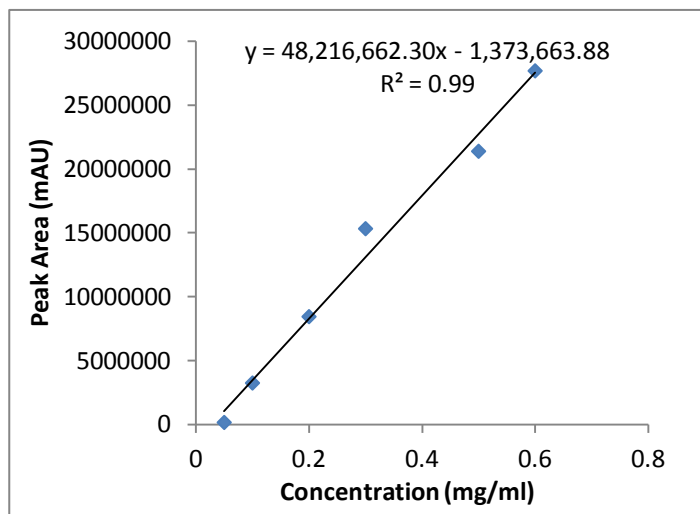


Figure 5 TLC of purified and standard Resveratrol.

### 5.7 HPLC of crude EA extract and purified Resveratrol fraction of #5VVSTL

The purity and concentration of purified Resveratrol was ascertained from the data of peak area vs concentration of standard Resveratrol (Figure 7). Different concentrations of standard Resveratrol were injected into HPLC and analyzed (Table 8, Figure 6). The concentration of purified fraction was 0.24 mg. The HPLC spectra of crude fraction showed one small peak at



Conc. (mg/ml)	Peak Area (mAU)	Retention Time (min)
0.1	3238206.52	35.28
0.2	8429410.14	35.27
0.3	15306900.98	35.30
0.5	21360449.36	35.40
0.6	27649124.55	35.27

Figure 6 Data of peak area (mAU) and concentration (mg/ml) of standard Resveratrol.

Table 8 Peak area and retention time of different concentrations of Resveratrol

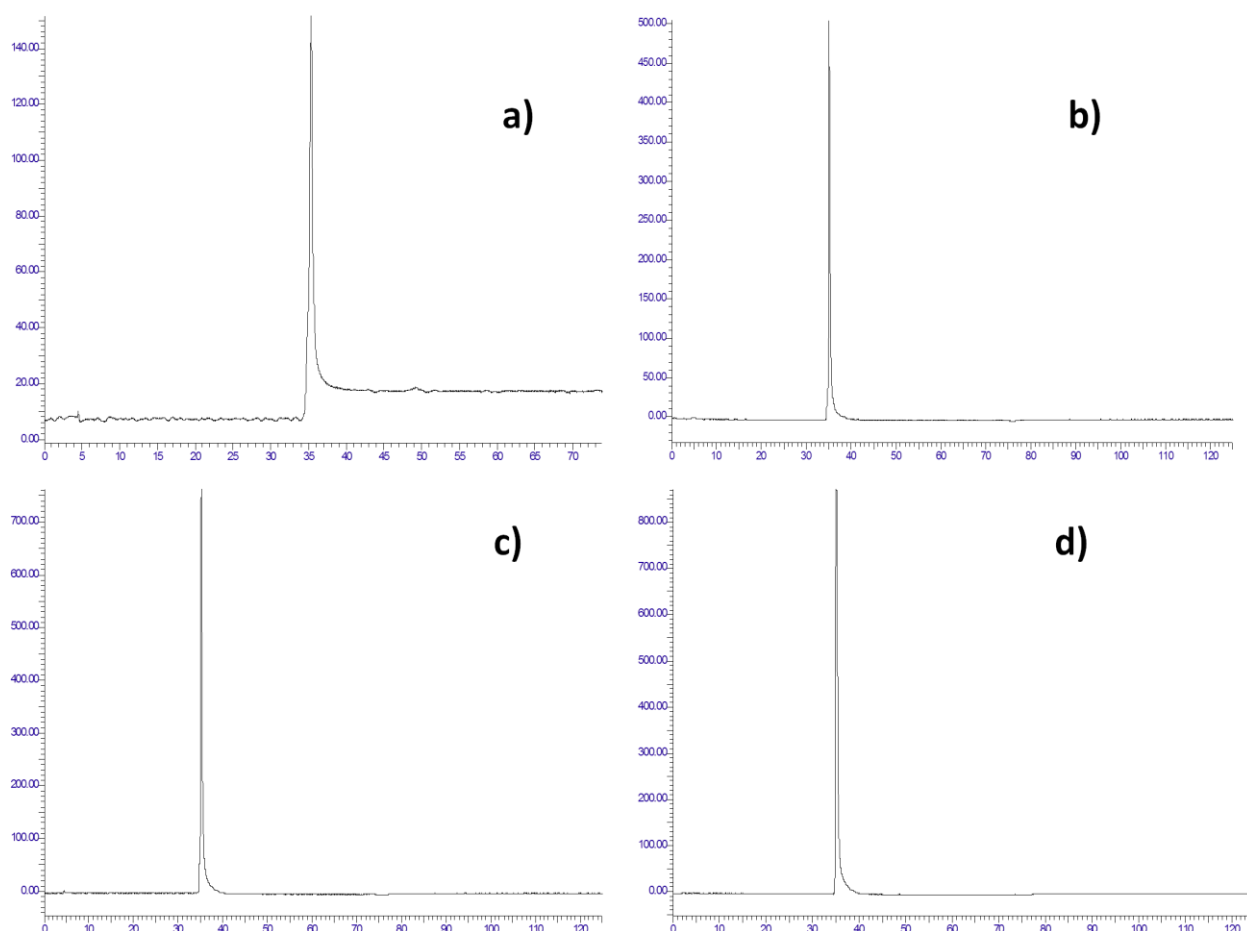


Figure 7 HPLC graphs of different concentrations of standard Resveratrol a) 0.1 mg/ml b) 0.2mg/ml c) 0.5mg/ml d) 0.6mg/ml

