Isolation and characterization of pancreatic lipase inhibitors from endophytic fungi

A
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in partial fulfilment for the award of degree
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Submitted by
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June 2016

CERTIFICATE

Certified that the thesis "Isolation and characterization of pancreatic lipase inhibitors from endophytic fungi" submitted by Ms. Mahiti Gupta, in partial fulfillment of the requirement for the award of the Degree of Doctor of Philosophy in the Department of Biotechnology, Thapar University, Patiala, is a record of candidate's own independent and original research work carried out by herself under our supervision and guidance. The material embodied in this thesis has not been submitted in part or full to any other University or Institute for the award of any degree.

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CANDIDATE'S DECLARATION

I, hereby declare that the work presented in the thesis entitled "Isolation and characterization of pancreatic lipase inhibitors from endophytic fungi" in the partial fulfillment of the requirement for the award of the Degree of Doctor of Philosophy at Department of Biotechnology, Thapar University, Patiala is an authentic record of my own work during the period from December 2012 to May 2015, under the supervision of Dr. Dinesh Goyal, Professor and Dr. Sanjai Saxena, Professor, Department of Biotechnology, Thapar University. This report has not been submitted for the award of any degree or certificate in this or any other university.

Place: Patiala

Date: 15th June 2016

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LIST OF PUBLICATIONS

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AWARDS

- Best Poster Award (2014) at 4th Biennial International conference on new developments in drug discovery from natural products and traditional medicines. National institute of pharmaceutical education and research (NIPER), Mohali, Punjab.
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- Awarded with 'Basic Science Research (BSR) fellowship for Meritorious students' by University Grants Commission (UGC) since 2010.

CONFERENCE PROCEEDINGS

ORAL PRESENTATION

- Gupta M, Saxena S and Goyal D (2015): Diversity and lipase inhibitory activity of endophytic fungal species associated with *Aegle marmelos*: A biosource for potential pancreatic lipase inhibitor. National symposium on mycological research–emerging trends, application and prospects and 41st annual meeting of mycological society of India. Department of Botany, Punjabi University, Patiala, Punjab.

POSTER PRESENTATIONS

- Gupta M, Saxena S, Goyal D (2013). Screening of pancreatic lipase inhibitors from endophytic fungi. National Conference on "Current Perspectives of Fungi in Health Care and Environment" (KAVAASTHA), Bangalore, Karnataka.
- Gupta M, Saxena S, Goyal D (2014). Endophytic fungi: a new source for pancreatic lipase inhibitor. International symposium on "Role of microbes in the 21st Century- A Global Scenario, University of Kolkata, Kolkata, West Bengal.
- Gupta M, Saxena S, Goyal D (2014). Endophytic fungi: a novel source for pancreatic lipase inhibitor. 4th Biennial International conference on new developments in drug discovery from natural products and traditional medicines. National institute of pharmaceutical education and research (NIPER), Mohali, Punjab.
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OTHER ABSTRACTS/ CONFERENCE ATTENDED

- Das N, Kapoor R, Gupta M, Aminedi R (2011): Effect of varying concentrations of cytokinin, N6-benzyladenine on microtuberization in the potato cultivars. 98th Indian Science Congress. SRM University, Chennai.
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List of Abbreviations

AMM	Adipocyte Maintenance Medium
AR	Analytical Reagent
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
CB1R	Cannabinoid Receptor Type 1
C/EBP	CCAAT/enhancer binding protein
CO_2	Carbon Dioxide
^{13}CNMR	Carbon Nuclear Magnetic Resonance
CNS	Central Nervous System
CVD	Cardio Vascular Diseases
DM	Differentiation Medium
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribose Nucleoside Triphosphate
DPX	Distrene Plasticiser Xylene
D_2O	Deuterated Water
ddW	Double Distilled Water
EC	Enzyme Commission
EGCG	Epigallocatechin-3-gallate
EMA	European Medicines Agency
ESI-MS	Electrospray Ionisation -Mass Spectrometry
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FFA	Free Fatty Acids
FTIR	Fourier transform infrared spectroscopy
GCPSR	Genealogical Concordance Phylogenetic Species Recognition
GI	Gastrointestinal
GR	Guaranteed Reagent

HCA	Hydroxycitric Acid
hCMV	Human Cytomegalovirus
HDAC	Histone Deacetylase
HMG- CoA	3-hydroxy-3-methyl-glutaryl-CoA reductase
^{1}HNMR	Proton Nuclear Magnetic Resonance
HPLC	High Performance Liquid Chromatography
HSL	Hormone Sensitive Lipase
IBMX	3-isobutyl-1-methylxanthine
IC_{50}	Inhibitory Concentration $_{50}$
ITS	Internal Transcribed Spacer
JSGW	Jain Scientific Glass Works
LB	Lineweaver Burk
LC-MS	Liquid Chromatography–Mass Spectrometry
LDL	Low Density Lipoprotein
NCBI	National Center for Biotechnology Information
MEGA	Molecular Evolutionary Genetics Analysis
MLST	Multi Locus Sequence Typing
MP	Maximum Parsimony
MTCC	Microbial Type Culture Collection
NCD	Non Communicable Diseases
NPY	Neuropeptide Y
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PEM	Pre-adipocyte Expansion Medium
Pen–strep	Penicillin–Streptomycin
PL	Pancreatic Lipase
pNPL	Para Nitro Phenol Laurate
pNPP	Para Nitro Phenol Palmitate
pNPS	Para Nitro Phenol Stearate
PPAR	Peroxisome Proliferation Activator Receptor
PPL	Porcine Pancreatic lipase
R_f	Retention Factor

RNase	Ribonuclease
rRNA	Ribosomal Ribo Nucleic Acid
RPB2	RNA Polymerase beta Subunit II
SD	Standard Deviation
SNA	Synthetischer Nahrstoffarmer Agar
SREBP	Sterol Regulatory Element Binding Protein
SRL	Sisco Research Laboratories
TCM	Traditional Chinese Medicine
EF–1α	Elongation Factor Alpha Promoter
THL	Tetrahydrolipstatin
TLC	Thin Layer Chromatography
TOF MS	Time Of Flight Mass Spectrometry
TR	Trypanothione Reductase
UV	Ultra Violet
USA	United States of America
USFDA	United States Food and Drug Administration
USD	United States Dollar
VLDL	Very Low Density Lipoprotein
V_{max}	Maximum Velocity
WHO	World Health organization

List of Symbols

%	Percentage
^{o}C	Degree Celsius
R^2	Correlation coefficient
OD	Optical density
rpm	Revolution per min
Kg/m^2	Kilograms per meter square
Kcal	Kilo calories
Nkat/l	Nanokatals per litre
KD	Kilodalton
w/v	Weight by volume
U/ml	Units per milliliter
U	Units
V	Volt
Kv	Kilovolt
Hz	Hertz
MHz	Mega hertz
bp	Base pair
Kb	Kilobase
µg	Microgram
mg	Milligram
g	Gram
Kg	Kilogram
µl	Microlitre
ml	Millilitre
l	Litre
nM	Nanomolar
µM	Micromolar
mM	Millimolar
M	Molar
s	Seconds

min	Minutes
h	Hour
ppm	Parts per million
nm	Nanometer
µm	Micrometer
mm	Millimeter
cm	Centimeter
Km	Kilometer
µg/µl	Microgram per microlitre
ng/ml	Nanogram per millilitre
µg/ml	Microgram per millilitre
µg/ml/min	Microgram per milliliter per minute
µl/min	Microlitre per minute
ml/min	Millilitre per minute
µM/min	Micromolar per minute
mM/min	Millimolar per minute
nmol/ml	Nanomole per millilitre
mM/min/g	Millimolar per minute per gram
J	Coupling Constant
K_i	Inhibition Constant
K_m	Michaelis Constant
$MgCl_2$	Magnesium Chloride
m/z	Mass to Charge Ratio
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
V_{max}	Maximum Velocity

Dedicated to...

My Uncle

Dr. D.C. Bansal

&

My Father

Mr. Ashok Gupta

LIST OF TABLES

Executive Summary

The present study was oriented to evaluate the potential of endophytic fungi to produce pancreatic lipase inhibitors. The cell free culture filtrates of 190 endophytic fungal isolates were screened for their pancreatic lipase inhibitory activity. The potential fungal culture filtrates were then partially purified using liquid-liquid fractionation. Crude aqueous residue of #6AMLWLS inhibited porcine pancreatic lipase with an IC_{50} of 2.12 µg/ml, which showed better potential than the positive control Orlistat (IC_{50} 2.73 µg/ml). The bioactive fungus (#6AMLWLS) was identified as *Fusarium incarnatum* both by morphological as well as molecular studies. Further TLC fractionation and silica gel column chromatography separated the crude residue into two pure bands (B1 and B2).

The pure band B1 competitively inhibited pancreatic lipase with an inhibition coefficient (K_i) of 7.1 µM. It was a white amorphous solid, highly polar having melting point greater than 300°C. In phytochemically testing B1 showed positive blue coloration with ninhydrin, suggesting it to be an amino acid. ^{1}HNMR, ^{13}CNMR spectroscopy suggested the bioactive compound to be a four carbon amino acid derivative and peaks in FTIR spectroscopy confirmed the respective functional groups. Band 1 exhibited ESI-MS ions at m/z 422 confirming four amino acids to be linked by peptide bonds. The compound was chemically named as 11-amino-2,5,8-triethoxy-4,7,10-trioxo-12-oxa-3,6,9-triazatetradecanoic acid and subsequently called as Fustat. A literature search revealed that this compound did not match with any reported lipase inhibitors. Fustat showed 60% inhibition of lipid accumulation in 3T3-L adipocytes exhibiting no cytotoxicity against 3T3-L adipocytes. The present study establishes endophytic fungi as an important resource for molecules, which could be used as inhibitors of pancreatic lipase, a major target for controlling diet induced obesity. Fustat exhibits similar potential as orlistat for its further evaluation using animal model for pharmaceutical drug development.

Chapter 1

Introduction

Obesity is a burgeoning major health concern all over the world. It is a complex multi-factorial disorder, characterized by excess accumulation of adipose tissue in terms of body mass index ratio of 30 kg/m^2 and above. Obesity results due to imbalance between calories consumed and calories utilized. The two prime reasons for overweight and obesity are; (a) increased intake of energy dense foods having high concentration of fats, salts and sugars, (b) decrease in physical activity, sedentary forms of work and increasing urbanization (WHO, 2000). The other possible causes of obesity are psychological in which people tend to eat more due to anxiety, when sad, bored or depressed. Some secondary causes of obesity are iatrogenic leading to hormonal imbalance in diseases like Cushing's syndrome, polycystic ovary syndrome and insulin tumors (Bujalska et al., 1997; Giovannucci, 1995; Gambineri et al., 2002).

The global trend of people suffering with obesity is alarming. In 2005, over 400 million people were obese, which is expected to become one billion in 2030 (Kelly et al., 2008). Obese individuals are more prone to non-communicable diseases (NCD) such as cardiovascular disease (CVD), stroke, type 2 diabetes, hypertension, reproductive and gastrointestinal (GI) cancers, osteoarthritis and sleep apnea. Globally every six deaths out of ten are due to NCD of which obesity is the primary reason and therefore it has been regarded as a disease, attracting medical therapy.

1.1 Therapeutic interventions for obesity management

Exercise therapy and pharmacotherapy are two basic interventions adopted for obesity management. Exercise therapy primarily involves weight management by doing physical exercise and simultaneously monitoring the calorie intake by taking specific low calorie diet regime in which high energy foods are replaced by nutritive supplements. The drawback of this therapy is high frequency of relapse of obesity when either the diet or exercise regimen is not strictly followed (Hill and Wyatt, 2005; Wadden et al., 1997). Pharmacotherapy has two approaches; one is traditional

and other is modern medicine.

1.2 Traditional medicine for obesity management

Traditional medicine comprises the use of plants as natural pharmaceutical aids. Several medicinal plants have been reported to possess anti–obesity properties in the ayurvedic literature (Gogte, 2002). A variety of herbal medicinal preparations or supplements have been developed based on the ayurvedic knowledge which is available in the market for obesity management. However, all of these supplements do not exhibit the same effect as they target different mechanisms of action. Broadly, these supplements have been classified on the basis of the effect they produce or on the mechanism of anti–obesity action. These plant products or supplements, like drugs act either peripherally, centrally or in combination on the receptors of the nervous system, thereby managing appetite, metabolism or calorie absorption.

Peripherally acting substances mediate their effects by reducing the calorie absorption in the GI system or by controlling systems outside the central nervous system (CNS). The herbs or plant extracts acting peripherally generally comprise of lipase inhibition, thermogenesis, down regulation of adipogenesis and lipolysis. Pancreatic lipase (PL) is the key enzyme responsible for the fat absorption in the intestine and therefore, the efficacy of herbal products/ supplements broadly classified as saponins, polyphenols, flavonoids and caffeine is determined by their ability to inhibit PL (Birari and Bhutani, 2007).

Adipocytes primarily store triglycerides and release them in the form of free fatty acids (FFA) when there is energy demand in the body. Synthesis of adipocytes is referred to as adipogenesis, a complex process regulated by several genes. Some primary adipogenic differentiation factors are peroxisome proliferator activator receptor (PPAR), CCAAT/ enhancer building protein (C/EBP) and sterol regulatory element binding protein (SREBP). Expression of PPAR-

γ is an important event during adipogenesis in the fat cells (Speigelman et al., 1997).

Phytochemicals like kaempferol, katechin, quercetin and dietary flavonoids present in fruits, vegetables and green tea have been found to down regulate the adipogenesis differentiation factor PPAR-γ, C/EBP-α and SREBP-1 (Murase et al., 1999; Chien et al., 2005). Resveratrol has been found to promote higher Sirtuin 1, which in turn promotes fat mobilization *via* repression of PPAR (Yang et al., 2008).

Lipolysis involves hydrolysis of triglycerides by which stored fat is reduced to combat obesity. Various flavonoids present in the leaves of *Nelumbo nucifera* are involved in regulation of β3–adrenergic receptor (Velusami et al., 2013). The receptors on CNS are primarily related to the sense of satiety within the body. Many herbal products act *via* the receptor thereby restricting the intake of food. Neuropeptide Y (NPY) expression increases food intake and body weight. Several natural appetite suppressants mediate the reduced expression of NPY. Natural hydroxycitric acid (HCA) obtained from *Garcinia cambogia* is a natural appetite stimulant which competitively inhibits adenosine-5′-triphosphate citrate lyase leading to decreased acetyl coenzyme A (CoA) production and decreased fatty acid synthesis. HCA is also implicated in the availability of serotonin which is a neurotransmitter regulating the eating behavior and appetite control (Igho et al., 2011).

Preference of people for herbal products for obesity management is because they have minimum or no side effects and are available without prescription besides perception of being 100% natural and safe. However, majority of herbal products in the market have limited published information, with no clinical evidence of their role in combating obesity despite several public claims of remarkable weight reducing effect (Chandrasekaran et al., 2012). Hence there is a need of a systematic, well-designed screening with generation of a reliable clinical data to develop a herbal weight loss product.

1.3 Modern pharmacotherapy for obesity

The prevalence of obesity, particularly among the young has increased suggesting that public health guidelines have not been sufficient in restricting the epidemic of obesity. It is very likely that drug based treatment of obesity will develop as an important therapeutic option and would primarily focus on effective drugs and well tolerated treatments for the people suffering with obesity. The drugs used currently act *via* decrease in appetite or increase satiety, are thermogenic agents and digestive inhibitors. The drugs decreasing appetite or increasing the satiety function *via* CNS such as norepinephrine (noradrenaline), serotonin (5–hydroxy–tryptamine), dopamine, or a combination of these. For short term treatment of obesity, benzphetamine and phendimetrazine have been used (Joo and Lee, 2014). These are anorectic sympathomimectic amines with pharmacological activities similar to amphetamines. Their potential side effects are cardiac dysrhythmias, tachycardia, and edema which restrict their long term use. Fluoxetine is another drug which decreases the appetite *via* serotonin uptake but was not approved by United States Food and Drug Administration (USFDA), because of inconsistent results in the clinical trial (Li and Cheng, 2009). Simbutramine was initially developed as an anti-depressant drug but it proved to be very effective to reduce body weight and appetite. The drug functions *via* CNS by increasing the satiety. The common side effect of long term use of Simbutramine is uncontrolled hypertension, coronary heart disease, cardiac dysrhythmias, congestive heart failure or stroke. It has been withdrawn from the market in 2010 (James et al., 2010).

Similarly, Rimonabant was given approval by European Medicine Agency (EMA) in Europe for the treatment of obesity as an adjunct to diet and exercise in obese people in 2006 (Christensen et al., 2007). However, USFDA never approved its use in America due to serious safety concerns. Rimonabant is a cannabinoid (CB)1–receptor which suppresses appetite and weight gain in

experimental animals as well as in human clinical trials. However, due to increasing reports of psychiatric problems such as anxiety, depression and suicidal tendencies, the drug was withdrawn from the market in 2008. This decision in turn rapidly led to the termination of several CB1–receptor antagonist based anti–obesity drug development programs including those for Rimonabant, Taranabant, Otenabant, Surinabant and Ibipinabant (Plieth, 2008).

The USFDA approved Orlistat, a PL inhibitor for the treatment of obesity was introduced in 1998. This drug reduces the fat absorption in the gut by ~30% (Borgstrom, 1988) and induces moderate weight loss to the tune of 3 Kg in one year. The consumption of Orlistat leads to reduced cardiovascular risk, due to lowering of low density lipoprotein (LDL), cholesterol, blood pressure and glycaemia (Broom et al., 2002; Torgerson et al., 2004). The side effects of Orlistat are limited, but include diarrhoea, flatulence, bloating, abdominal pain and dyspepsia. Till date, apart from Orlistat, limited drugs have received approval after initial rejection from USFDA. These are Locaserin which was initially rejected in 2010 owing to concerns on tumor growth in the pre–clinical studies but later approved in June 2012 and proposed to be marketed under the trade name of Belviq® (Vickers and Cheetham, 2007). The alteration of lipid metabolism preferably through the use of inhibitors of PL appears as a plausible and safest method of controlling the diet induced obesity in individuals.

1.4 Lipases as potential drug target

Lipases (EC.3.1.1.3, triacylglycerol acylhydrolases) are water–soluble enzymes that belong to the class of esterases. They play an important role in fat metabolism by cleaving glyceride ester bonds of long chain triglycerides into polar lipids. Lipases catalyze a number of acylation and deacylation reactions, but are best known for their hydrolysis and synthesis of triacylglycerols (Schmid and Verger, 1998). Most dietary fat is ingested as triglycerides (90–95%) which are hydrolyzed firstly in the mouth, followed by stomach where about 15–20% of the triglycerides are cleaved, and then

finally in the duodenum where the synergistic actions of gastric and colipase–dependent PL leads to the formation of monoglycerides and FFA. PL is the major lipid digesting enzyme secreted from the pancreas and is responsible for hydrolysing 50–70% of triglycerides converting them to monoglycerides and fatty acids. It removes fatty acid groups from α and β positions of dietary triglycerides (Crandall and Lowe, 2001).

$$\text{Triacylglycerol} + H_2O \longrightarrow \text{2–monoacylglycerol} + \text{2 fatty acid}$$

The larger fat droplets are coated by bile salts secreted from liver. Hence, these droplets are emulsified in smaller droplets thereby increasing the overall surface area where in duodenum they are broken down in monomers which are absorbed in lymphatic system. These monomers then again reform triglycerides and are stored as fats in adipose tissue. Lingual lipase, secreted by serous gland, digests approximately one third of the ingested fat. Gastric lipase secreted in response to mechanical stimulation, ingestion of food or sympathetic activation, accounts for the hydrolysis of 10–40% of dietary fat. These two enzymes, thus, potentially limit the nutritional impact of the inhibition of lipid absorption that could result from the reduction in the activity of PL alone. Another class of lipase is present in humans that are responsible for increase in size and number of adipose tissue. Lipoprotein lipase converts chylomicrons and very LDL to glycerol and FFA which enters the adipose tissue leading to increase in their size and number. Adipocyte triglyceride lipase and hormone sensitive lipase breaks triglycerides into diglycerides and then into monoglycerides, thereby releasing FFA that circulate in blood or undergo β oxidation to provide adenosine triphosphate (Morak et al., 2012).

1.5 Lipase inhibitors as potential drugs

One of the most important strategies in the treatment of obesity includes development of inhibitors that bind to the lipases responsible for digestion of triglycerides. This is an attempt to reduce

energy intake through GI mechanisms or lipogenesis, without altering any central mechanisms.

This can be done specifically at two points, firstly, in the duodenum where PL acts on ingested triglycerides thereby converting them to monoglycerides and FFA which then pass through enterocytes to form chylomicrons with lipoproteins (Yun, 2010). If the action of PL is partially or completely inhibited by help of certain pharmacophores, these triglycerides are excreted as such out of the body with stools (Frason and Rossner, 2000). As fat has been excreted, body now uses stored fat thereby causing loss of weight in an individual.

Secondly, in endothelial walls of capillaries and on the surface of adipose tissue cells where lipoprotein lipase act on chylomicrons and lipoproteins converting them to monoglycerides and fatty acids that enter into the adipose cells, where they are stored as fats and the process is called as lipogenesis (Albright and Stern, 1998). Certain molecules inhibiting the action of lipoprotein lipase can prevent fats to be stored in adipose tissue. These pharmacophores are called lipase inhibitors.

Orlistat (tetrahydrolipstatin (THL)) is the first and the only drug approved by USFDA is a new pharmacological class that limits triglyceride absorption (Fig. 1.1). It was approved by FDA in year 1998 for long term usage. It is a semi–synthetic hydrogenated derivative of naturally occurring compound produced by bacterium *Streptomyces toxitricini* (Suwailem et al., 2006). It causes acylation of the hydroxyl group of the serine residue present in the triad of the active site of the enzyme thereby causing a conformational change and permanently inhibiting action of lipase on triglycerides (Suwailem et al., 2006). Orlistat is lipophilic in nature, so it is sparsely soluble in the body and does not enter the blood circulation. Hence, its lipophilic nature makes it safe and it is

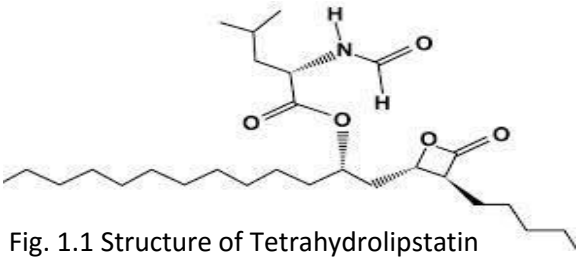


Fig. 1.1 Structure of Tetrahydrolipstatin

retained for a very less time in the body thereby reducing the action of PL by 30%. It has many side effects like steatorrhea (oily/ loose stools), abdominal pain, diarrhoea, fecal spotting, bloating and some hepatic toxicity effects (Singh et al., 2013).

Another lipase inhibitor that works exactly with the same mechanism of action as Orlistat but with lesser side effects is Cetilistat. Cetilistat is a novel highly lipophilic benzoxazinone (Fig. 1.2) inhibiting GI lipase and PL, which raises the possibility of a distinct clinical profile (Kopelman et al., 2007). In continued search for effective anti–obesity agents, several microbial and plant entities have been investigated to explore new chemical entities possessing PL inhibitory activity. The isolation of Orlistat (lipstatin) from actinobacterium *Streptomyces toxytricini* is considered as a landmark discovery in anti–obesity treatment, which has paved the way for finding novel molecules from microbial/ natural resources. Several other actinomycetes like *Streptomyces aburaviensis* produces ebelactone and *Boreostereum vibrans* produces vibralactone, which has potent PL inhibitory activity (Nonaka et al., 1996). All these compounds however are under clinical trials. Yeast and fungi like *Candida antarctica*, *Humicola lanuginosa*, *Schizophyllum* sp., *Leatriporus sulphureus* have also received much attention from pharmaceutical industry for being exploited as a source for anti–obesity drug (Slanc et al., 2004).

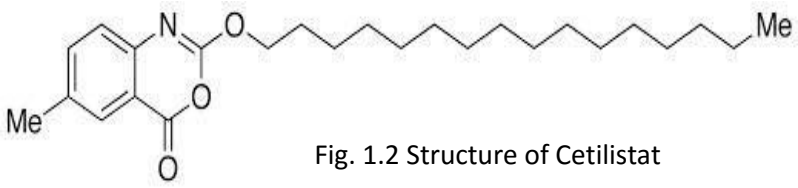


Fig. 1.2 Structure of Cetilistat

1.6 Endophytic fungi: an underexplored resource of pancreatic lipase inhibitors

The discovery of penicillin from fungus *Penicillium notatum* marked the golden era of antibiotics and since then fungi have been exploited extensively in development and use of some remarkable drugs such as cyclosporine, an immunosuppressant; penicillin's, cephalosporin's which are anti–bacterial antibiotics and griseofulvin which is an anti–fungal antibiotic (Berdy, 2005). Fungi like

Monascus ruber and *Aspergillus terreus* have also produced potent 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG–CoA) reductase inhibitors like mevastatin and lovastatin respectively, which are extensively being used as commercial cholesterol lowering drugs after relevant chemical modifications to improve their bioavailability (Endo, 2008). Fungi also exist inside the plants without any obvious indication of their existence and are referred to as endophytic fungi (Bacon and White, 2000; Schluz and Boyle, 2005). Endophytic fungi are diverse in nature and have profound impact over host plants. They increase the overall fitness of the plant by providing abiotic and biotic stress tolerance, decreasing water consumption and defending from pathogenic microorganisms (Rodriguez et al., 2009; White and Torres, 2010; White and Bacon, 2012). Endophytic fungi have been reported to be lucrative source of bioactive compounds possessing anti–bacterial, anti–cancer, cytotoxic, neuroprotective and antioxidant activities, some of which are under clinical trials (Tan and Zou, 2001; Strobel, 2003; Kaul et al., 2012). Bio–prospecting of endophytic fungi for screening and isolation of PL inhibitors is a nascent area with very scanty preliminary data. Methanol and dichloromethane extracts of some of the wood damaging mushrooms and macro fungi possess PL inhibitory activity (Slanc et al., 2004). There are no reports wherein endophytic fungi have been subjected to systematic screening for inhibition of PL. Thus, endophytic fungi offer immense possibilities of exploring hitherto unreported bioactive agents using a defined screening program based on a particular disease target. In the present study, endophytic fungi were systematically screened for their PL inhibitory potential to explore new lipase inhibitors for possible clinical evaluation as anti–obesity molecules or drugs.

Chapter 2

Present Approach

Endophytic fungi are extremely diverse group of microorganisms, ubiquitously existing in tissues of plants including trees, grasses and herbs without any over signatures of their presence or existence (Bacon and White, 2000). Endophytic fungi are believed to play a significant role in host plant association in biotic and abiotic stresses (Redman et al., 2002; Arnold et al., 2003; Zhang et al., 2006a; Bae et al., 2009). In certain cases the host plant tolerance to biotic stress has been attributed to host plant products produced by these endophytic fungi (Saikkonen et al., 1998; Tan and Zou, 2001; Strobel et al., 2004).

Endophytic fungi have also been found to produce putative phytochemicals such as campothecin (Puri et al., 2005) and podophyllotoxin (Puri et al., 2006; Kour et al., 2008). Apart from being source for putative phytochemicals of plant origin, endophytic fungi produces an array of molecules to maintain a chemical communication balance with the host plant in response to the signal molecules produced due to the biotic and abiotic stresses to which the host plant is exposed from time to time. These compounds do not match the plant metabolites but are of much interest due to their structural features or intriguing bioactivities, which could possibly be used directly or indirectly in pharmaceutical drug development.

Fungal natural products previously have been exploited for development in drugs like penicillin, griseofulvin, mevastatin, lovastatin and echinocandin. Endophytic fungi have also been reported to produce a variety of novel chemical structures, which have been primarily identified based on their anti–microbial, anti–parasitic, cytotoxic and neuroprotective activities (Strobel and Daisy, 2003; Strobel et al., 2004; Gunatilaka, 2006; Guo et al., 2008; Yu et al., 2010 ; Aly et al., 2010). Enodepside (Fujisawa Pharmaceutical Co. Ltd.) is an analogue of cyclodepsipeptide PF 1022A which was isolated from a sterile fungus existing in *Camellia japanoica* from Ibaraki Prefecture, Japan and is used as an anti–helminthic agent.

2.1 Hypothesis

Plant lipases (triacylglycerol lipase, lysosome associated lipase and phospholipase) play an important function in transportation and mobilization of stored lipids particularly during seed germination (Fuessner et al., 2001). It has also been well documented that plant lipases play a significant role in plant defense. Phospholipases in response to biotic stress are involved in the hydrolysis of phospholipids. Phospholipase A catalyzes the hydrolysis of phospholipids generating the corresponding FFA and lysophospholipid. The FFA released in turn play a role in Jasmonic acid biosynthesis which is released as a plant defense response (Shah, 2005).

In fungal system, lipids are the major constituents of membrane system apart from cell wall components and storage material, wherein they exist as lipid bodies. Lipid bodies also exist in the plant cells and provide carbon source for essential biological processes. It has been found that fungal endophytes are closely associated to these plant lipid bodies and probably play a role in carbon movement (Avila et al., 2012). It is hypothesized that these fungal endophytes in order to maintain their membrane integrity and stored/ reserve lipids produce certain molecules, which act on these plant lipases. It is expected that these signal molecules/ inhibitors produced by endophytic fungi may play a significant role in inhibiting the PL in human lipid metabolism hence altering the lipid metabolism and acting as anti–obesity drugs.

Based on the above hypothesis in the present study screening and identification of endophytic fungi for pancreatic lipase inhibitory activity was carried out followed by isolation, purification and biochemical characterization of the pancreatic lipase inhibitor.

Chapter 3

Review of Literature

Obesity is due to unnecessary fat deposition in adipose tissue causing various abnormalities which is increasing like an epidemic and the rising trends do not exhibit signs of abating. It has been projected that if the current scenario continues, by the end of year 2020 two individuals out of three will be overweight or obese (Obesity update, 2012). The management of obesity can be done by two different anti–obesity therapies viz. exercise therapy and drug therapy. Drug therapy is more convincing as there is relapse of weight gain after physical activity has been stopped. Drug therapy includes targeting drugs to CNS or peripheral nervous system eventually leading to satiety/ loss of hunger and PL inhibition respectively.

3.1 Obesity: a priority issue

Obesity, a "new world syndrome" is being widely spread in both developed and developing countries; such that it is replacing the traditional health concerns of infectious diseases and under nutrition therefore being a significant contributor to ill health (WHO, 2000). Obesity rates have increased by 2-3% in USA, Australia and United Kingdom, but it has grown at rapid rates in Canada, South Africa and China (Fig. 3.1). In the present scenario, India has lowest percentage of obese adults (Griffiths and Bentley, 2001) as India is still fighting with malnutrition. It is estimated that by year 2030, obesity in India will grow by 20% (Obesity update, 2014).

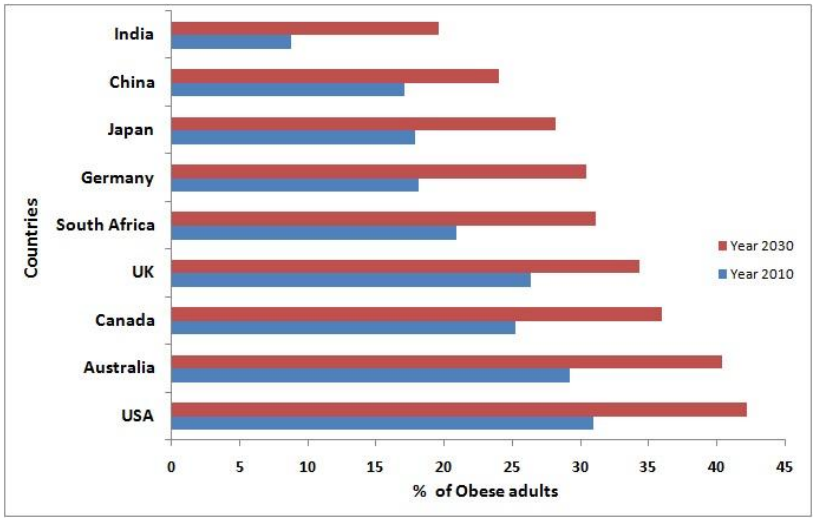


Fig. 3.1 World Scenario of obesity in year 2010 and 2030

The burden of obesity on the country's economy is increasing day by day and is now a worldwide concern. It has been estimated that obesity accounts for 0.7–2.8% of the country's total expenditure on healthcare. Further, it was also found that the medical expenditure of obese individuals is approximately 30% higher than their normal weight peers (Withrow and Alter, 2011). Pediatric/ childhood obesity has been recognized as a public health problem. Previously, a fat child was considered to be healthy who can withstand under–nourishment and various diseases; but in past decade, the increasing prevalence of childhood obesity has become a matter of public health crisis both nationally as well as internationally. Globally in the year 2010, approximately 42 million children under the age of five years were overweight of which 35 million were from the developing countries (WHO, 2009). In USA, about 25% children are overweight and about 15% are clinically obese (Livingstone, 2001). In developing nations, because of poor diet and lack of physical activity, the urban poor children are vulnerable, whereas the upper socioeconomic strata of the developing nations exhibit obesity in children due to adoption of western style of diet (James et al., 1997).

3.2 Adipogenesis

Adipocytes play an important role for the maintenance of good health. Adipogenesis is the process of formation of new fat cells which occurs due to storage of triglycerides leading to increase in cell size which eventually results in cell division, thereby enhancing the adipose tissue (Couillard et al., 2000). There are two modes of adipose tissue expansion, hypertrophy (increase in the cell size) and hyperplasia (increase in the cell number). Hypertrophy without hyperplasia leads to adipocytes that are metabolically unhealthy. Adipocytes are dynamic cells that perform certain critical functions in the body such as store fat, secrete specific endocrine hormones apart from their insulin sensitivity. Predominant types of adipocytes are brown and white. The white adipocytes store energy, while

the brown adipocytes dissipate energy in a heat generating process referred to as thermogenesis. Obesity is related to increase in the number of white adipocytes (Stephens, 2012).

3.3 Problems associated with obesity

Consumption of fat rich food is associated with high energy intake (Astrup, 2001; Little et al., 2007) which are considered to promote fat storage in the body as well as weight gain in human beings leading to obesity. Studies have indicated that habitual high fat intake modulates the GI responses such as slowing of gastric emptying, antropyrolic duodenal motility and GI hormonal secretions hence contributing to obesity (Bisset and Azpiroz, 2013).

Obesity is responsible for impairment of quality of life and serious medical implications such as hypertension, CVD, diabetes and osteoarthritis. The extra weight in obese persons induces disorders in the skeletal framework. Arthritis becomes a leading cause of physical disability due to the extra mechanical burden on spine, knees, hips and ankle (Falson et al., 1988; Circuttini et al., 1996). It is also indicated that obesity is involved in pathogenesis of osteoarthritis and can precede it by decades. Abdominal obesity results in pulmonary function abnormalities such as obesity hypoventilation syndrome and obstructive sleep apnea. Due to increase weight on the chest wall there is a decrease in respiratory compliance resulting in the increase of work of breathing and restricts ventilation (Strohl et al., 1998). Obese individuals are also predisposed to weight induced sleep apnea (Lazarus et al., 1997).

A change in waist size/ circumference has also been independently linked with sleep disordered breathing (Redline et al., 2003). The other obesity related metabolic abnormalities include insulin resistance, elevated serum uric acid (hyperureicemia) and increased inflammatory mediators. These all abnormalities may also result in cataract formation in obese people (Glynn et al., 1995; Hieler et al., 1998). Obese individuals release increased amounts of non–esterified fatty

acids, glycerol, hormones, pro–inflammatory cytokines and other factors which lead to the development of insulin resistance. When insulin resistance is accompanied by dysfunction of β–cells in pancreatic islets, it results in insulin failure to control blood glucose levels which leads to risk of development of type 2 diabetes (Kahn, 2001; Kahn et al., 2006). Obesity has also been identified as an independent risk factor for CVD, hypertension, coronary heart disease and heart failure (Kenchaiah et al., 2002; Lavie et al., 2008, 2009). It is also associated with cancer and accounts for 20% of all cancer cases. Analysis of 25 years data reveal that obesity is the cause of approximately 14% death in men and up to 20% cancer death in women (Calle et al., 2003). A study by the world cancer research fund and American Institute of Cancer Research provides convincing evidence between obesity, esophageal, pancreatic, colorectal, postmenopausal breast, endometrial and kidney cancers with probable evidence of gall bladder cancer (Wolin et al., 2010).

3.4 Management of obesity by drugs

Driven by chemistry but predominantly guided by pharmacology and the clinical sciences, the drug research has significantly contributed in the progress of medicine in the last century. Biochemically, the molecular targets which have been used for drug discovery and therapy have been classified into receptors, enzymes, hormones, factors and ion channels (Fig. 3.2, Drews, 2000). The drug research was shaped, nurtured and enriched by several new technologies which had a marked impact on drug discovery and therapy. Biochemistry had a great impact on drug research by introducing the concept of enzymes and receptors as drug

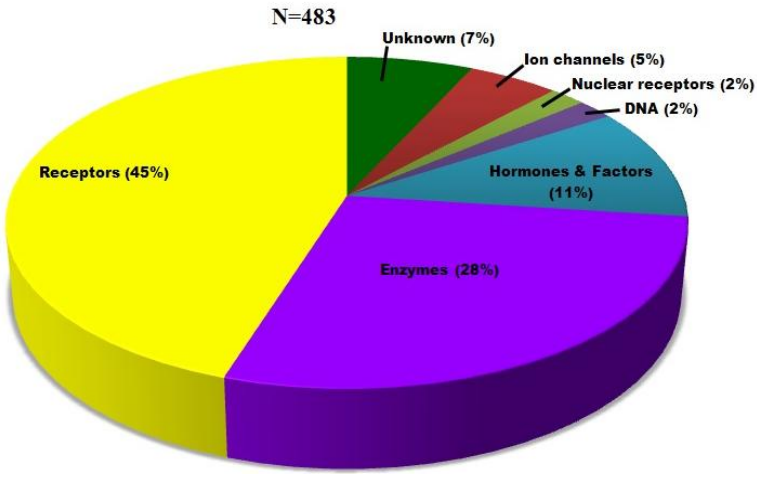


Fig. 3.2 Current targets for drug pharmacotherapy

targets. The earliest description of enzyme inhibitors began with the discovery of sulphanilamide as carboanhydrase inhibitor, which was active member of Prontosil (sulpha drug). Sulphanilamide led to the inhibition of carboanhydrase leading to an increase in natriuresis and excretion of water (Schwartz, 1949). Sulphanilamide was the driver for development of better carboanhydrase inhibitors such as acetazolamide and more effective diuretics like hydrochlorothiazide and furosemide (Maxwell and Eckhardt, 1990).

Since 1930's till date, over half a dozen drugs were prescribed to obese individuals for a period and withdrawn from the market due to their serious side effects (Derosa and Maffioli, 2012). The first drug introduced for obesity treatment was Dinitrophenol in 1933 which enhanced the metabolic rate but was responsible for fetal hyperthermia. This was followed by Amphetamines, which were popular between 1940's to 1960's as a short term treatment of obesity. The Amphetamines were later withdrawn due to increased risk of heart disease or hypertension with patients having the history, while those who tolerated the developed abuse leading to psychotic disorders. Phentermine was approved by USFDA in 1959, while Fenfluramine was approved in 1973.

Dexfenfluramine was developed as an alternative to Fenfluramine, which exhibited lesser side effects. Phentermine with Fenfluramine/ Dexfenfluramine was an effective combination which was responsible for over 10% weight loss (Weintraub et al., 1992). This combination was however withdrawn in 1997, due to increasing evidence of development of valvular heart disease in 30% of subjects who were administered the medicine (Conolly et al., 1997). Simbutramine was introduced in 1997 for the management of obesity including weight loss and maintenance of weight loss. This drug was recommended to be used with caution in patients with the history of hypertension, coronary heart disease, stroke or arrhythmia. A Simbutramine cardiovascular outcome trial revealed 16% rise in risk of having stroke or myocardial infarction in people taking Simbutramine,

which lead to market reauthorization of the drug in 2010 (FDA, 2010). Rimonabant was not approved in US/ Canada while it was withdrawn from Europe due to potential psychiatric disorders (Soyka, 2008).

Orlistat was the only drug which was approved in 100 countries for marketing. This drug is not an appetite suppressant but interferes with the GI lipase in the GI tract (Leung et al., 2003). Another PL inhibitor which is under phase III clinical trial is Cetilistat (Table 3.1) (Yamada et al., 2008). Lorcaserin has been approved as an anti-obesity drug by USFDA in 2012 (Smith et al., 2010).

3.5 Pancreatic lipase as drug targets

Lipases are ubiquitous enzymes playing a pivotal role in all aspects of fat and lipid metabolism in variety of organisms. In humans and other vertebrates, a variety of lipases control digestion, absorption and reconstitution of fat as well as lipoprotein metabolism (Desnuelle, 1986). In plants, during post germination, the metabolism of oil reserves provide energy and carbon skeleton for embryonic growth and is controlled by the action of lipases (Huang, 1987). Microorganisms such as bacteria and fungi are also known to produce a wide spectrum of extracellular lipid degrading enzymes to breakdown the insoluble lipid into soluble polar components to facilitate absorption (Lie et al., 1991). The digestion of lipids in the oral cavity by the action of lingual lipase continues in stomach with both lingual and gastric lipase in humans. Diacylglycerol and FFA are the digestion products of the gastric phase (Lein, 1994). Further, the lipids in the form of droplets enter the duodenum where they mix with bile and pancreatic juice (Iqbal and Hussain, 2009). PL (EC 3.1.1.3) is the principle lipolytic enzyme in the small intestine which cleaves the triglycerides yielding 2–monoglycerides and FFA.

Table 3.1 Current status of anti-obesity drugs with their mechanism of action (Elangbam, 2009; Ioannides-Demos et al., 2011; Kang and Park, 2012; Rodgers et al., 2012).

Year Introduced	Drug	Mechanism of action	Status
1930s	Dinitrophenol	Increases metabolic rate	Withdrawn—risk of neuropathy and cataracts
1936	Amphetamines: dexamphetamine, methamphetamine	Appetite suppression	Banned, restricted or discouraged—dependency and abuse potential, cardiovascular adverse effects
1959-US	Amphetamine-like analogues: Phentermine, diethylpropion, phenylpropanolamine	Appetite suppression	Diethylpropion—available for short-term use (≤12 weeks) Phentermine—available for short-term use (≤12 weeks) in some countries, withdrawn 2000 (UK) Phenylpropanolamine-withdrawn 2000 increased risk haemorrhagic stroke
1965	Aminorex	Appetite suppression	Withdrawn 1968—pulmonary hypertension
1970s	Mazindol	Appetite suppression	Discontinued 1993—Australia
1963-Europe 1973-US	Fenfluramine	Appetite suppression	Withdrawn 1997—valvular heart disease, pulmonary hypertension
1985-Europe 1996-US	Dexfenfluramine	Appetite suppression	Withdrawn 1997—valvular heart disease, pulmonary hypertension
1998-Europe and US	Orlistat	Decreased fat absorption	Also available *over-the-counter* in several countries
1997-US 2001-Europe	Sibutramine	Appetite suppression	Temporarily withdrawn 2002 Italy-concerns of raised risk of heart attacks and strokes, Increase in contraindications 2010-US, Australia Suspension of market authorization 2010
2006-Europe	Rimonabant	CB1R antagonist	Withdrawn 2009—potential of serious psychiatric disorders
	Cetilistat	PL inhibitor	Phase III
2010	Lorcaserin (Belviq)	Serotonin, dopamine, and norepinephrine reuptake inhibitor	Approved by the FDA in 2012

PL is responsible for the hydrolysis of 50–70% of total dietary fats and their inhibition could grossly affect the fat metabolism and thus play a significant role in the treatment of obesity (Thomson et al., 1997; Mukherjee, 2003; Shi and Burn, 2004). One of the promising strategies of obesity management is reduction in the energy intake through GI mechanisms without altering the central mechanisms, as dietary fats are not absorbed in the intestine unless subjected to the action of PL. Hence, PL appears to be a plausible target for determining the efficacy of molecules of natural and synthetic origin as anti-obesity agents (Birari and Bhutani, 2007).