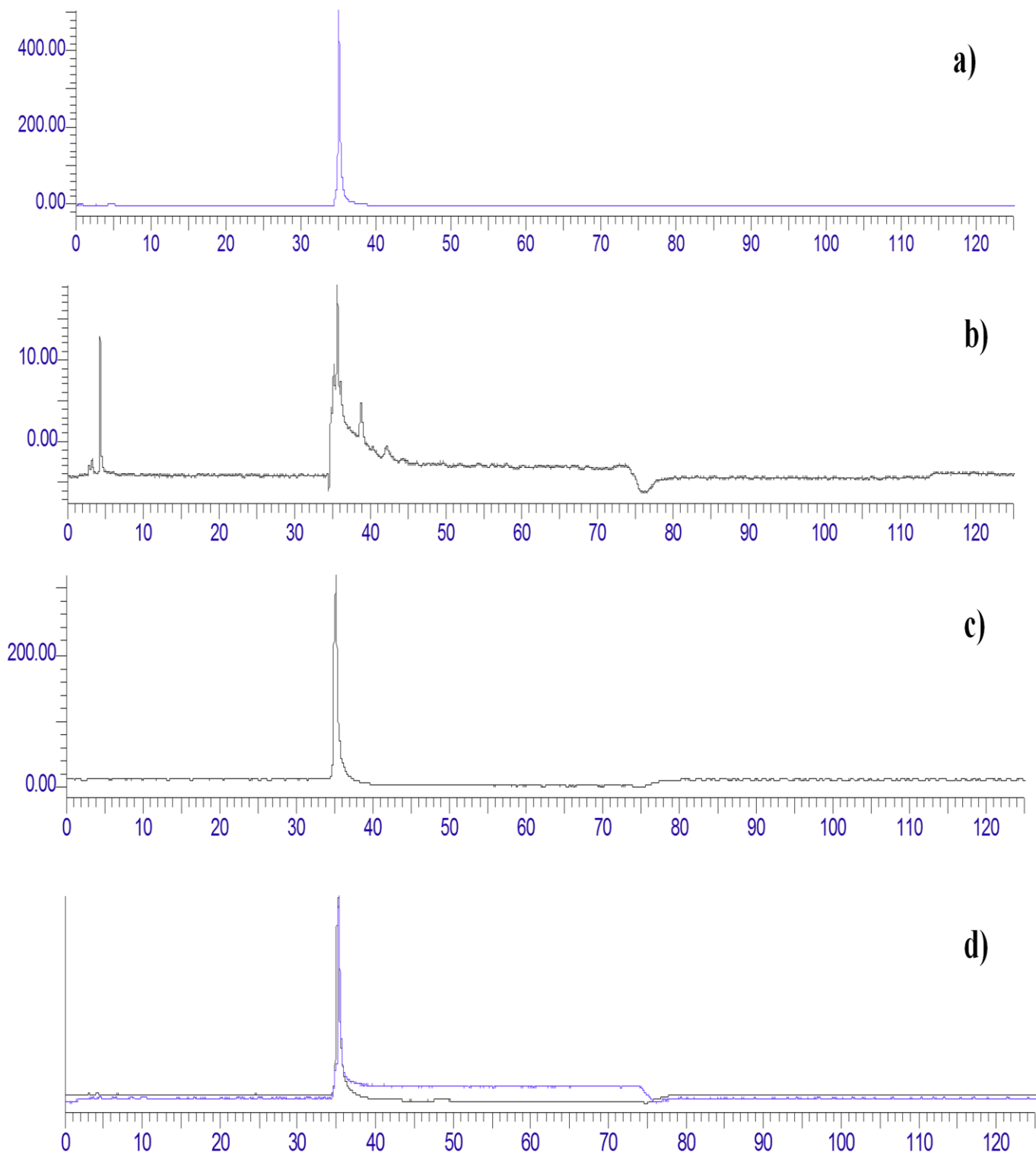


Figure 8 HPLC spectra of a) Standard Resveratrol b) crude ethyl acetate extract of #5VVSTL c) purified fungal Resveratrol from #5VVSTL d) combined spectra of purified Resveratrol along with standard Resveratrol.

with retention time of 5min and one prominent peak at retention time of 35 min comparable to that of single symmetric peak (Retention time- 35.2 min) of standard resveratrol along with many small peaks (Figure 8a, b). The HPLC spectra of purified Resveratrol revealed a single symmetric peak at retention time 35.2 min similar to that of standard resveretrol (Figure 8c, d)

## 5.8 Identification of endophytic fungi

The potential isolate #5VVSTL was identified on the basis of morphotaxonomy and Molecular taxonomy tools.

### 5.8.1 Morphotaxonomy

Colonies over PDA medium were pink in color, moderately growing ( $38.6 \pm 2.08$  mm), velutinous with wavy rough margins and radial crevices (Fig 9a). The fungus produced violet pigment at neutral pH whereas at 5.5 pH, the fungus did not produce violet pigment (Fig 9b). The fungus

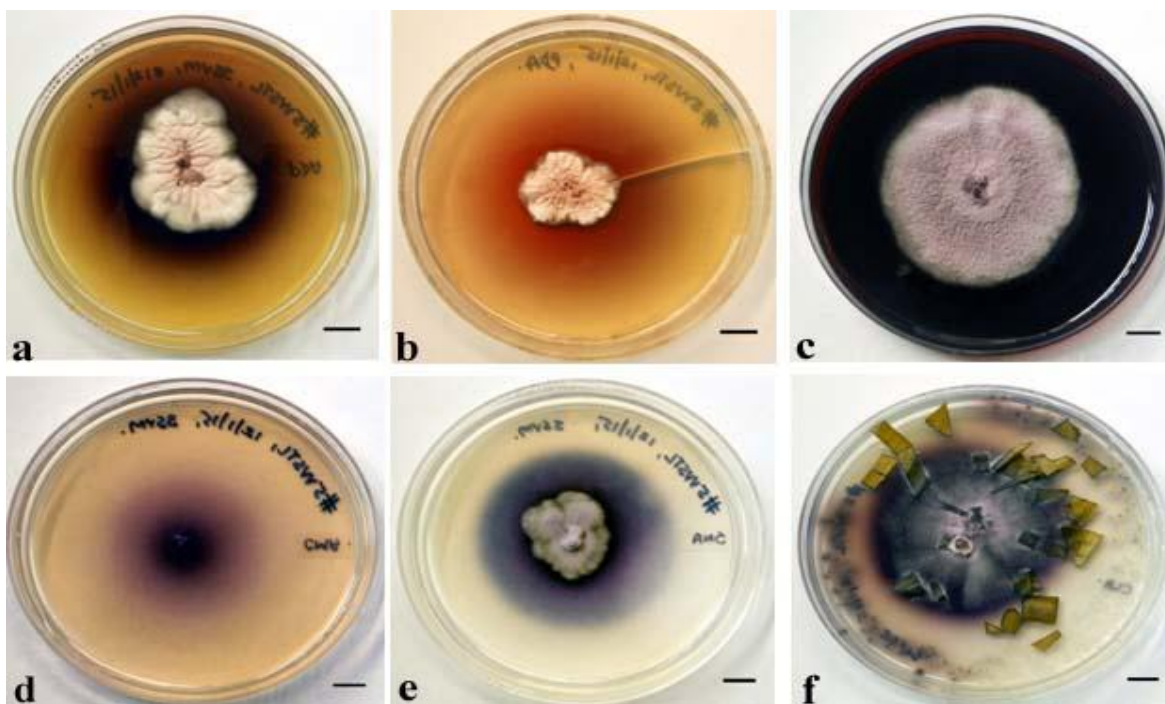


Figure 9 Colony morphology of *Quambalaria cyanescens* (#5VVSTL) over different media a) PDA (pH 7) pink velvety to powder like compact colonies with violet pigmentation b) PDA (pH 5.5) No violet pigment formation c) CDA (pH 7) Colony over CDA with violet pigment formation d) – f) Colony over CMA, SNA and GLA media respectively.

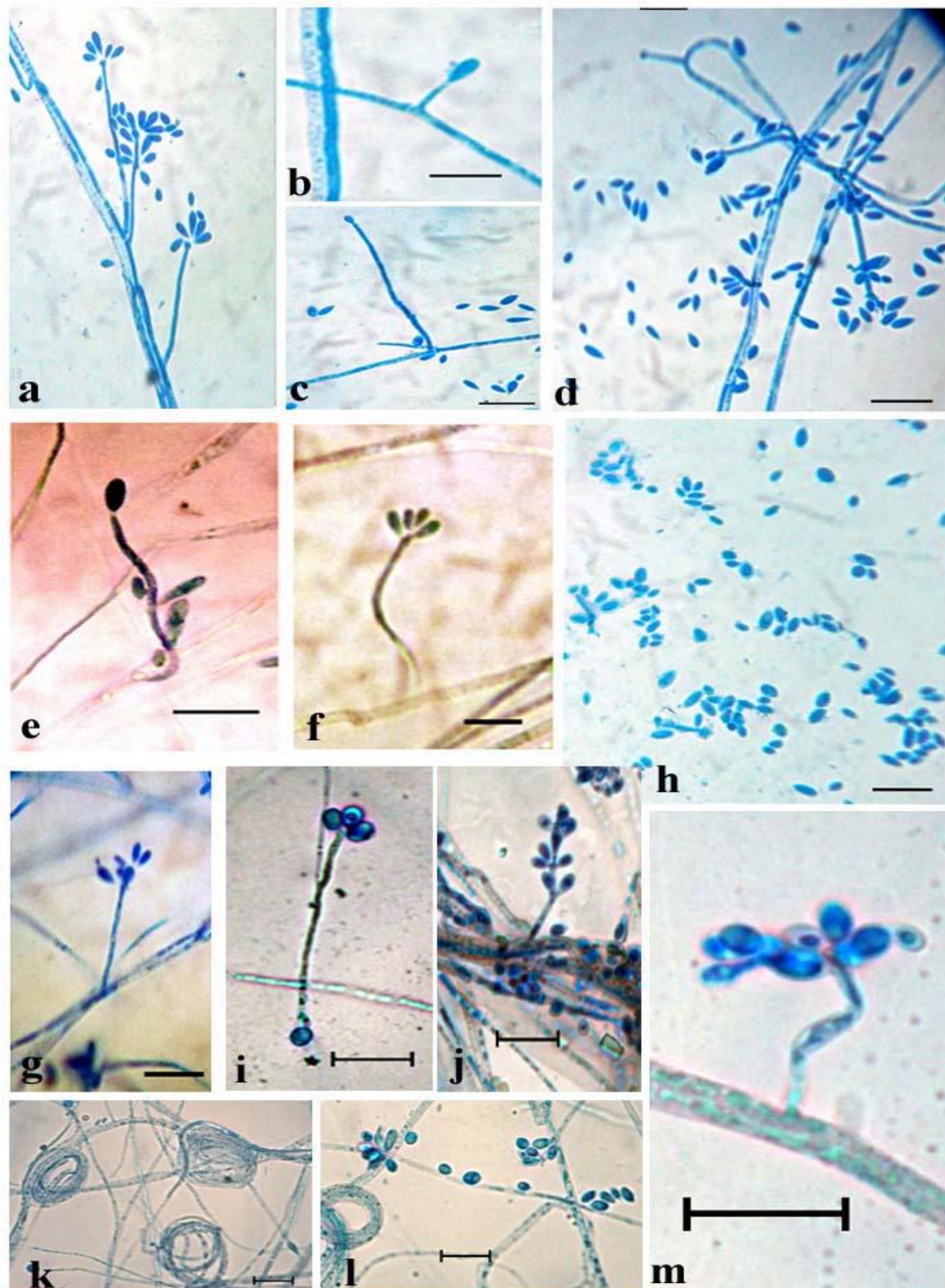


Figure 10 Microscopic features of *Quambalaria cyanescens* (#5VVSTL) over different media a- d: over SNA, a) cluster of primary and secondary conidia b)-c) variable sized conidiogenous cells d) Scattered conidia in medium; e-g: over CDA, e) Conidiogenous cells formed over CDA

medium f)-g) primary conidia cluster h: conidia over PDA medium i-j: over CMA, i) conidiogenous cells with geminating primary conidia formation j) primary and secondary conidia k-m: over GLA, k) hyphal coils l) conidia over GLA m) conidiogenous cells with conidia cluster.

formed pinkish-violet colored, floccose to woolly and moderately growing colonies ( $50.33 \pm 3.06$  mm) over CDA medium with violet pigment production (Fig 9c). Over CMA, SNA and GLA the fungus formed violet colored slow to moderately growing ( $40.3 \pm 1.15$  mm,  $24.67 \pm 1.15$  mm  $48.67$  mm respectively) velutinous colonies with pigment production (Fig 9 (d-f)).

Hyphae thick, coenocytic, long and branched with coils (Fig 10 k). Conidiogenous cells undifferentiated, variable in size (short to long) that terminates into cluster of conidia (Fig 5.9 b,c). Primary conidia germinates over conidiogenous cells (Fig 10 e-g, i). Primary conidia hyaline, non septate, ellipsoidal to fusiform or obovoid found in clusters (usually 4-8) with scars on either ends or only one end (Fig 10 d,h,l). Secondary conidia were found over primary conidia which are slightly larger in size (fig 10 a,j,m). Formation of secondary conidia were frequent.

## **5.8.2 Molecular identification**

### **5.8.2.1 Genomic DNA isolation and PCR amplification**

The genomic DNA isolation of the potent resveratrol producing endophytic fungi, #5VVSTL, was done and the size of the genomic DNA was found to be approximately more than 10 kb (Figure 11a). The concentration of DNA was estimated by taking the absorbance at 260 nm and the amount was  $25 \text{ ng}/\mu\text{l}$  using formula

$$\text{Concentration } (\mu\text{g/ml}) = \text{O.D.}_{260\text{nm}} \times 50 \mu\text{g/ml} \times \text{Dilution factor}$$

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 500 bp ladder. The size of the amplicon was found to be approximately 550 bp to 600 bp (Figure 11b). This size can be easily compared to the ITS region, which was amplified in order to characterize the fungi at molecular level.