3.6 Plants as a source of pancreatic lipase inhibitors

Natural plant products have been used as a source of medicines since times immemorial. All ancient systems of medicines such as Ayurveda, Siddha, Unani, traditional chinese medicine (TCM) and western traditional medicine involves the use of medicinal plants. Natural plant products offer a vast pool of PL inhibitors which could be converted into clinically useful products (Yun, 2010). The phytochemicals which exhibit lipase inhibitory activity have been broadly classified as saponins, polyphenols, terpenes (Birari and Bhutani, 2007). Sessiloside and chiisanoside are lupane type of saponins isolated from the leaves of *Accanthopanax sessiliflorus* and exhibit a strong PL inhibition under *in vitro* conditions (Yoshizumi et al., 2006). Platycodin saponins isolated from *Platycodin grandifolium* have been found to possess lipase inhibitory effects. The prominent of these is Platycodin D which exhibited PL competitively with a K_i of 180 µM (Zhao and Kim, 2004). Scabio saponins comprise a group of saponins viz. Scabio saponins E, Scabio saponins F, Scabio saponin I, Hookeroside A, Hookeroside B and Prosapogenin 1b which have been isolated from *Scabiosa tschiliensis.* Of these Prosapogenin 1b exhibited the strongest PL inhibition with a concentration of 129 µM (Zheng et al., 2004). *Dioscorea nipponica* produces Prosapogenin A and Gracillin which have a strong PL inhibitory activity with an IC_{50} of 2.48 µM and 32.6 µM respectively (Kwon et al., 2003).

Tea saponins have been found to inhibit PL in a dose dependent manner (Han et al., 2001). Ergoflavin is an ergochrome class of compound isolated from a leaf of medicinal plant *Mimusops elengi* (*Sapotaceae*), which possesses potential anti-cancer activity (Deshmukh et al., 2009).

Kaempferol and quercitin isolated from lentils (*Lens culinaris*) have been found to possess PL inhibitory activity with an IC_{50} of 115 µM and 74.5 µM respectively (Zhang et al., 2015a). Cyanidin-3-glucoside is a phenolic compound isolated from chokeberry (*Aronia melanocarpa* L.) and inhibits lipase with an IC_{50} of 2410 µM (Worsztynowicz et al., 2014). (-)-Epigallocatechin-3-gallate (EGCG) has been found to inhibit PL in a dose dependent manner, non-competitively with respect to substrate concentration with an IC_{50} of 7.5 µM (Grove et al., 2012). Luteolin 6-*C*-b-D-boivinopyranoside was isolated from methanolic extract of the leaves of *Eremochloa ophiuroides* (centipede grass) exhibited an IC_{50} value 18.5 µM (Lee et al., 2010a).

Polyphenolic extracts have also been evaluated for their potential to inhibit PL. Methanolic extract of rosemary (*Rosmarinus officinalis*) was found to exhibit PL inhibition with an IC_{50} value of 13.8 µg/ml (Bustanji et al., 2010). Polyphenols in white and green tea infusions have exhibited substantial inhibition of PL when screened at 25 µg gallic acid equivalent/ml (Gondion et al., 2010). Apple polyphenol extract has also been reported to possess *in vitro* PL inhibitory activity with an IC_{50} of 5.68 µg/ml (Sugiyama et al., 2007). Peanut shell extract has also shown to inhibit PL (Moreno et al., 2006). Leaf extract of *Nelumbo nucifera* has been found to inhibit PL activity with an IC_{50} of 4600 µg/ml. The inhibitory action of *Nelumbo nucifera* extract is attributed to the phenolic compounds present in them (Ono et al., 2006). Grape seed extract has been found to inhibit PL due to the presence of proanthocyanidins (Moreno et al., 2003).

Terpenes have also been reported to possess PL inhibitory activity. Methanolic extract of *Ginkgo biloba* has exhibited an *in vitro* PL inhibitory activity with IC_{50} of 16.5 µg/ml. Experimentally,

Ginkgolides A, B were found to inhibit PL significantly with an IC_{50} of 22.9 µg/ml (Bustanji et al., 2011a). PL is inhibited by Crocin isolated from *Gardenia jasminoids* competitively and reversibly with an IC_{50} of 28.63 µM (Sheng et al., 2006). Carnosic acid is an abietan type of diterpene isolated from the methanolic extract of *Salvia officinalis* leaves which competitively inhibits PL with an IC_{50} value of 36000 µM in a concentration dependent manner (Ninomiya et al., 2004).

Adisakwattana et al., (2012) investigated the effect of nine edible plants on the inhibition of PL and pancreatic cholesterol esterase activities, as well as the inhibition of cholesterol micelle formation, and bile acid binding. It was found that a strong PL inhibitory activity was exhibited by the mulberry leaf extract. 400 plant extracts were screened for their *in vitro* PL inhibition potential out of which only 44 extracts exhibited high activity of which only 4 extracts were actually found to possess the potential activity (Roh and Jung, 2012). Nine out of 23 plant extracts viz. *Anthemis palaestina* Boiss., *Salvia spinosa* L*., Ononis natrix* L, *Fagonia arabica* L., *Origanum syriaca* L., *Hypericum triquetrifolium* Turra, *Malva nicaeensis* L., *Chrysanthemum coronarium* L. and *Paronychia argentea* L. exhibited potential PL inhibitory activity with IC_{50} in the range of 107.7-342.7 µg/ml (Bustanji et al., 2011b). Similarly, methanolic extracts of 37 plants used in TCM were tested for their *in vitro* PL inhibition. Extracts of *Prunella vulgaris* and *Rheum palmatum* exhibited 74.7% and 53.8 % PL inhibition under *in vitro* conditions (Zheng et al., 2010). Slanc et al., (2009) has screened over 106 species of food and medicinal plants for their potential PL inhibitory activity. Only 10 extracts were identified to possess over 70% inhibition of the PL. Thus, a variety of natural products including crude plant extracts and isolated plant compounds can possibly prevent diet induced obesity by inhibition of PL (Table 3.2). The major limitation of plant products is the complex nature of extracts which makes the process of isolation tedious and their low content makes the process cost intensive and products expensive.

Table 3.2 Pancreatic lipase inhibitors from plants

Name of the plant	Compound name/ Extract	Class of Compound	Reference
Platycodon grandiflorus	Platycodin D	Saponins	Han et al., 2002
Dioscorea nipponica	Dioscin	Saponins	Kwon et al., 2003
Salvia officinalis	Carnosic acid	Terpenes	Ninomiya et al., 2004
Scabiosa tschiliensis	Prosapogenin	Saponins	Zheng et al., 2004
Panax japonicas	Chikusetsusaponins	Saponins	Han et al., 2005
Aesculus turbinate	Escins	Saponins	Kimura et al., 2006
Arachis hypogeal	Luteolin	Flavonoids	Moreno et al., 2006
Nelumbo nucifera	Leaf Extract	Phenolics	Ono et al., 2006
Gardenia jasminoides	Crocin and Crocetin	Terpenes	Sheng et al., 2006
Acanthopanax sessiliflorus	Sessiloside and Chiisanoside	Saponins	Yoshizumi et al., 2006
Afromomum meleguetta	Crude ethanolic extract	Complex mixture	Ekanem et al., 2007
Glycyrrhiza uralensis	Licochalcone A	Polyphenols	Won et al., 2007
Actinidia arguta	3-O-trans-p-coumaroyl actinidic acid	Terpenes	Jang et al., 2008
Rosmarinus officinalis	Methanol extract	Polyphenols	Bustanji et al., 2010
Ginkgo biloba	Ginkgolides A, B	Terpenes	Bustanji et al., 2011a
Bergenia crassifolia	(+)-catechin 3,5-di-O-gallate	Phenolics	Ivanov et al., 2011
Cudrania tricuspidata	Ethanolic leaf extract	Complex Mixture	Kim et al., 2012a
Sorbus commixta	Ethanol extract	Complex mixture	Lee et al., 2012
Viscum album	Ethanol extract	Complex mixture	Lee et al., 2012
Achyranthes aspera	Ethanolic Seed Extract	Complex mixture	Rani et al., 2012
Camellia sinensis	Methanol Leaf extract	Complex mixture	Sharma et al., 2012
Eugenia polyantha	Hydroxychavicol	Phenolics	Kato et al., 2013
Polygonum cuspidatum	Butanol fraction of ethanolic extract	Complex mixture	Kim et al., 2013
Cassia siamea	Cassiamin	Phenolics	Kumar et al., 2013
Terminalia paniculata	Ethanol extract	Complex mixture	Mopuri and Meriga, 2014
Aronia melanocarpa	Cyanidin-3-glucoside	Phenolics	Worsztynowicz et al., 2014
Phyllanthus niruri	Methanol extract	Complex mixture	Rahman et al., 2015

3.7 Pancreatic lipase inhibitors of bacterial origin

Streptomyces group amongst bacteria are prolific producers of bioactive compounds (Manivasagan et al., 2014). The literature survey also indicates that PL inhibitors have been predominantly reported from this group of microorganisms. Esterastin was isolated from the fermentation broth of actinomycetes *Streptomyces lavendulae* strain MD4-C1. Esterastin competitively inhibited the hog PL with IC_{50} value of 394 µM (Umezawa et al., 1978).

Streptomyces aburaviensis produces Ebelactone A and B which inhibited hog PL in a dose dependent manner (Umezawa et al., 1980). Valilactone was isolated from from *Streptomyces albolongus* which potently inhibited hog PL with an IC_{50} of 0.14 ng/ml (Kitahara et al., 1987). *Streptomyces toxytricini* (NRRL15443) was found to produce a specific inhibitor of PL lipstatin, which exhibited an IC_{50} of 0.14 µM (Weibel et al., 1987). Lipstatin is an irreversible inhibitor of PL. THL is a hydrogenated derivative of lipstatin which inhibits all the three lipases viz. gastric lipase, PL and carboxylester lipase under *in vitro* conditions. The commercial drug Orlistat, is a saturated derivative of lipstatin and is the only approved commercial lipase inhibitor till date.

Panclicins A/B/C/D/E from *Streptomyces* sp. NR 0619 isolated from soil sample collected from Yamaga-machi, Oita Prefecture, Japan, exhibited more potential PL inhibition as compared to THL. The IC_{50} of Panclicins A, B, C, D, and E for porcine pancreatic lipase (PPL) are 2.9, 2.6, 0.62, 0.66, and 0.89 µM respectively (Mutoh et al., 1994). Kekuda et al., (2011) reported PL inhibitory activity from *Streptomyces* species isolated from Western Ghat soil, Agumbe, Karnataka, India. The crude extract exhibited over 60% inhibition in PL activity at a concentration of 50,000 µg/ml. Similarly a *Streptomyces* sp. isolate (MTCC 5219) recovered from a soil sample of a cow barnyard in India was found to produce a new lipase inhibitor belonging to the class of enol acetate of p-amino phenyl acetaldehyde. This compound inhibited the hydrolysis of trioleate by PPL dose dependently with

IC_{50} of 7.46 µM (Tokdar et al., 2011). Extract from actinomycetes produces topoisomerase inhibitors, collismycins, cyclo–homononactic acid and cyclo nonactic acid which are novel source of anti-cancer and anti-microbial agents (Stadler et al., 2001).

3.8 Pancreatic lipase inhibitors from algae

Freshwater as well as marine algae have been screened for their potential to inhibit PL. British marine algae viz. *Ascophyllum nodosum*, *Fucus serratus*, *F. vesiculosus* and *Pelvetia canaliculata* have been found to inhibit the activity of the enzymes: lipase, trypsin and α-amylase. The inhibitors were isolated and identified by ^{1}H NMR spectroscopy as polyphenol having apparent molecular weights in the range of 30,000 to 100,000 daltons (Barwell et al., 1989).

Caulerpenyne was isolated from the ethyl acetate extract of marine alga *Caulerpa taxifolia* and was found to inhibit PL. Caulerpenyne is a competitive inhibitor of PL and the concentrations exhibiting 50% hydrolysis of the substrates triolein and 4-methylumbellierate oleate was found to be 2000 µM and 13 µM respectively (Bitou et al., 1999). *Caulerpa prolifera* ethanolic extract has been found to inhibit dog gastric lipase and human PL while screening marine fungi isolated from Tunisian coast (Rebah et al., 2008). Ethyl acetate extract of *Ecklonia cava* was found to inhibit PL under *in vitro* conditions. Isolation of the extract yielded Dieckol which exhibited a concentration dependent lipase inhibitory activity with an IC_{50} value of 350 µM (Kim et al., 2012b).

Ethanol extracts of the tropical red algae *Kappaphycus alvarezii*, *Kappaphycus striatus* and *Eucheuma denticulatum* were analyzed for their inhibitory effect on lipase activity using turbidimetric method. The ethanol extract of dried *Kappaphycus striatus* exhibited the highest reduction in lipase activity with 92% inhibition (Balasubramanium et al., 2013). More recently, chloroform fraction of methanol extract *Sargassum thunbergii* exhibited a potential lipase inhibitory activity with an IC_{50} value of 1980 µg/ml. Further analysis revealed that this fraction afforded three

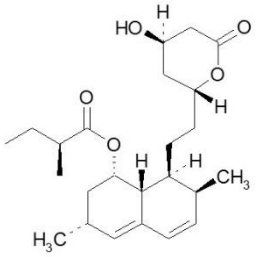
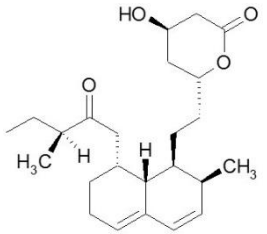
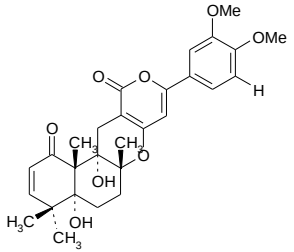
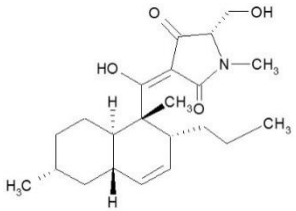
compounds viz. chlorophyll a, isofucosterol and saringosterol and the *in vitro* lipase inhibitory effect of the extract was attributed to the synergistic interactions between them (Kim et al., 2014). Recently, monogalactosyl diacylglyerols isolated from freshwater microalgae *Chlorella sorokiniana* have been reported to have potential *in vitro* PL inhibitory activity (Banskota et al., 2015).

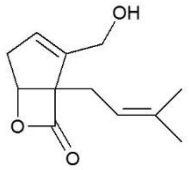
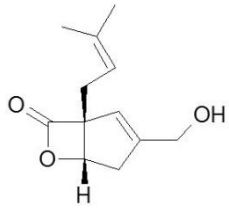
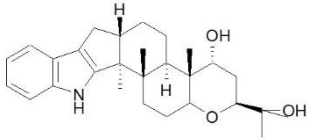
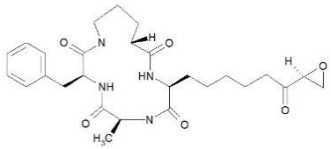
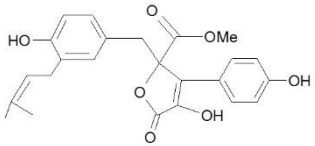
3.9 Fungi as a source of pancreatic lipase inhibitors

Fungi has been a source of variety of enzyme inhibitors which find applications in different therapeutic interventions such as antibiotics, anti–cancer, anti–hypercholesterolemic agents and for the treatment of diseases like Alzheimer's disease, diabetes, immunosuppression and in chemoprevention (Paterson, 2008). PL inhibitors have been predominantly reported from macrofungi. Already, an array of enzyme inhibitors has been discovered and many of them are being used as drugs/ therapeutic agents (Vandamme, 1994; Paterson, 2008).

Some novel enzyme inhibitors from fungi with pharmaceutical applications are given in Table 3.3. In a screening program dichloromethane and methanol extracts of 60 wood damaging fungi were screened for their *in vitro* PL inhibitory activity using the chromogenic substrate p-nitrophenyl palmitate. *Laetiporus sulphureus*, *Tylopilus felleus* and *Hygrocybe conica* exhibited the highest lipase inhibitory activities of 83 ± 5%, 96 ± 3% and 97 ± 5% respectively (Slanc et al., 2004). A novel compound Percyquinnin, a β-lactone has been obtained from the cultures of *Stereum complicatum*, which inhibited PL with IC_{50} of 2000 μM (Hopmann et al., 2003). Vibralactone, another unusual β-lactone produced by the polypore *Boreostereum vibrans* inhibited PL with an IC_{50} of 1.73 μM (Liu et al., 2006).

Table 3.3 Novel enzyme inhibitors from fungi with pharmaceutical applications

Name of the fungus	Compound/ Inhibitor produced	Potential Disease Target/ Enzyme Inhibited	Structure	Reference
Aspergillus terreus	Mevinolin	HMG–CoA reductase (Anti-hypercholestrolemic)		Alberts et al., 1980
Penicillium brevicompactum	Compactin	HMG–CoA reductase (Anti-hypercholestrolemic)		Endo, 1985
Penicillium species FO-4259	Arisugacins A & B	Acetylcholinesterase inhibitors (Alzheimer's Dementia)		Kuno et al., 1996
Fusarium heterosporum	Equisetin	HIV integrase inhibitor (Anti- HIV agent)		Singh et al.,1998

Stereum complicatum ST001837	Percyquinnin	Lipase inhibitor (anti–obesity)		Hopmann et al., 2003
Boreostereum vibrans	Vibralactone	PL inhibitor (anti–obesity)		Liu et al., 2006
Chaunopycnis alba	Terpendole E	Kinesin Eg5 inhibitor		Motoyama et al., 2012
Tolypocladium sp.	1-Alaninechlamydocin	Histone deacetylase (HDAC) inhibitor		Du et al., 2014
Aspergillus terreus MC751	Butyrolactone I	α-glucosidase inhibitor		Dewi et al., 2014

Fruiting bodies of six mushrooms viz. *Grifola frondosa*, *Hericium erinaceus*, *Agaricus blazei*, *Hypsizygus marmoreus*, *Lyophyllum shimeji*, *Pleurotus eryngii* and *Sparassis crispa* were extracted with water/ methanol and tested for their *in vitro* PL inhibition potential. The water extract of *Pleurotus eryngii* exhibited the maximum inhibition of PL under *in vitro* conditions (Mizutani et al., 2010).

Methanol extracts from fruiting bodies of 13 species of mushrooms were tested for their lipase inhibitory activities. Methanol extract of fruiting body of *Phellinus linteus* showed the highest lipase inhibitory activity (72.5%). The mycelial extract of *Phellinus linteus* also exhibited the highest lipase inhibitory activity under *in vitro* conditions. The partially purified lipase inhibitor from *P. linteus* as compared to commercial Orlistat (83% at 10 µg/ml), showed only 71.5% inhibitory activity against PL at the concentration 10 µg/ml (Lee et al., 2010b).

The extracts obtained with methanol, water and methanol: water (1:1) from 21 mushroom species were screened as potential sources of PL inhibitors using a standardized *in vitro* assay. Methanol: Water (1:1) extracts of *Lepiota procera* showed the highest inhibition activity closely followed by *Grifola frondosa, Pleurotus eryngii* and *Lyophyllum shimeji* but they did not exhibit any *in vivo* lipase inhibitory effects (Palanisamy et al., 2012).

3.10 Endophytic fungi as a source of pancreatic lipase inhibitors

The importance of endophytic fungi as a source of pharmaceutically and agrochemically active compounds came into limelight with the detection of taxol in the culture broth of the endophytic fungus *Taxomyces andreanae* that was isolated from *Taxus brevifolia,* the latter being the original source of this important anti–cancer drug (Stierle et al., 1993, 1995). This concept was experimentally extended to other pharmacologically important natural products such as Camptothecin (Puri et al., 2005; Amna et al., 2006), Podophyllotoxin (Puri et al., 2006; Eyberger et

al., 2006; Kour et al., 2008), Vinblastine (Ahmed et al., 2013) and Diosgenin. Apart from being the source of putative phytochemicals, endophytic fungi have also produced biologically active and structurally diverse natural products that are extraordinary in nature (Tan and Zou, 2001; Strobel and Daisy, 2003; Strobel et al., 2004; Gunatilaka, 2006; Zhang et al., 2006a; Aly et al., 2010; Kaul et al., 2012).

Cryptocin and Cryptocandin have been isolated from *Cryptosporiopsis quercina*, existing as an endophyte in *Tripterigeum wilfordii*, a medicinal plant native to Eurasia and possess potential anti–fungal activity (Strobel et al., 1999). Endophytic fungal extracts are being screened for their anti–bacterial as well as anti-fungal potential (Sandhu et al., 2014; Kumar et al., 2014) Recently, microsphaerol has been isolated from an endophytic fungi *Microsphaeropsis* sp. which has been isolated from the halotolerant herbaceous plant *Salsola oppositifolia* from Playa del Ingles (Gomera, Spain). The compound possesses good anti–bacterial activities against *Bacillus megaterium* and *Escherichia coli*, and good anti–algal and anti–fungal activities against *Candida fusca*, *Microbotryum violaceum* respectively (Hussain et al., 2015).

Endophytic fungi are also a source of novel anti–cancer agents apart from being source of putative phytochemicals with proven anti–cancer potential. Similarly, sclerotiorin isolated from the endophyte *Cephalotheca faveolata* possessed potential anti–proliferative effect on different cell lines and induced apoptosis in colon cancer cell line HCT 116 through the activation of pro-apoptotic protein BAX and down regulation of BLC-2 (Giridharan et al., 2012). A novel depsipeptide was isolated from an endophytic fungus *Phomopsis glabrae,* isolated from the leaves of *Pongamia pinnata*. The depsipetide PM181110, exhibited *in vitro* anti–cancer activity against 40 human cancer cell lines with a mean IC_{50} value of 0.089 µM and *ex vivo* efficacy towards 24 human tumor

xenografts (Verekar et al., 2014). Deacetyl-mycoepoxydiene is a novel microtubule inhibitor isolated from an endophytic *Phomosis* spp. (Zhu et al., 2015).

Endophytic fungi are also a prolific source of bioactive compounds, which inhibit specific enzymes commonly referred to as enzyme inhibitors. As several diseases are associated with abnormal enzyme activities, endophytic fungal metabolites are bound to yield valuable pharmaceutical compounds. Khafrefungin has been isolated from an unidentified sterile fungus isolated from a Costa Rican plant sample and was found to be a potent inhibitor of sphingolipid synthesis in fungal systems but does not affect the human sphingolipid biosynthesis. It has been found that Khafrefungin under *in vitro* condition inhibits inositol phosphoceramide synthase of *Candida albicans* with an IC_{50} of 600 µM (Mandala et al., 1997). Human cytomegalovirus (hCMV) is an opportunistic pathogen which causes disease in congenitally infected immune deficient infants and adults. One of the potent drug targets for this virus is hCMV protease as it is involved in the assembly of mature protein. Cytonic acids A and B have been identified as novel tridepsides isolated from the endophytic fungus *Cytonaema* sp. which inhibits hCMV protease (Guo et al., 2000a). Chloroform: methanol (1:1) fraction of *Fusarium* sp. IFB-121 which exists as an endophyte in healthy bark of *Quercus variabilis* yielded two xanthine oxidase inhibitory cerebrosides. The IC_{50} of Fusaruside was 43.8 µM, while that of other cerebroside being 55.5 µM (Shu et al., 2004). Extracts of culture filtrate of endophytic fungi have also been screened for inhibitors of acetylcholinesterase enzyme under *in vitro* conditions for treating the cognitive and functional symptoms of Alzheimer's disease (Rodrigues et al., 2005).

Trypanosoma and *Leishmania* are parasitic protozoans which are responsible for several diseases in tropical regions of the world. The enzyme trypanothione reductase (TR) protects *Trypanosoma* and *Leishmania* from oxidative stress and thus, is considered as a drug validated

target. *Alternaria* spp. (UFMGCB55) existing as an endophyte in *Trixis vauthieri* (*Asteraceae*), produces Altenusin, a biphenyl derivative which inhibits TR, thereby opening perspectives for designing more effective derivatives which could serve as drug leads for new chemotherapeutic agents to treat Trypanosomiasis and Leishmaniasis (Cota et al., 2008). The enzyme α-glucosidase plays an important role in the absorption of carbohydrates by hydrolyzing the glycosidic linkages in large carbohydrate molecules to yield glucose and related monosaccharides. It is considered as a valid drug target in management of type 2 diabetes. An endophytic fungus belonging to *Chaetomiaceae* family MEXU 27095, isolated from the Mexican medicinal plant *Hintonia latiflora* (*Rubiaceae*), produces three tridepsides identified as Thielavins A, J and K which have been found to inhibit *Saccharomyces cerevisiae* α–glucosidase in a concentration dependent manner with IC_{50} values of 23.8 μM, 15.8 μM and 22.1 μM respectively (Chavez et al., 2013). More recently, HDAC and protein kinase inhibitors have also been isolated from the endophytic fungus *Epicoccum nigrum* (Amrani et al., 2014).

3.11 Identification of fungi producing enzyme inhibitors

Fungi are the second largest group of microorganisms (1.5 million) of which only 5% have been described so far (Hawksworth, 1991; Perez-sierra and Henricot, 2002; Hibbett and Taylor, 2013). Traditionally, fungal taxonomy was solely based on identification of morphological features but since last two decades, morphological techniques have been substantiated by modern molecular phylogenetic methods which are more specific, reliable, sensitive and universally applicable (Sette et al., 2006; Morakotkarn et al., 2007). Internal transcribed spacer (ITS) region is the most frequently used locus for the molecular identification of fungi because they evolve very rapidly and exhibit greater variation between closely related species at genetic level (Guo et al., 2000b; Baayen et al., 2002; Lacap et al., 2003; Jeewon et al., 2004). The identification of various fungal groups

solely on the basis of ITS region sequence analysis is also not reliable due to its limitation in differentiating intra-species genetic variation. Moreover, various other coding gene regions are being employed for phylogenetic identification due to their highly conserved nature and low copy number characteristics. Molecular taxonomic researchers are currently utilizing multilocus genealogical concordance phylogenetic species recognition (GCPSR) method for the precise identification of fungi at species level. The GCPSR focuses on species identification through multigene genealogies and reciprocal monophyly to identify fungal species (Taylor et al., 2000). The identification of fungi producing enzyme inhibitors have been carried out using molecular as well as by morphological methods. Endophytic *Alternaria* species producing Altenusin, an inhibitor of trypanothione reductase of *Trypanosoma cruzi* was identified by amplifying the fungal DNA with ITS domains of rRNA gene using primers ITS1 and ITS4 (Cota et al., 2008). MEXU 27905 isolated from the leaves of *Hintonia latiflora* which produced α- glucosidase inhibitors Thielavins A, J and K were identified by ITS sequence to be a member of Chaetomiaceae family (Chavez et al., 2013). *Epicoccum nigrum* producing protein kinase and HDAC inhibitors were identified by carrying out the phylogenetic analysis of the amplified ITS region (Amrani et al., 2014). *Lasiodiplodia pseudotheobromae*, a xanthine oxidase inhibitor producing endophytic fungi which was identified using both morphological and ITS based molecular tool (Kapoor and Saxena, 2014).

3.12 Inhibition kinetics of enzyme inhibitors

As inhibitor slows down the enzyme reaction, it definitely has an effect on its kinetics. By studying the kinetics of enzyme reaction in presence and absence of an inhibitor, the mechanism by which the inhibitor impedes the action of the enzyme can be established. The inhibition of enzymes has been broadly classified into reversible and non–reversible inhibition. Further the reversible inhibition can be classified as competitive, non–competitive and un–competitive inhibition.

Mevinolin and ML-236B have been found to behave competitively with respect to HMG–CoA as evident from the dixon plots (Alberts et al., 1980). CPP-225,917 and CPP- 263,114 were mixed non–competitive type inhibitors of squalene synthase inhibitors as evident through Lineweaver Burk (LB) plot analysis (Dabrah et al., 1997). FYGL was found to be a competitive inhibitor of protein tyrosine phosphatase 1B activity has been considered to be a promising therapy approach to treat diabetes (Teng et al., 2011). Kinetic analysis has revealed that thielavins A, J and K were non–competitive inhibitors with K_i values of 27.8, 66.2 and 55.4 µM, respectively (Chavez et al., 2013). Kojic acid isolated from *A. niger* has been found to be a competitive inhibitor with a K_i of 85 µM (Vasantha et al., 2014). LB plot analysis of lactone of stachybotrydial isolated from *Stachybotrys* species FN298 exhibited a mixed type of inhibition of *Staphylococcus* dihydrofolate reductase with a K_i value of 7.4 µM (Kwon et al., 2014).

3.13 Summary

Literature suggests that obesity is a burgeoning problem worldwide and there is a great need for safe and cheap anti–obesity drugs. The present armamentarium of anti–obesity drugs are no doubt effective, but are bound with some unacceptable side effects leading to serious problems like heart attack and other life threatening diseases. PL is always the safest target for anti-obesity drugs. After 13 years of success of a PL inhibitor Orlistat, FDA has approved another anti-obesity drug Qsymia with a different mode of action but the salts contained in Qsymia have been prescribed by the doctors for weight loss years ago. Despite, good performance of Qsymia in clinical trials, it is not a step forward for treating obesity and hence it shows slow pace of researchers for anti–obesity drugs. A new pancreatic and gastric lipase inhibitor Cetilistat by Alizyme therapeutics is under phase III clinical trials and is found to have lesser side effects than Orlistat but is purely a synthetic drug. PL inhibitors from a prokaryotic source are combined with various side effects. Many plants have been

explored for PL inhibitors but large scale production may disturb the biodiversity. Thus bio–prospecting endophytic fungus for the production of PL inhibitors is a nascent area which needs to be exploited for anti–obesity drugs.

Chapter 4

Materials & Methods

4.1 Repository and maintenance of endophytic fungal isolates

A repository of endophytes isolated from different medicinal plants from the biodiversity hotspots of India existed in the laboratory. The fungal cultures were encoded based on the plant isolated, its part and the place from where it was collected. For example #6AMLWLS (where #6 refers to segment number; AM stands for *Aegle marmelos*; L refers to leaf, WLS refers to Wayanad Wild Life Sanctuary, Kerala (Place of sample collection) (Fig. 4.1).

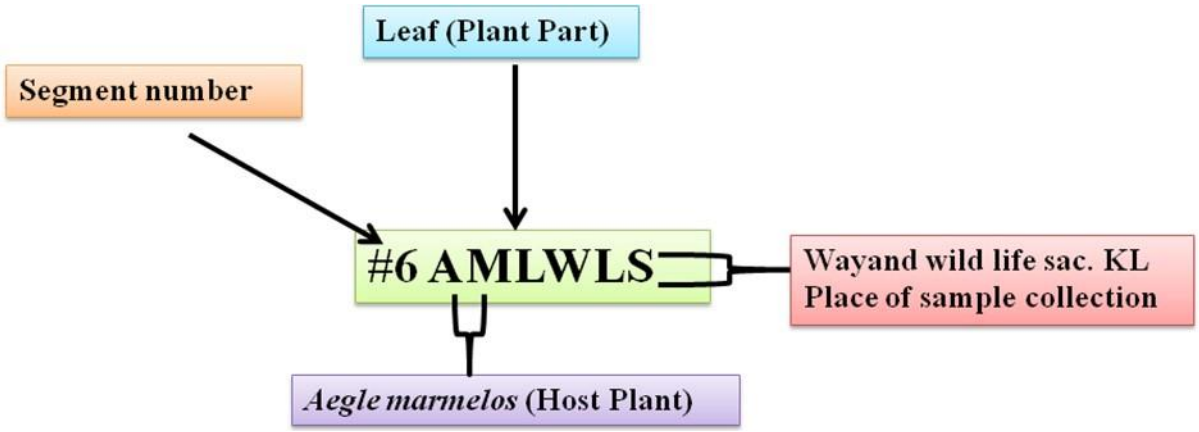


Fig. 4.1 Coding of the endophytic isolates

In the present study, 190 cultures of endophytic fungi were used which were maintained on potato dextrose agar (PDA) (Hi Media, India) slants containing 10% glycerol and stored at 4°C. The cultures were activated by transferring them to freshly prepared PDA plates and incubated at 26 ± 1°C for 6–7 days.

4.2 Production of culture filtrate

Liquid cultures of endophytic fungi were prepared by inoculating aseptically 5 mm disc of 7 day old culture in 25 ml of pre-sterilized (autoclaving at 121°C for 15 min) Richard's broth (composition: Sucrose – 50 g; Potassium Nitrate – 10 g; Potassium dihydrogen phosphate – 8 g, Magnesium Sulphate – 0.25 g; Ferric chloride – 0.002 g; Double distilled water (DDW) - 1000 ml, pH 4.5) followed by incubation at 26 ± 1°C at 120 rpm for 10 days. After the incubation period, the mycelial

mass was separated by filtration through Whatmann filter paper no.4 (Whatmann, GE health care Life Sciences, USA) followed by centrifugation at 9900 × g for 10 min at room temperature (Hitachi CF 15 RX II series, Japan). The supernatant was then passed through 0.22 µm nitrocellulose membrane (Whatmann, GE health care life sciences, USA) making it cell free. The cell-free broth(s) so obtained were stored at -20°C until further use (Raviraja et al., 2006).

4.3 Qualitative screening for porcine pancreatic lipase inhibition

To assess the inhibition of PPL two chromogenic plate assays were used containing olive oil (Figaro, Spain) as substrate and a dye. Different concentrations viz. 50 U, 100 U, 150 U, 200 U of PPL (Sigma Aldrich, USA) was used for standardization of the lipase concentration for their use in rhodamine olive oil and phenol red plate assays.

4.3.1 Rhodamine olive oil plate assay

The rhodamine olive oil plate assay was used for the primary screening of the PL inhibitory activity using cell free culture broths of endophytic fungi (Hamid et al., 2003). The rhodamine dye (SRL, India) forms a fluorescent complex with the fatty acids formed by the action of PPL on olive oil (substrate). 50 ml of pre–sterilized molten agarose (1.3%) (HiMedia, India) containing 2.5% olive oil, 0.3% rhodamine and 0.01% Tween 80 (HiMedia, India) was poured in a 120 mm sterile square petri plate (HiMedia, India) to make 4 ± 0.5 mm thick plates. The plates were allowed to solidify. After solidification wells were prepared using a sterile 5 mm cork borer. Subsequently, 35 µl of the master mix containing pre–incubated 15 µl of PPL (Stock 100 U/ml) and 20 µl of culture filtrates was dispensed into 5 mm wells and incubated at 37°C for 24 h. The control comprised of 15 µl of PPL and 20 µl of sterile Richard's broth. Appearance of orange colored halo under ultra UV rays indicated the PPL activity in the control while reduction in the diameter of halo as compared to control indicated PPL inhibition. All the tests were performed in triplicates and their mean and

standard deviation (SD) was calculated. Orlistat (Sigma Aldrich, Madison, USA) was used as a positive control for lipase inhibitory activity. The percentage inhibition was calculated with respect to control containing only lipase using formula

$$\% \textbf{ Inhibition} = \frac{(\mathbf{C}-\mathbf{T})}{\mathbf{C}} * \mathbf{100}$$

Where C = Mean reading of control (lipase+ buffer)

T = Mean reading of test (lipase+ buffer+ culture filtrate)

4.3.2 Phenol red olive oil plate assay

This assay assesses the change in color due to change in pH using a pH indicator dye phenol red. The medium is red in color due to neutral pH and it forms yellow halos (acidic) due to formation of FFA by the action of PPL on olive oil (Singh et al., 2006b). 50 ml of pre sterilized molten agar (2%) containing 2.5% olive oil, 0.01% filter sterilized phenol red (HiMedia, India) and 0.01% Tween 80 was poured in a 120 mm sterile square petri plate (HiMedia, India) to make 4 ± 0.5 mm thick plates. The pH of the media was adjusted to 7 giving it blood red color with sterile sodium hydroxide (NaOH, 2 M) (HiMedia, India). The plates were then allowed to solidify. After solidification wells were prepared by a sterile 5 mm cork borer. The pH of all the culture broths was set to neutral before performing this test using sterile NaOH (2 M)/ hydrochloric acid (HCl, 2 M). Subsequently, 35 µl of the master mix containing pre–incubated 15 µl of PPL (Stock 100 U/ml) and 20 µl of culture filtrates was dispensed into 5 mm wells and incubated at 37°C for 24 h. The control comprised of 15 µl of PPL and 20 µl of sterile Richard's broth. Appearance of yellow halo against red background indicated the PPL activity in the control while reduction the diameter of halo as compared to control indicated PPL inhibition. All the tests were performed in triplicates and their mean and SD was calculated. Orlistat was used as a positive control for lipase inhibitory activity. The percentage inhibition was calculated as described in the earlier section 4.3.1.

4.4 Standardization of assay conditions for optimal porcine pancreatic lipase activity

In order to arrive to the ideal *in vitro* assay conditions for optimal PPL activity different concentrations of substrate and enzyme were studied. Similarly the optimal pH and incubation time for maximum lipolytic activity was ascertained.

4.4.1 Effect of substrate concentration

Different concentrations of p-nitrophenyl laurate (pNPL) (Sigma Aldrich, USA) viz. 100 µM, 200 µM, 300 µM, 400 µM, 500 µM, 600 µM, 700 µM, 800 µM, 900 µM, 1000 µM of were dispensed in a 96 well microtiter plate (Tarsons, India) and subsequently 30 µl of PPL (50 U/ml) was added. Potassium phosphate buffer (pH 7.4) was added to make the final volume of 250 µl. The microtiter plate was then incubated at 37°C for 6 h and subsequently the amount of p-nitrophenol released was recorded at 410 nm using BIOTEK® Powerwave 340 microplate reader. The test was performed in triplicates and their mean ± SD values were calculated (Gogoi et al., 2008).

4.4.2 Effect of enzyme concentration

Different concentrations of PPL viz. 20 U, 40 U, 60 U, 80 U, 100 U, 120 U were used with the optimized substrate concentration to establish the optimal concentration of the enzyme in the assay. Potassium phosphate buffer (50 mM, pH 7.4) was added to make the volume to 250 µl. Thereafter the titer plate was incubated at 37°C for 6 h followed by quantification of the amount of p-nitrophenol released at 410 nm using a BIOTEK® Powerwave 340 microplate reader. The test was performed in triplicates and their mean ± SD values were calculated (Gogoi et al., 2008).

4.4.3 Optimization of the incubation time of the assay

Optimal concentrations of PPL and pNPL were incubated for different time intervals to optimize the assay time. The reaction volume was makeup to 250 µl in all wells using a potassium phosphate buffer (pH 7.4) and the microtiter plate incubated at 37°C for 6 h. The estimation of p-nitrophenol

was recorded at intervals of 1 h using BIOTEK® Powerwave 340 microplate reader at 410 nm. A curve was plotted between OD at 410 nm and time to determine the optimal time for best PPL activity. The experiment was performed in triplicates and their mean ± SD values were calculated (Brownlee et al., 2010).

4.4.4 Optimization of pH

Potassium phosphate buffer of different pH viz. 5.4, 6.2, 7.4, 8.0 and 9.6 were used to optimize the activity of lipase. The reaction mixture comprised of optimized concentration of the enzyme (PPL) and the substrate (pNPL) and the reaction volume was made up to 250 µl in all wells using potassium phosphate buffer at different pH and subsequently incubated for 3 h to record the PPL activity at 410 nm using BIOTEK® Powerwave 340 microplate reader. The experiment was performed in triplicates and their mean ± SD values were calculated. A curve was plotted between OD at 410 nm and pH to obtain the optimal enzyme activity (Brownlee et al., 2010).

4.4.5 Determination of Kinetic constants of porcine pancreatic lipase

The kinetic constants i.e K_m (Michaelis constant) and V_{max} (maxium velocity) of PPL were determined by modified protocol of Lewis and Liu (2012). The initial reaction rates with increasing concentration of pNPL from 100 µM to 2 mM and standard enzyme concentration of 20 U per reaction well were recorded. The potassium phosphate (50 mM) buffer at pH 7.4 was added to makeup the reaction volume to 250 µl. The titer plate was incubated at 37°C for 3 h and the kinetic read was recorded after every 30 min using BIOTEK® Powerwave 340 microplate reader at 410 nm. Non linear regression curve fit tool was used in Graph Pad Prism 5 to determine the K_m and V_{max} of the enzyme.

4.5 Quantitative screening of culture filtrates for porcine pancreatic lipase inhibition

The culture broths exhibiting positive results in the preliminary assays were confirmed for their PL inhibitory activity by a quantitative assay using pNPL as substrate. The PPL hydrolyses pNPL and releases p-nitrophenol which is measured at 410 nm. This assay uses a 96 well microtiter plate to assess the decrease in p-nitrophenol release by the test samples i.e culture filtrates against the control (Fig. 4.2). Stock solution of PPL (400 U/ml) was prepared by dissolving it in 50 mM potassium phosphate buffer (pH 7.4). 2 mM pNPL solution was prepared by dissolving it in 10% isopropanol (Merck Millipore, AR grade), 50 mM sodium orthophosphate buffer (pH 8.0) and 50 mM sodium deoxycholate (Sigma Aldrich, USA). 100 µl of test culture filtrates were pre–incubated with 30 µl of enzyme solution for 1 h at 37°C. The reaction is initiated by addition of 100 µl of pNPL and finally making up the volume to 250 µl by adding potassium phosphate buffer. Subsequently the microtiter plate was incubated at 37°C for 3 h. After incubation, the amount of p-nitrophenol released was recorded using BIOTEK® Powerwave 340 microplate reader at 410 nm. The reduction in the absorbance of test filtrates against control showed PL inhibition. The control comprised of 100 µl of Richards's broth, 30 µl of enzyme, 100 µl of pNPL and 20 µl of potassium phosphate buffer. A substrate blank of each test containing 100 µl of pNPL, 50 µl of potassium phosphate buffer and 100 µl of respective test filtrate was used. All tests were carried out in triplicates. The relative activity was expressed as percentage ratio of enzyme activity in the presence of inhibitor to the enzyme activity in the absence of enzyme inhibitor at the end 3 h of the enzyme reaction time (Lewis and Liu, 2010).