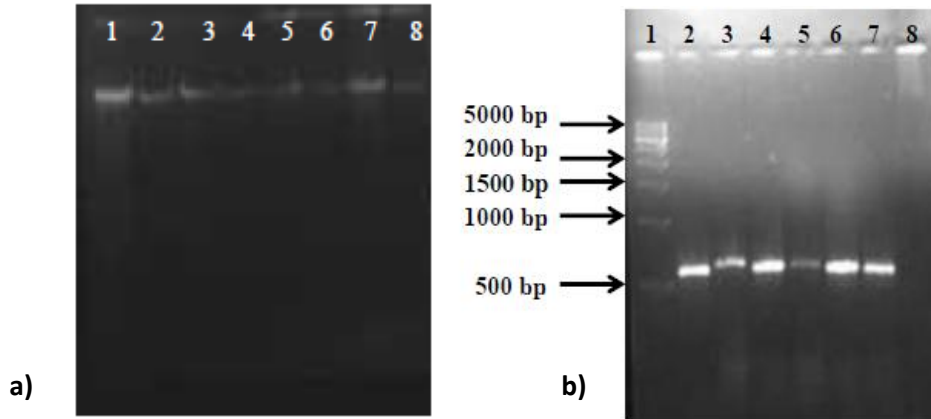


Figure 11 a) Genomic DNA isolation of #5VVSTL, Lane 1: 500bp ladder, Lane 2-8: DNA sample of #5VVSTL b) PCR amplicon of ITS region of #5VVSTL, Lane1 and 2: ITS amplicon, Lane 3-8: Blank

### 5.8.2.2 Sequence assembly, alignment and Phylogenetic Identification

The PCR amplicon of ITS region of #5VVSTL was sent for sequencing and final sequence was submitted to GenBank under the accession number KT200228. The positional homology of final sequence of #5VVSTL was established by subjecting it to sequence similarity search by using BLAST. BLAST analysis exhibited 99% sequence similarity with *Quambalaria cyanescens* species, followed by 95% sequence similarity with *Quambalaria pitereka*, *Quambalaria simpsoni* and 94% sequence similarity with *Quambalaria eucalypti* (Table 5.6)

The final sequence of #5VVSTL is as follows

#### Quambalaria sp #5VVSTL ITS region

```
GGGATCCGACCCTCACATTATCCACATACACTTGTGCACTGTTGGTTGGGTGTGAACCTTTGGTTTATCG
CCCGCCAACGCCAATTATACAAACTCTGAGAACTATGAATGTGAATTTAACTTGCCAATTTTTGGCGAAA
TTAAATACAACCTTTCGACAACGGATCTCTTGGTTCTCCCATCGATGAAGAACGCAGCGAATTGCGATAAG
TAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCTCCCTGGTATTCCTAG
GAGCATGCCTGTTTGAGTGTGATGAATTCCTCAAACCCCAATGTTTTGCAAAATTCATTGCTTGGGTTTG
GTTGTGGGCCTATATTGCTGCTTCACCGCAGCTGGCCTTAAAGATATTAGCTGGACTCTCCGAGACTCTT
GGATAGTTCCACTTGATGTGATAAAATTTGCATCGGTGGGTCAAGACTATGCTTGGGAAGTGTGAGAAGTC
TGCTTCTAATCCGGCGAGAGGTTCCATCCTCGACCAATTCATTTACAACCTCTGGCCTCAATCAGGTAGG
ACTACCCGCTGAACTTAAGCATATCAATAAGCGGGGGGA
```

S.No	Species Name	Accession no.	Query coverage (%)	Sequence similarity (%)
1	<i>Quambalaria cyanescens</i> AUMC6293	JQ425376	98	99
2	<i>Quambalaria cyanescens</i> AUMC6294	JQ425382	99	99
3	<i>Quambalaria cyanescens</i> IHEM5621	KP132549	97	99
4	<i>Quambalaria cyanescens</i> 11PU348	KF953496	96	99
5	<i>Quambalaria cyanescens</i>	KP641151	97	99
6	<i>Quambalaria cyanescens</i> CBS357.73	DQ317622	92	99
7	<i>Quambalaria eucalypti</i> UY1036	EU439922	99	94
8	<i>Quambalaria eucalypti</i> UY1718	EU439923	99	94
9	<i>Quambalaria pitereka</i> AV17-3	GQ258351	95	95
10	<i>Quambalaria pitereka</i> CMW23610	EF427372	92	95
11	<i>Quambalaria pitereka</i> BRIP48531	EF427371	91	95
12	<i>Quambalaria pitereka</i> BRIP448317	EF444854	92	94
13	<i>Quambalaria pitereka</i> BRIP48328	EF444872	91	95
14	<i>Quambalaria simpsonii</i> CBS12477	GQ303291	93	95
15	<i>Quambalaria simpsonii</i> CBS124772	GQ303290	92	95

Table 9 BLAST analysis of final sequence of #5VVSTL

The Maximum parsimony phylogenetic tree based upon the internal transcribed spacer (ITS) region sequence analysis was divided into 4 clades- Clade I which clusters 5 isolates of *Quambalaria pitereka* (Zhou et al., 2007) species with high bootstrap support, Clade II clustering 2 strains of *Quambalaria eucalypti* (Perez et al., 2008), Clade III which grouped *Quambalaria simpsonii* (Cheewangkoon et al., 2009) and Clade IV which clusters isolate under study viz. #5VVSTL with 6 strains of *Quambalaria cyanescens* (Fan et al., 2014; Irinyi et al., 2015) with significant bootstrap support value. *Muscodor albus* was chosen as outgroup to root the tree. #5VVSTL confirmed its placement in *Quambalaria cyanescens* species and was identified as *Quambalaria cyanescens*.