	1	2	3	4	5	6	7	8	9	10	11	12
A	C	C	C	C_7	C_7	C_7	C_{15}	C_{15}	C_{15}	C_{23}	C_{23}	C_{23}
B	Csb	Csb	Csb	C_8	C_8	C_8	C_{16}	C_{16}	C_{16}	C_{24}	C_{24}	C_{24}
C	C_1	C_1	C_1	C_9	C_9	C_9	C_{17}	C_{17}	C_{17}	C_{25}	C_{25}	C_{25}
D	C_2	C_2	C_2	C_{10}	C_{10}	C_{10}	C_{18}	C_{18}	C_{18}	C_{26}	C_{26}	C_{26}
E	C_3	C_3	C_3	C_{11}	C_{11}	C_{11}	C_{19}	C_{19}	C_{19}	C_{27}	C_{27}	C_{27}
F	C_4	C_4	C_4	C_{12}	C_{12}	C_{12}	C_{20}	C_{20}	C_{20}	C_{28}	C_{28}	C_{28}
G	C_5	C_5	C_5	C_{13}	C_{13}	C_{13}	C_{21}	C_{21}	C_{21}	C_{29}	C_{29}	C_{29}
H	C_6	C_6	C_6	C_{14}	C_{14}	C_{14}	C_{22}	C_{22}	C_{22}	C_o	C_o	C_o

Where C1-C14 = culture filtrate + buffer + enzyme + substrate, C (enzyme control) = buffer + enzyme + substrate, Csb (substrate control) = buffer + substrate, Co (positive control) = Orlistat + buffer + enzyme + substrate

Fig. 4.2 Template for pNPL assay

4.6 Partial purification of bioactive residue by liquid–liquid extraction

25 ml of cell free filtrates exhibiting a PL inhibitory activity of 70% and above in the quantitative screening were subjected to extraction by ethyl acetate (Merck Millipore, GR grade) in a ratio of 1:2 (filtrate: solvent) in a 250 ml glass separating funnel (Borosil, India). This process was repeated thrice and then the organic layer was pooled and dehydrated using anhydrous sodium sulphate (HiMedia, India). The remaining aqueous layer was further extracted sequentially with chloroform, petroleum ether, diethyl ether, dichloromethane and hexane (Merck Millipore, GR grade) (Fig. 4.3) following the procedure described above. The solvent fractions so obtained were evaporated using nitrogen blowout to obtain respective solvent residues at room temperature. The residues were weighed and stock solution was prepared using methanol and 5% dimethyl sulphoxide (DMSO) (Tokdar et al., 2011).

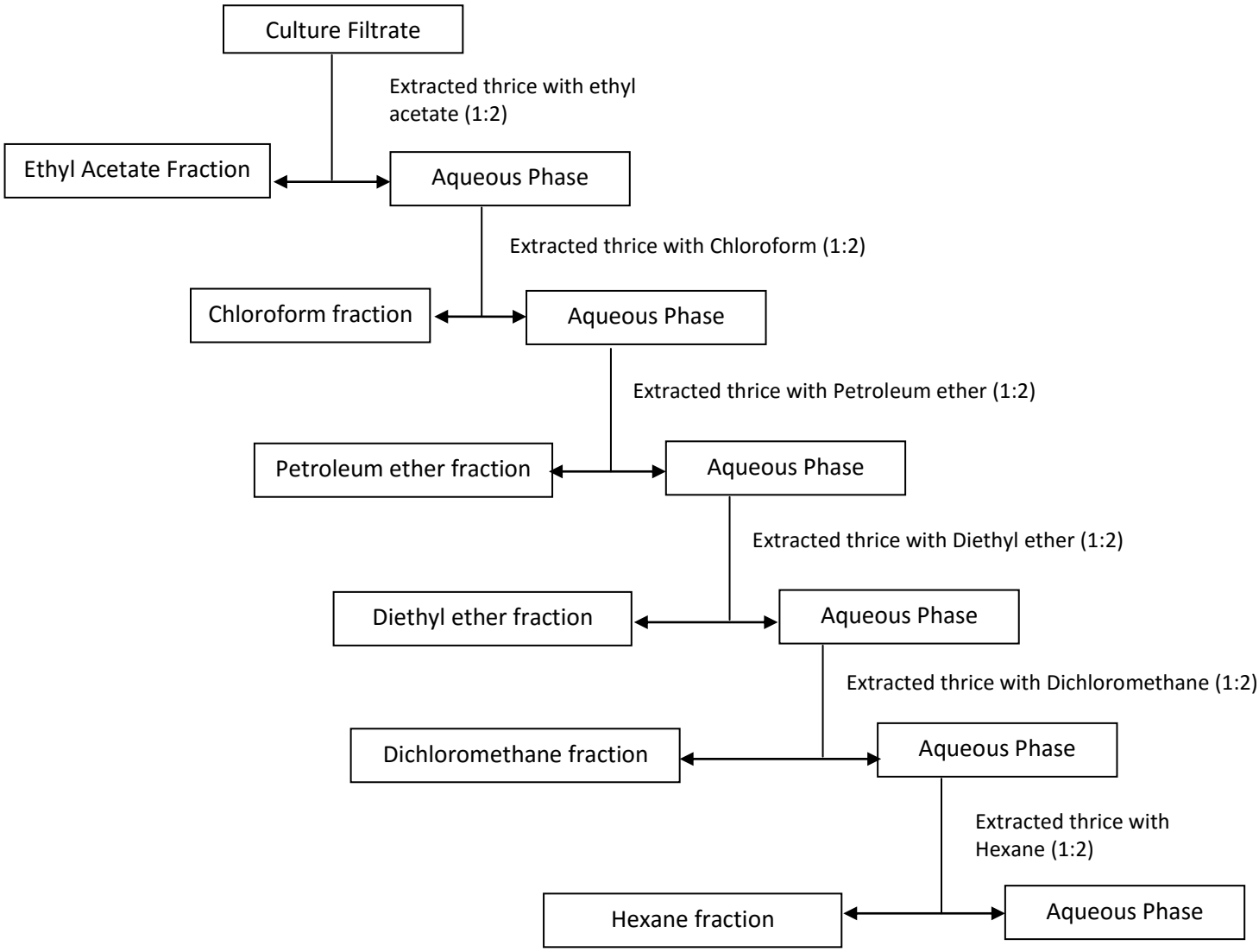


Fig. 4.3 Solvent extraction scheme of the culture filtrate exhibiting pancreatic lipase inhibition (≥70%)

4.7 Quantitative screening for inhibitory activity of partially purified bioactive residue

The ethyl acetate, chloroform, petroleum ether, diethyl ether, dichloromethane, hexane and aqueous (left after solvent extraction) residues were subsequently screened quantitatively for PL inhibition potential using a 96 well microtiter plate with pNPL as substrate. 40 µl of the each residue (stock solution: 1mg/ml) and 30 µl of PPL (400 U/ml) were pre-incubated at 37°C for 60 min. The reaction was then initiated using 100 µl of 2 mM pNPL, finally making up the volume 250 µl by adding potassium phosphate buffer (50 mM, pH 7.4). The plate was incubated at 37°C for 3 h. After

incubation, the amount of p-nitrophenol released was recorded using BIOTEK® Powerwave 340 microplate reader at 410 nm. The reduction in the absorbance of test filtrates against control showed PL inhibition. Control containing methanol and 5% DMSO were also used to see the inhibitory effect on the enzyme activity. Inhibition was expressed as a percentage relative to control. All tests were carried out in triplicates. The relative activity was expressed as percentage ratio of enzyme activity in the presence of inhibitors to the enzyme activity in the absence of enzyme inhibitors at the end of 3 h of the enzyme reaction time (Lewis and Liu, 2012).

4.8 Identification of potential endophytic fungus

Endophytic fungal isolate exhibiting the highest PL inhibitory activity against PPL was identified using classical morphotaxonomy and molecular tools.

4.8.1 Classical morphotaxonomy

The endophytic fungal isolate was grown on two media viz. PDA (HiMedia, India) and Synthetischer nahourstoffarmer agar (SNA) (HiMedia) and incubated at 26°C ± 2°C for 10-12 days with 12 h of photoperiod. The fungus was identified based upon morphological characteristics like colony size, texture and color.

4.8.1.1 Microscopic identification

The aerial mycelial mass was picked with a sharp needle and was placed over clean glass slide containing a drop of water. The mycelia mass was teased and separated into fine filaments with help of needle. A drop of lactophenol cotton blue (HiMedia, India) was added and then the cover slip was placed avoiding trapping of any air bubble. The glass slide was gently tapped twice. The slide was mounted using DPX. The slide was then observed under different magnifications (40X-1000X) of the compound microscope (Nikon, USA). The microscopic characters like hyphae, conidiophores and conidia were studied using a Nikon eclipse 50i microscope (Nikon, USA) coupled

with CCD camera and measurements carried out using NIS element software. At least 30 observations were made per structure (Booth 1971; Seifert 1996).

4.8.2 Molecular characterization

The identity of fungi was confirmed by using molecular tools like amplification of three different nuclear gene fragments by three different sets of primers and then creating a multi locus tree using Mega 5 software.

4.8.2.1 Isolation of DNA

Genomic DNA isolation was done by using the Wizard® Genomic DNA purification kit (Promega, Madison, USA) as per the manufacturer instructions. All the chemicals used were present in the kit. Firstly, 5 mg of fungal mass was crushed to fine powder using liquid nitrogen. This was followed by addition of 600 µl of cell lysis buffer. Subsequently 600 µl of nuclei lysis buffer is added and fungal mass powder is gently crushed. The mixture was then transferred to micro-centrifuge tube (Tarsons, India) and was then incubated at 65°C for 15 min in water bath with vortexing after every 5 min. After 15 min the micro-centrifuge tubes were brought to normal temperature and were subsequently centrifuged at 6300 × g for 5 min (Hitachi CF 15, RX II series, Japan). The supernatant was then transferred to fresh micro-centrifuge tubes and 3 µl of RNase (10 mg/ml) was added to them. These were then incubated at 37°C for 30 min. After 30 min, 600 µl of protein precipitating solution was added and was mixed gently by inverting the micro-centrifuge tubes for 5 min, subsequently centrifuging them at 14,200 × g for 3 min. The supernatant so obtained was transferred to fresh micro-centrifuge tubes containing 600 µl of isopropanol (HiMedia, molecular biology grade). Initially, the threads of DNA were observed in the tubes which were again centrifuged at 12,000 × g for 3 min for precipitation of the DNA. The DNA pellet was washed with 70% ethanol (Merck, AR grade) and was again centrifuged at 12,000 × g for 1 min. The pellets were

air dried and were dissolved in 50 µl of DNA rehydration buffer which were then kept at 4°C overnight. Gel electrophoresis (BioRad, USA) was carried out to check the quality of the DNA using 0.8% agarose (HiMedia, India) and 1X TAE buffer. The purity of DNA was checked using Thermo Scientific™ NanoDrop 2000 (Thermo Fisher Scientific, USA) at 260 nm and 280 nm. The quantity of DNA was estimated by Thermo Scientific™ NanoDrop 2000 at 280 nm.

4.8.2.2 Polymerase Chain Reaction

Three nuclear gene fragments viz. ITS, translation elongation factor (EF–1α), RNA polymerase β subunit II (RPB2) were selected for the present multi locus sequence typing (MLST) identification approach. The respective primer sequence of each loci is listed in Table 4.1.

Table 4.1 Primer sequences used for the amplification of genomic DNA

Primer name	Sequence (5′-3′)	Author
ITS1	TCCGTAGGTGAACCTCGG	White et al., 1990
ITS4	TCCTCCGCTTATTGATATGC	White et al., 1990
EF1T	ATGGGTAAGGAGGACAAGAC	O'Donnell *et al.,* 1998
EF2T	GGAAGTACCAGTGATCATGTT	O'Donnell *et al.,* 1998
RPB2-5f	GAYGAYMGWGATCAYTTYGG	Liu et al., 1999
RPB2-7cr	CCCATRGCTTGYTTRCCCAT	Liu et al., 1999

The polymerase chain reaction (PCR) reaction mixture composition for amplification of each loci was identical. Amplification was performed in 25 µl reaction mixture volume comprising of 1 µl of extracted genomic DNA, 0.8 µM of each primer pair (Xcleris labs Pvt. Ltd, Gujarat, India), 2.5 mM of dNTP (Genei, Bangaluru, India), 1.5 mM $MgCl_2$ (Genei, Bangaluru, India), 1.5 U of *Taq* DNA polymerase (Genei, Bangaluru, India). The thermal cycling parameters for ITS and RPB2 amplification was initial denaturation at 96°C for 5 min followed by 39 cycles of 95°C for 45 s, 60°C for 45 s, 72°C for 45 s followed by final extension at 72°C for 5 min. Whereas for EF–1α

amplification, the cycling parameters were: initial denaturation at 96°C for 5 min followed by 35 cycles of 95°C for 45 s, 56°C for 1 min, 72°C for 1.10 min followed by final extension at 72°C for 7 min. The amplified products of ITS–rDNA, EF–1α and RPB2 were sequenced at Xcleris Lab, Ahmedabad, Gujrat, India. The final consensus sequence of each locus was submitted in GenBank under accession numbers KC960885, KJ371936 and KM591682.

4.8.2.3 Sequence assembly and phylogenetic identification

The final sequence of each locus was subjected to basic local alignment search tool (BLAST) similarity search. MLST was employed for speciation of selected isolate. ITS, EF–1α and RPB2 sequence of 5 strains of *Fusarium incarnatum*, 4 strains of *F. equiseti* and *F. solani*, 2 strains of *F. chlamydosporum* along with selected isolate were used for phylogenetic tree construction.

The combined sequences of all the selected taxa were aligned using CLUSTAL W in MEGA 5 (Tamura et al., 2011). The aligned sequences were then subjected for maximum parsimony (MP) analysis to deduce evolutionary history. The evolutionary distances were computed by employing close neighbor-interchange algorithm (Nei and Kumar, 2000) with search level 3. All positions containing gaps and missing data were eliminated. 1000 bootstrap replicates were used to infer the evolutionary relationship.

4.9 Correlation between lipase inhibitory activity and fungal biomass

To evaluate the correlation between the growth of selected endophytic fungus and lipase inhibitory activity 25 ml of pre-sterilized Richard's broth in 250 ml of Erlenmeyer flask was inoculated with 5 mm mycelial plug of #6AMLWLS. Subsequently all the 20 flasks were incubated at 26 ± 1°C at 120 rpm for 10 days. After each day, two flasks were removed from the incubator, their contents were filtered using Whatmann no. 4 filter paper. The filtrate was tested for PL inhibition using quantitative plate assay while the mycelia and the spores were collected using Whatmann no. 4

filter paper were subjected to drying at 80°C overnight in a hot air oven to get the dry weight of the biomass. Triplicate reading for each flask was taken for estimating PL inhibitory activity (Pradeep et al., 2013).

4.10: Purification of bioactive residue

4.10.1 Thin layer chromatography of bioactive residue

The bioactive fraction was separated on thin layer chromatography (TLC) plates (Alumina Sheets GF_{254}, 5cm X 20cm, Merck) using different mobile phase combinations to achieve complete separation (Table 4.2) (Svendson and Verpoorte, 1984; Fleiger, 2003; Houghton, 2006; Mroczek, 2003). The fraction was loaded 1 cm above the TLC plate lower end which was then dipped into different mobile phases to achieve separation in the development chamber. The TLC plates were developed face down to a distance of 16 cm in horizontal sealed glass chamber (JSGW, India) after conditioning for 60 min with mobile phase vapor at 26°C. After development with the mobile phase, the solvent end run was marked. The TLC plates were evaporated to dryness at 28°C. Thereafter, the TLC plates were transferred to iodine chamber containing iodine crystals (SRL, India). Iodine vapors were used as the locating reagent to check the separation of the mixture. The separated components were detected as bands/ spots and their retention factor (R_f) value was calculated.

Table 4.2 Mobile phases used in thin layer chromatographic separation of the bioactive residue

Mobile phase code	Solvents used	Ratio
S1	Ethyl acetate: Hexane	9:1
S2	Ethyl acetate: Hexane	1:1
S3	Chloroform: Methanol	9:1
S4	Chloroform: Methanol	1:1
S5	Chloroform: Methanol: Water	1:1: drop
S6	Chloroform: Methanol: Ammonia	1:1: few drops
S7	Benzene: Methanol	1:1
S8	Petroleum ether: Methanol	1:1
S9	Petroleum Ether: Ethyl acetate	1:2
S10	Chloroform: Methanol	3.5:6.5
S11	Dichloromethane: Acetone	3:1

4.10.2 Purification of bioactive residue by column chromatography

Based on the best resolution obtained in TLC, column chromatography was carried out to fractionate the bioactive fraction. Briefly, silica gel (60-120 mesh, Merck) was dried in an oven for one hour at 100°C and subsequently 30 g of silica gel was wet packed with chloroform on to a glass column (30 x 3 cm, Borosil, India) fitted with a G0 filter. 540 mg of the bioactive residue was loaded on to the column. The elution was carried out using a gradient of $CHCl_3$- CH_30H (100:0 → 0:100). The fractions so obtained were analyzed by TLC using optimized solvent system, their R_f reconfirmed and tested for inhibition of PPL using quantitative enzyme assay as described in section 4.10 and 4.7 respectively (Chavez et al., 2015).

4.11 Phytochemical testing of crude residue and pure compound

Phytochemical analysis involves the assessment for the presence of broad chemical classes of compounds like saponins, glycosides, phenolics, alkaloids and tannins. Different phytochemical tests were done to establish the qualitative chemical profile of the crude and purified fraction.

4.11.1 Test for alkaloids

Two methods were used to confirm the presence of alkaloids in crude residue as well as pure compound obtained after column chromatography. In the first method few drops of Marquis reagent (100 ml of concentrated (95–98%) sulfuric acid to 5 ml of 40% formaldehyde) were added to 100 µl of the crude residue and purified compound (1 mg/ml in ddW). Turbidity or red precipitation indicated the presence of alkaloids (Harborne, 1973).

Similarly, 100 µl of crude residue and purified compound were mixed with few drops of Dragendorff's reagent (Bismuth sub-nitrate-1.7 g, glacial acetic acid-20 ml, water-80 ml and 50% solution of Potassium iodide in water-100 ml). Formation of turbidity or red precipitation indicated the presence of alkaloids (Oloyede, 2005).

4.11.2 Test for anthraquinones

To test the presence of anthraquinones in crude residue and purified compound, 500 µl of Benzene was added to 100 µl of each test sample. Subsequently 300 µl of 10% ammonia solution was added followed by vigorous shaking. The occurrence of a violet color in lower ammonical phase indicates the presence of free hydroxyl anthraquinones (Trease and Evans, 1996).

4.11.3 Test for tannins

Briefly few drops of ferric chloride reagent were added to 100 µl of crude residue and purified compound. The presence of a blue black precipitate indicates the presence of tannins (Trease and Evans, 1996).

4.11.4 Test for saponins

The ability of saponins to produce froth in aqueous solution was used as evidence to the presence of saponins in the extract. 100 µl of crude residue and purified compound were mixed with 200 µl of distilled water in a micro-centrifuge tube and subjected to vigorous vortexing. The appearance of froth upon slight warming of the vortexed mixture indicated the presence of saponins (Oloyede, 2005).

4.11.5 Test for flavonoids

The presence of flavonoids was tested by mixing 100 µl of crude residue and purified compound with 500 µl of water and ethanol mixture (1:1) and centrifuged. The solution was further mixed with 500 µl of concentrated HCl and 100 mg of zinc turnings. The presence of flavonoids was indicated by the appearance of pink/ magenta color within two minutes which could be extracted with butanol (Aynehchi et al., 1981).

4.11.6 Test for glycosides and glycolipids

To test the presence of glycosides and glycolipids, diphenylamine reagent was used. Diphenylamine reagent was prepared by adding 10 ml of 10% diphenylamine in ethanol, 100 ml HCl and 80 ml glacial acetic acid. This reagent was added to 100 µl of crude residue and purified compound, followed by heating for 30-40 min at 110°C. Appearance of blue spots/coloration indicates the presence of glycosides/ glycolipids (Narasimhan et al., 1982).

4.11.7 Test for carbohydrates

To test the presence of carbohydrates, 100 µl of the crude residue and purified compound were mixed with few drops of Molisch reagent (α-napthol in ethanol). After mixing, few drops of concentrated sulfuric acid were slowly added along the walls, without mixing, to form a layer. Appearance of a purple ring at the interface between the acid and test layers indicates presence of carbohydrates (Sawhney et al., 2011).

4.11.8 Test for amino acids

To confirm the presence of amino acids, ninhydrin solution was separately added to 100 µl of crude residue and purified compound in micro–centrifuge and then kept in boiling water bath for 5 min. Appearance of blue purple coloration confirms the presence of amino acids (Sawhney et al., 2011).

4.11.9 Test for fats

Briefly, 100 µl of crude residue and purified compound were separately mixed with sudan dye IV. Formation of red coloration confirms the presence of fats (Zhu et al., 2015a).

4.11.10 Test for terpenoids and steroids

1 mg of crude compound and purified compound was suspended in 1ml of chloroform in micro-centrifuge tube, placed in an ice bath for 10 minutes. Subsequently 1ml of acetic acid was added followed by a few drops of concentrated sulphuric acid along the walls. Appearance of pink or

pinkish brown ring/ colour indicates the presence of terpenoids and the appearance of bluish green or a rapid change from pink to blue color indicates the presence of steroids (Kantamreddi et al., 2010).

4.12 Structure elucidation of pure compound

4.12.1 Liquid chromatography mass spectrometry

Purified compound (band 1, R_f = 0.74) at a concentration of 1 mg/ml in water (Merck, HPLC grade) was analyzed in liquid chromatography mass spectrometry (LC–MS) system (Waters, Micromass Q–TOF micro using Waters Alliance 2795 separation module). Briefly 20 µl of band 1 was injected in LC–MS system using a Waters Symmetry–C18 chromatographic column (250 mm x 4.6 mm, 5 µm). The mobile phase contained a mixture of water (phase A): acetonitrile (phase B) 60:40. Equilibration of the column with solvent A for 10 min was performed.

The peaks obtained were analyzed using Time of Flight–Mass spectrometry (TOF–MS) (Waters 2795, Micromass Q–TOF micro) with electro spray ionization in positive mode (ES+). The ion optics used was capillary 3Kv conc 30 volt and 10 volt. The source block temperature was 120°C and desolvation temperature 200°C. The electrospray probe flow was adjusted to 400 µl/min and continuous mass spectra was recorded over a range M/Z 0 to 1000 with scan time of 1 sec and inter scan delay 0.1 s (Molina et al., 2015).

4.12.2 Fourier transform infrared spectroscopy

2 mg of compound (Band 1, R_f =0.74) was loaded onto the cell and a drop of water was added. IR spectrum was obtained on Bruker Vertex 80 Fourier transform infrared (FTIR) System using $CaCl_2$ cell with single point detector with range 7500-450 cm^{-1} using rapid scans of 65 spectra/s at 16 cm^{-1}. The spectral resolution was 0.2 cm^{-1} containing temperature control stage. For each spectrum, 32 scans were used (Houchuli et al., 1987).

4.12.3 Nuclear magnetic resonance spectroscopy

10 mg of the compound dissolved in 0.5 ml of deuterated DMSO/D_2O (Sigma Aldrich) was used for recording the nuclear magnetic resonance (NMR) spectra. NMR spectra were recorded at 400 MHz on a Bruker Avance II-400 NMR spectrometer (400.131 MHz proton and 100.525 MHz carbon frequencies) at 18°C. Proton and carbon 90° pulse widths were 10.9 sec and 8.37 sec µs respectively (Ado et al., 2013).

4.12.4 Melting point

2 mg of the column purified compound was packed in a capillary tube sealed at one end and placed in an oil bath heater with a thermometer. The temperature was read manually on the thermometer (Lapidot et al., 1967).

4.13 Kinetic studies on inhibition of porcine pancreatic lipase by pure compound

To study the K_m, V_{max} and K_i (inhibition constant) and the nature of inhibition LB plot and Dixon plot were used. Different concentrations (20, 50, 70, 120 µg/ml) of the inhibitor were incubated with 30 µl of PPL (400 U/ml) for 1 h at 37°C. The reaction was then started by adding different concentration of substrate (144, 176, 200, 240, 272, 320, 344, 400 µM) (pNPL). The microtiter plate was incubated at 37°C for 3 hours. The amount of p-nitrophenol released was recorded at 410 nm by BIOTEK® Powerwave 340 microplate reader. All the tests were performed in triplicates each having a substrate control. The values of K_m and V_{max} were calculated using a software Graph pad prism 5. LB plot was used to verify the type of inhibition and Dixon plot was used to calculate the K_i value of the pure compound (Mukherjee and Sengupta, 2013)

4.14. Anti adipogenesis and lipolytic effect of bioactive compound on 3T3 cell line cultures

4.14.1 3T3–L adipocytes cell culture

3T3–L cell line was obtained from American Type Culture Collection (ATCC) (Rockville, MD). 3T3–L is a continuous substrain of 3T3 (Swiss albino) developed thorough clonal isolation. 3T3-L pre-adipocyte differentiation was performed as per ATCC protocol. 3T3–L cell line was used as model to study the inhibitory effect of test compound against pre–adipocyte differentiation to adipocyte cells. Also the lipolytic activity of the compound was evaluated at 12 h and 24 h. Cells were seeded in a T–flask (HiMedia, India) and maintained with pre-adipocyte expansion medium (PEM) containing Dulbecco's Modified Eagle Medium (DMEM) (HiMedia, India) supplemented with 10% fetal bovine serum (FBS) (HiMedia, India) and 1% penicillin-streptomycin (pen-strep) (10,000 U pencillin–10 mg streptomycin) (HiMedia, India) for 48 h by incubating at 37°C and 5% CO_2 in a carbon dioxide incubator (WT-Binder) (Zebisch et al., 2012).

Subsequently, the similar volume of medium was replaced with differentiation medium (DM) (Day 0) containing DMEM supplemented with 10% FBS, 1% pen-strep, 0.5 mM 3-isobutyl-1-methylxanthine (prepared in 100% DMSO) (Sigma Aldrich, USA), 1 µM dexamethasone (prepared in 100% ethanol) (Sigma Aldrich, USA) and 1 µg/ml insulin (Sigma Aldrich, USA) and incubated for 48 h at 37°C in humidified atmosphere containing 5% CO_2 for 2 days (upto day 2).

After 2 days, DM was replaced with adipocyte maintenance medium (AMM) containing DMEM supplemented with 10% FBS, 1% pen-strep and 1 µg/ml insulin for 6 days (upto Day 8). AMM was changed after every 72 h. Finally, the cells are fully differentiated at day 8 after induction.

4.14.2 Inhibition of adipogenesis

To examine the effect on differentiation of adipocytes different concentrations (25, 50, 100, 200 µg/ml) of the test compound was added in the all three DM up to day 7. Esculetin at 100 µg/ml was kept as standard. Control was cells without test compound.

At the end of day 7, medium was removed and cells were washed with 50 mM phosphate buffer saline (PBS) solution. Quantification of triglycerides was done by Adipo Red™ reagent (Lonza, USA) as per manufacturer's instruction using Eclipse Cary fluorescence spectrophotometer (Varian, Australia), excitation at 485 nm and emission at 572 nm. The test was performed in 96 well black microtiter plate (HiMedia, India) (Karmase et al., 2013).

For qualitative observation, cells at end of 7^{th} day were washed with PBS and fixed with formalin (3% PBS) for 30 min, followed by staining with oil Red O for 30 min. Then the staining agent was removed and washed with PBS. The cells were visualized under Nikon Eclipse TS100 inverted microscope with Nikon Digital sight DS-U3 camera.

4.14.3 Estimation of lipolysis by glycerol release method

After incubation of cells in AMM for 8 days (upto day 10), adipocytes were washed with PBS. Cells were shifted to incubation solution (6.7 ml of bovine serum albumin (BSA) (30% in PBS) in 100 ml of Hank's balance salt solution) and different concentration of the test compound (50 and 100 µM) was added. Forskolin at 50 µM was kept as standard. After 12 and 24 h, 10 µl of supernatant was removed from the 96 well microtiter plate and glycerol reagent (Zen bio) was added. Absorbance was taken at 540 nm using Labsystem multiwell plate reader (USA). A standard curve containing different concentrations of glycerol (0, 100, 200, 500, 1000 µM) was prepared and quantified at 540 nm. The equation of the standard curve was used to calculate amount of glycerol released in control and test (Karmase et al., 2013).

Chapter 5

Results

5.1 Culture repository and maintenance of cultures

A total of 190 endophytic fungal isolates were procured from repository existing with Dr. Sanjai Saxena, Department of Biotechnology, Thapar University, Patiala, Punjab and were screened for their PL inhibitory activity. The fungal isolates, details of host plant with place of collection and identification used in the study are listed in Table 5.1 (appendix). The isolates exhibiting PL inhibitory activity are shown in Fig. 5.1. All fungal isolates were assigned their respective codes. The endophytic fungi showed different morphological and microscopic characteristics owing to their different class and species.

Fig. 5.1 Representative endophytic fungal isolates from various medicinal plants showing pancreatic lipase inhibitory activity (A,C,E,F,M,N,P- *Fusarium* spp.; D,L- *Lasiodiplodia* spp.; G-H- *Alternaria* spp.; J- *Penicillium* sp.; K- *Bionectria* sp.; O- *Pestalotiopsis* sp.; B,I- Unidentified).

Endophytic fungi used in the present study were mainly isolated from bark (38%) and stem (26%) of different medicinal plants (Fig. 5.2). Maximum isolates were from *Aegle marmelos* (33%) followed by *Taxus baccata* (24%) and *Cinnamomum malabaricum* (15%) (Fig. 5.3). Of the total identified isolates, 44.21% were hypomycetes, 17.89% were coelomycetes, 14.73% were ascomycetes and 1.57% were basidiomycetes, whereas remaining 21.57% were not identified (Fig. 5.4). The endophytic assemblage comprised of a number of cosmopolitan species such as *Fusarium*, *Alternaria*, *Pestalotiopsis*, *Aspergillus* and *Penicillium* that have been isolated from both temperate and tropical regions (Fig. 5.5). Amongst the uncommon endophytes were *Arthinium*, *Lasiodiplodia*, *Phaeoacremonium*, *Bionectria*, *Ascobolus* and *Schizophyllum* species. *Aegle marmelos* predominantly harboured *Fusarium, Botryosphaeria* and *Pestalotiopsis* as endophytic colonizer, whereas cinnamon plant was colonised by *Bionectria, Alternaria, Arthinium, Pestalotiopsis* species. Certain sterile volatile producing *Muscodor* species were obtained from cinnamon and bael plant. *Taxus baccata* exhibited similar endophytic colonization however *Pestalotiopsis* species was dominant followed by *Phomopsis* and *Penicillium*.

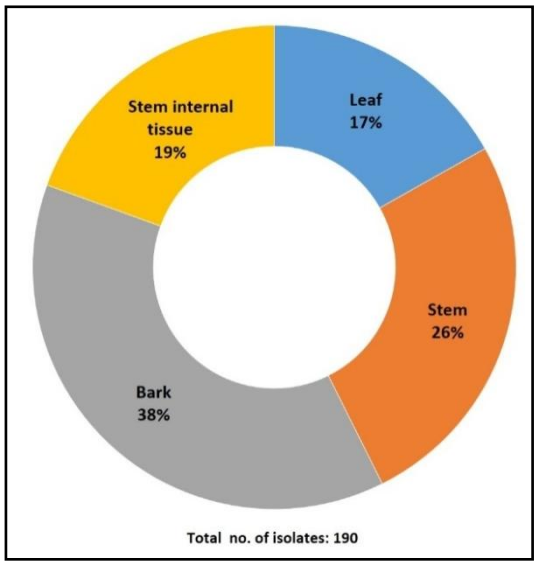


Fig. 5.2 Distribution of endophytic isolates (%) from different parts of medicinal plants

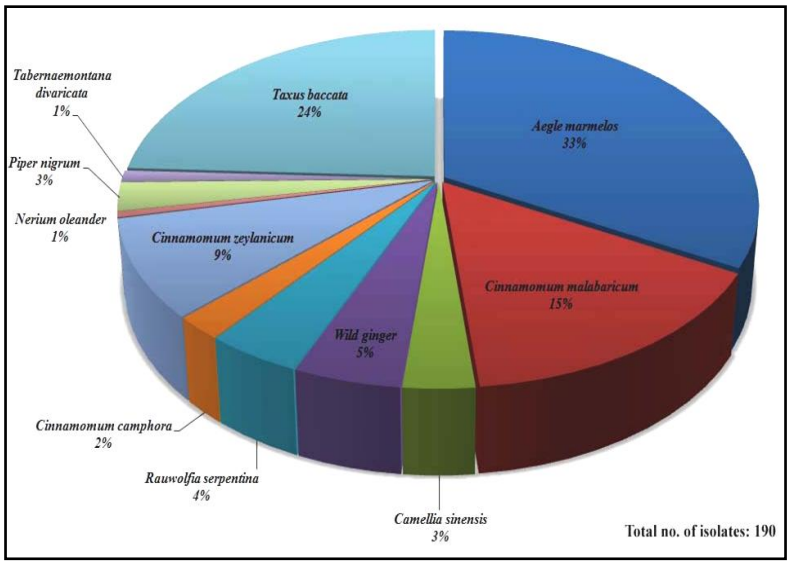


Fig. 5.3 Distribution of endophytic fungi (%) in different medicinal plants

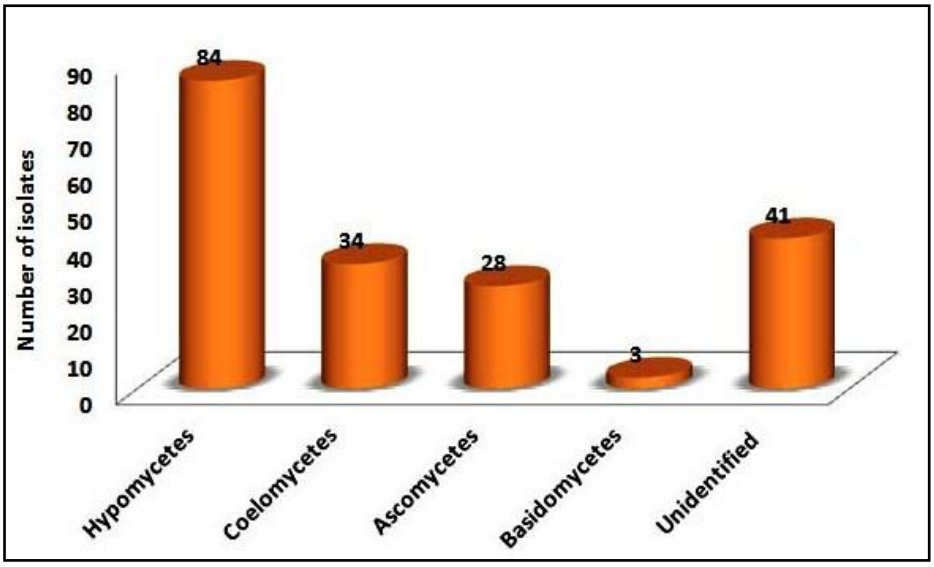


Fig. 5.4 Distribution of endophytic fungal isolates (%) in different classes of fungi

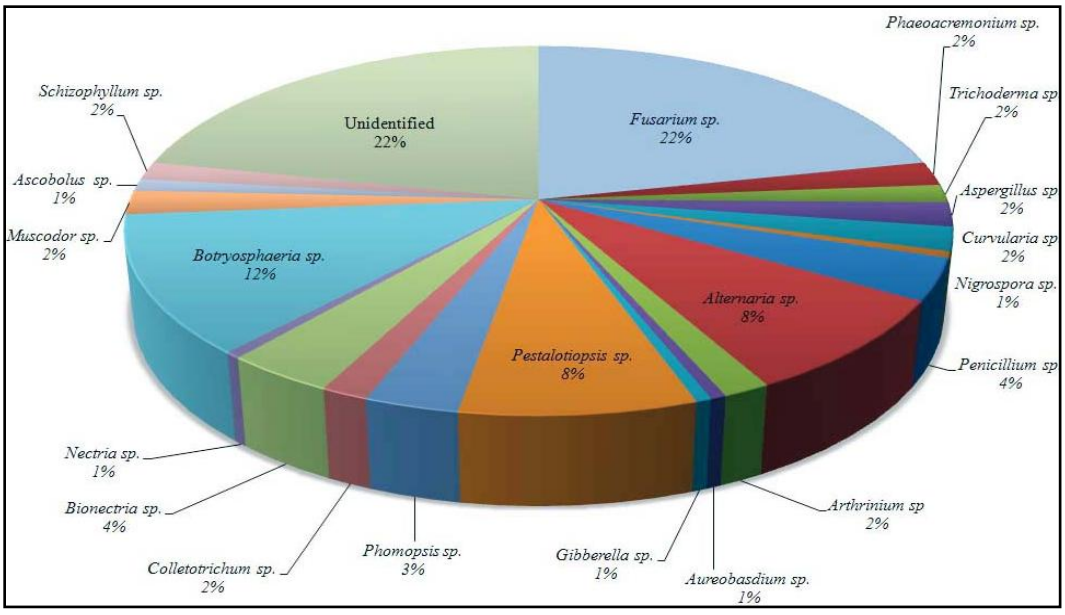


Fig. 5.5 Different genus of endophytic fungi (%)

The fungal isolates were preserved for long term storage on PDA slants, in grain and spawn cultures (Fig. 5.6). Subculturing after every 3 months was done in case of PDA slants, whereas grain and spawn cultures were stored upto 8 months.

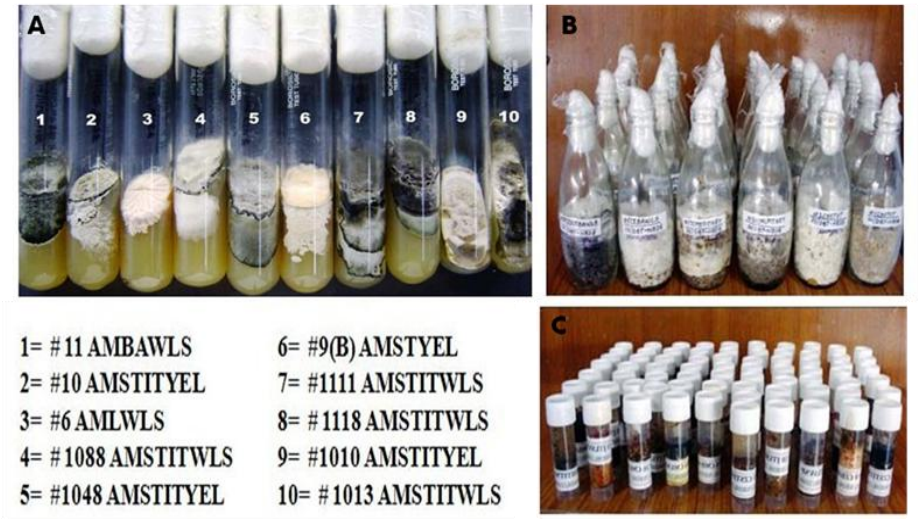


Fig. 5.6 Endophytic isolates preserved for long term storage by different methods
A- PDA slants, B- Grain stock cultures, C- Spawn stock cultures

5.2 Production of culture filtrate

Endophytic fungi were grown in Richard's broth and incubated at 28 ± 2 °C for 10 days at 120 rpm. Thereafter, pH, volume of the cell free culture filtrate and biomass produced by each fungus was determined (Table 5.2, Appendix, Fig. 5.7) for qualitative testing for PL inhibitory activity of crude culture filtrates and for studying the correlation between biomass and bioactivity.

Fig. 5.7 Production of culture filtrates by various fungal cultures

5.3 Qualitative screening for porcine pancreatic lipase inhibitory activity

The culture broth of all endophytic isolates was tested for their PPL inhibitory activity using the two chromogenic assays. The isolates exhibiting PPL inhibition above 50% are listed in Table 5.3. Out of 190 tested isolates, 35 isolates exhibited 50% inhibition of PPL where as 10 isolates showed 70% inhibition of PPL activity. *Fusarium incarnatum* (#6AMLWLS), an isolate from *Aegle marmelos* showed maximum PL inhibitory activity (96.52%) followed by *Penicillium* sp. (#57TBBALM, 94.72%) from *Taxus baccata*. Fungal isolates from *A. marmelos* showed good PL inhibitory activity such as *Botryosphaeria stevensii* (#59AMSTWLS, 86.42%), *Lasiodiploidia theobromae* (#1079AMSTWLS, 85.25%), *Fusarium* sp. (#1058AMSTITYEL, 83.79%, #5AMSTYEL, 83.71%). #33TBBALM inhibited PPL by 81.87% followed by *Sphaeropsis sapinea* (#8AMSTYEL, 72.30%, #1013AMSTITYEL, 70.06%) and *Fusarium* (#1CMSTITBRT, 69.80%).