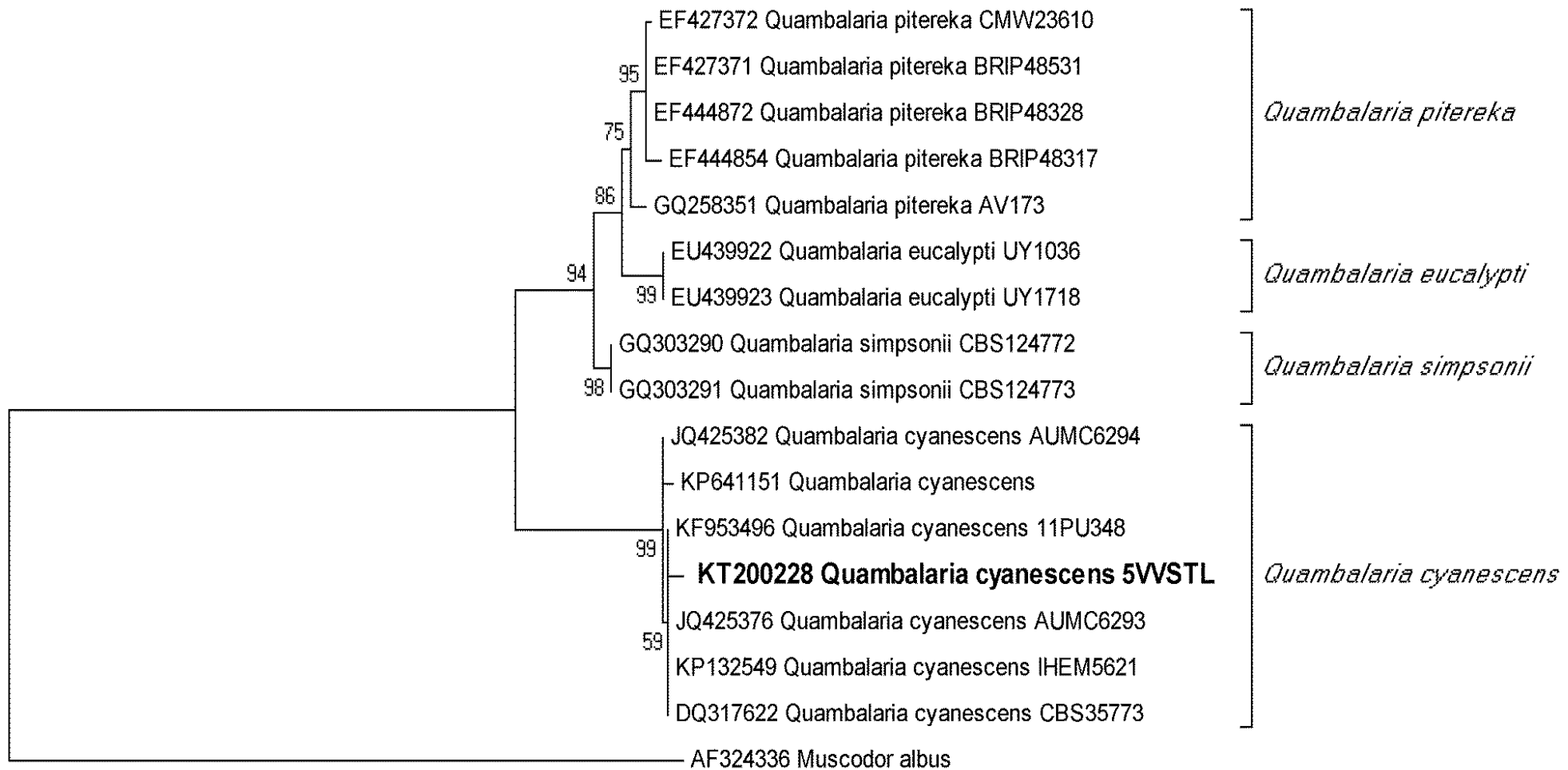


Figure 12 Maximum parsimony phylogenetic tree based on ITS region sequence. *Muscodor albus* was chosen as outgroup



# *Chapter 6*

---

## *Conclusion*

## **7. Conclusion**

From the current study we conclude that the *Quambalaria cyanescens* (#5VVSTL) is a significant producer of resveratrol. The results were confirmed through TLC and HPLC techniques. Further work on optimization and purification of the compound is warranted.

# *Chapter 7*

---

## *References*

## 8. References

1. Adrian M, Jeandet P, Douillet-Breuil AC, Tesson L, & Bessis R. (2000) Stilbene content of mature *Vitis vinifera* berries in response to UV-C elicitation. *Journal of Agricultural and Food Chemistry* 48(12): 6103-6105.
2. Al-Jumaily EFA, Hamid GS and Karima F Ali (2014) Synthesis and Total Phenol Content of New Resveratrol Derivative *American Journal of Alzheimer's Disease & Other Dementias* ISSN 2321-547X
3. Arnold AE & Lewis LC (2005) Ecology and evolution of fungal endophytes and their roles against insects. *Insect-Fungal Associations: Ecology and Evolution* Oxford University Press, New York, 74-96
4. Babu SK, Kumar KV & Subbaraju GV (2005) Estimation of trans-resveratrol in herbal extracts and dosage forms by high-performance thin-layer chromatography. *Chemical and pharmaceutical bulletin* 53(6): 691-693
5. Balan V, Miller GS, Kaplun L, Balan K, Chong ZZ, LiF Kaplun A, VanBerkum MF., Arking R, Freeman DC, Maiese K, Tzivion G (2008) Life span extension and neuronal protection by *Drosophila* nicotinamidase. *Journal of Biological Chemistry* 283:27810-27819
6. Baur JA and Sinclair DA (2006) Therapeutic potential of resveratrol the in vivo evidence *Nat Rev Drug Discov.* 5:493-506.
7. Beekwilder J, Wolswinkel R, Jonker H, Hall R, de Vos CHR, Bovy A (2006) Production of resveratrol in recombinant microorganisms. *Applied and Environmental Microbiology.* 72:5670–5672
8. Beher D 2009 Resveratrol is not a direct activator of SIRT1 enzyme activity. *Chemical Biology & Drug Design.* 74:619–624
9. Belguendouz, L, Fremont L and Linard A (1997) Resveratrol inhibits metal ion-dependent and independent peroxidation of porcine low-density lipoproteins. *Biochemical Pharmacology.* 53: 1347–1355
10. Bhat KP, Kosmeder JW & Pezzuto JM (2001) Biological effects of resveratrol. *Antioxidants and Redox Signaling.* 3(6): 1041-1064.
11. Borra MT, Smith, BC and Denu JM (2005) Mechanism of human SIRT1 activation by Resveratrol *Journal of Biological Chemistry.* 280(17): 17187-17195
12. Bradamante S, Barenghi L, & Villa A (2004) Cardiovascular protective effects of resveratrol. *Cardiovascular drug reviews.* 22(3): 169-188.

13. Burns J, Yokota T, Ashihara H, Lean ME & Crozier A (2002) Plant foods and herbal sources of resveratrol. *Journal of agricultural and food chemistry*. 50(11): 3337-3340.
14. Cai YZ, Luo Q, Sun M, Corke H (2004) Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. *Life Sciences* 74: 157-2184
15. Callemien D, Jerkovic V, Rozenberg R & Collin S (2005) Hop as an interesting source of resveratrol for brewers: optimization of the extraction and quantitative study by liquid chromatography/atmospheric pressure chemical ionization tandem mass spectrometry *Journal of agricultural and food chemistry*. 53(2): 424-429
16. Cantos E, García-Viguera C, de Pascual-Teresa S & Tomás-Barberán FA (2000) Effect of postharvest ultraviolet irradiation on resveratrol and other phenolics of cv Napoleon table grapes *Journal of Agricultural and Food Chemistry*. 48(10): 4606-4612.
17. Cassidy A, Hanley B, & Lamuela-Raventos RM (2000) Isoflavones ignans and stilbenes origins, metabolism and potential importance to human health. *Journal of the Science of Food and Agriculture*. 80(7): 1044-1062
18. Changsha Nutramax Inc (2009) Polygonum cuspidatum extract resveratrol 20%~99%
19. Chattopadhyay I, Biswas K, Bandyopadhyay U, Banerjee RK (2004) Turmeric and curcumin Biological actions and medicinal applications. *Current science (India)*. 87: 44-53
20. Cheewangkoon R, Groenewald JZ, Summerell BA, Hyde KD, To-anun C & Crous PW (2009) *Myrtaceae* a cache of fungal biodiversity. *Persoonia*. 23: 55–85.
21. Chen RS, Wu PL & Chiou RYY (2002) Peanut roots as a source of resveratrol. *Journal of agricultural and food chemistry*. 50(6): 1665-1667.
22. Chen WL, Weng YM, Tseng CY (2003) Antioxidative and antimutagenic activities of healthy herbal drinks from chinese medicinal herbs. *American Journal of Chinese Medicine* 31: 523-532
23. Chen YB, Sun BX & Chen JX (2007) Study on the stability of resveratrol in rhizoma polygoni cuspidati. *Journal of Chinese medicinal materials*. 30(7): 805-807
24. Constant J (1997) Alcohol, ischemic heart disease, and the French paradox. *Clinical cardiology*. 20(5): 420-424.
25. Dercks W & Creasy L L (1989) Influence of fosetyl AI on phytoalexin accumulation in the Plasmopara viticola grapevine interaction. *Physiological and Molecular Plant Pathology*. 34(3): 203-213