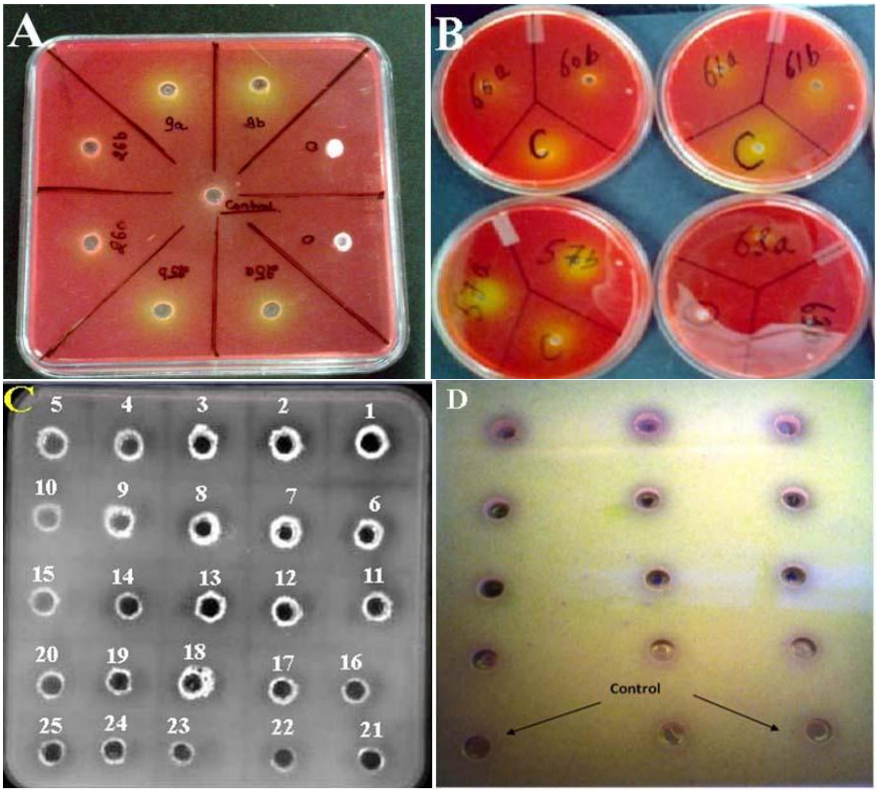


Fig. 5.8 Phenol red assay (A–B) and rhodamine olive oil plate assay (C–D) for qualitative determination of pancreatic lipase inhibitory activity

Table 5.3 Fungal isolates showing *in vitro* pancreatic lipase inhibition (≥ 50%) in rhodamine and phenol red plate assays

S.No.	Culture Code	Rhodamine plate assay		Phenol red plate assay	
		Zone Diameter Mean ± SD (mm)	% Inhibition	Zone Diameter Mean ±SD (mm)	% Inhibition
1.	**#1079AMSTWLS**	**1.05±0.07**	**81.32**	**2.63±0.02**	**85.25**
2.	**#5AMSTYEL**	**1.04±0.04**	**81.50**	**2.9±0.01**	**83.71**
3.	#6AMSTYEL	2.28±0.01	59.63	7.92±0.06	55.51
4.	**#8AMSTYEL**	**1.36±0.04**	**75.89**	**4.93±0.01**	**72.30**
5.	#9AMSTYEL	1.96±0.04	65.25	6.06±0.05	65.98
6.	**#1013AMSTITYEL**	**1.79±0.03**	**68.20**	**5.33±0.01**	**70.06**
7.	**#1058AMSTITYEL**	**1.11±0.02**	**80.38**	**2.89±0.01**	**83.79**
8.	#1070AMSTITYEL	2.25±0.04	60.11	6.56±0.06	63.15
9.	#1005AMLBRT	2.05±0.01	64.67	6.51±0.03	64.12
10.	**#59AMSTWLS**	**0.83±0.05**	**85.34**	**2.42±0.03**	**86.42**
11.	#27AMSTWLS	1.89±0.02	66.55	6.05±0.01	66.01
12.	#25AMSTWLS	2.49±0.08	55.91	8.77±0.01	50.76
13.	#37(b)AMSTWLS	2.15±0.03	61.82	6.90±0.01	61.24
14.	#1104AMSTITWLS	2.07±0.05	63.36	6.15±0.01	65.48
15.	#42AMSTITWLS	2.17±0.02	61.58	6.81±0.02	61.77
16.	**#6AMLWLS**	**0.19±0.00**	**96.57**	**0.62±0.01**	**96.52**
17.	#2004AMBARS	2.31±0.03	57.20	6.97±0.01	55.13
18.	#2102AMBARS	2.21±0.03	60.76	5.87±0.00	67.02
19.	#18CMBANEY	2.19±0.12	61.51	6.87±0.04	61.29
20.	#4CMBABRT	2.15±0.03	65.94	6.96±0.01	58.90
21.	#2CMLNEY	2.41±0.09	57.21	8.03±0.02	54.92
22.	#44CMSTNEY	2.21±0.03	60.76	5.87±0.00	67.02
23.	#79CMSTITNEY	1.94±0.05	65.60	6.00±0.01	66.29
24.	**#1CMSTITBRT**	**2.10±0.08**	**62.77**	**5.38±0.02**	**69.80**
25.	#1(b)WGSTNEY	2.07±0.04	63.36	5.95±0.06	66.57
26.	# 1CSSTOT	2.64±0.16	53.13	9.34±0.44	47.55
27.	#31TBBALM	2.85±0.01	51.80	8.19±0.06	54.01
28.	**#33TBBALM**	**0.86±0.00**	**84.75**	**3.23±0.07**	**81.87**
29.	#35TBBALM	2.48±0.06	56.09	8.18±0.12	54.03
30.	#50TBBALM	2.06±0.04	63.48	6.40±0.09	64.03
31.	#54TBBALM	2.18±0.01	61.35	6.96±0.06	60.92
32.	**#57TBBALM**	**0.39±0.05**	**93.09**	**0.94±0.15**	**94.72**
33.	#89TBBALM	1.98±0.01	64.83	6.19±0.10	65.22
34.	#91TBBALM	2.01±0.01	64.42	6.02±0.04	66.16
35.	#93TBBALM	2.22±0.02	60.64	6.25±0.39	64.89
	Orlistat	0.21±0.01	96.34	0.64±0.03	96.40
	Control (lipase+buffer)	5.64±0.01		17.8±0.01	

*Bias- 0.32 (acceptable 0 to 0.5), SD of bias- 3.66, 95% limits of agreement from -6.86 to 7.5 (Bland Altman analysis). Hence both the assays have insignificant difference.

Phenol red plate assay clearly depicted a yellow halo zone around the well (Fig. 5.8 (A) - wells 25 a,b, 9 a,b) indicating PPL activity, whereas no yellow zone indicated inhibition of the PPL

activity (Fig. 5.8 (A) – well 26 a,b having extract of #6AMLWLS). Similarly, in Fig. 5.8 (B) the well–marked with 'C' represents the activity of standard PPL, whereas the wells 57 a,b exhibit a bigger yellow halo as compared to the control well suggesting the extract of #2104CZSTITG have a higher lipase activity than the control thereby indicating that the endophytic fungus to be a lipase producer. Orlistat was taken as a positive control marked as "O" showing complete inhibition of PL in the qualitative plate assay.

In rhodamine olive oil plate assay (Fig. 5.8 C-D) the wells 1,2 and 3 exhibit the halo formed by standard PPL (Fig. 5.8C) while the wells 7, 8 and 9 exhibit the halos formed by the lipase producing *Alternaria* sp. (#2104CZSTITG). Very small halos were observed in well no. 21, 22 and 23 indicating the inhibition of PPL by the extract of #6AMLWLS. Fig. 5.8 (D) depicts the halos formed by standard PPL. Bland Altman analysis was used to find if there is any significant difference between the inhibition percentage of two plate assays. Insignificant bias of 0.32 was found between the two plate assays thus suggesting that the results obtained in both the tests have significant similarity and can be used interchangeably.

5.4 Standardization of assay conditions for optimal porcine pancreatic lipase activity

Assay conditions including optimal substrate and enzyme concentration, incubation time, pH and kinetic constants were standardized for obtaining maximum PPL activity in quantitative estimation.

5.4.1 Effect of substrate concentration

Firstly, the substrate concentration is optimized with a constant enzyme concentration. It is clearly evident from Fig. 5.9 that as the concentration of substrate increases from 100 μM to 800 μM, there is exponential increase in PL activity. The enzyme activity was maximum at 800 μM of the pNPL concentration after which the activity becomes constant even if the substrate concentration is further increased.

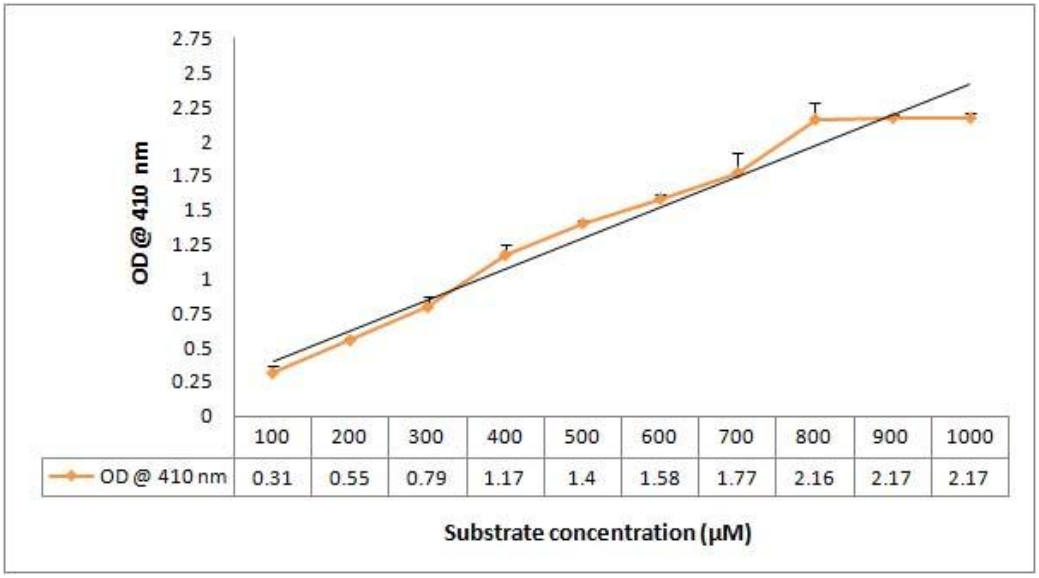


Fig. 5.9 Effect of substrate concentration

5.4.2 Effect of enzyme concentration

The amount of enzyme needed to disintegrate standard substrate concentration fully into the products was optimized. Different enzyme concentrations were tested against optimized substrate concentration (800 µM of pNPL). Fig. 5.10 clearly shows that enzyme activity greatly increases with increase in concentration of enzyme till 12U/ 250 µl, after which it becomes almost constant.

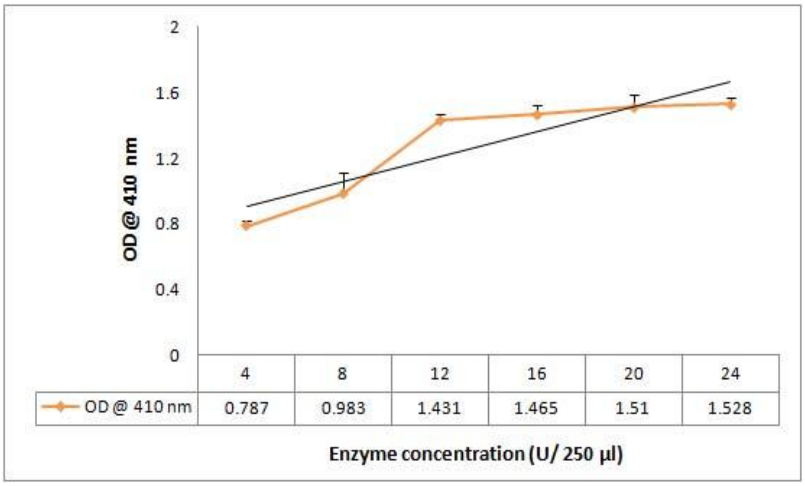


Fig. 5.10 Effect of enzyme concentration

Hence, 12 U/ 250 µl of enzyme completely utilizes the optimized substrate concentration (800 µM) to produce p–nitrophenol which was quantified spectrophotometrically.

5.4.3 Optimization of the incubation time of the assay

The incubation period for the maximum enzyme activity was optimized at optimum concentration of enzyme and substrate. As shown in Fig. 5.11, there was an insignificant change in OD between 3 to 6 h and very minor change from 6 to 23 h indicating that the optimum activity of enzyme was achieved in 3 h and this incubation time is sufficient for conversion of substrate into products.

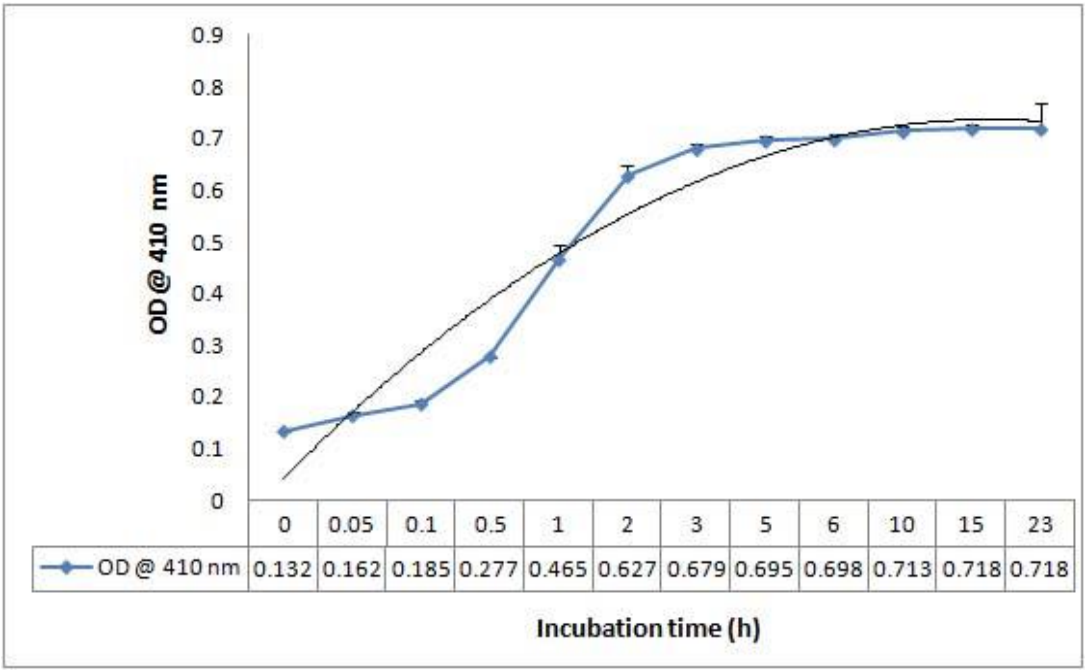


Fig. 5.11 Optimization of incubation time for enzyme assay

5.4.4 Optimization of pH

As PL enzyme activity was taken in consideration, the pH range from slightly acidic (5.4) to basic (9.6) was evaluated to assess the optimum PPL activity using optimized enzyme concentration, substrate and incubation time. Fig. 5.12 exhibited that the PPL worked best in the pH range of 7.4-8.0.

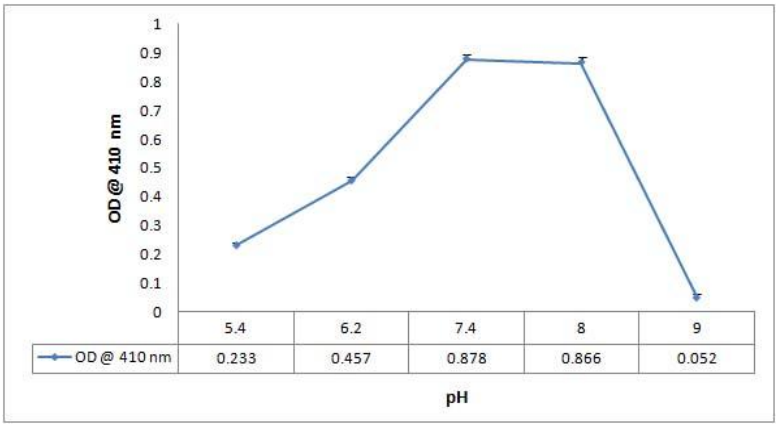


Fig. 5.12 Optimization of pH for optimal activity of enzyme

The enzyme activity was found very low at pH 5.4 which considerably increased till pH 7.4. It remained constant till pH 8.0 and decreased considerably at pH 9.0. The maximum PL activity was found at the pH of intestines (7.4-8.0). Hence assay conditions for maximum PPL activity were optimized at 800 µM substrate, 12 U/ 250 µl of PPL in buffer of pH 7.4 with incubation time of 3 h.

5.4.5 Determination of the kinetic constants of porcine pancreatic lipase

Different concentrations of substrate were plotted against the reciprocal of substrate and velocity for the linear regression of the Michealis Menten equation. The points where the linear line cut through the X-axis and Y-axis were $1/V_{max}$ and $-1/K_m$. Therefore using LB plot the K_m and V_{max} of PPL was found to be 846 µM and 0.2267 µM/min under the above assay conditions (Fig. 5.13).

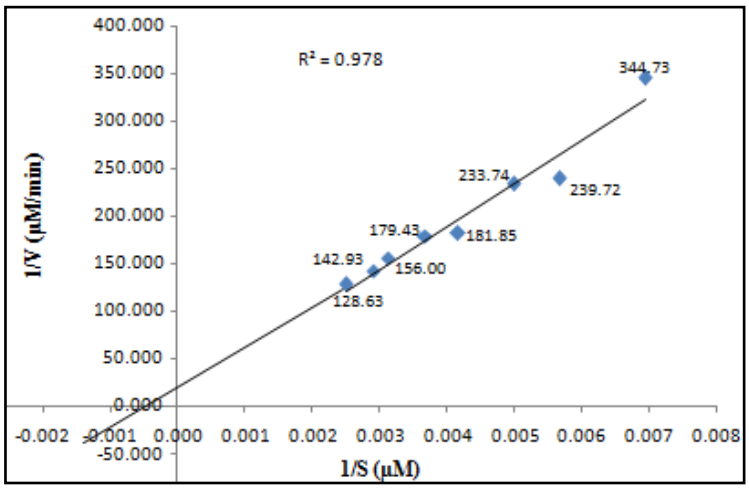


Fig. 5.13 Lineweaver Burk plot representing K_m and V_{max} (reciprocal of substrate at X-axis and reciprocal of velocity at Y-axis).

5.5 Quantitative screening of culture filtrates for porcine pancreatic lipase inhibition

The cultures showing PPL inhibitory activity greater than 70% in qualitative screening were selected for quantitative screening. The percentage inhibition (I%) of PL was calculated using formula:

$$\text{Inhibitory acitivity (I\%)} = 100 - \frac{(\text{B} - \text{b})}{(\text{A} - \text{a})} * 100$$

Where, A is the activity without inhibitor; 'a' is the negative control without inhibitor and enzyme; B is the activity with inhibitor; and b is the negative control with inhibitor and enzyme.

Table 5.4 Percentage inhibitory activity (I%) in ten selected fungal culture broths

Culture Code	B (Mean ± SD)	B (negative control)	B-b	% Inhibition
#1013AMSTITYEL	1.362 ± 0.00	0.766	0.596	69.97
#1CMSTITBRT	1.359 ± 0.00	0.766	0.593	70.14
#8AMSTYEL	1.338 ± 0.00	0.782	0.556	72.01
#5AMSTYEL	1.292 ± 0.00	0.540	0.51	79.36
#33TBBALM	0.951 ± 0.04	0.621	0.33	83.38
#1058AMSTYEL	1.234 ± 0.00	0.915	0.319	83.94
#1079AMSTWLS	0.973 ± 0.01	0.694	0.279	85.95
#59AMSTWLS	1.021 ± 0.00	0.787	0.234	88.19
#57TBBALM	1.041 ± 0.00	0.904	0.137	93.07
#6AMLWLS	0.855 ± 0.00	0.841	0.014	99.29

The culture filtrate of *Fusarium incarnatum* (#6AMLWLS) exhibited maximum inhibition of PPL followed by *Pencillium* sp. (#57TBBALM), *Botryosphaeria stevensii* (#59AMSTWLS), *Lasiodiplodia theobromae* (#1079AMSTWLS) and *Fusarium semitectum* (#1058AMSTYEL). Corresponding results were obtained in the qualitative plate assay (Table 5.4). #33TBBALM showed greater inhibition potential than *Fusarium moniliforme* (#5AMSTYEL) and *Sphaeropsis sapinea* (#8AMSTYEL). Dunnett's analysis showed significant difference between the control (containing lipase and

substrate) and inhibition percentage of selected endophytic fungal isolates thereby confirming the selection of above isolates for partial purification. The level of significance was 95% (Table 5.5).

Table 5.5 ANOVA and Dunnett's multiple comparison tests to assess the inhibition of ten selected isolates

One way ANOVA (level of significance)	Dunnett's Multiple Comparison Test					Inference
	Treatments	Mean Difference	q value	p value	95% CI of difference	
p<0.0001	Control vs #57TBBALM	1.810	90.20	p<0.05	1.745 to 1.875	Significant
p<0.0001	Control vs #59AMSTWLS	1.713	85.36	p<0.05	1.648 to 1.778	Significant
p<0.0001	Control vs #33TBBALM	1.618	80.60	p<0.05	1.553 to 1.682	Significant
p<0.0001	Control vs #5AMSTYEL	1.196	59.58	p<0.05	1.131 to 1.260	Significant
p<0.0001	Control vs #1058AMSTYEL	1.629	81.15	p<0.05	1.564 to 1.693	Significant
p<0.0001	Control vs #1079AMSTWLS	1.669	83.15	p<0.05	1.604 to 1.733	Significant
p<0.0001	Control vs #8AMSTYEL	1.392	69.34	p<0.05	1.327 to 1.456	Significant
p<0.0001	Control vs #1013AMSTITYEL	1.351	67.32	p<0.05	1.286 to 1.416	Significant
p<0.0001	Control vs #1CMSTITBRT	1.355	67.50	p<0.05	1.290 to 1.419	Significant
p<0.0001	Control vs #6AMLWLS	1.934	96.35	p<0.05	1.869 to 1.998	Significant

5.6 Partial purification of bioactive residue by liquid-liquid extraction

The highest yield was found in the remaining aqueous extract of all the culture filtrates as compared to their organic residue. Maximum yield was obtained in the aqueous extract of #6AMLWLS (11 mg) followed by #1CMSTITBRT (9.66 mg) and #1058 AMSTITYEL (8.13 mg). The ethyl acetate residue of #5AMSTYEL and #1079AMSTWLS yielded 7.8 mg and 6.14 mg of the residue respectively (Table 5.6).

Table 5.6 Weight of residue left after solvent extraction of selected endophytic fungi

Culture Code	Weight of the extracted residue (mg) in different solvents ***							
	EA*	DEE*	PE*	DCM*	CHL*	HEX*	CTC*	Aqueous
#57TBBALM	1.94 ± 0.00	3.32 ± 0.00	4.65 ± 0.00	3.22 ± 0.00	2.39±0.00	6.44 ± 0.00	1.09±0.00	8.13 ± 0.00
#59AMSTWLS	3.56 ± 0.00	1.44 ± 0.01	2.11 ± 0.00	1.07 ± 0.00	3.39 ± 0.00	2.64 ± 0.00	-	6.96 ± 0.00
#33TBBALM	4.87 ± 0.00	1.23 ± 0.00	3.75 ± 0.00	1.67 ± 0.00	7.72 ± 0.00	4.23 ± 0.00	-	7.65 ± 0.00
#5AMSTYEL	7.89 ± 0.00	2.43 ± 0.00	4.41 ± 0.00	2.16 ± 0.00	1.43 ± 0.00	1.14 ± 0.00	-	7.22 ± 0.00
#1058AMSTYEL	2.36 ± 0.00	1.55 ± 0.00	4.68 ± 0.00	3.39 ± 0.00	1.46 ± 0.00	2.19 ± 0.00	1.11 ± 0.00	8.13 ± 0.00
#1079AMSTWLS	6.14 ± 0.00	4.69 ± 0.00	1.71 ± 0.00	1.85 ± 0.00	6.94 ± 0.00	4.12 ± 0.00	0.59 ± 0.01	7.14 ± 0.00
#8AMSTYEL	4.80 ± 0.00	3.32 ± 0.00	2.29 ± 0.00	2.63 ± 0.00	5.21 ± 0.00	2.47 ± 0.00	1.42 ± 0.01	6.78 ± 0.00
#1013AMSTITYEL	3.59 ± 0.00	2.76 ± 0.00	3.28 ± 0.00	2.97 ± 0.00	5.98 ± 0.00	1.25 ± 0.00	0.68 ± 0.00	4.13 ± 0.00
#1CMSTITBRT	3.74 ± 0.00	1.19 ± 0.00	1.91 ± 0.00	1.79 ± 0.00	7.43 ± 0.00	2.23 ± 0.00	1.53 ± 0.01	9.66 ± 0.00
#6AMLWLS	2.90 ± 0.00	1.41 ± 0.00	3.49 ± 0.00	2.35 ± 0.00	6.45 ± 0.00	3.55 ± 0.00	0.24 ± 0.00	11.02± 0.03

*EA- Ethyl Acetate; DEE- Diethylether; PE- Petroleum ether; DCM- Dichloromethane; CHL- Chloroform; HEX- Hexane; CTC- Carbon tetrachloride

Initially the fungal broth was 25 ml; * Mean ± SD of the extracted fraction

5.7 Quantitative screening for pancreatic lipase inhibitory activity of partially purified residues

All the solvent residues were then subjected to quantitative PL inhibitory assay as mentioned in section 4.7. As evident from the Table 5.7, the highest PL inhibitory activity was present in the aqueous residue (left after solvent extraction) of #6AMLWLS, which exhibited complete inhibition of PL in quantitative assay. This was followed by ethyl acetate residue of #57TBBALM (95%), #59AMSTWLS (89%), #33TBBALM (85.6%), #1058AMSTYEL (84.6%), #8AMSTYEL (77.7%) and #1013AMSTITYEL (71.5%) and the diethyl ether residues of #5AMSTYEL (81.5%) and #1CMSTITBRT (75%).

Table 5.7 Pancreatic lipase inhibitory activity of different solvent residues of selected cultures

Culture Code	% Inhibition of PL***						
	EA*	DEE*	PE*	DCM*	CHL*	HEX*	Aqueous
#57TBBALM	**94.9** ± 0.00	15.1 ± 0.00	22.0 ± 0.00	-	-	-	8.24±0.00
#5AMSTYEL	-	**81.5** ± 0.00	7.3 ± 0.20	17.7 ± 0.09	-	-	7.34±0.09
#33TBBALM	**85.6** ± 0.00	-	1.1 ± 0.00	7.1 ± 0.00	8.4 ± 0.00	4.0 ± 0.00	-
#59AMSTWLS	**88.6** ± 0.00	12.4 ± 0.00	-	-	-	-	3.0 ±0.01
#1058AMSTYEL	**84.6** ± 0.00	29.5 ± 0.00	12.0 ± 0.02	-	0.8 ± 0.00	-	-
#1079AMSTWLS	**57.5** ± 0.05	16.3 ± 0.08	35.4 ± 0.19	15.7 ± 0.46	16.5 ± 0.05	17.2 ± 0.37	12.0±0.11
#8AMSTYEL	**77.7** ±0.00	10.6 ± 0.01	1.7 ± 0.10	-	-	6.5 ± 0.03	13.0 ±0.00
#1013AMSTITYEL	**71.5** ± 0.04	-	14.6 ± 0.02	1.7 ± 0.13	2.3 ± 0.00	1.74 ± 0.00	-
#1CMSTITBRT	20.1 ± 0.06	**75.0** ± 0.00	-	9.72 ± 0.02	-	4.40 ± 0.14	-
#6AMLWLS	-	06.3 ± 0.006	13.0 ± 0.00	27.62± 0.00	26.0 ± 0.00	-	**100**±0.001

*EA-Ethyl acetate; *DEE- Diethyl ether; *PE- Petroleum ether; DCM- Dichloromethane, *CHL- Chloroform; *HEX- Hexane;

***Mean ± SD of triplicate readings, the working concentration of the each solvent residue was 200 µg/ml

Tukey's analysis (Table 5.8) of different extracted fractions of various endophytic fungi suggested that there is a significant difference between PL inhibitory potential of #6AMLWLS and all other fungal extracts except #57TBBALM. Hence both #6AMLWLS and #57TBBALM were taken into consideration for further studies.

Table 5.8 Tukey's multiple comparison between the different solvent residues of selected cultures

One way ANOVA (level of significance)	Tukey's Multiple Comparison Test					Inference
	Treatments	Mean Difference	q value	p <0.05	95% CI of difference	
p<0.0001	#57TBBALM EA vs #5AMSTYEL DEE	-0.160	6.62	Yes	-0.2823 to -0.03771	Significant
p<0.0001	#57TBBALM EA vs #33TBBALM EA	-0.107	4.45	No	-0.2300 to 0.01462	Not Significant
p<0.0001	#57TBBALM EA vs #59AMSTWLS EA	-0.069	2.85	No	-0.1913 to 0.05329	Not Significant
p<0.0001	#57TBBALM EA vs #1058AMSTYEL EA	-0.166	6.86	Yes	-0.2883 to -0.04371	Significant
p<0.0001	#57TBBALM EA vs #1079AMSTWLS EA	-0.573	23.85	Yes	-0.6986 to -0.4540	Significant
p<0.0001	#57TBBALM EA vs #8AMSTYEL EA	-0.210	8.68	Yes	-0.3323 to -0.08771	Significant
p<0.0001	#57TBBALM EA vs #1013AMSTITYEL EA	-0.290	12.00	Yes	-0.4123 to -0.1677	Significant
p<0.0001	#57TBBALM EA vs #1CMSTITBRT DEE	-0.245	10.14	Yes	-0.3673 to -0.1227	Significant

p<0.0001	#57TBBALM EA vs #6AMLWLS AQ	0.078	3.24	No	-0.04396 to 0.2006	Not Significant
p<0.0001	#57TBBALM EA vs Control	-1.320	54.61	Yes	-1.442 to -1.197	Significant
p<0.0001	#5AMSTYEL DEE vs #33TBBALM EA	0.052	2.16	No	-0.06996 to 0.1746	Not Significant
p<0.0001	#5AMSTYEL DEE vs #59AMSTWLS EA	0.091	3.76	No	-0.03129 to 0.2133	Not Significant
p<0.0001	#5AMSTYEL DEE vs #1058AMSTYEL EA	-0.006	0.24	No	-0.1283 to 0.1163	Not Significant
p<0.0001	#5AMSTYEL DEE vs #1079AMSTWLS EA	-0.416	17.23	Yes	-0.5386 to -0.2940	Significant
p<0.0001	#5AMSTYEL DEE vs #8AMSTYEL EA	-0.050	2.06	No	-0.1723 to 0.07229	Not Significant
p<0.0001	#5AMSTYEL DEE vs #1013AMSTITYEL EA	-0.130	5.37	Yes	-0.2523 to -0.007711	Significant
p<0.0001	#5AMSTYEL DEE vs #1CMSTITBRT DEE	-0.085	3.51	No	-0.2073 to 0.03729	Not Significant
p<0.0001	#5AMSTYEL DEE vs #6AMLWLS AQ	0.238	9.86	Yes	0.1160 to 0.3606	Significant
p<0.0001	#5AMSTYEL DEE vs Control	-1.160	48.0	Yes	-1.282 to -1.037	Significant
p<0.0001	#33TBBALM EA vs #59AMSTWLS EA	0.038	1.60	No	-0.08362 to 0.1610	Not Significant
p<0.0001	#33TBBALM EA vs #1058AMSTYEL EA	-0.058	2.41	No	-0.1806 to 0.06396	Not Significant
p<0.0001	#33TBBALM EA vs #1079AMSTWLS EA	-0.468	19.40	Yes	-0.5910 to -0.3464	Significant
p<0.0001	#33TBBALM EA vs #8AMSTYEL EA	-0.102	4.23	No	-0.2246 to 0.01996	Not Significant
p<0.0001	#33TBBALM EA vs #1013AMSTITYEL EA	-0.182	7.54	Yes	-0.3046 to -0.06004	Significant
p<0.0001	#33TBBALM EA vs #1CMSTITBRT DEE	-0.137	5.68	Yes	-0.2596 to -0.01504	Significant
p<0.0001	#33TBBALM EA vs #6AMLWLS AQ	0.186	7.69	Yes	0.06371 to 0.3083	Significant
p<0.0001	#33TBBALM EA vs Control	-1.212	50.15	Yes	-1.334 to -1.090	Significant
p<0.0001	#59AMSTWLS EA vs #1058AMSTYEL EA	-0.097	4.01	No	-0.2193 to 0.02529	Not Significant
p<0.0001	#59AMSTWLS EA vs #1079AMSTWLS EA	-0.507	21.00	Yes	-0.6296 to -0.3850	Significant
p<0.0001	#59AMSTWLS EA vs #8AMSTYEL EA	-0.141	5.83	Yes	-0.2633 to -0.01871	Significant
p<0.0001	#59AMSTWLS EA vs #1013AMSTITYEL EA	-0.221	9.14	Yes	-0.3433 to -0.09871	Significant
p<0.0001	#59AMSTWLS EA vs #1CMSTITBRT DEE	-0.176	7.28	Yes	-0.2983 to -0.05371	Significant
p<0.0001	#59AMSTWLS EA vs #6AMLWLS AQ	0.147	6.09	Yes	0.02504 to 0.2696	Significant
p<0.0001	#59AMSTWLS EA vs Control	-1.251	51.75	Yes	-1.373 to -1.128	Significant
p<0.0001	#1058AMSTYEL EA vs #1079AMSTWLS EA	-0.410	16.98	Yes	-0.5326 to -0.2880	Significant
p<0.0001	#1058AMSTYEL EA vs #8AMSTYEL EA	-0.044	1.82	No	-0.1663 to 0.07829	Not Significant
p<0.0001	#1058AMSTYEL EA vs #1013AMSTITYEL EA	-0.124	5.13	Yes	-0.2463 to -0.001711	Significant
p<0.0001	#1058AMSTYEL EA vs #1CMSTITBRT DEE	-0.079	3.26	No	-0.2013 to 0.04329	Not Significant
p<0.0001	#1058AMSTYEL EA vs #6AMLWLS AQ	0.244	10.11	Yes	0.1220 to 0.3666	Significant
p<0.0001	#1058AMSTYEL EA vs Control	-1.154	47.74	Yes	-1.276 to -1.031	Significant
p<0.0001	#1079AMSTWLS EA vs #8AMSTYEL EA	0.366	15.16	Yes	0.2440 to 0.4886	Significant
p<0.0001	#1079AMSTWLS EA vs #1013AMSTITYEL EA	0.286	11.85	Yes	0.1640 to 0.4086	Significant
p<0.0001	#1079AMSTWLS EA vs #1CMSTITBRT DEE	0.331	13.71	Yes	0.2090 to 0.4536	Significant
p<0.0001	#1079AMSTWLS EA vs #6AMLWLS AQ	0.654	27.09	Yes	0.5324 to 0.7770	Significant
p<0.0001	#1079AMSTWLS EA vs Control	-0.743	30.76	Yes	-0.8657 to -0.6212	Significant
p<0.0001	#8AMSTYEL EA vs #1013AMSTITYEL EA	-0.080	3.310	No	-0.2023 to 0.04229	Not Significant
p<0.0001	#8AMSTYEL EA vs #1CMSTITBRT DEE	-0.035	1.448	No	-0.1573 to 0.08729	Not Significant

p<0.0001	#8AMSTYEL EA vs #6AMLWLS AQ	0.288	11.93	Yes	0.1660 to 0.4106	Significant
p<0.0001	#8AMSTYEL EA vs Control	-1.110	45.92	Yes	-1.232 to -0.9875	Significant
p<0.0001	#1013AMSTITYEL EA vs #1CMSTITBRT DEE	0.045	1.862	No	-0.07729 to 0.1673	Not Significant
p<0.0001	#1013AMSTITYEL EA vs #6AMLWLS AQ	0.368	15.24	Yes	0.2460 to 0.4906	Significant
p<0.0001	#1013AMSTITYEL EA vs Control	-1.030	42.61	Yes	-1.152 to -0.9075	Significant
p<0.0001	#1CMSTITBRT DEE vs #6AMLWLS AQ	0.323	13.38	Yes	0.2010 to 0.4456	Significant
p<0.0001	#1CMSTITBRT DEE vs Control	-1.075	44.47	Yes	-1.197 to -0.9525	Significant
p<0.0001	#6AMLWLS AQ vs Control	-1.398	57.85	Yes	-1.520 to -1.276	Significant

5.7.1 Calculation of IC_{50} of two potential isolates using quantitative plate assay

Different concentrations of partially purified residue of selected fungi viz. *Fusarium* sp. (#6AMLWLS) and *Penicillium* sp. (#57TBBALM) were tested using quantitative pNPL assay. The IC_{50} values of #6AMLWLS and #57TBAALM were found to be 2.12 µg/ml and 3.69 µg/ml respectively whereas the IC_{50} value of the positive control Orlistat was found to be 2.73 µg/ml. Hence it could be clearly deciphered from the results that #6AMLWLS aqueous residue was possessing better dose response concentration than Orlistat and #57TBBALM (Fig. 5.14). The IC_{50} values were calculated using Graph Pad Prism 5.

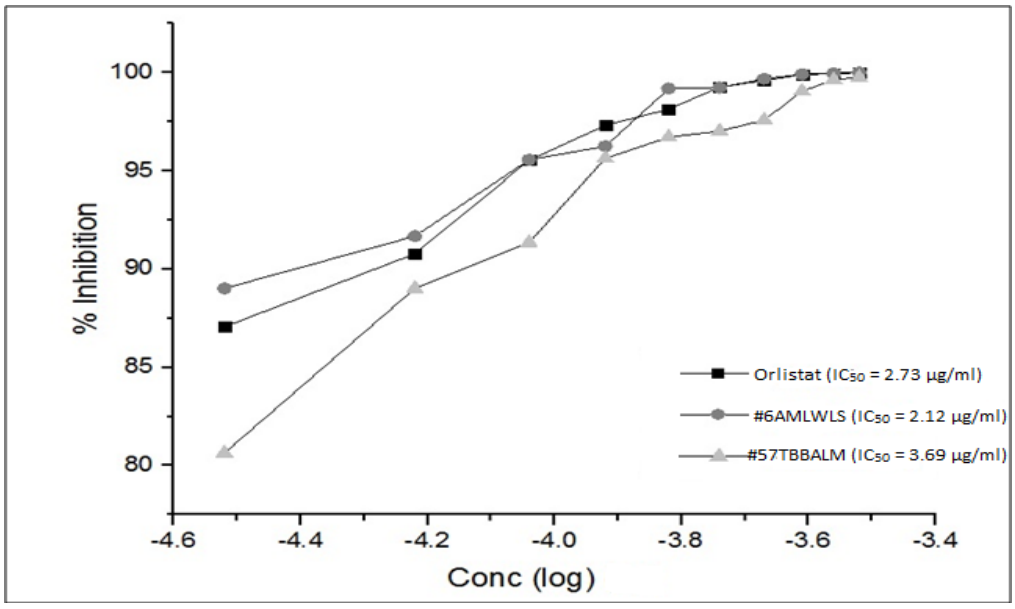


Fig. 5.14 Dose response curves of #6AMLWLS, #57TBBALM and Orlistat

5.8 Identification of potential endophytic fungus

5.8.1 Morphotaxonomy

On PDA surface, colonies grow moderately (55.67 ± 2.08), with floccose aerial mycelium, initially white in colour and later becoming light pink to avellaneous and reverse is orange to peach colored with rough margins (Fig. 5.15A,B). Hyphae (1.96(-2.57 ± 0.65-) 4.32µm) were thick, septate, long and branched. Conidiophores were present in the aerial mycelium (Fig. 5.15C). Conidia present in sporodichia straight to fusiform. Conidia developed over aerial conidiophores are generally borne singly on scattered denticle, fusiform to falcate in shape, usually 3-7 septate (15.93(-23.5 ± 3.69-) 27.67) µm × (2.63(-3.26 ± 0.3-) 3.97 µm) (Fig. 5.15D,E) and microconidia were absent. Intercalary chlamydospores were rare and present in chain (Fig. 5.15F).

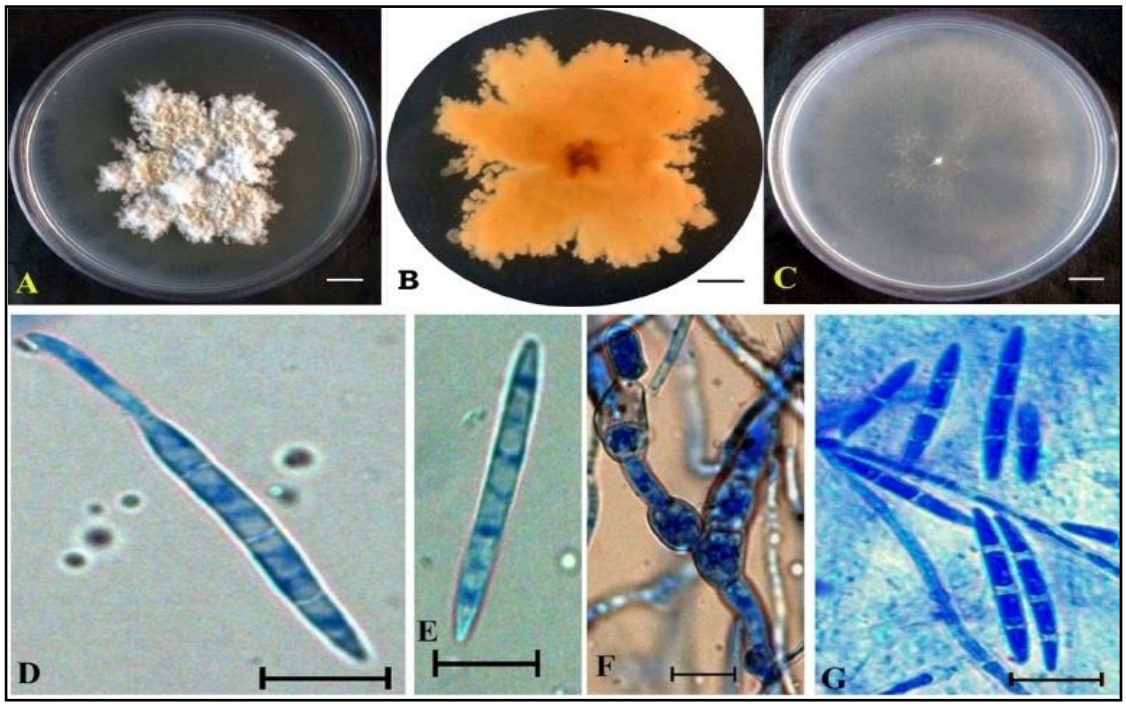


Fig. 5.15 A) Morphological and microscopic features of #6AMLWLS. A) Colony morphology on PDA, Front view, B) Colony reverse side, C) Colony over SNA, D-E) Macroconidia over PDA, F) Intercalary chlamydospores, G) Macroconidia on SNA medium

On SNA medium, colonies were moderate to rapidly growing, white in color, downy to floccose (Fig 5.15 C). Hyphae (1.69(-2.56 ± 0.48-) 3.35 µm) septate and thick. Macroconidia (22.62(-26.18±2.47-) 32.74 µm × 1.97(-2.78 ± 0.39-) 3.21 µm) fusiform to slightly curved with foot cell, mostly 3–4 septate (Fig 5.15G) and microconidia were absent. Based on these morphological characteristics the fungus was identified as *Fusarium incarnatum* (Seifert, 1996).

5.8.2 DNA isolation and molecular identification

Genomic DNA of #6AMLWLS was isolated (Fig. 5.16A) having concentration of 80 ng/µl. The purity of the DNA was checked by measuring absorbance at 260 nm and 280 nm. The ratio (A_{260}/A_{280}) was 1.76. For the identification of the potential bioactive isolate, the most widely used ITS–rDNA amplification was done and an amplicon of approximate size of 500 bp was observed (Fig. 5.16B). The sequence data exhibited homology with *Fusarium* sp. with 96% identity (Table 5.9).

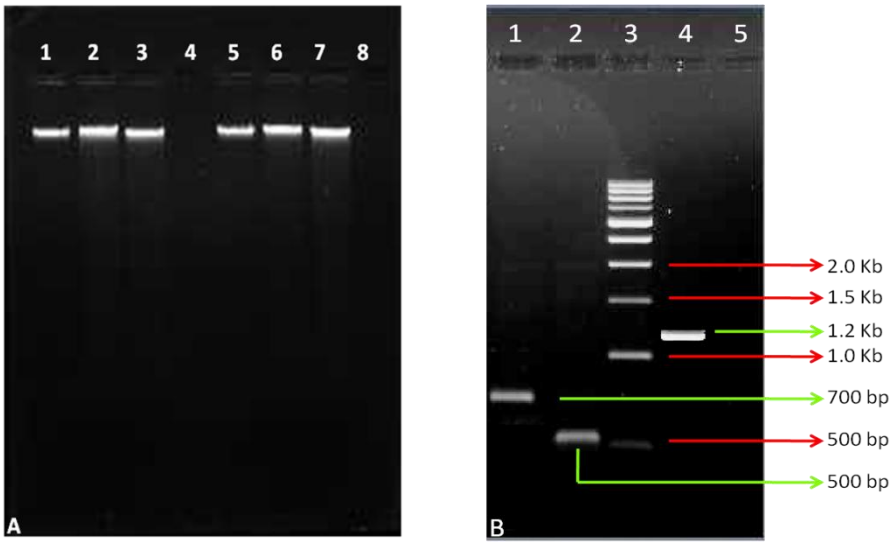


Fig. 5.16A Genomic DNA of #6AMLWLS, B PCR amplification; Lane 1: TEF region (700 bp), Lane 2: ITS1-5.8-ITS4 (500 bp), Lane 3: 500 bp ladder, Lane4: RPB region (1.2 kb) amplification

Although ITS–rDNA provide sufficient sequence data, but in case of some fungi the ITS sequence alone does not help in accurate identification of the fungus (Atkins and Clark, 2004; Rakeman et al., 2005) and therefore requires more highly conserved region sequence analysis.