26. Docherty JJ, McEwen HA, Sweet TJ, Bailey E and Booth T D (2007): Resveratrol inhibition of *Propionibacterium acnes*. *Journal of Antimicrobial Chemotherapy*. 59(6):1182-1184
27. Dreyfuss MM & Chapela IH (1994) Potential of fungi in the discovery of novel, low-molecular weight pharmaceuticals. *Biotechnology*. 26: 49.
28. Ector B J, Magee JB, Hegwood CP & Coign MJ (1996) Resveratrol concentration in muscadine berries juice pomace purees seeds and wines. *American Journal of Enology and Viticulture*. 47(1): 57-62.
29. Fan X, Xiao M, Kong F, Kudinha T, Wang H & Xu YC (2014) A Rare Fungal Species *Quambalaria cyanescens* Isolated from a Patient after Augmentation Mammoplasty – Environmental Contaminant or Pathogen. *PLoS ONE*. 9(10).
30. Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, 783-791
31. Fermont L (2000) Biological effects of resveratrol. *Life Sciences*. 66: 663– 73
32. Frémont L Belguendouz L & Delpal S (1999) Antioxidant activity of resveratrol and alcohol-free wine polyphenols related to LDL oxidation and polyunsaturated fatty acids. *Life sciences*. 64(26): 2511-2521
33. Gennaro M, Gonthier P & Nicolotti G (2003) Fungal endophytic communities in healthy and declining *Quercus robur* L and *Q. cerris* L trees in northern Italy. *Journal of Phytopathology*. 151(10): 529-534
34. Goodwin JS (1984) Immunologic effects of non-steroidal anti-inflammatory drugs. *Journal Of Medicinal Chemistry*. 77:7-15
35. Guo L D, Hyde K D and Liew E C Y (2000) Identification of endophytic fungi from *Livistona chinensis* based on morphology and rDNA sequences. *New Phytologist*;t (147): 617-630.
36. Gusman J, Malonne H, Atassi G (2001) A reappraisal of the potential chemopreventive and chemotherapeutic properties of resveratrol. *Carcinogenesis* 22: 1111–1117
37. Harney SK, Edwards F S and Allen M F (1997) Identification of arbuscular mycorrhizal fungi from *Artemisia californica* using the polymerase chain reaction. *Mycologia*. (89): 547-55
38. Ibern Gómez M, Roig Pérez S, Lamuela-Raventós RM & de la Torre-Boronat MC (2000) Resveratrol and piceid levels in natural and blended peanut butters. *Journal of agricultural and food chemistry*. 48(12) 6352-6354

39. Irinyi L, Lackner M, de Hoog S & Meyer W (2015) DNA barcoding of fungi causing infections in humans and animals. *Fungal Biology*.
40. Jang JH & Surh YJ (2001) Protective effects of resveratrol on hydrogen peroxide-induced apoptosis in rat pheochromocytoma (PC12) cells. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*. 496(1): 181-190.
41. Jang M, Cai L, Udeani GO, Slowing KV, Thomas CF, Beecher CW & Pezzuto JM (1997) Cancer chemopreventive activity of resveratrol a natural product derived from grapes. *Science*. 275(5297): 218-220
42. Jeandet P, Douillat-Breuil AC, Bessis R, Deborel S, Sbaghi M and Adrain M (2002) Phytoalexins from Vitaceae biosynthesis phytoalexin gene expression in transgenic plants antifungal activity and metabolism. *Journal of Agricultural and Food Chemistry's*. 50: 2731-2741
43. Junling Shi & Qin Zeng & Yanlin Liu & Zhongli Pan (2012) Alternaria sp MG1 a resveratrol-producing fungus isolation identification and optimal cultivation conditions for resveratrol production. *Applied Microbiology and Biotechnology*. 95:369–379
44. Karuppagounder SS, Pinto JT, Xu H, Chen H, Leal MF & Gibson GE (2009) Dietary supplementation with resveratrol reduces plaque pathology in a transgenic model of Alzheimer's disease. *Neurochemistry international*. 54(2), 111-118
45. Kaufmann SH (1989) Induction of endonucleolytic DNA cleavage in human acute myelogenous leukemia cells by etoposide camptothecin and other cytotoxic anticancer drugs a cautionary note. *Cancer Research*. 49(21): 5870-5878
46. Kiselev KV, Bulgakov VP (2009) Stability of the rolC gene and its expression in 15-year-old cell cultures of Panax ginseng. *Applied Biochemistry and Microbiology* 45:252–258
47. Kiselev KV, Dubrovina AS, Veselova MV, Bulgakov VP, Fedoreyev SA & Zhuravlev YN (2007) The rolB gene-induced overproduction of resveratrol in Vitis amurensis transformed cells. *Journal of biotechnology*. 128(3): 681-692
48. Kornreich DA & Saif MW (2012) Epistaxis Secondary to Panitumumab in a Patient with Colon Cancer. *Anticancer research*. 32(11): 4983-4985
49. Lancon A, Delma D, Osman H, Thenot JP, Jannin B & Latruffe N (2004) Human hepatic cell uptake of resveratrol: involvement of both passive diffusion and carrier-mediated process. *Biochemical and biophysical research communications*. 316(4): 1132-1137.
50. Lancon A, Delma D, Osman H, Thenot JP, Jannin B & Latruffe N (2004) Human hepatic cell uptake of resveratrol: involvement of both passive diffusion and carrier-mediated process. *Biochemical and biophysical research communications*. 316(4): 1132-1137.

51. Lançon A, Hanet N, Jannin B, Delmas D, Heydel JM, Lizard G & Latruffe N (2007) Resveratrol in human hepatoma HepG2 cells: metabolism and inducibility of detoxifying enzymes. *Drug metabolism and disposition*. 35(5): 699-703.
52. Langcake P and Pryce RJ (1976) The production of resveratrol by *Vitis vinifera* and other members of the Vitaceae as a response to infection or injury. *Physiological Plant Pathology*. 9: 77–86
53. Lee SS, Lee SM, Kim M, Chun J, Cheong YK & Lee J (2004) Analysis of trans-resveratrol in peanuts and peanut butters consumed in Korea. *Food research international*. 37(3): 247-251.
54. Li JY, Harper JK, Grant DM, Tombe BO, Bashyal B, Hess WM & Strobel GA (2001) Ambuic acid, a highly functionalized cyclohexenone with antifungal activity from *Pestalotiopsis* spp. and *Monochaetia* sp. *Phytochemistry*. 56(5): 463-468.
55. Lim CG, Fowler ZL, Hueller T, Schaffer S & Koffas MA (2011) High-yield resveratrol production in engineered *Escherichia coli*. *Applied and environmental microbiology*. 77(10): 3451-3460.
56. Mahady GB & Pendland SL (2000) Resveratrol inhibits the growth of *Helicobacter pylori* in vitro. *The American journal of gastroenterology*. 95(7): 1849.
57. Malele RS, Zvikomborero MC (2014) Estimation of trans-resveratrol in grape berry skin extract by High-Performance Thin-Layer Chromatography (HPTLC). *International Journal of Health Sciences and Research*. Jan31;2(1):213-23.
58. Medina-Bolivar F, Condori J, Rimando AM, Hubstenberger J, Shelton K, O’Keefe SF & Dolan MC (2007) Production and secretion of resveratrol in hairy root cultures of peanut. *Phytochemistry*. 68(14): 1992-2003.
59. Morales M, Bru R, Garcia-Carmona F, Barceló AR & Pedreno MA (1998) Effect of dimethyl- $\beta$ -cyclodextrins on resveratrol metabolism in Gamay grapevine cell cultures before and after inoculation with shape *Xylophilus ampelinus*. *Plant Cell, Tissue and Organ Culture*. 53(3): 179-187.
60. Movahed A, Nabipour I, Lieben Louis X, Thandapilly SJ, Yu L, Kalantarhormozi M & Netticadan T (2013) Antihyperglycemic Effects of Short Term Resveratrol Supplementation in Type 2 Diabetic Patients. *Evidence-Based Complementary and Alternative Medicine*, 2013.
61. Nalawade SM, Sagare AP, Lee CY, Kao CL & Tsay HS, (2003) Studies on tissue culture of Chinese medicinal plant resources in Taiwan and their sustainable utilization. *Botanical Bulletin of Academia Sinica*. 44: 79-98.

62. Nei M & Kumar S (2000) *Molecular evolution and phylogenetics*. Oxford University Press.
63. Newman DJ & Cragg G.M (2007) Natural products as sources of new drugs over the last 25 years. *Journal of Natural Products*. 70: 461-477.
64. Pace-Asciak CR, Hahn S, Diamandis EP, Soleas G & Goldberg DM (1995) The red wine phenolics trans-resveratrol and quercetin block human platelet aggregation and eicosanoid synthesis: implications for protection against coronary heart disease. *International journal of clinical chemistry*. 235(2): 207-219.
65. Palsamy P & Subramanian S (2009) Resveratrol, a natural phytoalexin, normalizes hyperglycemia in streptozotocin-nicotinamide induced experimental diabetic rats. *Biomedicine & Pharmacotherapy*. 62: 598–605.
66. Park J & Boo YC (2013) Isolation of resveratrol from *vitis viniferae* caulis and its potent inhibition of human tyrosinase. *Evidence-Based Complementary and Alternative Medicine*.
67. Parker JA, Arango M, Abderrahmane S, Lambert E, Tourette C, Catoire H & Néri C (2005) Resveratrol rescues mutant polyglutamine cytotoxicity in nematode and mammalian neurons. *Nature genetics*. 37(4), 349-350.
68. Paul B, Masih I, Deopujari J & Charpentier C (1999) Occurrence of resveratrol and pterostilbene in age-old darakchasava, an ayurvedic medicine from India. *Journal of ethnopharmacology*. 68(1): 71-76.
69. Pérez CA, De Beer ZW, Altier NA, Wingfield MJ & Blanchette RA (2008) Discovery of the eucalypt pathogen *Quambalaria eucalypti* infecting a non-Eucalyptus host in Uruguay. *Australasian Plant Pathology*. 37(6): 600-604.
70. Petrini O (1991) Fungal endophytes of tree leaves. *Microbial ecology of leaves*. 179-197.
71. Petrovski G, Gurusamy N, & Das DK (2011) Resveratrol in cardiovascular health and disease. *Annals of the New York Academy of Sciences*. 1215(1): 22-33.
72. Plescia OJ, Smith H & Grinwich K (1975) Subversion of immune system by tumor cells and role of prostaglandins. *Proceedings of the National Academy of Sciences*. 72(5): 1848–1851
73. Puri SC, Nazir A, Chawla R, Arora R, Riyaz-ul-Hasan S, Amna T & Qazi GN (2006) The endophytic fungus *Trametes hirsuta* as a novel alternative source of podophyllotoxin and related aryl tetralin lignans. *Journal of biotechnology*. 122(4), 494-510.

74. Richard T, Pawlus AD, Iglésias ML, Pedrot E, Waffo-Teguo P, Mérillon JM & Monti JP (2011) Neuroprotective properties of resveratrol and derivatives. *Annals of the New York Academy of Sciences*. 1215(1): 103-108.
75. Rodrigues KF, Manfred H & Christa W (2000) Antimicrobial activity of secondary metabolites produced by endophytic fungi from *Spondias mombin*. *Journal of Basic Microbiology*. 40(4): 261-267
76. Sanders TH, McMichael RW & Hendrix KW (2000) Occurrence of resveratrol in edible peanuts. *Journal of agricultural and food chemistry*. 48(4): 1243-1246.
77. Saxena S & Srivastava A (2014) Resveratrol: Biological Activities and Therapeutic Potential.
78. Sette LD, Passarini MRZ , Delarmelina C , Salati F & Duarte MCT (2006) Molecular characterization and antimicrobial activity of endophytic fungi from coffee plants. *World Journal of Microbiology and Biotechnology*. (22): 1185-1195.
79. Sharma S, Misra CS, Arumugam S, Roy S, Shah V, Davis J, Shirumalla RK & Ray (2011) Anti-sdiabetic activity of resveratrol, a known SIRT1 activator in a genetic model for type-2 diabetes. *Phytotherapy Research*. 25: 67-73.
80. Sheth S, Jajoo S, Kaur T, Mukherjea D, Sheehan K, Rybak LP & Ramkumar V (2012) Resveratrol Reduces Prostate Cancer Growth and Metastasis by Inhibiting the Akt/MicroRNA-21 Pathway. *PLoS ONE*, 7(12).
81. Slichenmyer WJ & Von Hoff DD (1991). Taxol: a new and effective anti-cancer drug. *Anti-Cancer Drugs*. 2(6): 519-530.
82. Sobolev VS, & Cole RJ (1999) trans-Resveratrol content in commercial peanuts and peanut products. *Journal of Agricultural and Food Chemistry*. 47(4): 1435-1439.
83. Soleas GJ, Diamandis EP, & Goldberg DM (1997) Resveratrol: a molecule whose time has come? And gone?. *Clinical biochemistry*. 30(2): 91-113.
84. Strobel G & Daisy B (2003) Bioprospecting for microbial endophytes and their natural products. *Microbiology and Molecular Biology Reviews*. 5:535–544
85. Strobel G, Daisy B, Castillo U & Harper J (2004) Natural products from endophytic microorganisms. *Journal of Natural Products*.67: 257-268.
86. Strobel GA, Ezra D & Hess WM (2004) New endophytic isolates of *Muscodora albus*, a volatile-antibiotic-producing fungus. *Microbiology*. 150: 4023–4033.
87. Sun y, Wang Q, Simonyi and Sun y (2010) Resveratrol as a therapeutic agent for the neurodegenerative diseases. *Molecular Neurobiology*. 41:375–383.