Molecular phylogenetics needs much more access to highly conserved, low copy number, protein encoding nuclear genes for representation of evolutionary history scenario.

Table 5.9 Blast hits of ITS, EF 1α, RPBII gene of #6AMLWLS with nucleotide sequences in NCBI database

Accession no.	Description	% similarity	Query coverage	e-value
	#6AMLWLS ITS1-5.8-ITS4 gene			
HQ718414	*Fusarium equiseti* strain	96	100%	0.0
KF913195	*Fusarium* sp.	96	99	0.0
KJ412504	*Fusarium equiseti* isolate JG55	96	99	0.0
FJ459976	*Fusarium equiseti* isolate T34	96	99	0.0
GQ352488	*Fusarium* SP. 141GP/S	96	99	0.0
KC513507	*Fusarium equiseti* strain MTAM	96	100	0.0
KJ412509	*Fusarium equiseti* isolate JG65	96	99	0.0
AB425996	*Fusarium equiseti* genes	96	99	0.0
KJ412508	*Fusarium equiseti* isolate JG64	96	99	0.0
HQ718416	*Fusarium equiseti* strain Fe3	100	96	0.0
	#6AMLWLS TEF1α gene			
KF499580	*Fusarium incarnatum* strain CBS 791.70	94	100	0.0
JF740821	*Fusarium* sp. NRRL 52745	94	100	0.0
JF740814	*Fusarium* sp. NRRL 52737	94	100	0.0
JF740864	*Fusarium* sp. NRRL 52796	94	100	0.0
KF993975	*Fusarium incarnatum* isolate DEB29	94	97	0.0
HM852057	*Fusarium cf. incarnatum* CS-2011	94	97	0.0
JXs269001	*Fusarium incarnatum* isolate CC08-67S-4	94	97	0.0
JF740852	*Fusarium* sp. NRRL 52784	93	100	0.0
JF740780	*Fusarium* sp. NRRL 52697	93	100	0.0
JF740756	*Fusarium* sp. NRRL 25135	93	100	0.0
	#6AMLWLS RPBII gene			
KC999855	*Fusarium* sp. NK-2013	100	100	0.0
JX885465	*Fusarium incarnatum* strain R2	99	100	0.0
KC999856	*Fusarium* sp. SS-2012a	96	100	0.0
KF255548	*Fusarium incarnatum* strain CBS 133024	99	82	0.0
KF255544	*Fusarium incarnatum* strain CBS 132317	99	82	0.0
KF255542	*Fusarium incarnatum* strain CBS 132194	99	82	0.0
KF255545	*Fusarium incarnatum* strain CBS 132894	99	82	0.0
GQ915494	*Fusarium* sp. NRRL 31160	98	83	0.0
EF470121	*Fusarium* sp. NRRL 20425	99	78	0.0
EF470081	*Fusarium* sp. NRRL 43370	99	78	0.0

Gene fragment EF–1α and RPB2 were amplified using specific primer pair set and an amplicon of approximate size of 700 bp in case of EF–1α gene and 1.2 Kb in RPB2 gene was observed (Fig. 5.16B). The EF–1α region exhibited 94% identity, whereas RPB2 gene exhibited 98–99% identity with *Fusarium incarnatum.* The respective sequences have been submitted in NCBI data base with accession no. KC960885, KM591682, KJ371936 (Fig. 5.17, Appendix).

For proper speciation of the isolate, MLST study based on combined dataset of ITS, EF–1α and RPB2 were used. The MP tree was constructed based on combined dataset (Fig. 5.18). The consistency index is (0.825), the retention index is (0.917), and the composite index is 0.812 (0.757) for all sites and parsimony–informative sites. The MP tree was divided into 4 different clades of respective *Fusarium species* viz. *Fusarium incarnatum* clade, *F. equiseti, F. chlymadosporum* and *F. solani* clade. The isolate #6AMLWLS was clustered in *Fusarium incaranatum* clade thereby confirming its placement in *Fusarium incarnatum* species. *Alternaria* sp. was chosen as outgroup to root the tree.

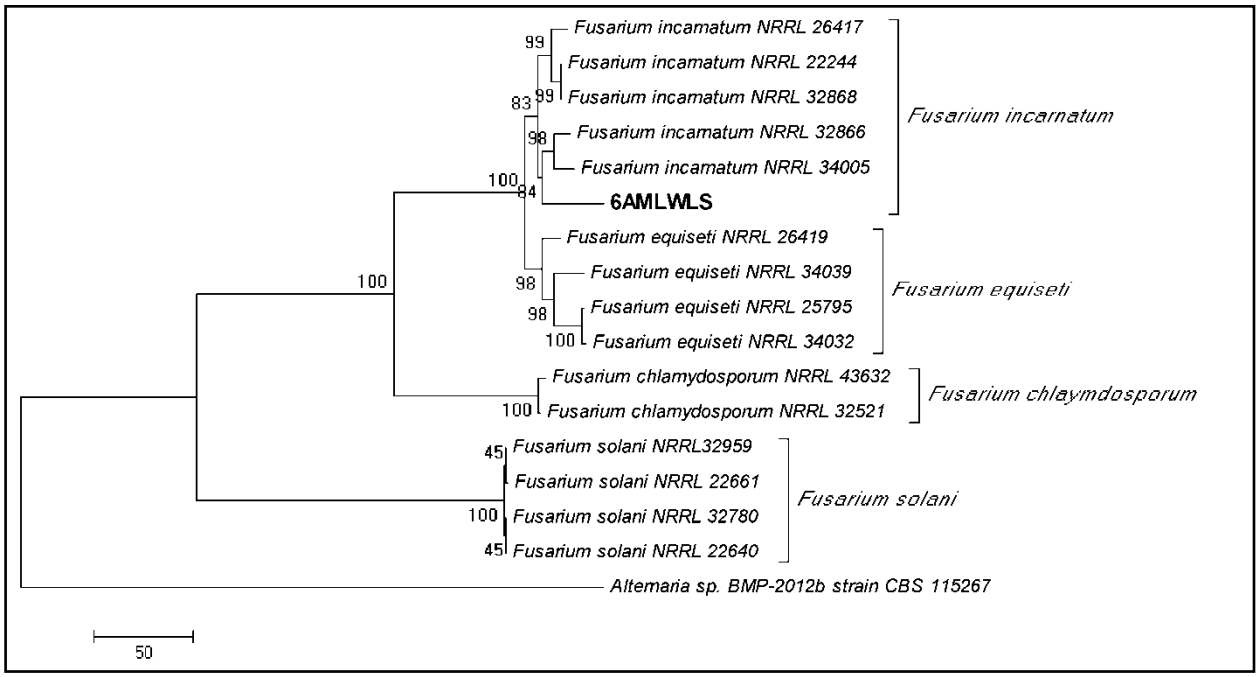


Fig. 5.18 Multilocus sequence typing tree of three loci of #6AMLWLS

5.9 Correlation between lipase inhibitory activity and fungal biomass

As evident from Fig. 5.19, the PL inhibitory activity was growth associated. It started from 2nd day and increased till 8th day after which there was no change in the biomass and PL inhibitory activity.

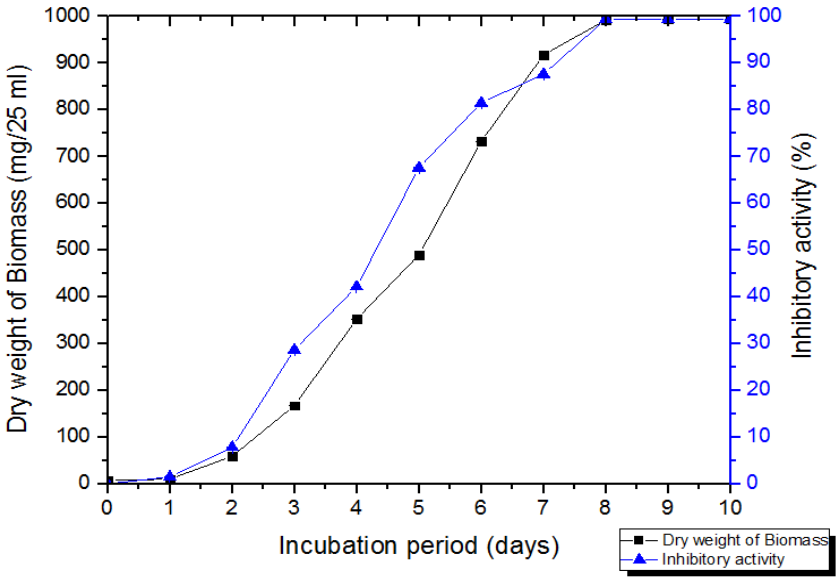


Fig. 5.19 Biomass production and percentage inhibitory activity of #6AMLWLS

5.10 Purification of bioactive residue

5.10.1 Thin layer chromatographic fractionation of bioactive residue

The bioactive aqueous residue of #6AMLWLS resolved into two bands of R_f 0.74 (band 1) and 0.86 (band 2) over silica gel TLC plate when developed using methanol: chloroform (65:35) as the mobile phase (Fig. 5.20A).

5.10.2 Purification of bioactive compound by column chromatography

Based on the resolution of the aqueous fraction into two bands as mentioned in section 5.10, silica gel column chromatographic separation (Fig. 5.20B) was carried out as per the conditions presented in section 4.10.1. In all, 105 different fractions were collected according to sample size which were then subsequently analyzed using TLC. Fractions 56–70 (15 fractions) gave a single band of R_f 0.86

on TLC plates, which were then pooled together yielding 137.62 mg of compound. Similarly, fractions 76-85 (10 fractions) also showed a single band of R_f 0.74 when analysed on TLC plates and they were also pooled to give a total yield of 60.24 mg of a separate compound. 5 fractions i.e fractions 71-75 co-eluted both the bands of R_f 0.86 and 0.74 thereby yielding a mixture of two compounds weighing 6.44 mg (Table 5.10, Appendix). These three pooled fractions were subjected to quantitative pNPL assay for estimating their PL inhibition potential (Fig. 5.20C-D).

Band 1 (R_f 0.74) showed potential PL inhibitory activity whereas band 2 (R_f 0.86) did not exhibit any activity. The mixed fraction however exhibited PL inhibition due to presence of band 1.

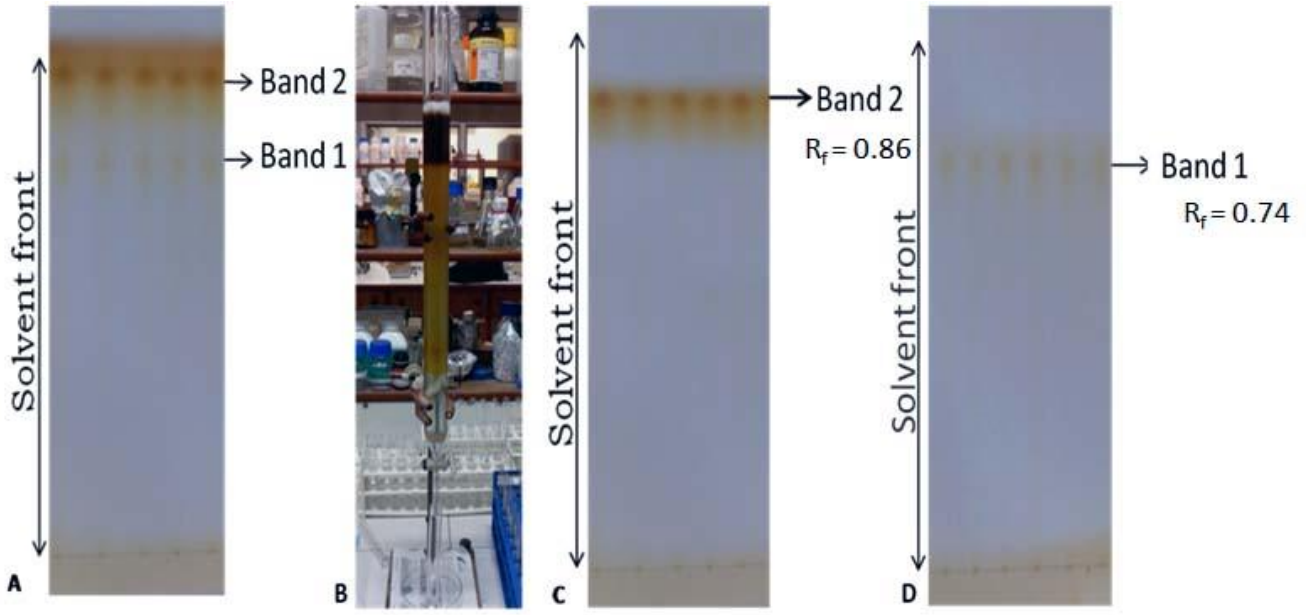


Fig. 5.20 Purification of crude residue by thin layer and column chromatography (A) Silica plate of crude residue showing two bands (B) Packed silica column loaded with crude fraction (C-D) Column fractions on silica plates

5.11 Phytochemical testing of crude residue and pure compound

The phytochemical testing of crude residue revealed the presence of amino acids/proteins, glycosides and terpenoids. The fraction formed a pinkish brown ring indicating the presence of terpenoids, whereas blue coloration suggested the presence of amino acids (ninhydrin) and

glycosides (diphenylamine reagent) (Table 5.11, Fig. 5.21). The pure bioactive compound (band 1) on the other hand, gave blue color with ninhydrin confirming the presence of amino acids.

Table 5.11 Phytochemical nature of crude residue and pure compound

S. no	Biochemical test	Crude	Pure compound (Band 1)
1	Alkaloid	-ve	-ve
2	Amino acid	+ve	+ve
3	Anthraquinone	-ve	-ve
4	Carbohydrate	-ve	-ve
5	Fats	-ve	-ve
6	Favanoid	-ve	-ve
7	Glycoside	+ve	-ve
8	Saponin	-ve	-ve
9	Steroid	-ve	-ve
10	Tannin	-ve	-ve
11	Terpenoid	+ve	-ve

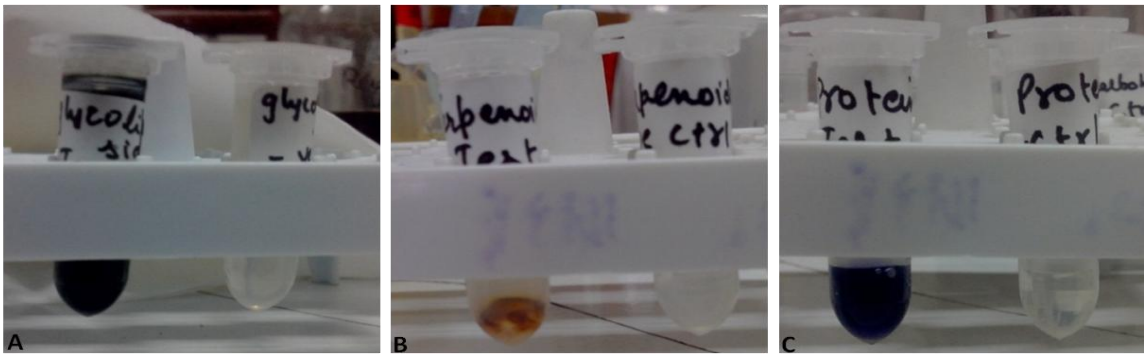


Fig. 5.21 Phytochemical test of crude and pure compound (A) glycosides (B) terpenoids (C) amino acids

5.12 Structure elucidation of bioactive compound

The structure of the bioactive compound was elucidated using various analytical techniques. The single peak of LC at 15 seconds confirmed the purity of the compound (Fig. 5.22). ESI–MS spectra of the bioactive compound gave a molecular mass of 422 (Fig. 5.23). The base ion peak represents $[M+2H]^+$ of a single amino acid which was found to be at 121 thereby suggesting the compound to be a tetrapeptide. The IR spectra of the bioactive compound confirmed the presence of different functional groups viz. C–H bend (1106.50 cm^{-1}) and C–H stretching (2922 cm^{-1}), carboxyl group (1624 cm^{-1}), aliphatic amines (1026 cm^{-1}) and N–H stretch (3691 cm^{-1}) (Fig. 5.24). NMR (^{13}C) clearly revealed the presence of a carboxyl group whereas ^{1}HNMR (Fig. 5.25) showed the presence of

methyl and O-linked ethyl group. ^{1}H NMR (dDMSO) showed signals from 1.1 to 8.2 parts per million. The triplet at 1.1 is due presence of methyl group that has been split into triplet by two protons of corresponding $–CH_2$ group. The quartet at 3.4 represents splitting of $–CH_2$ protons by three protons of methyl group. A singlet at 4.5 is represented by –OCH attached to carboxyl group at one end and NH-C=O at the other. The singlet at 8.2 represents the peak of -COOH group whereas at 2.1 represents the peak of -NH. ^{13}C NMR (D_2O) shows four signals clearly explaining the presence of four carbon atoms (Fig. 5.26). The signal at 14.3 is due to $–CH_3$ group and at 62.1 is due to $–CH_2$. Peak at 53.8 is due to –CH and at 173.3 is due to COOH/-CO of the peptide bond.

Based on above analytical data, proposed chemical name of the bioactive compound is **11-amino-2,5,8-triethoxy-4,7,10-trioxo-12-oxa-3,6,9-triazatetradecanoic acid** and the common name given to this moiety is Fustat as it is an inhibitor of PPL isolated from endophytic *Fusarium incarnatum* (Fig. 5.27). SciFinder/literature search has revealed that this compound is hitherto unreported a novel lipase inhibitor.

11-amino-2,5,8-triethoxy-4,7,10-trioxo-12-oxa-3,6,9-triazatetradecanoic acid: Physiochemical properties are listed in Table 5.12. White amorphous solid; 16.1% yield; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.21 (s, 1H, OH), 4.51 (s, 1H, CH), 3.48 (q, J= 4.0 Hz, 2H), 2.10 (s, 1H, NH), 1.12 (t, J= 8.0 Hz, 3H); ^{13}C NMR (100 MHz, D_2O) δ 14.31, 53.81, 62.16, 173.3.; Mass calculated for $C_{16}H_{30}N_4O_9$ $[M]^+$ 422.2; found 422.1.

Table 5.12 Physiochemical properties of Fustat

Characteristics	**Compound data**
Appearance	White amorphous solid
Solubility	Soluble in water, sparsely soluble in methanol and DMSO
Nature	Highly polar
TLC solvent	Methanol : Chloroform (65:35)
Melting point	Above 300°C
Biochemical characterization	Ninhydrin positive
λ max	435 nm

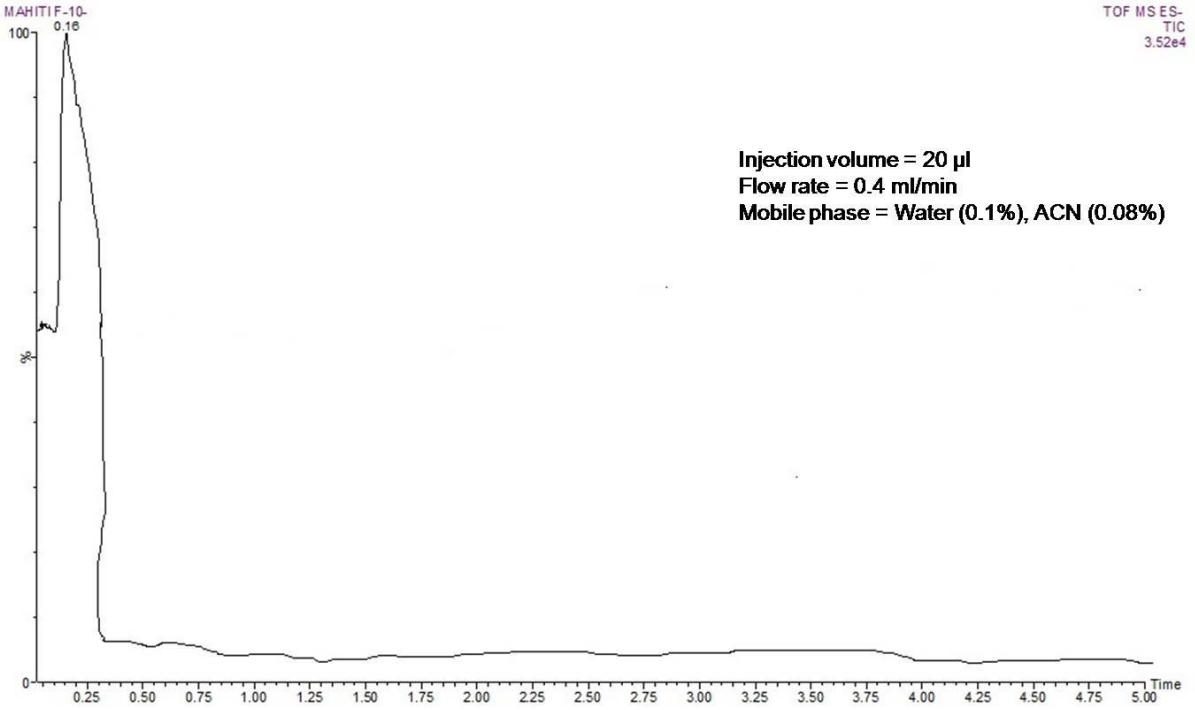


Fig. 5.22 LC peak of bioactive compound

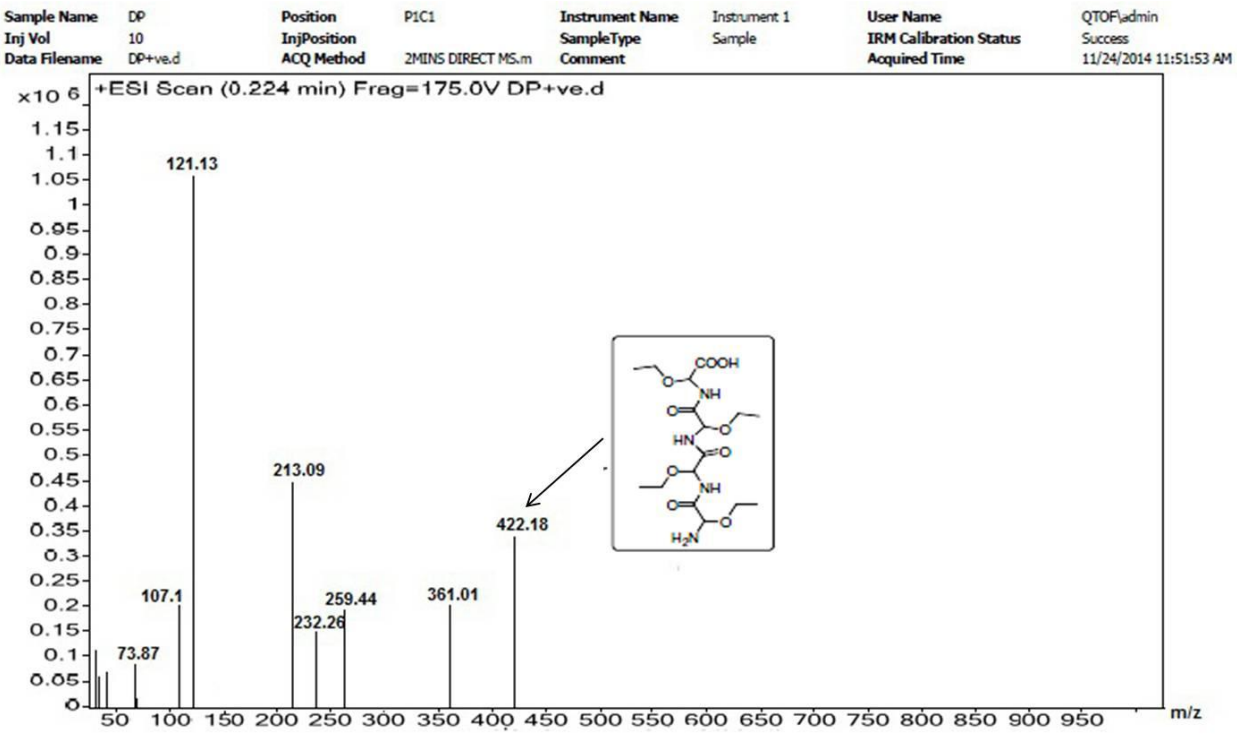


Fig. 5.23 ESI-MS spectra of the bioactive compound

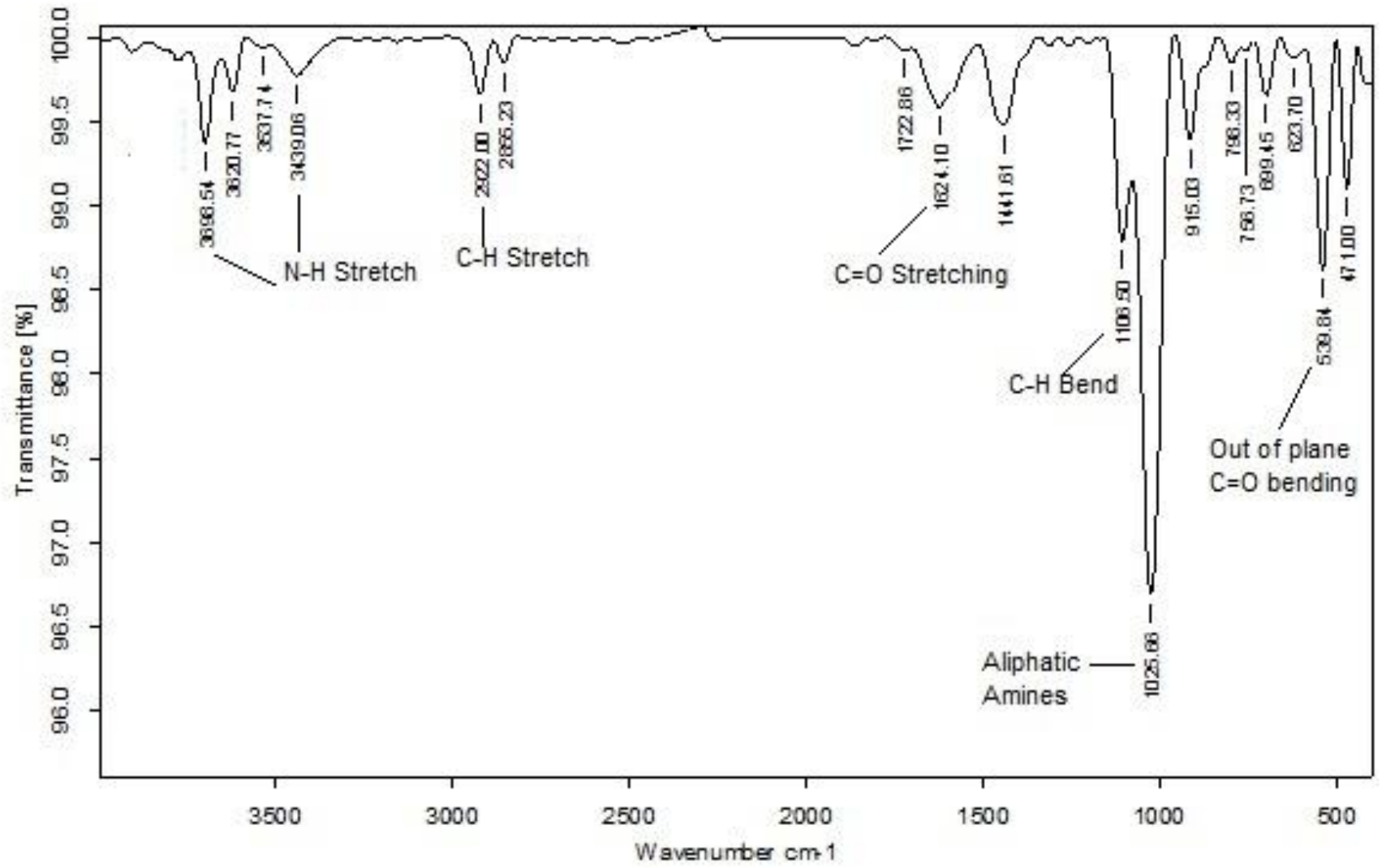


Fig. 5.24 FTIR spectrum of bioactive compound

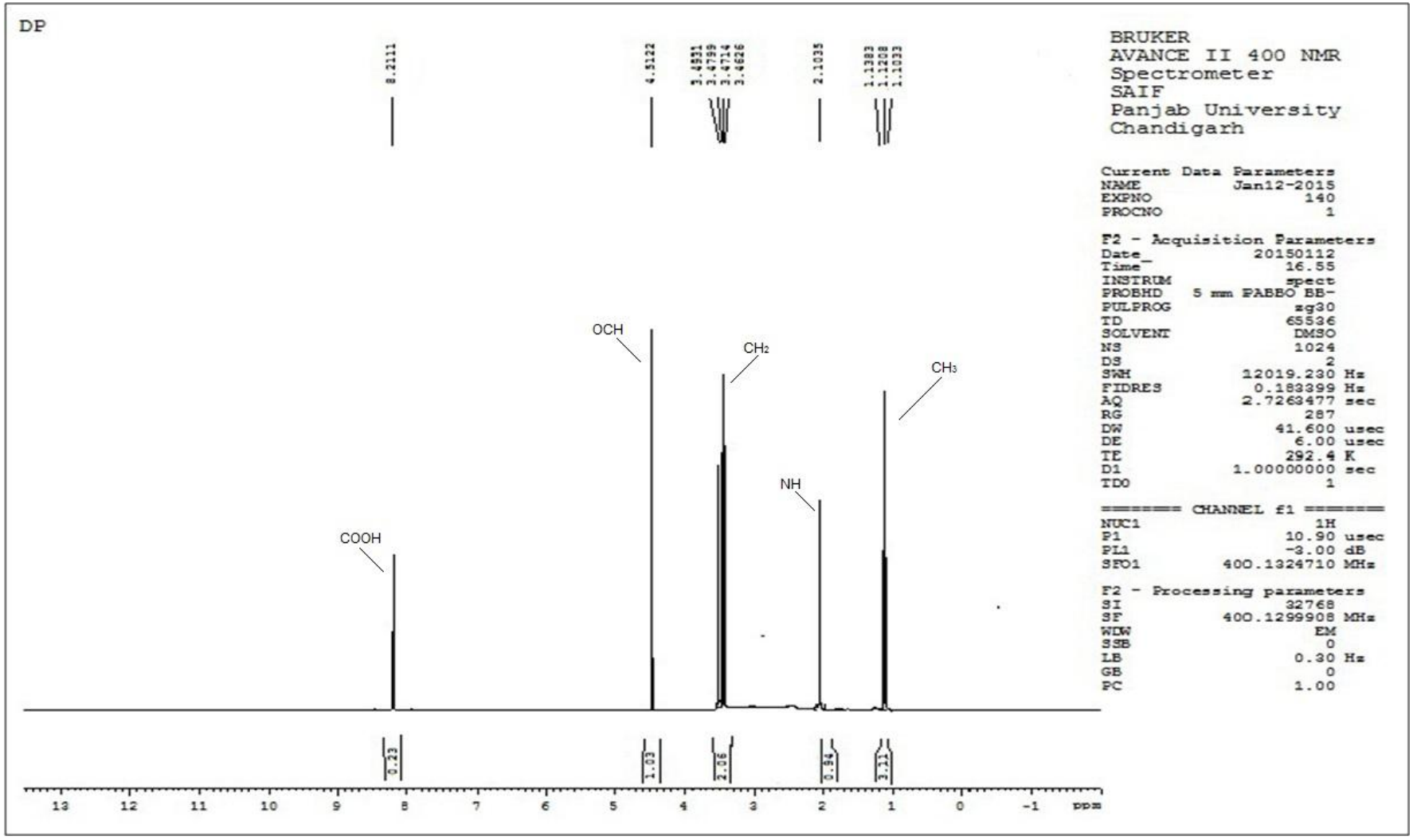


Fig. 5.25 ^{1}H NMR spectrum of bioactive compound

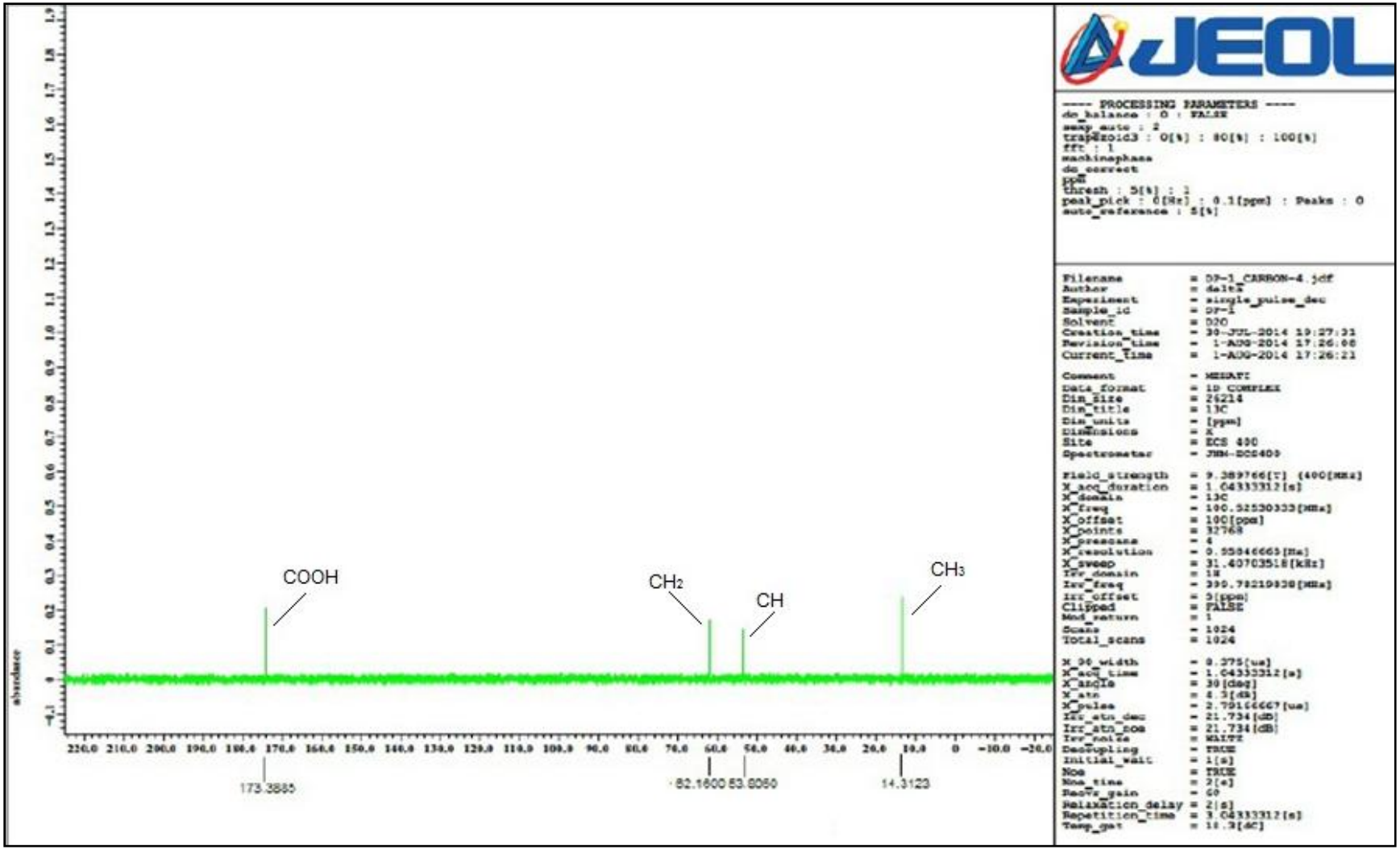


Fig. 5.26 ^{13}C NMR spectrum of bioactive compound

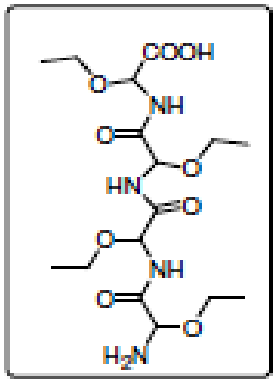


Fig. 5.27 Chemical structure of Fustat

5.13 Kinetic studies on the inhibition of pancreatic lipase by purified bioactive compound

5.13.1 Lineweaver Burk plot of pancreatic lipase inhibition by purified bioactive compound

In order to determine the nature of inhibition, PPL was incubated with different concentrations of inhibitor and substrate. The results illustrated in Fig. 5.28 (A,B) shows that increasing the concentration of inhibitor resulted in a family of lines with a common intercept at 1/v resulting in increase in K_m with different slope values. However, as inhibitor concentration was increased (20, 50, 70, 120 µg/ml), V_{max} value was found to be same which indicated the inhibition to be competitive.

The K_i of Fustat was less than Orlistat at same concentrations indicating better inhibition of PPL than Orlistat.

The inhibition constant K_i was calculated using formula (Kakkar et al., 1999)

$$\mathrm{Ki} = \frac{\mathrm{Km} * \mathrm{I}}{\mathrm{Kmapp} - \mathrm{Km}}$$

Where K_m = Michealis Menten constant

K_{mapp}= Michealis Menten constant in presence of inhibitor

I = Inhibition concentration

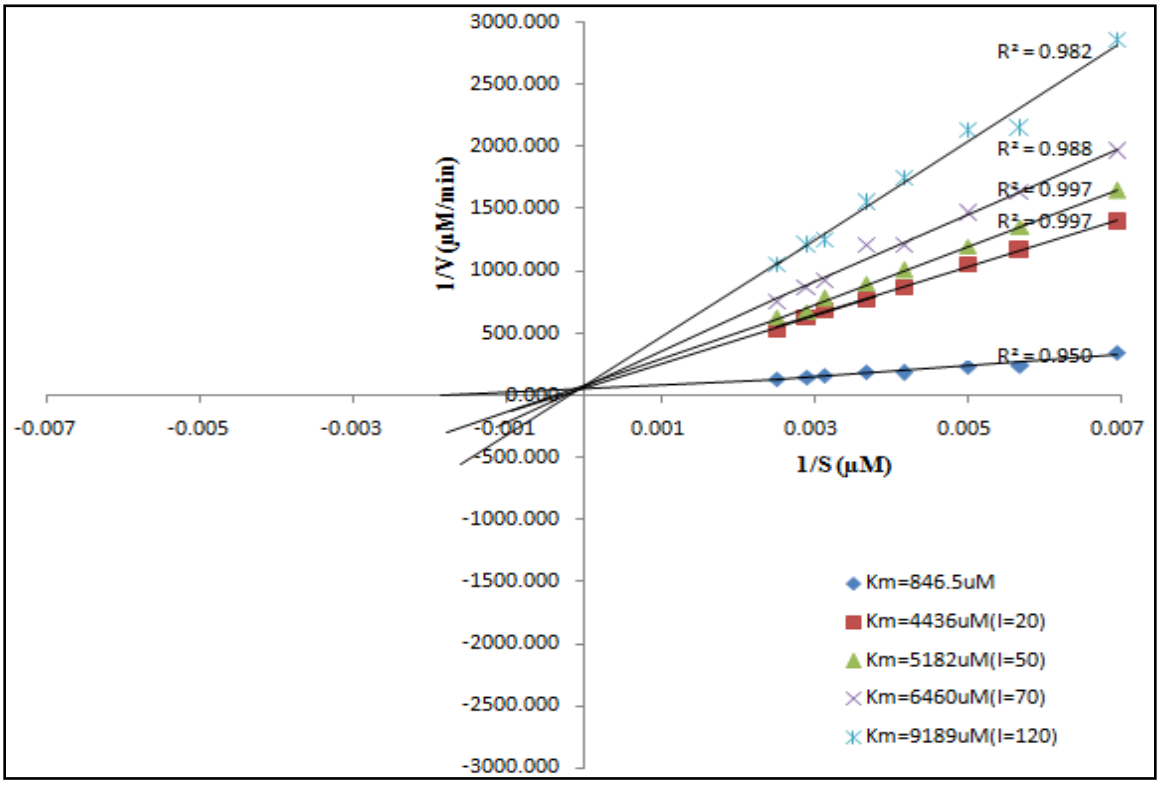


Fig. 5.28A Lineweaver Burk plot representing the competitive inhibition by Fustat

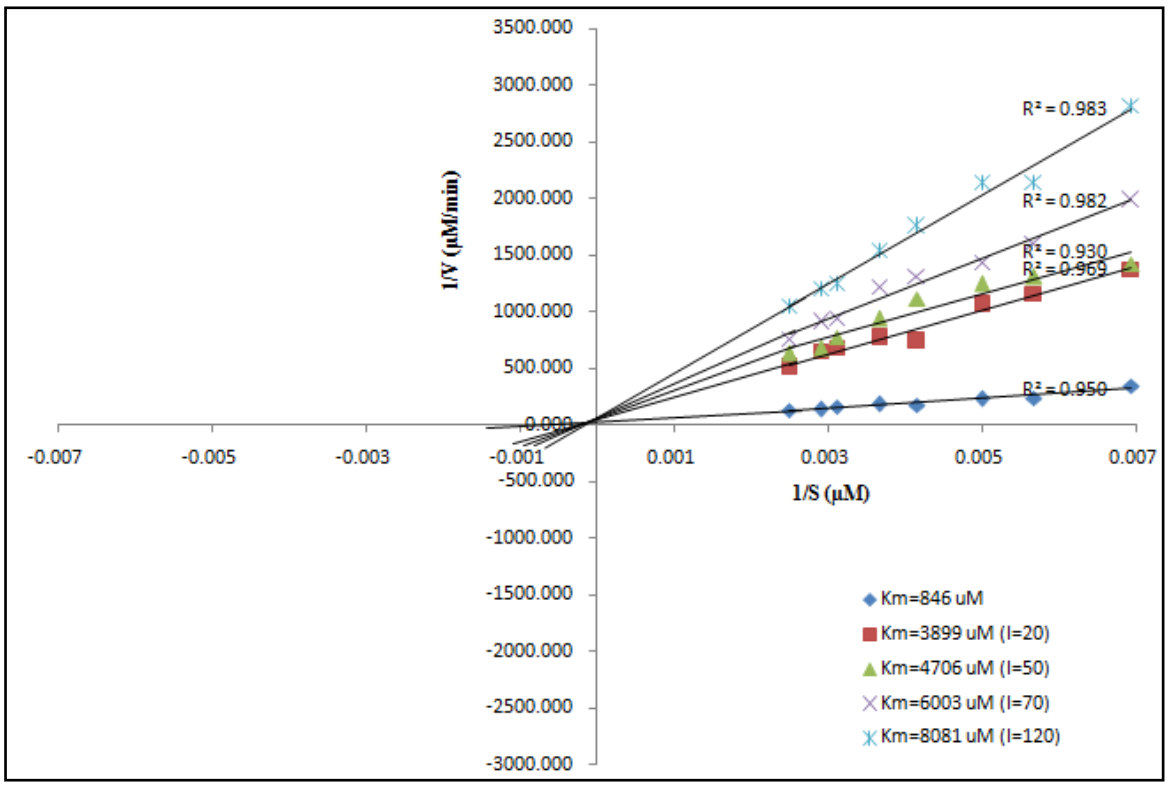


Fig. 5.28B Lineweaver Burk plot showing competitive inhibition by Orlistat

5.13.2 Dixon plot of porcine pancreatic lipase inhibition by bioactive compound

Dixon plot method is a reliable method for determination of K_i in competitive inhibition. In this approach the reciprocal velocity, 1/v is plotted against the inhibitor concentration (I) at two or more values of S, the substrate concentration. For each value of S, the points lie on a straight line, and then the lines are extrapolated at different S values such that they intersect at a single point, for which $I = -K_i$ and $1/v = 1/V$ (Bowden and Eisenthal, 1974). The K_i of the Fustat calculated from Dixon Plot was 7.1 µM (Fig. 5.29).

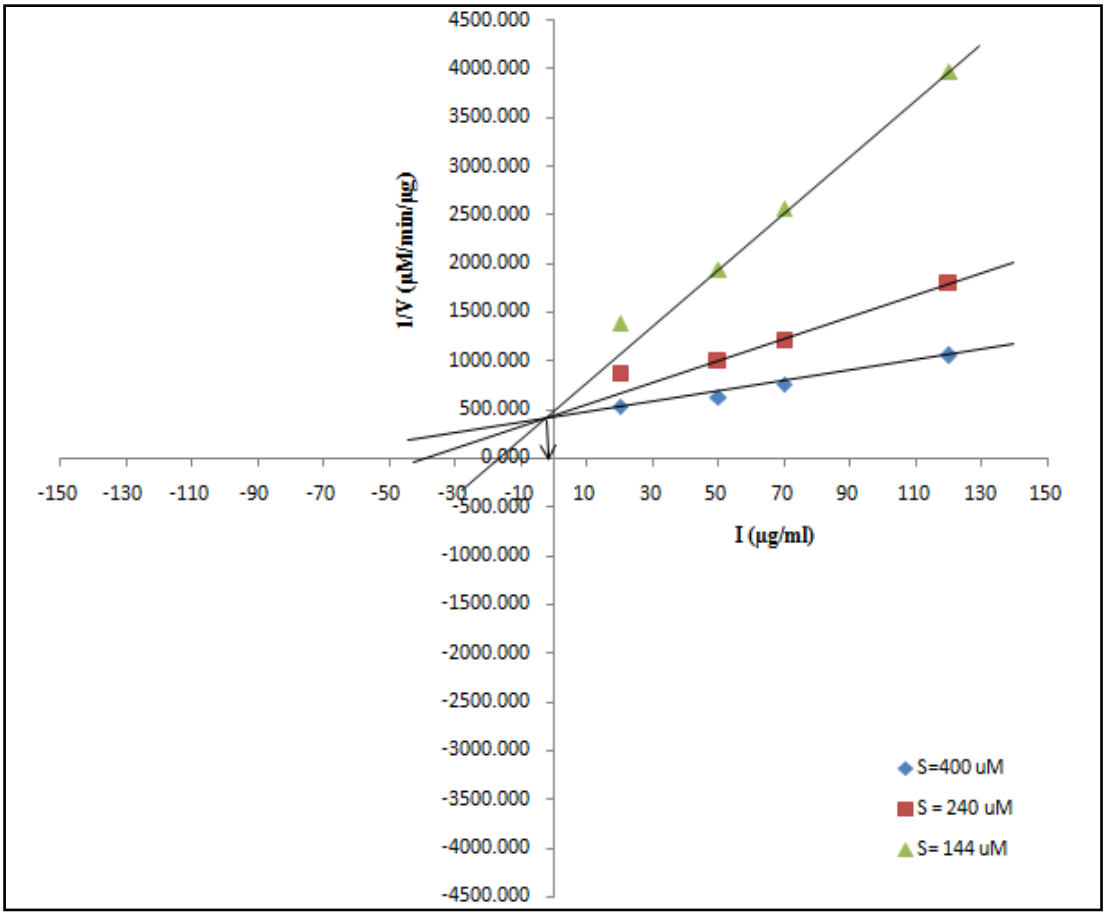


Fig 5.29 Dixon plot between different concentrations of the inhibitor and reciprocal of velocity (The point of intersection of lines (arrow) shows $-K_i$ value on inhibitor concentration)

5.14 Anti adipogenesis and lipolytic effect of bioactive compound on 3T3 cell line

5.14.1 Inhibition of adipogenesis

Fustat inhibited adipogenesis by 62.4% at the concentration of 200 µg/ml. Tukey's test analysis (Table 5.13) revealed a significant difference in inhibition of adipogenesis by negative control and various concentrations of Fustat (50, 100, 200 µg/ml). The inhibition of adipogenesis by Esculetin (positive control) and Fustat did not exhibit any significant difference at a concentration of 100 µg/ml.

Table 5.13 Tukey's multiple comparison showing % reduction of lipid content in adipocytes

One way ANOVA (level of significance)	Tukey's Multiple Comparison Test					Inference
	Treatments	Mean Difference	q value	p < 0.05	95% CI of difference	
p<0.0001	control vs 25 ug/ml	24.63	3.468	No	-6.427 to 55.69	Ns
p<0.000	control vs 50 ug/ml	31.85	4.485	Yes	0.7925 to 62.91	*
p<0.0001	control vs 100 ug/ml	51.51	7.252	Yes	20.45 to 82.57	***
p<0.0001	control vs 200 ug/ml	62.40	8.786	Yes	31.34 to 93.46	***
p<0.0001	control vs Esculetin (100 ug/ml)	58.77	8.274	Yes	27.71 to 89.83	***
p<0.0001	25 ug/ml vs 50 ug/ml	7.220	1.017	No	-23.84 to 38.28	Ns
p<0.000	25 ug/ml vs 100 ug/ml	26.88	3.784	No	-4.183 to 57.94	Ns
p<0.0001	25 ug/ml vs 200 ug/ml	37.77	5.318	Yes	6.709 to 68.83	*
p<0.0001	25 ug/ml vs Esculetin (100 ug/ml)	34.13	4.806	Yes	3.075 to 65.19	*
p<0.0001	50 ug/ml vs 100 ug/ml	19.66	2.767	No	-11.40 to 50.72	Ns
p<0.0001	50 ug/ml vs 200 ug/ml	30.55	4.301	No	-0.5115 to 61.61	Ns
p<0.0001	50 ug/ml vs Esculetin (100 ug/ml)	26.91	3.789	No	-4.145 to 57.97	Ns
p<0.0001	100 ug/ml vs 200 ug/ml	10.89	1.534	No	-20.17 to 41.95	Ns
p<0.0001	100 ug/ml vs Esculetin (100 ug/ml)	7.259	1.022	No	-23.80 to 38.32	Ns
p<0.0001	200 ug/ml vs Esculetin (100 ug/ml)	-3.633	0.5116	No	-34.69 to 27.43	Ns

The intracellular oil globule staining with oil red O stain indicated a very high number of oil globules in the control (untreated) cells, while those treated with Fustat significantly reduced the

number of oil globules suggesting decrease in lipid accumulation. Fustat significantly reduced oil accumulation in adipocytes at a concentration of 200 µg/ml (Fig. 5.30). Fustat was non–cytotoxic even at higher concentrations of 200 µM. This experiment indicated that Fustat inhibits the process of adipogenesis that increases with the increase in concentration of Fustat.

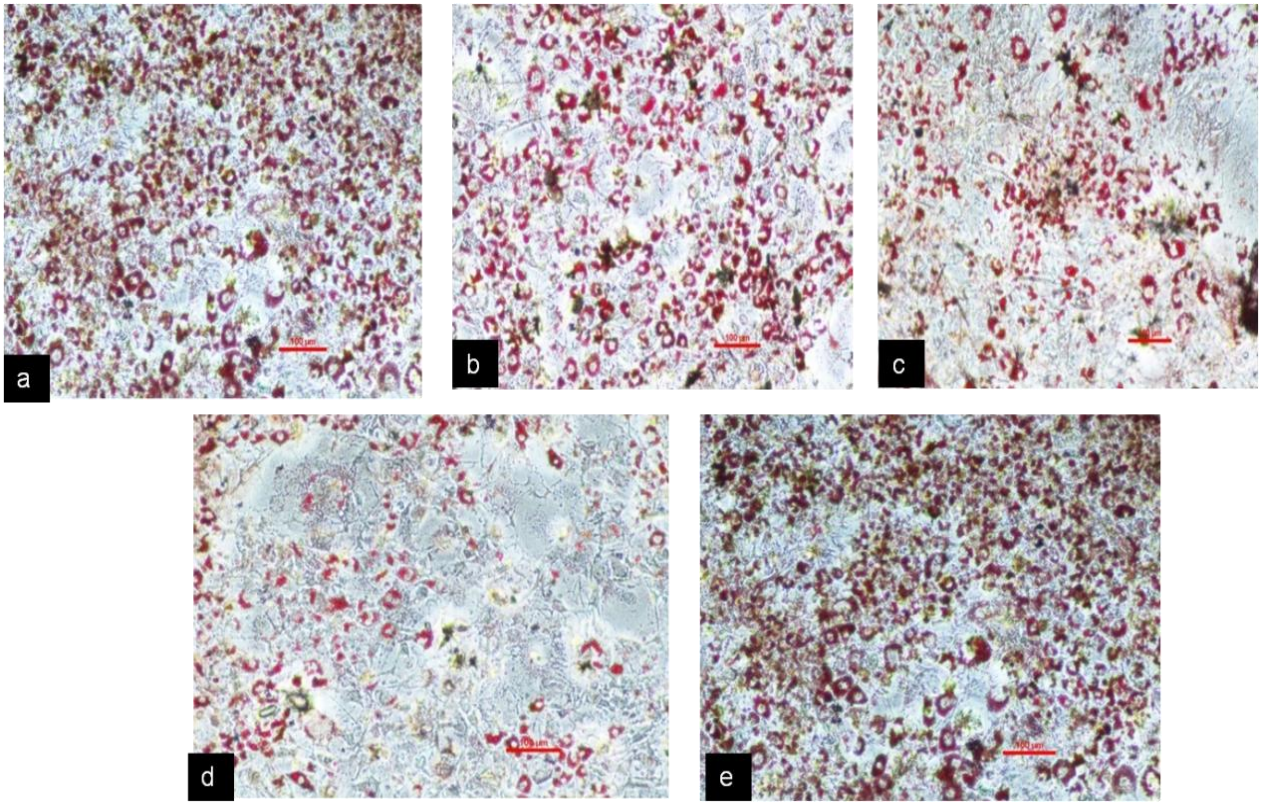


Fig. 5.30 Oil O red staining showing lipid accumulation in adipocytes. Test compound a. 25 µg/ml b. 50 µg/ml c. 100 µg/ml d. 200 µg/ml e. Control (0 µg/ml)

5.14.2 Lipolysis

The ability of Fustat to enhance lipolysis of stored fat from adipocytes was evaluated using Forskolin as control. The amount of glycerol release was quantified using the equation of standard curve of glycerol (Fig. 5.31).

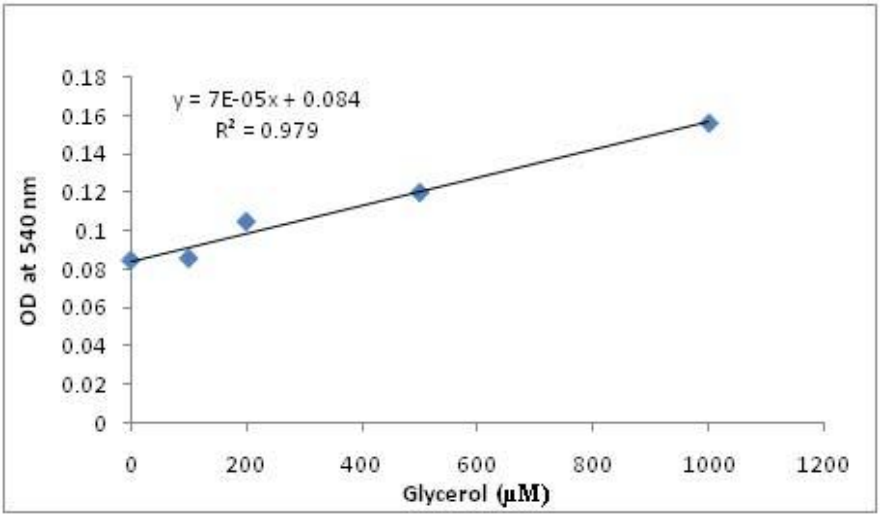


Fig. 5.31 Standard curve of glycerol

The free glycerol release was quantified at 12 h and 24 h. No significant result was seen after 12 h but after 24 h increase in glycerol concentration was seen by Fustat at the concentration of 100 µM. Time dependent and concentration dependent increase in release of free glycerol was observed for the Fustat and Forskolin. BSA (2%) was used as control here in experiment to see the normal release of glycerol (Fig. 5.32)

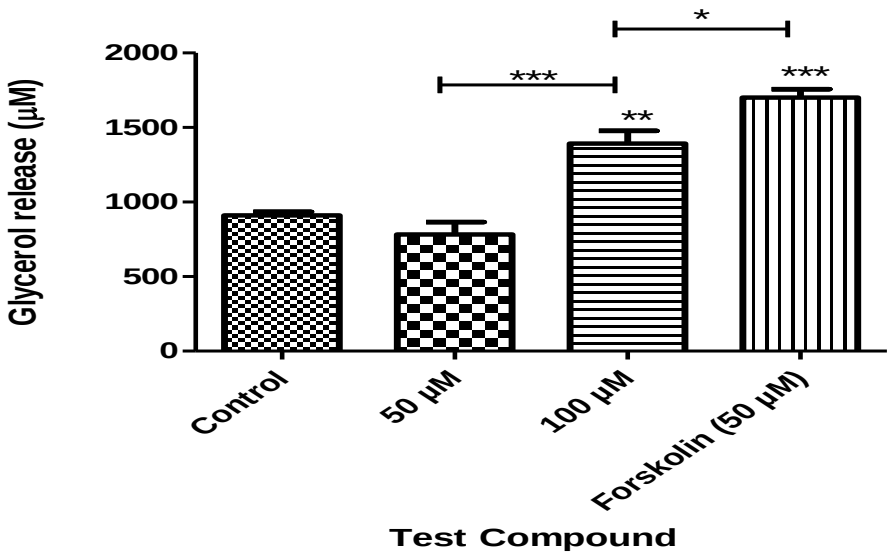


Fig. 5.32 Effect of Fustat and Forskolin in lipolysis in adipocytes. *- p<0.01, **- p<0.05, ***-p<0.001

Table 5.14 Tukey's test showing level of significance among different concentrations of test compound in lipolysis

One way ANOVA (level of significance)	Tukey's Multiple Comparison Test					Inference
	Treatments	Mean Difference	q value	p < 0.05	95% CI of difference	
p<0.0001(***)	Control vs 50 µM	128.6	1.931	No	-151.0 to 408.1	Ns
p<0.0001(***)	Control vs 100 µM	-482.1	7.242	Yes	-761.7 to -202.6	**
p<0.0001(***)	Control vs Forskolin (50 µM)	-789.3	11.86	Yes	-1069 to -509.7	***
p<0.0001(***)	50 µM vs 100 µM	-610.7	9.173	Yes	-890.3 to -331.2	***
p<0.0001(***)	50 µM vs Forskolin (50 µM)	-917.9	13.79	Yes	-1197 to -638.3	***
p<0.0001(***)	100 µM vs Forskolin (50 µM)	-307.1	4.613	Yes	-586.7 to -27.59	*

Fustat exhibited moderate lipolytic activity as compared to control containing 2% BSA solution. Tukey's statistical analysis (Table 5.14) showed that there is insignificant glycerol release by 50 µM of the test compound as compared to control whereas 100 µM of the test compound showed glycerol release comparable to 50 µM Forskolin. The above results revealed that Fustat is only a PL inhibitor with moderate inhibition of adipogenesis and it exhibited very less lipolytic activity even after 24 h.

Chapter 6

Discussion

Obesity is a daunting health problem in the world today, which is responsible for approximately 2.8 million deaths annually (WHO, 2015). Modern pharmacotherapy appears to be a better option for efficacious treatment of obesity since the molecule of interest works on a defined pharmacological target thereby interrupting the lipid metabolic pathway which is responsible for obesity. Despite high social demand for an effective treatment of obesity and extensive research both in pharmaceutical industry and academia to date only Orlistat has been approved by USFDA as a drug for the treatment of diet induced obesity.

Orlistat is a bioactive natural product initially isolated from actinobacterium *Streptomyces toxytricini* and inhibits PL. However Orlistat has a plethora of side effects which restrict its prolonged use and thus there is a need of new PL inhibitors with better pharmacological properties and safer drug profiles. This has stimulated research for identifying new PL inhibitors from natural sources.

Endophytic fungi from last two decades have attained remarkable recognition in the field of drug discovery and development as producers as well as sustainable sources of unique and diverse bioactive compounds which can be directly or indirectly used as potent drugs for combating major global diseases (Strobel and Daisy, 2003; Aly et al., 2010; Zhang et al., 2015b; Zheng et al., 2016). However endophytic fungi have not been explored so far for their anti-obesity properties. PL has been reported to be one of the safest pharmacological drug target for the treatment of obesity (Birari and Bhutani, 2007; Lunagariya et al., 2014). Thus, the present study was oriented to screen and isolate PL inhibitors from the endophytic fungi isolated from medicinal plants. Yet another premise of taking up this study is the possible association of endophytic fungi with plant lipid bodies for their probable participation in carbon movement. However to maintain their membrane integrity and stored lipids in the above process these endophytic fungi express signal molecules/ inhibitors, which act on plant lipases (Avila et al., 2012).

6.1 Screening of potential fungi for pancreatic lipase inhibitory activity

Suitable modifications of qualitative plate assays for detection of lipase activity using rhodamine and phenol red was carried out for preliminary screening PPL inhibitory potential of the endophytic fungi. This is the very first report of development of qualitative plate assays for screening PPL inhibitors. In the preliminary screening, culture filtrate of 35 endophytic fungi exhibited more than 50% PPL inhibition in the qualitative assays suggesting higher propensity of occurrence of PL inhibitors as compared to plant extracts. Roh and Jung (2012) have reported 11% PPL inhibitory activity of the 400 crude plant extracts screened. Similar results were obtained by Gholamhosenian et al., (2010) wherein 4 out of 100 plant extracts exhibited PPL inhibitory activity.

The isolate #6AMLWLS was exhibiting the maximum PL inhibition in both rhodamine and phenol red assay which was isolated from *Aegle marmelos*. *Aegle marmelos* or Bael is a medicinal plant possessing anti-hyperglycemic (Kamalakannan and Prince, 2003) and anti-obesity activity (Karmase et al., 2013). Another important observation in the present study was that majority of endophytic fungi which exhibited PL inhibition were isolated from *Aegle marmelos*, which confirmed the much hypothesized belief that endophytic fungi mimic the properties of their host plant (Gunatilaka, 2006; Aly et al., 2013). Endophytic fungi of *Taxus baccata* has been largely screened for their potential to produce the anti-cancer compound Taxol and related intermediate compounds such as deacetyl baccatin (Sreekanth et al., 2009; Mirjalali et al., 2012; Garyali et al., 2013; Somjaipeng et al., 2015). Only Lovastatin (a HMG CoA inhibitor) production has been reported from *Aspergillus niger* PN2 isolated from healthy tissues of *Taxus baccata* (Raghunath et al., 2012). The present study for the first time reports PPL inhibitory activity from an endophytic *Penicillium* species isolated from *Taxus baccata* (Gupta et al., 2015a).

Extract of endophytic fungi have been screened for inhibition of different enzymes such as HMG CoA reductase (Raghunath et al., 2012), Xanthine oxidase (Huang et al., 2007; Kapoor and Saxena, 2014), Cholinesterase (Rodriguez et al., 2005), Cyclooxygenase-2 inhibitors (Ju et al., 2015). However to the best of our knowledge the current study is the very first report to screen endophytic fungi for PL inhibition (Gupta et al., 2015a; Gupta et al., 2015b). More recently Leptin inhibitors from fungal endophytes have been explored as a possible intervention for the treatment of obesity (Chandramouli et al., 2016).

Thus based on preliminary screening, culture broths of selected 10 endophytic isolates were assayed for quantitative estimation of PPL inhibition. A pre-requisite for quantitative PPL inhibition was ascertaining the optimal conditions of PPL activity using chromogenic substrate pNPL. The other chromogenic substrates which are generally used are pNPP (p-nitrophenol palmitate) and pNPS (p-nitrophenol stearate) (Syed et al., 2010). The assay conditions for optimal PPL activity comprised of pH 7.4, temperature 37°C and incubation time of 3 hrs which corresponded to studies by Senior and Isselbacher (1963) and Mattson and Volpenhein (1972). Further the optimal concentration of substrate was 800 uM while enzyme was 12 U/250 µl of reaction volume.

In the present study culture filtrate of #6AMLWLS and #57TBBALM exhibited the best activity amongst the 10 endophytic fungi evaluated for PPL inhibition using quantitative assay. However, using 1-way ANOVA and post hoc analysis, all the culture filtrates exhibited a significant PPL inhibitory activity when compared to the control (without any inhibitor). Thus, in order to select the best isolate for isolation and characterization of the PPL inhibitor, partial purification of culture filtrates of the selected fungi using liquid-liquid extraction was carried out and the solvent residues so obtained was reanalysed for their quantitative PPL inhibition. Total inhibition (100%) of PPL activity was observed in the aqueous fraction of #6AMLWLS closely followed by ethyl acetate fraction of #57TBBALM and #59AMSTWLS. For the first time aqueous

solution after solvent extraction has exhibited 100% PPL inhibitory activity which could possibly be attributed to a hydrophilic moiety. Methanol water extract of edible mushroom viz. *Lepiota procera*, *Grifola frondosa*, *Pleurotus eryngii* and *Lyophyllum shimeji* exhibited a much less PL inhibition as compared to #6AMLWLS (Palaniswamy et al., 2012).

Ethyl acetate extract residues of several endophytic fungi have been reported to inhibit different enzymes such as topoisomerase (Xialoing et al., 2010), Beta-secretase 1 (Harun et al., 2011), α-amylase and β-galactosidase (Pavithra et al., 2014), α-glucosidase (Singh et al., 2015; Cui et al., 2016). In our study also ethyl acetate extract of endophytic *Penicillium* species (#57TBBALM) exhibited potential PPL inhibition. However when we statistically compared the aqueous extract of #6AMLWLS and ethyl acetate extract of #57TBBALM we did not find a significant difference in their PPL inhibitory potential and thus we further analysed them for their IC_{50} values on the basis of which further selection of potential isolate for isolation and characterization could be carried out. IC_{50} is half of the maximal inhibitory concentration of a substance wherein the specific biological or biochemical function is reduced to half. This parameter is generally used to measure the effectiveness of drugs. In the present study the IC_{50} of aqueous extract of #6AMLWLS was much better as compared to the positive control i.e Orlistat and ethyl acetate extract of #57TBBALM. Similar IC_{50} values were obtained with Panclicins A and B which were isolated from *Streptomyces* species NR0169 (Mutoh et al., 1994). Panclicins are analogues of tetrahydrolipistatin which is a derivative of lipstatin isolated from *Streptomyces* species (Weibel et al., 1987). The aqueous fraction of #6AMLWLS also significantly exhibited better IC_{50} values when compared to different plant extracts namely mangosteen (*Garcinia mangostana*) pericarp extract (Adnyana et al., 2015), *Ginkgo biloba* (Bustanji et al., 2011a), methanolic extract of rosemary (Bustanji et al., 2010), *Salvia officinalis* (Ninomiya et al., 2004). Water extract of Walnut (*Juglans regia*) shell exhibited a much higher IC_{50} value for PL inhibition as compared to aqueous extract of #6AMLWLS (Yang et al., 2014). Phytochemicals like

Hesperidin, Diosgenin, Licochalcone A have significant PL inhibition activity based on their IC_{50} values, however aqueous extract of #6AMLWLS exhibited a much lower IC_{50} value suggesting that further purification would lead to a potent PPL inhibitor (Kawaguchi et al., 1997; Kwon et al., 2003; Won et al., 2007). A few compounds from plants have exhibited a potential activity as compared to the aqueous extract of the endophytic isolate #6AMLWLS, but the major issue with plant based moieties pertains to their supply or synthesis and therefore microbial (fungal/ bacterial) sources appear to be a better option, as production can be achieved through mass production methods. Vibralactone, a fused beta-lactone isolated from *Boreostereum vibrans* exhibited a PL inhibition with IC_{50} of 0.4 µg/ml (Liu et al., 2006) while Percyquinin isolated from the basidiomycete *Stereum complicatum* which exhibited an IC_{50} value of 2 µm for PL inhibition (Hopmann et al., 2003). However, these two compounds are yet to be taken up for pre-clinical and clinical studies.

Phytochemical analysis provides an idea of the classes of compounds existing in an extract on the basis of which an isolation and purification method could be developed. In the present study the aqueous extract of the endophyte #6AMLWLS reported the presence of terpenes and amino acids/ proteins. Terpenes like Ginkgolides A, B and Bilobalide were found to inhibit PL (Bustanji et al., 2011a). Water extract of fructus of *Gardenia jasminoides* have yielded two terpenes Crocin and Crocetin which exhibited a potential PL inhibition (Lee et al., 2005). The other terpenes which have been reported to possess potent PL inhibition include 3-O-trans-p-coumaroyl actinidic acid from *Actinidia arguta*, Cornosol and Carnosic acid from methanol extract of Salvia *officinalis* leaves (Ninomiya et al., 2004; Jang et al., 2008). Endophytic fungi have also been reported to produce terpenes the predominant being sesquiterpenes which exhibit anti-cancer, anti-fungal and anti-bacterial properties, however none of them have been reported to possess enzyme inhibition properties (Souza et al., 2011). Proteins have also been tested for their PL inhibitory properties in the presence and absence of co-lipase and bile salts.

The different proteins which have inhibited the PL activity include melittin, ovalbumin, myoglobin, β-lactoglobulin, serum albumin and soyabean proteins (Gargouri et al., 1984). Other basic proteins which have been found to inhibit PL are protamine, purothionin and histones (Tsujita et al., 1996). ε- Polylysine has been found to inhibit PL (Kido et al., 2003). Thus the potential inhibitory effect on PPL might be due to synergistic effect of these terpene and protein moieties in the extract could be evaluated separately when the process of purification is attempted.

6.2 Identification of potential fungi

Thus, the endophytic fungi exhibiting potential PL inhibitory activity was #6AMLWLS. This organism was identified using morphological as well as molecular tools. Morphologically the endophyte belonged to the genus *Fusarium*, however determining species is a contentious issue and precise taxonomy remain a challenge for the fungal taxonomists (Chandra et al., 2011). Predominantly *Fusarium* species are homoplastic morphologically thus there is a limitation to distinguish between two closely related species based on the description of taxa which is solely based on color, texture and appearance of colony, shape and size of conidia and conidiophores (Skovgaard et al., 2003). Presently morphological studies accompanied by phylogenetics are used to define species in the *Fusarium* genus. The fungi are distinguished into two broad classes based on their colony growth rate on PDA. #6AMLWLS is a moderately growing *Fusarium* species which gives rise to conidiophores which in turn bear macroconidia. In *Fusarium incarnatum* the conidia are borne on aerial hyphae. Shape of macroconidia and proliferation of conidiogenous cells is a critical feature for distinguishing among the species. The macroconidia in *F. incarnatum* possess 3-7 septate macroconidia which vary from fusiform to falcate in shape. Similarly marcoconidia of *F. culmorum* and *F. sambucinium* are wedge shaped whereas in *F. acuminatum* are broad below the centre. The shape of macroconidia also varied from sickle shaped in the case of *F. equiseti*, to narrow and straight in *F. moniliformae,* to straight and

robustic in *F. solani* whereas it is 4-6 celled fusiform in *F. incarnatum*. Microconidia also varies in shape from comma shape in *F. oxysporum* and *F. solani,* to globose in *F. sporotrichioides*, *F. chalmydosporum* and *F. poae,* to lemon shape in *F. tricinctum* whereas it is completely absent in *F. incarnatum* (Booth, 1971; Gerlach and Nirenberg, 1982; Nelson et al., 1983; Seifert, 1996). The major shortcoming of the morphological approach is that the number of differentiating morphological characters is too less than the number of species that need to be distinguished. In biological species concept, species is defined as members of population that potentially or actually interbreed in nature and not according to the similarity of appearance. Since most of the *Fusarium* species exist as anamorphs and rarely produce a sexual stage, the application of biological species concept becomes impractical. However phylogenetic species concept has become more relevant in the recent past and has provided new insights to fungal systematics (Summerell et al., 2010). Today evolutionary history, genetic diversity and speciation of various *Fusaria* lineages is very well defined by employing multilocus GCPSR schemes (Taylor et al., 2000; O'Donnell et al., 2012). Previous studies have reported the identification of various *Fusarium* species based on single nuclear genes such as EF-1a (Skovgaard et al., 2003; Kristensen et al., 2005), ITS (Gurjar et al., 2009), RBP2 (O'Donnell et al., 2007) and β-tubulin (Azor et al., 2009). The EF-1α and RPB2 genes are the most appropriate sequences for inference of phylogenetic diversity and evolutionary dynamics of speciation as they are highly conserved as well as have a low copy number of characteristics for species level identification of *Fusarium* genus (Geiser et al., 2004; Sun et al., 2007). Thus, #6AMLWLS was identified based on the combined dataset of ITS, EF-1a and RPB2 for its phylogenetic placement. The phylogenetic analysis revealed that the isolate #6AMLWLS belonged to the *Fusarium incarnatum* species complex.

Endophytic *Fusarium* species has been reported to be a prolific producer of phytomedicinals as well as novel bioactive compounds. *F. solani* existing as an endophyte in

Apodytes dimidiata has been found to produce Camptothecin, 10-hydroxycamptothecin and 9-methoxycamptothecin which serves as precursor for synthesis of two clinically important anti-cancer drugs Topotecan and Irinotecan (Shweta et al., 2010). *F. proliferatum,* an endophyte in *Dysoxylem binectariferum* has been found to produce Rohitukine, a chromane alkaloid possessing anti-cancer, anti-inflammatory and immunomodulatory properties. Flavopiridol, a semisynthetic derivative of Rohitukine is a cyclin dependent kinase inhibitor and currently in clinical phase III trials (Mohana et al., 2012). Similarly Ginkgolide B is being produced from *F. oxysporum* which exists as an endophyte in *Ginkgo biloba* (Cui et al., 2012). Endophytic *F. oxysporum* isolated from *Juniperus recurva* has been found to produce podophyllotoxin which was initially reported from the plant *Podophyllum hexandrum* and is a precursor for anti-cancer drugs like Etoposide and Teniposide (Kour et al. 2008). Several unusual alkaloids have been isolated from culture broth of *F. incarnatum* which exists as an endophyte in the mangrove plant *Aegiceras corniculatum* (Ding et al., 2012). *F. tricinctum* isolated as an endophyte from *Salicornia bigelovii* has been found to produce Fusarticin which has potent anti-microbial activities (Zhang et al, 2015b). Fusaristatins A and B have been isolated from endophytic *Fusarium* species YG-45 existing in *Maackia chinensis* and exhibited moderate topoisomerase I and II inhibition (Shiono et al., 2007). Another endophytic *Fusarium* species which is reported to produce cerebrosides named as Fusaruside have been found to inhibit the enzyme Xanthine oxidase (Shu et al., 2004). However the current study is first report of an endophytic *F. incarnatum* #6AMLWLS which has exhibited a PPL inhibitory activity. Till date PL inhibitors have been reported from macroscopic fungi like *Boreosterum vibrans* and *Tylopilus felleus*, *Hygrocybe conica* and *Laetiporous sulphureus* (Slanc et al., 2004; Liu et al., 2006).

A significant impact has been observed on the quantity and quality of fungal secondary metabolites with variation in their growth environment. In the present study Richard's broth exhibited the best PL inhibitory activity when compared to other growth media. Richard's broth

has been used as a growth medium to evaluate the anti-microbial efficacy of endophytic fungi isolated from *Butea monosperma* (Tupped and Shishupala, 2014). *F. incarnatum* (#6AMLWLS) exhibited a positive correlation between the PL inhibitory activity and biomass production on Richard's broth. Shukla et al., (2014) also screened natural as well as synthetic media for production of anti-microbial compound by *F. rosenum*. The production of maximum biomass as well as anti-microbial metabolite was reported in Richard's broth. The optimal growth and secondary metabolite production by *F. incarnatum* #6AMLWLS was observed on 8^{th} day in the present study while it was observed on 9^{th} day in case of endophytic *F. solani* isolated from *Tylophora indica* (Merlin et al., 2013). Thus, there is a change in the pH of media, substrate utilization, and viability of the cells during the growth of microorganism for production of bioactive metabolites (Srivastava et al., 2011).

6.3 Purification of bioactive compound

TLC and column chromatography are two most widely used methods for the isolation and purification of bioactive compounds from the lead fraction identified using bioassay guided fractionation of the culture filtrate of the endophytic fungi. During this study the aqueous fraction of *F. incarnatum* (#6AMLWLS) resolved into two bands with R_f value of 0.74 and 0.86. The PL inhibitory activity was associated with band 1 while the other band did not exhibit any PL inhibition. These were re-chromatographed on silica gel column using different gradients of chloroform and methanol to check further fractions. The fractions of the column were cross checked by TLC to ensure purity. TLC has been used for identification of bioactive fraction from endophytic *Aspergillus terreus* MP15 which exhibited a promising anti-bacterial activity against gram positive bacteria (Yin et al., 2015). For identification of Vincristine and Vinblastine producing endophytic fungi, methanolic extract of the endophytic isolates along with standard compounds have been chromatographed using TLC (Palem et al., 2015). TLC has also been used to analyse the chemical content of crude extracts of endophytic fungi which formed the

selection of a specific fungal endophyte for further purification and isolation of the bioactive compounds using column chromatography (Akay et al., 2014). Phytochemical analysis of the band 1 obtained after column chromatography indicated the presence of amine group suggesting it to be a peptide/protein.

6.4 Structure elucidation of bioactive compound

The presence of amine group was further confirmed by structural elucidation which involved different techniques like ESI-MS, FTIR and NMR. On the basis of these analytical techniques the band 1 of *F. incarnatum* (#6AMLWLS) was found to be tetrapeptide comprising of four threonine residues. This compound was named as Fustat. Chemically Fustat is 11-amino-2, 5, 8- triethoxy-4, 7, 10-trioxo-12-oxa-3, 6, 9-triazatetradecanoic acid based on its ESI-MS, FTIR and NMR data. This compound so far has not been reported in the literature as well as in chemical databases and is considered as a new chemical entity. Fusaristatins A and B are two cyclic lipopetides which have already been reported to possess moderate topoisomerase I and II inhibitory potential (Shiono et al., 2007). Two cyclic depsipeptides have been reported from fermentation broth of an endophytic fungus isolated from *Kandelia candel* (Huang et al., 2007). Recently an anti-diabetic peptide has been reported from endophytic *Aspergillus awamorii* (Singh and Kaur, 2016). ESI-MS, FTIR and NMR have been used for characterization of different enzyme inhibitors isolated from endophytic fungi such as *Xylaria feejeensis*, *Cladosporium* species, *Colletorichum* species TSC13, *Phomopsis vexans*, *Guignardia* species KcF8 (Artanti et al., 2014; Ai et al., 2014; Chavez et al., 2015; Singh et al., 2015; Parthasarthy and Sathiyabama, 2015).

Fustat is a white amorphous, highly polar solid having a melting point above 300^{o}C. In general amino acids and short peptides generally possess high melting point. The appearance of the pure compound largely depends on its chemical nature for instance Vibralactone was purified from *Boreostereum vibrans* as colourless oil, whereas Valilactone and Ebelactone were obtained as colourless needles with melting point of 57°C and 86°C respectively (Umezawa et

al., 1980; Kitahara et al., 1987). Kinetic studies on Fustat established that it acts as a competitive inhibitor with a K_i of 7.1 µm and thus proved to be better under *in vitro* conditions as compared to the current drug Orlistat. Some synthetic peptides displayed by a phage library were also evaluated for their PL inhibitory potential of which only one heptapeptide proved to possess PL inhibitory activity with apparent K_i of 16 µm which is much higher when compared to Fustat (Lunder et al., 2005). Derivatives of *Monascus* pigments have also been evaluated for lipase inhibition owing to its success as a source of drugs for treatment of hypercholesterolemia. H-Pen derivative (Penicillamine) exhibited the highest PL inhibitory activity of the four derivatives. The inhibition was non-competitive with a K_i of 20.7 µM (Kim et al., 2007). Platycodin D isolated from the plant *Platycodin grandifolium* was found to be a competitive inhibitor of PL with a K_i of 180 µM (Zhao and Kim, 2004). Licochalcone A isolated from the plant *Glycyrrhiza uralensis* exhibited a much higher K_i and was found to be a non-competitive inhibitor (Won et al., 2007). Most of the compounds either synthetic peptides, derivatives of natural products from fungi or phytochemicals from plants exhibited a higher inhibition constant when compared to Fustat. Thus, Fustat is a potent PL inhibitor which is better than the present drug Orlistat (tetrohyrolipstatin).

Further to understand the role of Fustat in lipid metabolism apart from PL inhibition in the present study, anti-adipogenesis and lipolytic effects were evaluated using 3T3 cell lines. Fustat exhibited a moderate inhibition in adipogenesis which is within the acceptable limits i.e between 50-70%. This aspect is important since if adipogenesis is completely inhibited then the fat will be stored in other tissue which will lead to ectopic accumulation of lipid and development of insulin resistance thereby causing type 2 diabetes (Stephens, 2012). Many herbal extracts have been found to possess anti-adipogenesis effects (Arumugam et al., 2008; Velusami et al., 2013; Kodali et al., 2014; Muguli et al., 2015). Aqueous and ethanolic leaf extract of *Aegle marmelos* also inhibited adipocyte differentiation and hypertrophy (Karmase et al.,

2013; Garg and Singh, 2014). Lipolysis is a process in which triglycerides are converted into glycerol and free fatty acid by the action of hormone sensitive lipase. In the present study it was found that Fustat did not have any effect on the process of lipolysis suggesting that it specifically inhibiting PL. *Nelumbo nucifera* water and methanol extracts exhibited 1.9 and 3.5 fold increase in glycerol production at a concentration of 50 µg/ml and 100 µg/ml respectively (Velusami et al., 2013). Karmase et al., (2013) reported the lipolytic effect of Umbelliferone and Esculetin isolated from the dichloromethane extract of leaves of *Aegle marmelos* in a rat model. Another important aspect of this study was that no cytotoxicity was observed with Fustat suggesting it to be toxicologically safer. Since Fustat is made up of four residues of Threonine which is an essential amino acid, it is expected that the compound would be safe in toxicological studies. Thus it can be inferred that Fustat modulates the lipid metabolism by inhibiting PL and by causing moderate inhibition of adipogenesis which leads to loss in body weight.

Thus the present study establishes that endophytic fungi could be a novel resource for screening PL inhibitors and Fustat is a promising candidate for further pre-clinical evaluation using animal model for possible use as an anti-obesity drug with lesser side effects as compared to Orlistat. The diet induced obese rat model appears to be the best for carrying out the pre-clincal and safety evaluation of Fustat (Vikers et al., 2011). Further docking studies of Fustat and PL can provide an insight in improving the structure to enhance its PL inhibition.

Chapter 7

Conclusions

The present study establishes that endophytic fungi could be a promising resource for exploring PL inhibitors for their possible development into an anti–obesity drug for diet induced obesity.

1. Cell free culture filtrates of 190 fungal isolates from different medicinal plants were screened qualitatively using two new plate assays viz. rhodamine and phenol red olive oil plate assays. About 70% of the isolates from different class of fungi showed PL inhibition. Endophytic isolates belonging to hypomycetes (42.96%) were the most potent lipase inhibitors followed by ascomycetes (15.55%), coelomycetes (20%) and basidiomycetes (1.48%) respectively. 20% of the endophytic fungal isolates exhibiting lipase inhibitory activity remained unidentified.
2. Culture filtrates showing inhibitory potential ≥ 70% were then screened quantitatively for their inhibitory activity and were partially purified using solvent extraction. Partial purification led to increase in inhibitory activity in all the selected fungi except in *Lasiodiplodia theobromae* (#1079AMSTWLS) in which activity was due to synergistic effect of two or more compounds.
3. *Fusarium incarnatum* (#6AMLWLS) and *Penicillium* sp. (#57TBBALM) gave best results and were considered for calculating IC_{50}. The IC_{50} for partially purified fraction of #57TBBALM was 3.69 µg/ml and of #6AMLWLS was 2.12 µg/ml that was better than IC_{50} of pure salt of Orlistat (IC_{50}= 2.72 µg/ml).
4. The potential fungus was identified by morphological and molecular tools as *Fusarium incarnatum* (#6AMLWLS), an endophyte in *Aegle marmelos,* a plant already known for its anti-obesity properties in Ayurveda.
5. The potential bioactive fraction was then purified using gradient column chromatography and the inhibition coefficient (K_i) was 7.1 µM. Purified compound was a tetrapeptide with novel amino acids having molecular mass of 422. The compound was novel hitherto unknown molecule and was

chemically named as 11-amino-2,5,8-triethoxy-4,7,10-trioxo-12-oxa-3,6,9-triazatetradecanoic acid (Fustat).

6. Fustat was tested on 3T3-L adipocytes for inhibition of adipogenesis and lipolysis. It showed 60% inhibition of lipid accumulation in adipocytes leading to inhibition of adipogenesis, whereas lipolysis was seen at high concentration (100 μM) of Fustat. Cell viability was 100%, showing non toxic nature of Fustat.
7. Fustat further warrants it's testing on obese mice model for further development into anti–obesity drug as it has been found to be non-cytotoxic. As threonine is an essential amino acid and Fustat is a tetrapeptide containing threonine derivative, it might be safe for human use. The potential of Fustat to inhibit PL is even greater than Orlistat, moreover it is highly polar so it might get readily absorbed in the body hence increasing its efficacy to inhibit PL. Softwares like preclinical ADME screening facility and Lexi-complete PDA are available for evaluating pharmacokinetics and drug interaction with cytochrome P450 enzymes.

Chapter 8

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Chapter 9

Appendix

MEDIA COMPOSITION

Potato Dextrose Agar

Ingredients	Quantity (g/l)
Infusion from potatoes	200.0
Dextrose	20.0
Agar	15.0

Final pH at 25° C (5.6±0.2)

Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 min.