88. Szmitko PE & Verma S (2005) Antiatherogenic potential of red wine: clinician update. *American Journal of Physiology-Heart and Circulatory Physiology*. 288(5): H2023-H2030.
89. Takaoka M (1939) Resveratrol, a new phenolic compound from *Veratrum grandiflorum*. *Nippon Kagaku Kaishi*, 60: 1090-1100.
90. Tamura K, Peterson D, Peterson N, Stecher G, Nei M & Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular biology and evolution*. 28(10): 2731-2739.
91. Terzibasi, Valenzano DR & Cellerino (2007) The short lived fish *Nothobranchius furzeri* as a new model system for ageing studies. *Experimental Gerontology*. 42:81-89
92. Sanders TH, McMichael RW & Hendrix KW (2000) Occurrence of resveratrol in edible peanuts. *Journal of agricultural and food chemistry*. 48(4): 1243-1246.
93. Tissenbaum H & Guarente L (2001) Increased dosage of Sir-2 gene extends lifespan in *Caenorhabditis elegans*. *Nature* 410:227-230.
94. Valenzano DR & Cellerino (2006) Resveratrol and the pharmacology of ageing : a new vertebrate model to validate an old molecule. *Cell Cycle* 5: 1027-1032.
95. Vastano BC, Chen Y, Zhu N, Ho CT, Zhou Z & Rosen RT (2000) Isolation and Identification of Stilbenes in Two Varieties of *Polygonum cuspidatum*. *Journal of agricultural and food chemistry*. 48(2): 253-256.
96. Vicente M.F. & Cabello A (2001): Antimicrobial activity of ergokonin A from *Trichoderma longibrachiatum*. *Journal of Applied Microbiology*. 91(5): 806-813.
97. Vuorela P, Leinonen M, Saikku P, Tammela P, Rauha JP, Wennberg T & Vuorela H (2004) Natural products in the process of finding new drug candidates. *Current medicinal chemistry*. 11(11): 1375-1389.
98. Wang DG, Liu WY & Chen GT (2013) A simple method for the isolation and purification of resveratrol from *Polygonum cuspidatum*. *Journal of Pharmaceutical Analysis*. 3(4): 241-247.
99. Wang FW, Jiao RH, Cheng AB, Tan SH & Song YC (2007) Antimicrobial potentials of endophytic fungi residing in *Quercus variabilis* and brefeldin A obtained from *Cladosporium* sp. *World Journal of Microbiology and Biotechnology*. 23(1): 79-83.
100. Wang L, Xu M, Liu C, Wang J & Xi H (2013) Resveratrols in Grape Berry Skins and Leaves in *Vitis* Germplasm. *PLoS ONE* 8(4): e61642.

101. Wang QJ, Rottinghaus, Simonyi, Lubahn D, Sun gy & Sun y (2002) Resveratrol protects against global cerebral ischemic injury in gerbils. *Brain Research* 958:439- 447.
102. Wang QS, Simonyi, Rottinghaus, Sun gy & Sun y (2004): Resveratrol protects against neurotoxicity induced by kainic acid. *Neurochemical Research*. 29: 2105-21
103. White TJ, Bruns T, Lee SJWT & Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: a guide to methods and applications* 18: 315-322.
104. Wolter F, Akoglu B, Clausnitzer and Stein J (2001) Down regulation of the cyclin D1/Cdk4 complex occurs during resveratrol-induced cell cycle arrest in colon cancer cell lines. *Journal of Nutrition*. 131: 2197-2203
105. Wong JC & Fiscus RR (2015) Resveratrol at anti-angiogenesis/anticancer concentrations suppresses protein kinase G signaling and decreases IAPs expression in HUVECs. *Anticancer research* 35(1): 273-281.
106. Yuan HF, Zhai C, Yan XL, Zhao DD, Wang JX, Zeng Q & Pei XT (2012) SIRT1 is required for long-term ;growth of human mesenchymal stem cells. *Journal of molecular medicine* 90(4):389-400.
107. Zenser TV, Mattammal MB, Wise RW, Rice JR, Davis B.B (1983) Prostaglandin H synthase-catalyzed activation of benzidine: a model to assess pharmacologic intervention of the initiation of chemical carcinogenesis. *Journal of Pharmacology and Experimental Therapeutics* 227(3):545–550.
108. Zhang Y, Fournier J, Pointing SB & Hyde KD (2008) Are *Melanomma pulvis-pyrius* and *Trematosphaeria pertusa* congeneric. *Fungal Diversity*. (33): 47-60.
109. Zhou X, De Beer ZW, Xie Y, Pegg GS & Wingfield M J (2007) DNA-based identification of *Quambalaria pitereka* causing severe leaf blight of *Corymbia citriodora* in China. *Fungal Diversity* 25:245-254.
110. Zou J, Huang, Chen, Wei, Cao K & Wu, JM (2000) Effects of resveratrol on oxidative modification of human low density lipoprotein. *Chinese medical journal*. 113: 99–102.
111. Zou J, Huang Z, Chen Q, Wei H, Heish TC & Wu, JM (1999) Resveratrol inhibits copper ion-induced and azo compound-initiated oxidative modification of human low density lipoprotein. *International Journal of Biochemistry and Molecular Biology*. 47: 1089–1096.

# Appendix

## Media

### 1. Czapek dox broth

Sucrose – 30g

NaNO<sub>3</sub> – 3g

K<sub>2</sub>HPO<sub>4</sub> – 1g

KCl – 0.5g

MgSO<sub>4</sub> · 7H<sub>2</sub>O – 0.5g

FeSO<sub>4</sub> · 7H<sub>2</sub>O – 0.01g

Distilled H<sub>2</sub>O – 1L

Final pH ( at 26°C)- 7.0±0.2

Autoclave at 121°C for 15 min

### 2. Corn meal agar

Corn meal – 50g

Agar – 15g

Distilled H<sub>2</sub>O – 1L

Final pH (at 26°C) 6.0±0.2

Autoclave at 121°C for 15 min

### 3. Grape leaf agar

Grape leaves – 1g

Agar – 15g

Distilled H<sub>2</sub>O – 1L

Final pH (at 26°C) 7.0±0.2

Autoclave at 121°C for 15 min

### 4. Malt extract agar

Malt extract – 30gm

Agar – 15gm

dH<sub>2</sub>O – 1L

Final pH - 7.6 ± 0.2 at 37°C

Autoclave at 121°C for 15 min

### 5. Potato Dextrose Agar

Potatoes - 200g

Dextrose – 20g

Agar – 15g

Final pH ( at 26°C)- 5.6±0.2

Autoclave at 121°C for 15 min

### 6. Synthetischer nährstoffarmer agar

KNO<sub>3</sub> – 1g

K<sub>2</sub>HPO<sub>4</sub> – 1g

KCl – 0.5g

MgSO<sub>4</sub> · 7H<sub>2</sub>O – 0.5g

Glucose – 0.2g

Saccharose – 0.2g

Agar – 20g

Distilled H<sub>2</sub>O – 1L

Final pH (at 26°C) 7.0±0.2

Autoclave at 121°C for 15 min

### 7. Water agar

Agar – 20gm

Distilled H<sub>2</sub>O – 1L

Autoclave at 121°C for 15 min

## Buffers

### 1. 50XTAE

Tris base – 242g

Glacial acetic acid – 57.1mL

0.5M EDTA – 10mL

Distilled H<sub>2</sub>O – 1L

### 2. 1X TE

Tris-HCl (pH 8.0) -10 mM

EDTA - 0.1 mM, Distilled H<sub>2</sub>O 100ml