Richards Broth

Ingredients	Quantity (g/l)
Sucrose	5.0
Potassium Nitrate	1.0
KH_2PO_4	0.8
Magnesium sulphate	0.25
Ferric chloride	0.002

Final pH at 25° C (4.5±0.2)

Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 min

Pre-adipocyte Expansion Medium (PEM)

Ingredients	Quantity (ml)
DMEM	89.0
FBS	10.0
Pen-Sep (10 KU:10 mg)	1.0

Differentiation Medium (DM)

Ingredients	Quantity (ml)
DMEM	89.0 ml
FBS	10.0 ml
Pen-Sep (10KU:10mg)	1.0 ml
IBMX	0.5 mM
Dexamethasone	1.0 µM
Insulin	100 µg

Adipocyte Maintenance Medium (AMM)

Ingredients	Quantity (ml)
DMEM	89.0 ml
FBS	10.0 ml
Pen-Sep (10KU:10mg)	1.0 ml
Insulin	100 µg

BUFFERS AND SOLUTIONS

Potassium Phosphate Buffer

Ingredients	Volume (ml)
KOH (0.2M)	35.0 ml
KH2PO4 (0.02M)	50.0 ml
DDW	15.0 ml

Final pH at 25°C (7.4)

Phosphate Buffered Saline

Potassium Phosphate (monobasic) 1M	15.6 ml
Potassium Phosphate (dibasic) 1M	34.4 ml
Sodium Chloride 5M	30.0 ml
DDW	920.0 ml

Sodium hydrogen orthophosphate Buffer (0.5M/100ml)

Ingredients	Quantity (g)
Na_2HPO_4	5.95 g

Final pH at 25°C (8.0) with HCl

MOLECULAR BIOLOGY REAGENTS

Tris EDTA (1X)

Tris	10 mM
EDTA	1 mM

Final pH at 25°C (8.0)

TAE Buffer (50X)

Tris base (2M)	242.0 g/l
Glacial acetic acid (1M)	57.1 ml
EDTA (0.5M), pH 8.0	100 ml
Sterile DDW	842.9 ml

TAE Buffer (1X)

TAE Buffer (50X)	2 ml
Sterile DDW	98 ml

Ethidium Bromide (EtBr)

EtBr	10.0 mg
Sterile DDW	1.0 ml

6X Loading Buffer

Bromophenol blue	0.025 g
Glycerol	3.0 ml
Sterile DDW	7.0 ml

0.8% Agarose

Agarose	0.8 g
TAE buffer (1X)	100 ml

1.5% Agarose

Agarose	1.5 g
TAE buffer (1X)	100 ml

Table 5.1 Endophytic fungal isolates used in the study: their host plant, place of collection of plant and tentative identification

S.No.	Culture Code	Host plant	Plant part	Location	Tentative identification
1.	#2AMSTYEL	*Aegle marmelos*	Stem	Yelandur, Karnataka	*Fusarium moniliforme*
2.	#3AMSTYEL	*Aegle marmelos*	Stem	Yelandur, Karnataka	*Phaeoacremonium rubrigenum*
3.	#4AMSTYEL	*Aegle marmelos*	Stem	Yelandur, Karnataka	*Fusarium moniliforme*
4.	#5AMSTYEL	*Aegle marmelos*	Stem	Yelandur, Karnataka	*Fusarium moniliforme*
5.	#6AMSTYEL	*Aegle marmelos*	Stem	Yelandur, Karnataka	*Fusarium incarnatum*
6.	#7AMSTYEL	*Aegle marmelos*	Stem	Yelandur, Karnataka	*Fusarium equiseti*
7.	#7(a)AMSTYEL	*Aegle marmelos*	Stem	Yelandur, Karnataka	*Fusarium solani*
8.	#8AMSTYEL	*Aegle marmelos*	Stem	Yelandur, Karnataka	*Sphaeropsis sapinea*
9.	#9AMSTYEL	*Aegle marmelos*	Stem	Yelandur, Karnataka	*Pestalotiopsis microspora*
10.	#17AMSTYEL	*Aegle marmelos*	Stem	Yelandur, Karnataka	*Fusarium* sp.
11.	#22AMSTYEL	*Aegle marmelos*	Stem	Yelandur, Karnataka	*Fusarium incarnatum*
12.	#32AMSTYEL	*Aegle marmelos*	Stem	Yelandur, Karnataka	*Botryosphaeria* sp.
13.	#1003AMSTYEL	*Aegle marmelos*	Stem	Yelandur, Karnataka	*Diplodia* sp.
14.	#23AMSTYEL	*Aegle marmelos*	Stem	Yelandur, Karnataka	*Botryosphaerea* sp.
15.	#1032AMSTYEL	*Aegle marmelos*	Stem	Yelandur, Karnataka	*Sphaeropsis sapinea*
16.	#23(b)AMSTYEL	*Aegle marmelos*	Stem	Yelandur, Karnataka	*Lasiodiplodia gonubiensis*
17.	#9(b)AMSTYEL	*Aegle marmelos*	Stem	Yelandur, Karnataka	*Fusarium equiseti*
18.	#15AMSTYEL	*Aegle marmelos*	Stem	Yelandur, Karnataka	*Botryosphaeria* sp.

19.	#18AMSTYEL	*Aegle marmelos*	Stem	Yelandur, Karnataka	*Alternaria alternate*
20.	#1048AMSTYEL	*Aegle marmelos*	Stem	Yelandur, Karnataka	*Lasiodiplodia pseudotheobromae*
21.	#1013AMSTITYEL	*Aegle marmelos*	Stem internal tissue	Yelandur, Karnataka	*Sphaeropsis sapinea*
22.	#1010AMSTITYEL	*Aegle marmelos*	Stem internal tissue	Yelandur, Karnataka	*Fusarium oxysporum*
23.	#1011AMSTITYEL	*Aegle marmelos*	Stem internal tissue	Yelandur, Karnataka	*Penicillium chrysogenum*
24.	#1058AMSTITYEL	*Aegle marmelos*	Stem internal tissue	Yelandur, Karnataka	*Fusarium semitectum*
25.	#1069AMSTITYEL	*Aegle marmelos*	Stem internal tissue	Yelandur, Karnataka	*Fusarium equiseti*
26.	#1070AMSTITYEL	*Aegle marmelos*	Stem internal tissue	Yelandur, Karnataka	*Fusarium equiseti*
27.	#1022AMSTITYEL	*Aegle marmelos*	Stem internal tissue	Yelandur, Karnataka	*Fusarium lateritium*
28.	#17AMLBRT	*Aegle marmelos*	Leaf	BRT wildlife sanctuary	*Fusarium oxysporum*
29.	#1006AMLBRT	*Aegle marmelos*	Leaf	BRT wildlife sanctuary	*Fusarium commune*
30.	#1007AMLBRT	*Aegle marmelos*	Leaf	BRT wildlife sanctuary	*Fusarium commune*
31.	#1016AMLBRT	*Aegle marmelos*	Leaf	BRT wildlife sanctuary	*Fusarium chlamydosporum*
32.	#1017AMLBRT	*Aegle marmelos*	Leaf	BRT wildlife sanctuary	*Fusarium semitectum*
33.	#4AMLBRT	*Aegle marmelos*	Leaf	BRT wildlife sanctuary	*Fusarium oxysporum*
34.	#9AMLBRT	*Aegle marmelos*	Leaf	BRT wildlife sanctuary	*Fusarium culmorum*
35.	#1005AMLBRT	*Aegle marmelos*	Leaf	BRT wildlife sanctuary	*Alternaria marmelos*
36.	#1004AMLBRT	*Aegle marmelos*	Leaf	BRT wildlife sanctuary	*Botryosphaeria stevensii*
37.	#59AMSTWLS	*Aegle marmelos*	Stem	Wayanad, Kerala	*Botryosphaeria stevensii*
38.	#27AMSTWLS	*Aegle marmelos*	Stem	Wayanad, Kerala	*Alternaria alternate*
39.	#28AMSTWLS	*Aegle marmelos*	Stem	Wayanad, Kerala	*Botryosphaeria* sp.
40.	#32AMSTWLS	*Aegle marmelos*	Stem	Wayanad, Kerala	*Phaeoacremonium rubrigenum*

41.	#25AMSTWLS	*Aegle marmelos*	Stem	Wayanad, Kerala	*Alternaria solani*
42.	#20AMSTWLS	*Aegle marmelos*	Stem	Wayanad, Kerala	Unidentified
43.	#37(a)AMSTWLS	*Aegle marmelos*	Stem	Wayanad, Kerala	*Phaeoacremonium rubrigenum*
44.	#37(b)AMSTWLS	*Aegle marmelos*	Stem	Wayanad, Kerala	*Trichoderma viride*
45.	#39AMSTWLS	*Aegle marmelos*	Stem	Wayanad, Kerala	*Botryosphaeria* sp.
46.	#1104AMSTITWLS	*Aegle marmelos*	Stem internal tissue	Wayanad, Kerala	*Lasiodiplodia gonubiensis*
47.	#1111AMSTITWLS	*Aegle marmelos*	Stem internal tissue	Wayanad, Kerala	*Barriopsis iraniana*
48.	#1079AMSTWLS	*Aegle marmelos*	Stem internal tissue	Wayanad, Kerala	*Lasiodiplodia theobromae*
49.	#1082AMSTITWLS	*Aegle marmelos*	Stem internal tissue	Wayanad, Kerala	*Lasiodiplodia theobromae*
50.	#1088AMSTITWLS	*Aegle marmelos*	Stem internal tissue	Wayanad, Kerala	*Lasiodiplodia pseudotheobromae*
51.	#42AMSTITWLS	*Aegle marmelos*	Stem internal tissue	Wayanad, Kerala	*Phomopsis* sp.
52.	#61AMLWLS	*Aegle marmelos*	Leaf	Wayanad, Kerala	*Trichoderma viride*
53.	#6AMLWLS	*Aegle marmelos*	Leaf	Wayanad, Kerala	*Fusarium incarnatum*
54.	#16AMLWLS	*Aegle marmelos*	Leaf	Wayanad, Kerala	*Muscodor kashayum*
55.	#11AMBAWLS	*Aegle marmelos*	Bark	Wayanad, Kerala	*Aureobasdium* sp.
56.	#22AMBAWLS	*Aegle marmelos*	Bark	Wayanad, Kerala	*Aspergillus niger*
57.	#23AMBAWLS	*Aegle marmelos*	Bark	Wayanad, Kerala	*Penicillium chrysogenum*
58.	#24AMBAWLS	*Aegle marmelos*	Bark	Wayanad, Kerala	*Fusarium solani*
59.	#29AMBAWLS	*Aegle marmelos*	Bark	Wayanad, Kerala	*Trichoderma viride*
60.	#33AMSTWLS	*Aegle marmelos*	Bark	Wayanad, Kerala	*Penicillium rubrigenum*
61.	#2004AMBARS	*Aegle marmelos*	Bark	Rohini Sukhna, WB	*Aspergillus niger*
62.	#2013AMBARS	*Aegle marmelos*	Bark	Rohini Sukhna, WB	*Penicillium chrysogenum*

63.	#2102AMBARS	*Aegle marmelos*	Bark	Rohini Sukhna, WB	*Fusarium semitectum*
64.	#18CMBANEY	*Cinnamomum malabaricum*	Bark	Neyyar,Kerala	*Botryosphaeria* sp.
65.	#2CMBANEY	*Cinnamomum malabaricum*	Bark	Neyyar,Kerala	Unidentified
66.	#14CMBANEY	*Cinnamomum malabaricum*	Bark	Neyyar,Kerala	*Alternaria* sp.
67.	#12CMBANEY	*Cinnamomum malabaricum*	Bark	Neyyar,Kerala	*Botryosphaeria* sp.
68.	#20CMBANEY	*Cinnamomum malabaricum*	Bark	Neyyar,Kerala	*Botryosphaeria* sp.
69.	#4CMBABRT	*Cinnamomum malabaricum*	Bark	BRT wildlife sanctuary	*Bionectria* sp.
70.	#9CMBABRT	*Cinnamomum malabaricum*	Bark	BRT wildlife sanctuary	Unidentified
71.	#12CMBABRT	*Cinnamomum malabaricum*	Bark	BRT wildlife sanctuary	*Clonostachys* sp.
72.	#16CMBABRT	*Cinnamomum malabaricum*	Bark	BRT wildlife sanctuary	*Bionectria* sp.
73.	#18CMBABRT	*Cinnamomum malabaricum*	Bark	BRT wildlife sanctuary	*Clonostachys* sp.
74.	#28CMBABRT	*Cinnamomum malabaricum*	Bark	BRT wildlife sanctuary	Unidentified
75.	#2CMLNEY	*Cinnamomum malabaricum*	Leaf	Neyyar,Kerala	Unidentified
76.	#37CMLNEY	*Cinnamomum malabaricum*	Leaf	Neyyar,Kerala	*Bionectria* sp.
77.	#1CMLNEY	*Cinnamomum malabaricum*	Leaf	Neyyar,Kerala	Unidentified
78.	#31CMLNEY	*Cinnamomum malabaricum*	Leaf	Neyyar,Kerala	*Clonostachys* sp.
79.	#29CMLNEY	*Cinnamomum malabaricum*	Leaf	Neyyar,Kerala	*Bionectria* sp.
80.	#4CMLBRT	*Cinnamomum malabaricum*	Leaf	BRT wildlife sanctuary	Unidentified
81.	#40CMLBRT	*Cinnamomum malabaricum*	Leaf	BRT wildlife sanctuary	Unidentified
82.	#27CMLBRT	*Cinnamomum malabaricum*	Leaf	BRT wildlife sanctuary	Unidentified
83.	#44CMSTNEY	*Cinnamomum malabaricum*	Stem	Neyyar,Kerala	*Arthrinium* sp.
84.	#4CMSTNEY	*Cinnamomum malabaricum*	Stem	Neyyar,Kerala	*Arthrinium* sp.

85.	#1622CMSTITNEY	*Cinnamomum malabaricum*	Stem internal tissue	Neyyar,Kerala	*Curvularia* sp.
86.	#54(b)CMSTITNEY	*Cinnamomum malabaricum*	Stem internal tissue	Neyyar,Kerala	*Pestalotiopsis* sp.
87.	#96CMSTITNEY	*Cinnamomum malabaricum*	Stem internal tissue	Neyyar, Kerala	*Pestalotiopsis* sp.
88.	#21CMSTITNEY	*Cinnamomum malabaricum*	Stem internal tissue	Neyyar,Kerala	*Arthrinium* sp.
89.	#79CMSTITNEY	*Cinnamomum malabaricum*	Stem internal tissue	Neyyar,Kerala	*Curvularia* sp.
90.	#1CMSTITBRT	*Cinnamomum malabaricum*	Stem internal tissue	BRT wildlife sanctuary	*Fusarium* sp.
91.	#1CMLBRT	*Cinnamomum malabaricum*	Leaf	BRT wildlife sanctuary	Unidentified
92.	#2CMLBRT	*Cinnamomum malabaricum*	Leaf	BRT wildlife sanctuary	Unidentified
93.	#1CSSTOT	*Camellia sinensis*	Stem	Ooty, Tamil Nadu	*Schizophyllum* sp.
94.	#2CSSTOT	*Camellia sinensis*	Stem	Ooty, Tamil Nadu	*Schizophyllum* sp.
95.	#4CSSTOT	*Camellia sinensis*	Stem	Ooty, Tamil Nadu	*Colletotrichum* sp.
96.	# 5CSSTOT	*Camellia sinensis*	Stem	Ooty, Tamil Nadu	*Botryosphaeria* sp.
97.	# 6CSSTOT	*Camellia sinensis*	Stem	Ooty, Tamil Nadu	*Schizophyllum* sp.
98.	# 7CSSTOT	*Camellia sinensis*	Stem	Ooty, Tamil Nadu	*Botryosphaeria* sp.
99.	#1(a)WGSTNEY	*Wild ginger*	Stem	Neyyar,Kerala	*Pestalotiopsis* sp.
100.	#1(b)WGSTNEY	*Wild ginger*	Stem	Neyyar,Kerala	*Colletotrichum* sp.
101.	#7 WGSTNEY	*Wild ginger*	Stem	Neyyar,Kerala	*Colletotrichum* sp.
102.	#15(a)WGSTNEY	*Wild ginger*	Stem	Neyyar,Kerala	*Fusarium* sp.
103.	#11WGSTNEY	*Wild ginger*	Stem	Neyyar,Kerala	*Pestalotiopsis* sp.
104.	#12WGSTNEY	*Wild ginger*	Stem	Neyyar,Kerala	*Pestalotiopsis* sp.
105.	#13WGSTNEY	*Wild ginger*	Stem	Neyyar,Kerala	*Fusarium* sp.
106.	#14(a)WGSTNEY	*Wild ginger*	Stem	Neyyar,Kerala	*Fusarium* sp.

107.	#14(b)WGSTNEY	*Wild ginger*	Stem	Neyyar,Kerala	*Fusarium* sp.
108.	#1RSBANEY	*Rauwolfia serpentina*	Bark	Neyyar,Kerala	Unidentified
109.	#14RSBANEY	*Rauwolfia serpentina*	Bark	Neyyar,Kerala	*Fusarium* sp.
110.	#16RSBANEY	*Rauwolfia serpentina*	Bark	Neyyar,Kerala	Unidentified
111.	#7RSLBRT	*Rauwolfia serpentina*	Leaf	BRT wildlife sanctuary	*Fusarium* sp.
112.	#10RSLBRT	*Rauwolfia serpentina*	Leaf	BRT wildlife sanctuary	*Fusarium* sp.
113.	#11RSLBRT	*Rauwolfia serpentina*	Leaf	BRT wildlife sanctuary	*Fusarium* sp.
114.	#15RSLBRT	*Rauwolfia serpentina*	Leaf	BRT wildlife sanctuary	*Fusarium* sp.
115.	#16RSLBRT	*Rauwolfia serpentina*	Leaf	BRT wildlife sanctuary	*Fusarium* sp.
116.	#1CCBD	*Cinnamomum camphora*	Bark	Darjeeling, West Bengal	*Fusarium* sp.
117.	#1CCSTITD	*Cinnamomum camphora*	Stem internal tissue	Darjeeling, West Bengal	*Muscodor darjeelingensis*
118.	# 2CCSTITD	*Cinnamomum camphora*	Stem internal tissue	Darjeeling, West Bengal	*Muscodor tigerii*
119.	#1639CCSTITD	*Cinnamomum camphora*	Stem internal tissue	Darjeeling, West Bengal	*Muscodor* sp.
120.	#5CZBAWLS	*Cinnamomum zeylanicum*	Bark	Wayanad, Kerala	*Fusarium* sp.
121.	#28CZBAWLS	*Cinnamomum zeylanicum*	Bark	Wayanad, Kerala	*Curvularia* sp.
122.	#2104CZSTITG	*Cinnamomum zeylanicum*	Stem internal tissue	Guwahati, Assam	*Alternaria* sp.
123.	#2106CZSTITG	*Cinnamomum zeylanicum*	Stem internal tissue	Guwahati, Assam	*Curvularia* sp.
124.	#2112CZSTITG	*Cinnamomum zeylanicum*	Stem internal tissue	Guwahati, Assam	*Nigrospora* sp.
125.	#2116CZSTITG	*Cinnamomum zeylanicum*	Stem internal tissue	Guwahati, Assam	*Alternaria* sp.
126.	#2117CZSTITG	*Cinnamomum zeylanicum*	Stem internal tissue	Guwahati, Assam	*Alternaria* sp.
127.	#2122CZSTITG	*Cinnamomum zeylanicum*	Stem internal tissue	Guwahati, Assam	Unidentified
128.	#2123CZSTITG	*Cinnamomum zeylanicum*	Stem internal tissue	Guwahati, Assam	Unidentified

129.	#2125CZSTITG	*Cinnamomum zeylanicum*	Stem internal tissue	Guwahati, Assam	Unidentified
130.	#2130CZSTITG	*Cinnamomum zeylanicum*	Stem internal tissue	Guwahati, Assam	Unidentified
131.	#2131CZSTITG	*Cinnamomum zeylanicum*	Stem internal tissue	Guwahati, Assam	Unidentified
132.	#2143CZSTITG	*Cinnamomum zeylanicum*	Stem internal tissue	Guwahati, Assam	*Alternaria* sp.
133.	#2158CZSTITG	*Cinnamomum zeylanicum*	Stem internal tissue	Guwahati, Assam	*Pestalotiopsis* sp.
134.	#2161CZSTITG	*Cinnamomum zeylanicum*	Stem internal tissue	Guwahati, Assam	Unidentified
135.	#2162CZSTITG	*Cinnamomum zeylanicum*	Stem internal tissue	Guwahati, Assam	*Aspergillus* sp.
136.	#2168CZSTITG	*Cinnamomum zeylanicum*	Stem internal tissue	Guwahati, Assam	*Aspergillus* sp.
137.	#16NOBASVNP	*Nerium oleander*	Bark	Silent Valley National Park, Kerala	*Gibberella* sp.
138.	#1PNLNEY	*Piper nigrum*	Leaf	Neyyar,Kerala	*Alternaria* sp.
139.	#2PNLNEY	*Piper nigrum*	Leaf	Neyyar,Kerala	*Alternaria* sp.
140.	#5PNLNEY	*Piper nigrum*	Leaf	Neyyar,Kerala	*Alternaria* sp.
141.	#7PNLNEY	*Piper nigrum*	Leaf	Neyyar,Kerala	*Alternaria* sp.
142.	#8PNLNEY	*Piper nigrum*	Leaf	Neyyar,Kerala	*Alternaria* sp.
143.	#2TMDSTYEL	*Tabernaemontana divaricata*	Stem	Yelandur, Karnataka	*Fusarium* sp.
144.	#4TMDSTYEL	*Tabernaemontana divaricata*	Stem	Yelandur, Karnataka	*Fusarium* sp.
145.	#6TBBALM	*Taxus baccata*	Bark	Almorah, Uttrakhand	Unidentified
146.	#7TBBALM	*Taxus baccata*	Bark	Almorah, Uttrakhand	Mycelia sterile
147.	#8TBBALM	*Taxus baccata*	Bark	Almorah, Uttrakhand	*Pestalotiopsis* sp.
148.	#11TBBALM	*Taxus baccata*	Bark	Almorah, Uttrakhand	Unidentified
149.	#13TBBALM	*Taxus baccata*	Bark	Almorah, Uttrakhand	Unidentified

150.	#14TBBALM	*Taxus baccata*	Bark	Almorah, Uttrakhand	Unidentified
151.	#17TBBALM	*Taxus baccata*	Bark	Almorah, Uttrakhand	*Fusarium* solani
152.	#20TBBALM	*Taxus baccata*	Bark	Almorah, Uttrakhand	*Alternaria* sp.
153.	#21TBBALM	*Taxus baccata*	Bark	Almorah, Uttrakhand	*Phomopsis* sp.
154.	#22TBBALM	*Taxus baccata*	Bark	Almorah, Uttrakhand	Unidentified
155.	#25TBBALM	*Taxus baccata*	Bark	Almorah, Uttrakhand	*Phomopsis* sp.
156.	#26TBBALM	*Taxus baccata*	Bark	Almorah, Uttrakhand	Unidentified
157.	#27TBBALM	*Taxus baccata*	Bark	Almorah, Uttrakhand	Unidentified
158.	#28TBBALM	*Taxus baccata*	Bark	Almorah, Uttrakhand	Unidentified
159.	#30TBBALM	*Taxus baccata*	Bark	Almorah, Uttrakhand	Unidentified
160.	#31TBBALM	*Taxus baccata*	Bark	Almorah, Uttrakhand	*Ascobolus* sp.
161.	#33TBBALM	*Taxus baccata*	Bark	Almorah, Uttrakhand	Mycelia sterile
162.	#35TBBALM	*Taxus baccata*	Bark	Almorah, Uttrakhand	*Phomopsis* sp.
163.	#36TBBALM	*Taxus baccata*	Bark	Almorah, Uttrakhand	*Alternaria* sp.
164.	#40TBBALM	*Taxus baccata*	Bark	Almorah, Uttrakhand	*Pestalotiopsis* sp.
165.	#41TBBALM	*Taxus baccata*	Bark	Almorah, Uttrakhand	*Pestalotiopsis* sp.
166.	#42TBBALM	*Taxus baccata*	Bark	Almorah, Uttrakhand	*Pestalotiopsis* sp.
167.	#43TBBALM	*Taxus baccata*	Bark	Almorah, Uttrakhand	*Fusarium* sp.
168.	#44TBBALM	*Taxus baccata*	Bark	Almorah, Uttrakhand	Unidentified
169.	#46TBBALM	*Taxus baccata*	Bark	Almorah, Uttrakhand	*Ascobolus* sp.
170.	#48TBBALM	*Taxus baccata*	Bark	Almorah, Uttrakhand	Unidentified
171.	#50TBBALM	*Taxus baccata*	Bark	Almorah, Uttrakhand	Unidentified

172.	#53TBBALM	*Taxus baccata*	Bark	Almorah, Uttrakhand	Unidentified
173.	#54TBBALM	*Taxus baccata*	Bark	Almorah, Uttrakhand	*Nectaria* sp.
174.	#57TBBALM	*Taxus baccata*	Bark	Almorah, Uttrakhand	*Penicillium* sp.
175.	#65TBBALM	*Taxus baccata*	Bark	Almorah, Uttrakhand	*Pestalotiopsis* sp.
176.	#67TBBALM	*Taxus baccata*	Bark	Almorah, Uttrakhand	Unidentified
177.	#69TBBALM	*Taxus baccata*	Bark	Almorah, Uttrakhand	Unidentified
178.	#73TBBALM	*Taxus baccata*	Bark	Almorah, Uttrakhand	Unidentified
179.	#80TBBALM	*Taxus baccata*	Bark	Almorah, Uttrakhand	Unidentified
180.	#89TBBALM	*Taxus baccata*	Bark	Almorah, Uttrakhand	Unidentified
181.	#91TBBALM	*Taxus baccata*	Bark	Almorah, Uttrakhand	*Phomopsis* sp.
182.	#93TBBALM	*Taxus baccata*	Bark	Almorah, Uttrakhand	*Pestalotiopsis* sp.
183.	#94TBBALM	*Taxus baccata*	Bark	Almorah, Uttrakhand	Unidentified
184.	#98TBBALM	*Taxus baccata*	Bark	Almorah, Uttrakhand	*Penicillium* sp.
185.	#100TBBALM	*Taxus baccata*	Bark	Almorah, Uttrakhand	*Penicillium* sp.
186.	#113TBBALM	*Taxus baccata*	Bark	Almorah, Uttrakhand	*Pestalotiopsis* sp.
187.	#114TBBALM	*Taxus baccata*	Bark	Almorah, Uttrakhand	*Penicillium* sp.
188.	#118TBBALM	*Taxus baccata*	Bark	Almorah, Uttrakhand	Unidentified
189.	#120TBBALM	*Taxus baccata*	Bark	Almorah, Uttrakhand	*Pestalotiopsis* sp.
190.	#405TBBALM	*Taxus baccata*	Bark	Almorah, Uttrakhand	*Phomopsis* sp.

Table 5.2 Volume of cell free culture filtrate, its pH and biomass of endophytic fungi produced

S.No.	Culture code	Fungal mass (g)/25 ml*	Vol. of culture filtrate (ml)*	pH*
1.	#2AMSTYEL	1.11±0.09	15.8±0.22	7.9±0.03
2.	#3AMSTYEL	0.93±0.01	16.1±0.18	4.67±0.02
3.	#4AMSTYEL	1.04±0.03	16.2±0.15	5.12±0.02
4.	#5AMSTYEL	0.85±0.02	16.8±0.12	4.33±0.03
5.	#6AMSTYEL	1.45±0.08	16.7±0.22	4.98±0.07
6.	#7AMSTYEL	1.21±0.06	16.2±0.14	4.68±0.01
7.	#7(a)AMSTYEL	1.66±0.03	15.1±0.17	7.12±0.02
8.	#8AMSTYEL	1.92±0.03	14.4±0.23	6.23±0.03
9.	#9AMSTYEL	0.45±0.06	19.7±0.28	5.59±0.02
10.	#17AMSTYEL	1.65±0.02	14.9±0.11	6.92±0.02
11.	#22AMSTYEL	1.42±0.01	16.4±0.16	3.11±0.04
12.	#32AMSTYEL	0.65±0.01	18.5±0.15	5.67±0.03
13.	#1003AMSTYEL	1.34±0.04	14.3±0.09	4.53±0.01
14.	#23AMSTYEL	1.26±0.10	14.1±0.21	4.5±0.00
15.	#1032AMSTYEL	1.54±0.02	16.3±0.18	8.12±0.01
16.	#23(b)AMSTYEL	0.77±0.03	17.8±0.19	3.92±0.01
17.	#9(b)AMSTYEL	0.72±0.01	18.1±0.07	7.67±0.02
18.	#15AMSTYEL	1.12±0.04	16.5±0.11	8.01±0.01
19.	#18AMSTYEL	1.31±0.05	16.2±0.13	5.66±0.02
20.	#1048AMSTYEL	1.87±0.02	17.1±0.18	6.54±0.01
21.	#1013AMSTITYEL	1.79±0.03	14.6±0.14	5.76±0.01
22.	#1010AMSTITYEL	0.91±0.01	18.4±0.15	5.54±0.03
23.	#1011AMSTITYEL	0.87±0.02	18.5±0.12	4.91±0.02
24.	#1058AMSTITYEL	1.43±0.06	17.9±0.13	7.64±0.01
25.	#1069AMSTITYEL	1.36±0.03	17.5±0.13	5.5±0.04
26.	#1070AMSTITYEL	1.77±0.02	14.9±0.16	4.81±0.02
27.	#1079AMSTWLS	0.34±0.01	18.2±0.17	4.73±0.01
28.	#1022AMSTITYEL	0.37±0.05	18.5±0.18	5.12±0.02
29.	#17AMLBRT	0.78±0.07	17.4±0.12	5.73±0.01
30.	#1006AMLBRT	1.14±0.01	17.1±0.14	5.92±0.02
31.	#1007AMLBRT	0.87±0.02	17.3±0.12	5.67±0.01
32.	#1016AMLBRT	0.76±0.02	18.2±0.16	6.02±0.02
33.	#1017AMLBRT	0.95±0.01	17.9±0.11	6.13±0.01
34.	#4AMLBRT	0.57±0.05	16.3±0.13	4.89±0.03
35.	#9AMLBRT	0.49±0.02	18.3±0.12	6.72±0.02
36.	#1005AMLBRT	1.10±0.03	16.8±0.15	6.04±0.01
37.	#1004AMLBRT	0.67±0.04	17.4±0.14	6.52±0.01
38.	#59AMSTWLS	0.75±0.06	17.2±0.11	5.31±0.04
39.	#27AMSTWLS	0.34±0.05	17.5±0.12	6.34±0.03

40.	#28AMSTWLS	0.57±0.05	17.3±0.14	6.98±0.02
41.	#32AMSTWLS	1.35±0.02	15.9±0.16	5.81±0.02
42.	#33AMSTWLS	1.15±0.03	18.9±0.13	6.01±0.01
43.	#25AMSTWLS	1.76±0.02	18.6±0.13	4.89±0.03
44.	#20AMSTWLS	1.79±0.01	19.1±0.14	4.76±0.01
45.	#37(a)AMSTWLS	1.23±0.07	18.6±0.14	5.43±0.03
46.	#37(b)AMSTWLS	0.59±0.02	19.3±0.15	5.12±0.02
47.	#39AMSTWLS	0.41±0.03	18.6±0.13	4.23±0.04
48.	#1104AMSTITWLS	0.92±0.01	15.9±0.14	4.78±0.03
49.	#1088AMSTITWLS	0.76±0.03	18.1±0.14	5.03±0.02
50.	#1111 AMSTITWLS	1.12±0.01	15.9±0.11	4.83±0.01
51.	#1082 AMSTITWLS	0.81±0.02	16.3±0.08	5.53±0.03
52.	#42AMSTITWLS	1.15±0.01	16.9±0.13	6.42±0.02
53.	#61AMLWLS	1.04±0.02	17.3±0.12	3.91±0.01
54.	#6AMLWLS	0.98±0.01	20.6±0.21	6.4±0.02
55.	#16AMLWLS	0.87±0.01	16.1±0.18	5.52±0.01
56.	#11AMBAWLS	1.21±0.01	14.3±0.16	4.91±0.09
57.	#22AMBAWLS	0.97±0.02	15.8±0.16	7.60±0.01
58.	#23AMBAWLS	0.59±0.01	16.9±0.12	4.60±0.02
59.	#24AMBAWLS	1.06±0.04	15.3±0.13	4.41±0.02
60.	#29AMBAWLS	1.14±0.02	15.4±0.16	4.83±0.01
61.	#2004AMBARS	1.32±0.00	15.9±0.16	5.87±0.02
62.	#2013AMBARS	1.42±0.10	13.3±0.12	4.69±0.01
63.	#2102AMBARS	0.56±0.02	20.0±0.13	3.54±0.02
64.	#18CMBANEY	1.37±0.01	19.6±0.12	4.44±0.02
65.	#2CMBANEY	0.96±0.11	17.8±0.15	4.57±0.01
66.	#14CMBANEY	1.14±0.01	18.1±0.12	3.91±0.02
67.	#12CMBANEY	1.36±0.01	18.6±0.17	6.03±0.03
68.	#20CMBANEY	0.66±0.05	19.3±0.13	5.06±0.04
69.	#4CMBABRT	0.52±0.03	19.4±0.22	3.98±0.05
70.	#9CMBABRT	0.71±0.02	19.8±0.14	4.52±0.01
71.	#12CMBABRT	1.23±0.01	18.3±0.13	5.87±0.01
72.	#16CMBABRT	0.34±0.04	17.2±0.14	5.69±0.02
73.	#18CMBABRT	0.53±0.05	16.9±0.17	4.65±0.02
74.	#28CMBABRT	1.35±0.09	16.5±0.15	6.32±0.08
75.	#2CMLNEY	1.42±0.04	17.6±0.15	7.45±0.02
76.	#37CMLNEY	1.25±0.05	17.7±0.18	4.42±0.05
77.	#1CMLNEY	0.58±0.08	18.2±0.12	3.98±0.04
78.	#31CMLNEY	1.22±0.07	15.9±0.11	4.01±0.04
79.	#29CMLNEY	0.77±0.02	16.6±0.18	5.43±0.03
80.	#4CMLBRT	0.87±0.01	14.3±0.12	4.49±0.07

81.	#40CMLBRT	0.56±0.09	16.1±0.13	4.19±0.08
82.	#27CMLBRT	1.05±0.02	17.4±0.12	6.62±0.02
83.	#44CMSTNEY	1.66±0.01	15.9±0.12	4.45±0.00
84.	#4CMSTNEY	1.32±0.01	16.2±0.11	3.98±0.04
85.	#1622CMSTITNEY	1.41±0.02	15.7±0.12	5.67±0.02
86.	#54(b)CMSTITNEY	0.89±0.03	16.4±0.16	4.83±0.03
87.	#96CMSTITNEY	0.78±0.04	17.1±0.12	7.75±0.02
88.	#21CMSTITNEY	0.64±0.05	16.2±0.12	7.53±0.03
89.	#79CMSTITNEY	1.12±0.03	15.8±0.14	7.41±0.04
90.	#1CMSTITBRT	1.06±0.02	16.3±0.12	6.92±0.01
91.	#1CMLBRT	0.82±0.02	17.3±0.13	6.83±0.02
92.	#2CMLBRT	0.57±0.03	16.4±0.13	4.42±0.01
93.	#1CSSTOT	1.54±0.01	15.7±0.14	3.94±0.02
94.	#2CSSTOT	1.65±0.04	14.9±0.19	4.86±0.03
95.	#4CSSTOT	1.43±0.04	15.0±0.18	4.65±0.02
96.	# 5CSSTOT	1.32±0.01	16.7±0.19	4.58±0.04
97.	# 6CSSTOT	0.87±0.01	16.9±0.16	3.93±0.03
98.	# 7CSSTOT	0.76±0.02	16.4±0.14	3.74±0.02
99.	#1(a)WGSTNEY	0.69±0.03	18.1±0.20	4.32±0.01
100.	#1(b)WGSTNEY	0.54±0.07	16.9±0.12	4.56±0.01
101.	#7 WGSTNEY	1.23±0.05	17.4±0.13	4.31±0.02
102.	#15(a)WGSTNEY	1.64±0.04	18.2±0.12	4.34±0.03
103.	#11WGSTNEY	1.42±0.06	19.1±0.08	3.32±0.02
104.	#12WGSTNEY	1.57±0.02	20.2±0.17	3.67±0.04
105.	#13WGSTNEY	1.43±0.04	16.3±0.18	3.74±0.07
106.	#14(a)WGSTNEY	1.91±0.02	16.4±0.13	3.87±0.04
107.	#14(b)WGSTNEY	2.01±0.08	15.7±0.09	5.43±0.02
108.	#1RSBANEY	1.34±0.03	18.2±0.13	5.64±0.03
109.	#14RSBANEY	1.42±0.04	15.2±0.15	4.78±0.01
110.	#16RSBANEY	1.53±0.02	17.7±0.16	3.92±0.06
111.	#7RSLBRT	1.15±0.01	16.3±0.19	4.65±0.01
112.	#10RSLBRT	1.67±0.01	16.9±0.13	4.12±0.01
113.	#11RSLBRT	1.73±0.02	17.2±0.14	3.87±0.01
114.	#15RSLBRT	1.82±0.03	13.1±0.17	7.13±0.02
115.	#16RSLBRT	0.96±0.05	13.6±0.19	7.45±0.02
116.	#1CCBD	0.74±0.02	16.4±0.21	4.56±0.01
117.	#1CCSTITD	1.37±0.01	14.3±0.11	4.89±0.01
118.	# 2CCSTITD	1.42±0.02	17.8±0.14	7.12±0.02
119.	#1639CCSTITD	0.83±0.01	17.4±0.17	7.34±0.01
120.	#5CZBAWLS	0.45±0.02	17.5±0.13	6.96±0.03
121.	#28CZBAWLS	1.56±0.03	17.3±0.16	6.45±0.03

122.	#2104CZSTITG	1.89±0.01	16.9±0.12	5.43±0.04
123.	#2106CZSTITG	1.41±0.01	16.2±0.15	5.79±0.05
124.	#2112CZSTITG	1.35±0.01	16.4±0.13	4.32±0.02
125.	#2116CZSTITG	0.69±0.03	16.8±0.14	7.13±0.05
126.	#2117CZSTITG	0.76±0.03	18.1±0.17	5.44±0.03
127.	#2122CZSTITG	1.22±0.02	17.6±0.18	5.96±0.04
128.	#2123CZSTITG	1.43±0.01	14.5±0.11	4.37±0.02
129.	#2125CZSTITG	0.65±0.07	15.4±0.12	3.86±0.02
130.	#2130CZSTITG	0.92±0.06	15.9±0.13	3.92±0.02
131.	#2131CZSTITG	1.44±0.06	16.3±0.14	3.73±0.01
132.	#2143CZSTITG	1.32±0.08	18.7±0.15	4.78±0.01
133.	#2158CZSTITG	1.27±0.07	20.0±0.17	4.14±0.06
134.	#2161CZSTITG	1.61±0.05	16.7±0.13	5.78±0.04
135.	#2162CZSTITG	1.43±0.04	17.2±0.17	5.69±0.04
136.	#2168CZSTITG	1.45±0.02	17.4±0.16	7.32±0.01
137.	#16NOBASVNP	0.79±0.06	18.1±0.13	6.42±0.02
138.	#1PNLNEY	0.41±0.02	18.4±0.13	6.18±0.01
139.	#2PNLNEY	1.39±0.01	18.2±0.14	4.37±0.03
140.	#5PNLNEY	1.45±0.02	16.9±0.12	5.59±0.03
141.	#7PNLNEY	0.92±0.01	16.3±0.14	6.62±0.01
142.	#8PNLNEY	0.61±0.03	15.1±0.14	4.39±0.01
143.	#2TMDSTYEL	1.54±0.02	14.9±0.15	5.77±0.02
144.	#4TMDSTYEL	1.48±0.01	15.9±0.19	6.89±0.04
145.	#6TBBALM	1.01±0.01	18.1±0.20	5.14±0.03
146.	#7TBBALM	0.98±0.04	19.2±0.13	5.02±0.01
147.	#8TBBALM	1.24±0.02	16.4±0.15	4.56±0.03
148.	#11TBBALM	1.14±0.01	16.2±0.14	6.70±0.03
149.	#13TBBALM	1.13±0.01	16.2±0.12	5.41±0.05
150.	#14TBBALM	0.61±0.02	20.3±0.15	5.52±0.05
151.	#17TBBALM	0.76±0.01	18.9±0.12	5.02±0.07
152.	#20TBBALM	0.73±0.01	17.9±0.13	6.03±0.06
153.	#21TBBALM	0.64±0.03	18.6±0.12	5.85±0.04
154.	#22TBBALM	0.78±0.04	19.4±0.14	5.75±0.03
155.	#25TBBALM	0.66±0.01	19.2±0.12	5.03±0.02
156.	#26TBBALM	1.32±0.02	15.7±0.15	5.91±0.05
157.	#27TBBALM	0.49±0.01	20.4±0.12	4.92±0.07
158.	#28TBBALM	0.65±0.02	18.1±0.15	5.81±0.06
159.	#30TBBALM	1.39±0.01	17.9±0.17	5.32±0.04
160.	#31TBBALM	0.19±0.05	20.3±0.13	5.07±0.01
161.	#33TBBALM	0.72±0.05	18.9±0.12	6.02±0.02
162.	#35TBBALM	0.74±0.02	18.9±0.12	5.43±0.03

163.	#36TBBALM	1.24±0.01	16.5±0.15	5.16±0.04
164.	#40TBBALM	1.21±0.03	16.8±0.14	5.66±0.03
165.	#41TBBALM	0.58±0.04	18.9±0.16	5.63±0.07
166.	#42TBBALM	0.56±0.02	19.3±0.13	5.51±0.04
167.	#43TBBALM	0.41±0.01	19.3±0.14	4.82±0.01
168.	#44TBBALM	0.91±0.05	18.8±0.12	6.02±0.01
169.	#46TBBALM	0.47±0.06	20.1±0.15	4.91±0.05
170.	#48TBBALM	0.44±0.07	19.7±0.17	6.54±0.05
171.	#50TBBALM	0.82±0.04	18.5±0.16	5.92±0.02
172.	#53TBBALM	0.13±0.02	20.3±0.12	5.64±0.01
173.	#54TBBALM	0.27±0.01	19.8±0.12	4.92±0.06
174.	#57TBBALM	1.03±0.01	19.4±0.13	5.81±0.07
175.	#65TBBALM	0.44±0.04	19.3±0.12	5.46±0.01
176.	#67TBBALM	1.24±0.04	15.8±0.14	5.32±0.02
177.	#69TBBALM	0.87±0.05	16.4±0.15	5.79±0.04
178.	#73TBBALM	1.14±0.01	16.8±0.16	6.01±0.08
179.	#80TBBALM	0.77±0.03	17.1±0.17	5.49±0.09
180.	#89TBBALM	1.21±0.01	17.2±0.14	5.34±0.02
181.	#91TBBALM	0.54±0.02	18.8±0.13	6.08±0.04
182.	#93TBBALM	0.81±0.04	18.4±0.17	5.70±0.02
183.	#94TBBALM	0.79±0.02	18.1±0.13	6.03±0.01
184.	#98TBBALM	1.32±0.01	17.9±0.15	5.22±0.03
185.	#100TBBALM	1.64±0.04	17.2±0.14	5.42±0.02
186.	#113TBBALM	0.97±0.02	18.3±0.12	5.97±0.01
187.	#114TBBALM	0.42±0.03	18.2±0.11	6.14±0.01
188.	#118TBBALM	1.36±0.05	16.5±0.09	6.32±0.01
189.	#120TBBALM	0.61±0.01	18.5±0.12	6.22±0.04
190.	#405TBBALM	0.32±0.01	18.9±0.15	5.43±0.05

*Mean ± SD of the triplicate experiment set

** The volume of media in each flask initially was 25 ml and pH was 4.5

Table 5.10 TLC analysis and PL inhibition by quantitative assay of the fractions collected from the column chromatography

Fraction No.	Solvent gradient ($CHCl_3$:CH_3OH)	TLC analysis	Yield	Inhibitory activity
1.	99:1	Blank	No Yield	No activity
2.	97:3	Blank	No Yield	No activity
3.	95:5	Blank	No Yield	No activity
4.	93:7	Blank	No Yield	No activity
5.	90:7	Blank	No Yield	No activity
6.	87:13	Blank	No Yield	No activity
7.	85:15	Blank	No Yield	No activity
8.	82:18	Impurities moving with solvent front		No activity
9.	80:20	Impurities moving with solvent front		No activity
10.	79:21	Impurities moving with solvent front		No activity
11.	78:22	Impurities moving with solvent front		No activity
12.	77:23	Impurities moving with solvent front		No activity
13.	76:24	Impurities moving with solvent front	Pooled as one fraction (F0)and the yield was 184.31 mg	No activity
14.	75:25	Impurities moving with solvent front		No activity
15.	74:26	Impurities moving with solvent front		No activity
16.	73:27	Impurities moving with solvent front		No activity
17.	73:27,72:28	Impurities moving with solvent front		No activity
18.	72:28	Impurities moving with solvent front		No activity
19.	71:29	Impurities moving with solvent front		No activity
20.	70:30	Impurities moving with solvent front		No activity
21.	70:30, 69.5:30.5	Blank	No Yield	No activity
22.	69.5:30.5	Blank	No Yield	No activity
23.	69:31	Blank	No Yield	No activity
24.	69:31, 68.5:31.5	Blank	No Yield	No activity
25.	68.5:31.5	Blank	No Yield	No activity

26.	68:32	Blank	No Yield	No activity
27.	67.5:32.5	Blank	No Yield	No activity
28.	67.5:32.5, 67:33	Blank	No Yield	No activity
29.	66.5:33.5	Blank	No Yield	No activity
30.	66.5:33.5, 66:34	Blank	No Yield	No activity
31.	66:34	Blank	No Yield	No activity
32.	65.5:34.5	Blank	No Yield	No activity
33.	65.5:34.5, 65:35	Blank	No Yield	No activity
34.	65:35	Blank	No Yield	No activity
35.	64.5:35.5	Blank	No Yield	No activity
36.	64.5:35.5, 64:36	Blank	No Yield	No activity
37.	64:36	Blank	No Yield	No activity
38.	63.5:36.5	Blank	No Yield	No activity
39.	63.5:36.5, 63:37	Blank	No Yield	No activity
40.	63:37, 62.5:37.5	Blank	No Yield	No activity
41.	62.5:37.5	Blank	No Yield	No activity
42.	62:38	Blank	No Yield	No activity
43.	62:38, 61.5:38.5	Blank	No Yield	No activity
44.	61.5:38.5	Blank	No Yield	No activity
45.	61:39	Blank	No Yield	No activity
46.	61:39, 60.5:39.5	Blank	No Yield	No activity
47.	60.5:39.5	Blank	No Yield	No activity
48.	60:40	Blank	No Yield	No activity
49.	60:40, 59.5:40.5	Blank	No Yield	No activity
50.	59.5:40.5, 59:41	Blank	No Yield	No activity
51.	59:41, 58.5:41.5	Blank	No Yield	No activity
52.	58.5:41.5	Blank	No Yield	No activity

53.	58:42	Blank	No Yield	No activity
54.	58:42, 57.5:42.5	Blank	No Yield	No activity
55.	57.5:42.5	Blank	No Yield	No activity
56.	57:43	Fraction with Rf = 0.86		6.34±0.45
57.	57:43, 56.5:43.5	Fraction with Rf = 0.86		6.34±0.45
58.	56.5:43.5	Fraction with Rf = 0.86		6.34±0.45
59.	56:44	Fraction with Rf = 0.86		6.34±0.45
60.	56:44, 55.5:44.5	Fraction with Rf = 0.86		6.34±0.45
61.	55.5:44.5	Fraction with Rf = 0.86		6.34±0.45
62.	55:45	Fraction with Rf = 0.86	Pooled as one fraction (F1) having yield = 137.62 mg	6.34±0.45
63.	55:45, 54.5:45.5	Fraction with Rf = 0.86		6.34±0.45
64.	54.5:45.5	Fraction with Rf = 0.86		6.34±0.45
65.	54:46	Fraction with Rf = 0.86		6.34±0.45
66.	54:46, 53.5:46.5	Fraction with Rf = 0.86		6.34±0.45
67.	53.5:46.5	Fraction with Rf = 0.86		6.34±0.45
68.	53:47	Fraction with Rf = 0.86		6.34±0.45
69.	53:47, 52.5:47.5	Fraction with Rf = 0.86		6.34±0.45
70.	52.5:47.5	Fraction with Rf = 0.86		6.34±0.45
71.	52:48	Fraction with 2 bands Rf =0.86 and 0.74	Pooled as one fraction (F12) having yield = 6.44 mg	99.81±0.29
72.	52:48, 51.5:48.5	Fraction with 2 bands Rf =0.86 and 0.74		99.81±0.29
73.	51.5:48.5	Fraction with 2 bands Rf =0.86 and 0.74		99.81±0.29
74.	51:49	Fraction with 2 bands Rf =0.86 and 0.74		99.81±0.29
75.	51:49, 50.5:49.5	Fraction with 2 bands Rf =0.86 and 0.74		99.81±0.29
76.	50.5:49.5	Fraction with Rf = 0.74		99.81±0.29
77.	50:50	Fraction with Rf = 0.74		99.81±0.29
78.	50:50, 49.5:50.5	Fraction with Rf = 0.74		99.81±0.29
79.	49.5:50.5	Fraction with Rf = 0.74	Pooled as one fraction (F2) having yield = 60.24 mg	99.81±0.29
80.	49:51	Fraction with Rf = 0.74		99.81±0.29
81.	49:51, 48.5:51.5	Fraction with Rf = 0.74		99.81±0.29
82.	48:52	Fraction with Rf = 0.74		99.81±0.29
83.	48:52, 47.5:52.5	Fraction with Rf = 0.74		99.81±0.29
84.	47.5:52.5	Fraction with Rf = 0.74		99.81±0.29
85.	47:53	Fraction with Rf = 0.74		99.81±0.29

86.	47:53, 46.5:53.5	Blank	No Yield	No activity
87.	46.5:53.5	Blank	No Yield	No activity
88.	46:54	Blank	No Yield	No activity
89.	46:54, 45.5:54.5	Blank	No Yield	No activity
90.	45.5:54.5	Blank	No Yield	No activity
91.	45:55	Blank	No Yield	No activity
92.	45:55, 44.5:55.5	Blank	No Yield	No activity
93.	44.5:55.5	Blank	No Yield	No activity
94.	44:56	Blank	No Yield	No activity
95.	44:56, 43.5:56.5	Blank	No Yield	No activity
96.	43.5:56.5	Blank	No Yield	No activity
97.	43:57	Blank	No Yield	No activity
98.	43:57, 42.5:57.5	Blank	No Yield	No activity
99.	42:58	Blank	No Yield	No activity
100.	42:58, 41.5:58.5	Blank	No Yield	No activity
101.	41.5:58.5	Blank	No Yield	No activity
102.	41:59	Blank	No Yield	No activity
103.	41:59, 40.5:59.5	Blank	No Yield	No activity
104.	40.5:59.5	Blank	No Yield	No activity
105.	40:60	Blank	No Yield	No activity

#6AMLWLS ITS1-5.8S-ITS2 region (Accession no. KC960885.1)

5´TTCCGTAGGTGAACCTGCGGAGGGATCATTACCGAGTTTACAACTCCCAAACCCCGGGCAACATATAATCAATGCCACGGCGGA
TCCGCCCGTGCCCCGTAAAACGGGACGGCCCGCCCGAGGACCCCTAAACTCTGTTTTTAGTGGAACTTCTGAGTAAAACAAACAAA
TAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGA
ATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCT
CAAGCTCAGCTTGGTGTTGGGCTCGCGGTAACCCGCGTTCCCCAAATCGATTGGCGGTCACGTCGAGCTTCCAAGCGTAGTAATCT
CACCTCGTTACTGGTAA3´

#6AMLWLS RPBII gene, partial cds (Accession no. KM591682.1)

5´CTCTTCCGTGGCATTATAAGGCGAATGAACACTGAGCTTTCCAACTATCTCAAACGTTGCGTTGAGTCTAACCGGCATTTCAAC
CTGAACGTTGCCATCAAGCCAGGAACCCTGTCCAAsTGGTCTCAAGTACTCCCTGGCTACTGGTAACTGGGGAGATCAAAAGAAGG
CTGCAAGCTCCACAGCTGGTGTCTCTCAGGTGTTGAACAGATATACATTTGCTTCCACTCTTTCACATTTGCGACGTACCAACACA
CCCATTGGTCGTGATGGTAAGCTTGCGAAGCCTCGGCAGCTGCACAACACTCACTGGGGCTTGGTGTGCCCTGCCGAGACACCAGA
AGGTCAGGCTTGTGGTCTGGTCAAGAACTTGTCGCTGATGTGCTATGTGAGTGTGGGTTCGCCAGCTGAGCCTTTGATCGAGTTCA
TGATCAACAGAGGTATGGAAGTTGTTGAAGAATACGAACCAACAAGATATCCTCACGCAACCAAGGTTTTCGTCAACGGAAGTTGG
GTCGGCGTCCACCCTGATCCCAGGCACCTGGTGAACTCCGTCCTGGACACACGACGAAAGTCTTACGTCCAGTTCGAGGTTTCCCT
TGTTCGTGACATCCGTGACCGTGAATTCAAGATTTTCTCTGATGCAGGCCGAGTCATGAGACCAGTCTTCACAGTGCAACAAGAGG
ATGATTACGAAACCGGCATCAATAAGGGACAGCTAGTATTGACAAAGGAGCTCGTGAACAAGATTGCCCAGGAGCAGGCAGAGCCA
CCTGCTGATCCATCTGAGAAGATTGGATGGGAGGGCCTCATCCGCTCTGGAGCTGTGGAGTATCTCGACGCCGAGGAAGAAGAAAC
CGCAATGATTTGCATGACGCCTGAAGATCTCGAAATCTATCGTGAACAGAAGCAGGACGAGGTTAATCTTACAGAGGAAGAGAAGC
GGGCCAAGCAGGAAGCAGAGAAGAGGGAGCAAGAGGAAGAACGCAACAAGCGTCTGAAGACGAAGGTCAACCCCACAACTCACATG
TACACACATTGTGAGATTCATCCCAGTATGATTCTGGGTATTTGTGCCAGTATCATTCCCTTCCCTGATCACAACCAGGTATGTAT
TCCCACCCTCGATGCAAGTGAGCCTA3´

#6AMLWLS TEF-1α gene, partial cds (Accession no. KJ371936.1)

5´GACTCACCTTAACGTCGTTGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACTACTCACGATGAGATGCGTAT
CTGTAGTCATCCGCCCCGGATGGGGCGGGGTGAGTTCAACTTGATATATATGCTGACTAGATTAAAAAGACCGGTCACTTCTACCA
GTGCGGTGGTATCGACAAGCGAACCATCGAGAAGTTCGAGAAGGATGGTTTCCATTTCCCTCGATCGCACGCCCTCTACCCACCGA
TCCATCAGTCGAATCAGTTACGACGATATATGCGCCTGTTACCCCGCTCGAGTACAAAATCTTGCGGTTCAACCGTTTTTTTTGGT
GGGGTTTCAACCCCGCTACTCGAGCGACAGACGTTTGCCCTCTTCCCACAAACTCATTTCTTGTGCATCACGTGTCCATCAGCCAC
TAACCACGCGACAAGAAGCCGCCGAGCTCGGTAAGGGTTCTTTCAAGTACGCCTGGGTTCTTGACAAGCTCAAGGCTGAGCGAGAG
CGTGGTATCACCATCGATATCGCCCTCTGGAAGTTCGAGACTCCTCGCTACTATGTCACCGTCATTGGTACGTTATCATCACTTAC
ACTCAATACTTTCCCATGCCATGCACTTCAGACGCTCCCGGTCACCGTGATTTCATCAAGAACATCACTGGTACTTCCCA3′

Fig. 5.17 ITS1-5.8S-ITS2 region, RPBII (partial cds) and EF-1α (partial cds) of #6AMLWLS

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RESEARCH ARTICLE

Potential pancreatic lipase inhibitory activity of an endophytic *Penicillium* species

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Abstract

Pancreatic lipase (PL) is considered as one of the safest target for diet-induced anti-obesity drug development. Orlistat is the only PL inhibitor approved for anti-obesity treatment till date. In the process of exploration of new PL inhibitors, we have screened culture filtrates of 70 endophytic fungi of medicinal plants using qualitative as well as quantitative *in-vitro* PL assays. The qualitative assays indicated potential PL inhibition in only three isolates, namely #57 TBBALM, #33 TBBALM and #1 CSSTOT. Only ethyl acetate extracts of the culture filtrates of these isolates exhibited the PL inhibition. #57 TBBLAM ethyl acetate extract of culture filtrate exhibited potential PL inhibition with an IC_{50} of 3.69 μg/ml which was comparable to the positive control, i.e. Orlistat exhibiting IC_{50} value of 2.73 μg/ml. Further molecular phylogenetic tools and morphological studies were used to identify the isolate #57 TBBALM as *Penicillium* species.

Keywords

Endophytic fungi, ITS rDNA, *p*-nitrophenol laurate, rhodamine assay, *Taxus baccata*

History

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Introduction

Obesity is a disease resulting from improper balance between energy intake and expenditure and is increasingly becoming a major cause of preventable mortality. In the present millennium, it has become a major global health threat with more than 1 billion overweight adults of which nearly 300 million have been designated as clinically obese[1,2]. The primary cause of obesity is the sedentary lifestyle, stress and high-calorie food intake. Obesity is also a precursor to variety of serious diseases like hypertension, hyperlipidemia, atherosclerosis and type II diabetes[3]. Obesity is being recognized as a distinct medical problem hence obesity therapies are going to be an integral part of medicine and healthcare industry. Thus, targeting novel metabolic pathways for obesity treatment is a major research focus in pharmaceutical and biopharmaceutical industry.

One of the key enzymes in lipid metabolism is pancreatic lipase (PL). PL hydrolyzes approximately 50–70% of total triglycerides present in food into glycerol and fatty acids which are eventually absorbed in the small intestine. Hence, PL appears to be a valuable therapeutic target for the treatment of diet induced obesity in humans. Xenical (Orlistat, Roche, Basel, Switzerland), a PL inhibitor isolated from the actinobacterium *Streptomyces toxytricini* has become one of the best selling drugs for treatment of diet-induced obesity. It reduces approximately 30–40% of fat absorption from the meals[4]. However it possesses side effects like oily stools, flatulence, faecal urgency, abdominal cramps, leaking of oil from the rectum leading to oily spotting. The success of orlistat has stimulated research for identifying new enzyme inhibitors from natural sources which may lack some unpleasant side effects of orlistat.

The discovery of Penicillin from the fungi *Penicillium notatum* marked the golden era of antibiotics and since then fungi have been exploited extensively in development of some remarkable drugs like Cyclosporine (an immunosuppressant) and Penicillin's, Cephalosporin's (β-lactams antibiotics) and Griseofulvin (antifungal antibiotic). Fungi like *Monascus ruber* and *Aspergillus terreus* have also produced potent 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors like Mevastatin and Lovastatin, which are extensively being used as commercial cholesterol lowering drugs. Fungi also exist inside the plants without any indication of their existence. These are known as endophytic fungi. Endophytic fungi have been reported to be lucrative sources of bioactive compounds possessing antibacterial, anti-cancer, cytotoxic, neuroprotective and anti-oxidant activities some of which are under clinical trials[5].

Bio-prospecting of endophytic fungi for production of PL inhibitors is a nascent area with very scanty preliminary data. Methanol and dichloromethane extracts of some of the wood damaging mushrooms and macrofungi possess PL inhibitory activity[6]. However, endophytic fungi have not been exploited for search of PL inhibitors. We carried out an *in vitro* screening program of PL inhibition by cell-free culture filtrate of 70 endophytic fungi of plants inhabiting in the Western Ghats and north-east regions of India.

Here, we report the potential of a lead extract from the culture filtrate of endophytic fungal isolate #57 TBBALM which induced complete inhibition of PL activity and identification of the fungi using molecular and morphological approaches for its exploitation in the isolation of the PL inhibitor and its evaluation as a pharmacophore for treatment of obesity.

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Materials and methods

Isolation of endophytic fungi

The plant material (bark) was collected from conserved forest area of Western Ghats and North eastern Himalayas. Each collected sample was properly labelled and placed in a sterile bag and stored at 4 °C till further use. Isolation was done following a modified procedure described by[7]. Briefly, the plant samples were washed in running tap water for 5 min and then sequentially surface sterilized with 1% sodium hypochlorite for 3 min followed by 70% ethanol for 1 min and then 30% ethanol for 30 s. The surface sterilized plant subsequently allowed to surface dry aseptically and then cut into 1–3 mm segments with the help of sterile blade. These segments were then placed over Potato Dextrose Agar (PDA) with ventral side facing the medium and incubated at 26 ± 1 °C for 10 d with 12 h light/dark cycles. The germinating fungal hyphae were picked from the fine tipped sterile needle and sub-cultured over PDA. The isolates were maintained as pure cultures over PDA slants supplemented with 10% glycerol.

Production of culture filtrates

Liquid cultures of endophytic fungi were prepared by the procedure of Raviraja et al.[8]. Concisely, the method comprised of inoculation of 100 ml pre-sterilized Richard's Broth (composition: Sucrose – 5 g; Potassium Nitrate – 1 g; Potassium dihydrogen phosphate – 800 mg, Magnesium Sulphate – 250 mg; Ferric chloride – 2 mg; pH 4.5) aseptically with 5 mm mycelial disc of 7-d-old culture of endophytic fungus followed by incubation at 26 ± 1 °C, 120 rpm for 15 d. After the culmination of incubation period, the mycelial mass was separated using Whatmann filter paper no.4 followed by centrifugation at 1000 rpm for 10 min at room temperature. The supernatant was then passed through 0.22 µm nitrocellulose membrane making it cell free. The cell-free broths were stored at −20 °C until subjected to qualitative screening for PL inhibition.

Qualitative screening for PL inhibitors

Two chromogenic plate assays were used for screening the lipase inhibitory activity where the PL inhibition was indicated by decrease in halo (Rhodamine-olive oil plate assay) and change in color (Phenol-red olive oil plate assay).

Rhodamine olive oil plate assay

The assay was used for the preliminary screening of the PL inhibitory activity using the cell-free culture broth of the endophytic fungi. The assay was carried as per the method of[9]which involved preparation of 4 mm thick olive-agarose plate consisting of 2.5% olive oil, 1.3% agarose and 0.3% rhodamine as indicator. After solidification, 5 mm wells were punched using a sterile cork borer. Subsequently, 35 µl of the master-mix containing pre-incubated 15 µl of porcine PL (Stock = 40 U/ml) and 20 µl of culture filtrates was dispensed into 5 mm wells and incubated at 37 °C for 24 h. The control comprised of 15 µl of porcine PL and 20 µl of sterile saline. Appearance of orange-colored halo under ultra violet (UV) rays indicated the PL activity in the control while reduction the diameter of halo as compared to control indicated PL inhibition. All the tests were performed in triplicates and their mean and SD was calculated. Orlistat was used as a positive control for lipase inhibitory activity.

Phenol red olive oil plate assay

The assay assessed the change in color due to pH indicator as a result of fatty acid formation by the PL activity by hydrolysis of olive oil[10]. Briefly, the assay plates were prepared using olive oil as substrate (2.5%) and phenol red (0.01%) as pH indicator dye with 2% agar. Wells measuring 5 mm were punched aseptically and 35 µl of master mix having the same composition as mentioned in the rhodamine assay was dispensed and plates were incubated at 37 °C for 24 h. The control comprised of 15 µl of porcine PL (Stock = 40 U/ml) and 20 µl of sterile saline. The change in color from red to orange infers lipase production and decrease in the halo formation as compared to the test indicates lipase inhibitory potential of the culture filtrates/solvent extracts. All the tests were performed in triplicates and their mean and SD was calculated. Orlistat was used as positive control.

Liquid–liquid extraction

Liquid–liquid extraction procedure was adopted for extracting bioactive metabolites from the culture filtrate of endophytic fungi. The cell-free culture filtrate was extracted thrice with ethyl acetate in the ratio 2:1. The organic layer so obtained was pooled and then dehydrated using anhydrous sodium sulphate. The remaining aqueous layer was then extracted with chloroform and hexane following the same procedure. The solvent was evaporated using nitrogen blowout to obtain ethyl acetate, chloroform and hexane extract residue at room temperature. The residue so obtained was weighed and a stock solution was prepared using methanol and stored at −20 °C till further use.

Quantitative screening of PL inhibition

The culture filtrate and ethyl acetate residue of selected endophytic fungi exhibiting potential inhibition in the *in vitro* plate assays were further evaluated quantitatively using a spectrophotometric assay. Briefly, the assay comprises of using PNPL (*p*-nitrophenyl laurate) as substrate for PL activity. Hydrolysis of the substrate releases *p*-nitrophenyl which is a chromogen of yellow color measured spectrophotmetrically at 410 nm. The reduction in intensity of yellow color is indicative of PL inhibition. Cell-free culture filtrates of endophytic fungi were pre-incubated with PL (prepared in potassium phosphate buffer, pH 7.4) at 37 °C for 1 h prior to assaying the activity. The reaction was started by adding 100 µl of PNPL (2 mM) as a substrate and 20 µl enzyme (40U) and rest of the volume is made up to 250 µl using buffer. After incubation at 37 °C for 3 h, the amount of *p*-nitrophenol released in the reaction was measured at 410 nm using a Bioteck Powerwave 340 plate reader. The control comprised of the enzyme and substrate without cell-free culture filtrate and orlistat was used as a positive control in the assay. All the tests were performed in triplicates. The percentage inhibition (I) was given by the following formula;

$$\text{Inhibitory activity (I)} = 100 - ((\text{B} - \text{b})/(\text{A} - \text{a}) \times 100).$$

where ''A'' is the activity without inhibitor; ''a'' is the negative control without inhibitor; ''B'' is the activity with inhibitor; and ''b'' is the negative control with inhibitor[11].

Identification of PL inhibiting endophytic fungi

The potential isolate was identified using microscopic as well as molecular methods.

Microscopic identification of the bioactive fungi

The bioactive endophytic fungus was grown over PDA and incubated at 26 °C for 5 d. The morphological structures like the colony size, texture, color and microscopic characters like the hyphae, conidia, stipe, phialides, were observed and recorded. The microscopic characters were studied using a polarising

Table 1. Endophytic fungi from different medicinal plants used during the study with their corresponding inhibition activities in two chromogenic *in vitro* qualitative assays.

Culture code	Tentative identification	Host plant	Part of the plant	Rhodamine assay	Phenol red assay
#1 CCBD	*Fusarium* sp	*Cinnamomum camphora*	Bark	−−	−−
#1 CCSTITD	*Muscodor* sp	*C. camphora*	Stem internal tissue	++	++
#5 CZBAWLS	*Fusarium* sp	*Cinnamomum zeylanicum*	Bark	+	+
#2104 CZSTITG	*Alternaria* sp	*C. zeylanicum*	Stem internal tissue	−−	−−
#2106 CZSTITG	*Curvularia* sp	*C. zeylanicum*	Stem internal tissue	−−	−−
#2112 CZSTITG	*Nigro spora* sp	*C. zeylanicum*	Stem internal tissue	−−	−−
#2117 CZSTITG	*Alternaria* sp	*C. zeylanicum*	Stem internal tissue	−−	−−
#2125 CZSTITG	*Unidentified*	*C. zeylanicum*	Stem internal tissue	−−	−−
#2131 CZSTITG	*Unidentified*	*C. zeylanicum*	Stem internal tissue	−−	−−
#2143 CZSTITG	*Alternaria* sp	*C. zeylanicum*	Stem internal tissue	−−	−−
#2158 CZSTITG	*Pestalotiopsis* sp	*C. zeylanicum*	Stem internal tissue	−−	−−
#2161 CZSTITG	*Unidentified*	*C. zeylanicum*	Stem internal tissue	−−	−−
#2162 CZSTITG	*Aspergillus* sp	*C. zeylanicum*	Stem internal tissue	+	++
#2168 CZSTITG	*Aspergillus* sp	*C. zeylanicum*	Stem internal tissue	−−	−−
# 1 CSSTOT	*Schizophyllum* sp	*Camellia sinensis*	Stem	+++	+++
# 2 CSSTOT	*Schizophyllum* sp	*C. sinensis*	Stem	++	+++
# 7 CSSTOT	*Colletotrichum* sp	*C. sinensis*	Stem	+	++
#16 NOBASVNP	*Fusarium* sp	*Nerium oleander*	Bark	++	+
#1 PNLNEY	*Alternaria* sp	*Piper nigrum*	Leaf	−−	−−
#2 PNLNEY	*Alternaria* sp	*P. nigrum*	Leaf	+	+
#8 PNLNEY	*Alternaria* sp	*P. nigrum*	Leaf	++	+++
#14 RSBANEY	*Fusarium* sp	*Rauwolfia serpentina*	Bark	−−	−−
#16 RSLBRT	*Fusarium* sp	*R. serpentina*	Leaf	+	+
#2 TMSDTYEL	*Fusarium* sp	*Tabernaemontana divaricata*	Stem	−	−
#4 TMSDTYEL	*Fusarium* sp	*T. divaricata*	Stem	+	+
#7 TBBALM	*Mycelia sterile*	*Taxus baccata*	Bark	++	++
#8 TBBALM	*Pestalotiopsis* sp	*T. baccata*	Bark	−−	−−
#11 TBBALM	*Unidentified*	*T. baccata*	Bark	+	+
#13 TBBALM	*Unidentified*	*T. baccata*	Bark	−−	−−
#14 TBBALM	*Unidentified*	*T. baccata*	Bark	+	+
#17 TBBALM	*Fusarium* sp	*T. baccata*	Bark	−−	+
#20 TBBALM	*Alternaria* sp	*T. baccata*	Bark	++	++
#21 TBBALM	*Phomopsis* sp	*T. baccata*	Bark	−−	−−
#22 TBBALM	*Unidentified*	*T. baccata*	Bark	−−	−−
#25 TBBALM	*Phomopsis* sp	*T. baccata*	Bark	+	+
#26 TBBALM	*Unidentified*	*T. baccata*	Bark	−−	−−
#27 TBBALM	*Unidentified*	*T. baccata*	Bark	−−	−−
#28 TBBALM	*Unidentified*	*T. baccata*	Bark	−−	−−
#30 TBBALM	*Unidentified*	*T. baccata*	Bark	−−	−−
#31 TBBALM	*Ascobolus* sp	*T. baccata*	Bark	++	++
#33 TBBALM	*Mycelia sterilia*	*T. baccata*	Bark	+++	+++
#35 TBBALM	*Phomopsis* sp	*T. baccata*	Bark	++	++
#36 TBBALM	*Alternaria* sp	*T. baccata*	Bark	+	+
#40 TBBALM	*Pestalotiopsis* sp	*T. baccata*	Bark	−−	++
#41 TBBALM	*Pestalotiopsis* sp	*T. baccata*	Bark	−−	−−
#42 TBBALM	*Pestalotiopsis* sp	*T. baccata*	Bark	+	+
#43 TBBALM	*Fusarium* sp	*T. baccata*	Bark	−−	−−
#44 TBBALM	*Unidentified*	*T. baccata*	Bark	−−	−−
#46 TBBALM	*Ascobolus* sp	*T. baccata*	Bark	+	+
#48 TBBALM	*Unidentified*	*T. baccata*	Bark	−−	−−
#50 TBBALM	*Unidentified*	*T. baccata*	Bark	++	++
#53 TBBALM	*Unidentified*	*T. baccata*	Bark	−−	−−
#54 TBBALM	*Nectria* sp	*T. baccata*	Bark	++	++
#57 TBBALM	*Penicillium* sp	*T. baccata*	Bark	+++	++++
#65 TBBALM	*Pestalotiopsis* sp	*T. baccata*	Bark	−−	−−
#67 TBBALM	*Unidentified*	*T. baccata*	Bark	+	+
#69 TBBALM	*Unidentified*	*T. baccata*	Bark	−−	−−
#73 TBBALM	*Unidentified*	*T. baccata*	Bark	−−	−−
#80 TBBALM	*Unidentified*	*T. baccata*	Bark	−−	−−
#89 TBBALM	*Unidentified*	*T. baccata*	Bark	++	+
#91 TBBALM	*Phomopsis* sp	*T. baccata*	Bark	++	+
#93 TBBALM	*Pestalotiopsis* sp	*T. baccata*	Bark	++	++
#94 TBBALM	*Unidentified*	*T. baccata*	Bark	−−	−−
#98 TBBALM	*Penicillium* sp	*T. baccata*	Bark	+	+
#100 TBBALM	*Penicillium* sp	*T. baccata*	Bark	−−	−−
#113 TBBALM	*Pestalotiopsis* sp	*T. baccata*	Bark	−−	−−
#114 TBBALM	*Penicillium* sp	*T. baccata*	Bark	+	+
#118 TBBALM	*Unidentified*	*T. baccata*	Bark	+	+
#120 TBBALM	*Pestalotiopsis* sp	*T. baccata*	Bark	+	+
#405 TBBALM	*Phomopsis* sp	*T. baccata*	Bark	−−	−−
control(lipase+ RB)					
Orlistat				++++	++++

(+) sign indicates the severity of PL inhibition.

optical microscope (Olympus BX-51 P, Tokyo, Japan) coupled with CCD camera and measurements carried out using Image J software. At least 30 observations were made per structure[12,13].

ITS-based molecular taxonomy and phylogenetic analysis

For the genomic DNA isolation, about 0.1–0.2 g of cultured mycelia was scrapped off from the 3-to-4-d-old culture with sterile inoculation loop and crushed to very fine powder in pestle and mortar using liquid nitrogen. Further DNA extraction was done by using the Wizard® Genomic DNA purification kit (Promega, Madison, WI) as per the manufacturer instructions.

The ITS1, 5.8S, ITS2 rDNA sequence was amplified using Bio Rad thermocycler. PCR reaction was carried out by using universal ITS primers[14]. Amplification was performed in 25 µl reaction mixture containing 1 µl of extracted genomic DNA, 10 µM of each primer, 2.5 mM of dNTP, 1.5U of Taq DNA Polymerase in 10 X Taq buffer containing 25 mM $MgCl_2$. The PCR cycling conditions consisted of initial denaturation at 96 °C for 5 min followed by 39 cycles of 95 °C for 45 s, 60 °C for 45 s, 72 °C for 45 s followed by final extension at 72 °C for 5 min. PCR products (500–600 bp) were sent for sequencing to Chromus Biotech Labs (Bangalore, India). The accession number of the sequences in NCBI Genbank is KF537624.

Sequence assembly, alignment and phylogenetic analysis

Sequence similarity search for the obtained sequences of #57 TBBALM was performed using the BLAST algorithm against the non-redundant database maintained by the National Center for Biotechnology Information (NCBI). The phylogenetic analysis of #57 TBBALM involved 17 sequences which comprised of 1 sequence under study, 15 sequences from BLAST search which are representative sequences of *Penicillium species* and 1 *Muscodor* species chosen as outgroup. All ambiguous positions were removed from each sequence pair. These sequences were then aligned using Clustal W option in MEGA 5 (Tempe, AZ)[15]. The aligned sequences were then trimmed using the primer sequences so as to make the alignment uniform. The aligned files of the sequences were exported to FASTA as well as MEGA format. The matrix was analyzed by the Neighbor-Joining method[16] using the Kimura-2-parameter model[17] to calculate the evolutionary distances, and 1000 bootstrap replicates were taken into account to infer the consensus tree for the representation of evolutionary history.

Results

Qualitative screening

In the present study, 70 endophytic fungal isolates were screened for the PL Inhibitory potential. Approximately, 66% cultures were isolated from bark of *Taxus baccat*a while 34% cultures were isolated from different parts. *Cinnamomum zeylanicum*, *Camellia sinensis*, *Piper nigrum*, *Rauwolfia serpentina*, *Tabernaemontana divaricata* and *Nerium oleander*. Dominant species among the endophytic isolates were of *Pestalotiopsis*, *Alternaria*, *Fusarium* and *Penicillium*. Three sterile fungi were also reported during the isolation procedure (Table 1). In the rhodamine as well as phenol red plate assay it was found that #57 TBBALM exhibited the maximum enzyme inhibition (Tables 2 and 3). The other common organisms selected for further quantitative evaluation of the inhibitory potential of the PL were #33 TBBALM and #1 CSSTOT.

Quantitative screening

Further spectrophotometric assay ascertained the quantitative reduction in PL activity by the crude solvent extracts of culture filtrates of #57 TBBALM, #33 TBBALM and #1 CSSTOT which were compared to the positive control, Orlistat. The IC_{50} values of Orlistat and ethyl acetate fraction of #57 TBBALM culture filtrate was 2.73 µg/ml and 3.69 µg/ml, respectively (Figure 1). The inhibition pattern of ethyl acetate fraction of #57 TBBALM culture filtrate was quite similar to that of orlistat. Further the IC_{50} values of ethyl acetate fraction of #33 TBBALM and #1 CSSTOT were found to be 16.64 µg/ml and 42.5 µg/ml, respectively. Chloroform and hexane extracts of #57 TBBALM, #33 TBBALM and #1 CSSTOT did not exhibit any PL inhibitory activity. These results suggest that the ethyl acetate fraction of #57 TBBALM possess a bioactive compound which has potential PL inhibitory activity.

Table 2. *In vitro* porcine pancreatic lipase inhibitory activity of the culture filtrates in phenol red plate assay.

Culture code	Enzyme activity* as halo formed (diameter mm)	% Inhibition
Control	24.4 ± 0.3	0
ORLISTAT	10 ± 0.1	59.0 ± 0.2
#57 TBBALM	16 ± 0.5	34.6 ± 2.5
#1 CSSTOT	16.5 ± 0.4	32.5 ± 1.6
#8 PNLNEY	16.5 ± 0.5	32.5 ± 2.5
#2 CSSTOT	16.7 ± 0.5	31.6 ± 2.4
#33 TBBALM	17.3 ± 0.1	29.2 ± 0.9
#50 TBBALM	17.7 ± 0.2	27.4 ± 0.2
#1 CCSTITD	17.9 ± 0.2	26.8 ± 0.2
#54 TBBALM	18 ± 0.1	26.5 ± 0.2
#7 CSSTOT	18 ± 0.2	26.5 ± 0.2
#2162 CZSTITG	18 ± 0.2	26.4 ± 0.2
#93 TBBALM	19 ± 0.2	22.6 ± 0.3
#7 TBBALM	19 ± 0.1	22.4 ± 0.3
#40 TBBALM	19 ± 0.2	22.1 ± 0.3
#20 TBBALM	19.1 ± 0.3	21.8 ± 0.3
#35 TBBALM	19.2 ± 0.1	21.5 ± 0.3
#31 TBBALM	19.2 ± 0.1	21.5 ± 0.3
#2 PNLNEY	20.3 ± 0.3	16.9 ± 0.5
#14 TBBALM	20.3 ± 0.1	16.8 ± 1.3
#4 TMSDTYEL	21 ± 0.2	14.4 ± 0.5
#16 RSLBRT	21 ± 0.1	14.2 ± 1.3
#11 TBBALM	21.3 ± 0.4	13.0 ± 1.1
#5 CZBAWLS	21.3 ± 0.1	12.9 ± 1.3
#16 NOBASVNP	21.6 ± 0.4	11.6 ± 2.9
#91 TBBALM	22.0 ± 0.1	9.9 ± 2.7
#98 TBBALM	22.1 ± 0.1	9.7 ± 1.3
#89 TBBALM	22.2 ± 0.1	9.2 ± 1.4
#118 TBBALM	22.2 ± 0.1	9.0 ± 1.4
#36 TBBALM	22.3 ± 0.1	8.8 ± 1.5
#42 TBBALM	22.3 ± 0.1	8.7 ± 1.5
#114 TBBALM	22.4 ± 0.1	8.3 ± 1.5
#67 TBBALM	22.6 ± 0.2	7.6 ± 0.6
#46 TBBALM	22.8 ± 0.2	6.8 ± 0.6
#25 TBBALM	23.4 ± 0.2	4.3 ± 0.7
#2106 CZSTITG	23.5 ± 0.1	3.8 ± 0.7
#2112 CZSTITG	23.8 ± 0.4	2.6 ± 0.9
#1 CCBD	23.8 ± 0.4	2.4 ± 0.9
#17 TBBALM	24.0 ± 0.1	1.9 ± 1.6

*Stock concentration of enzyme used 40 U/ml or 0.6 U per well.

ITS based molecular taxonomy and phylogenetic analysis (Figure 2)

The phylogenetic tree based upon the ITS1-5.8S-ITS2 region sequence of #57 TBBALM using MEGA5 comprised of three clades. Clade I consisted of *Penicillium lapidosum*, *P. thomii*, *P. sclerotiorum*, type strains, namely *P. tricolor*, *P. cyclopium* and two strains of *P. polonicum* The clade II clustered the two strains of *P. melanoconidium*, three strains of *P. viridicatum*, and one strain each of *P. verrucosum* and *P. neoechinulatum*. On the other

hand, #57 TBBALM along with *Penicillium* sp BMP3038 diverged as a sister clade from clade I and clade II with significantly high bootstrap support value thereby confirming its placement in the genus *Penicillium*.

Table 3. Inhibitory activity of the endophytic culture filtrates against Pl in rhodamine plate assay.

Culture code	Enzyme activity* as halo formed (diameter mm)	% Inhibition
Control	10 ± 0.7	
ORLISTAT	5.2 ± 0.1	48.0 ± 5.3
#57 TBBALM	5.4 ± 0.1	46.1 ± 5.5
#33 TBBALM	5.7 ± 0.0	43.1 ± 8.0
#7 TBBALM	6.4 ± 0.1	35.6 ± 7.0
#35 TBBALM	6.5 ± 0.1	35.2 ± 7.2
#31 TBBALM	6.7 ± 0.2	33.5 ± 5.4
#89 TBBALM	7.0 ± 0.0	30.2 ± 9.8
#91 TBBALM	7.0 ± 0.1	30 ± 7.8
#1 CSSTOT	7.2 ± 0.2	28 ± 6.1
#93TBBALM	7.2 ± 0.2	28 ± 6.1
#20 TBBALM	7.3 ± 0.2	27.4 ± 6.3
#50 TBBALM	7.5 ± 0.5	25.3 ± 0.5
#54 TBBALM	7.7 ± 0.6	23.3 ± 1.2
#8 PNLNEY	7.9 ± 0.4	21.1 ± 1.1
#98 TBBALM	8.2 ± 0.4	18 ± 3.5
#118 TBBALM	8.3 ± 0.0	17.4 ± 11.7
#120 TBBALM	8.4 ± 0.1	16.1 ± 19.8
#67 TBBALM	8.4 ± 0.1	16 ± 19.8
#11 TBBALM	8.4 ± 0.2	16 ± 7.8
#46 TBBALM	8.5 ± 0.4	15 ± 3.9
#2 CSSTOT	8.6 ± 0.3	14.2 ± 6.1
#114 TBBALM	8.6 ± 0.13	14 ± 9.5
#14 TBBALM	8.6 ± 0.2	14 ± 9.1
#16 NOBASVNP	8.6 ± 0.4	14 ± 4.0
#1 CCSTITD	8.6 ± 0.1	13.6 ± 10.0
#42 TBBALM	8.6 ± 0.1	13.6 ± 10.0
#25 TBBALM	8.7 ± 0.3	13.3 ± 6.2
#36 TBBALM	8.8 ± 0.0	12.3 ± 12.4
#2162 CZSTITG	8.7 ± 0.1	11.4 ± 10.2
#5 CZBAWLS	9.0 ± 0.0	10 ± 12.6
#40 TBBALM	9.4 ± 0.0	No activity
#7 CSSTOT	9.4 ± 0.3	No activity
#2 PNLNEY	9.4 ± 0.2	No activity
#4 TMSDTYEL	9.6 ± 0.4	No activity
#16 RSLBRT	9.6 ± 0.3	No activity
#17 TBBALM	9.7 ± 0.4	No activity

*Stock concentration of enzyme used 40 U/ml or 0.6 U per well.

Morphotaxonomy

Over PDA medium the fungus forms flat, velutinous, fast growing (18–20 mm) colonies which are initially white later turning to green color on incubation at 26 ± 2 °C for 5 d. The colony is pale colored from reverse. It forms septate, hyaline hyphae (1.51)−2.59 ± 0.56−(3.69) µm. Long broad stipe (2.22)–3.64 ± 0.87−(5.47) µm arises from the hyphae either singly or synmeta. Metulae (8.75)−10.73 ± 1.58−(13.92) µm × (1.76) −2.79 ± 0.52−(4.23) µm arises from the stipe which carries flask shaped phialides. Metulae are 3–4 in number. Each metulae gives rise to four phialides (5.51)−7.75 ± 1.61−(10.82) µm × (1.15)−2.13 ± 0.45−(2.79) µm which forms brush like clusters known as penicilli. The conidia (2.19)−2.95 ± 0.51−(4.09) µm are darkly stained, unicellular, globose shaped arranged in basipital chain. (Figures 3A and B).

Discussion and conclusion

Our present findings implicate that the ethyl acetate extract of culture filtrate of #57 TBBALM exhibits a promising porcine PL inhibitory activity when compared to plant extracts, namely grape seed extract[18] and *Nelumbo nucifera* extract[19]. Further, in terms of IC_{50} values, the ethyl acetate extract of #57 TBBALM exhibited a potential lipase inhibitory activity when compared to Carnosic acid[20] and Hersperidin[21]isolated from *Salvia officinalis* and citrus fruits, respectively.

It is expected further purification of the ethyl acetate fraction would improve the inhibition kinetics of porcine PL which would be helpful in arriving to the exact IC_{50} of the pure compound. Microbial compounds like Vibralactone[22] and Percyquinin[23] have exhibited better IC_{50} values as compared to their crude extracts.

Thus, endophytic fungi also offer to be a promising under-explored resource for screening potential PL inhibitors. Till date, there exists no report on endophytic fungi producing PL inhibitor. The present study is a pioneer work wherein we have explored the potential PL inhibitory activity of endophytic fungi which were isolated from different medicinal plants. Based on the qualitative and quantitative *in vitro* assays, it was found that an isolate from *Taxus baccata*, #57 TBBALM possess potential porcine PL inhibitory activity. Hence, this activity of the ethyl acetate extract of the culture filtrate of the endophytic fungus #57 TBBALM identified as *Penicillium* species can be further exploited. Further purification and characterization of this

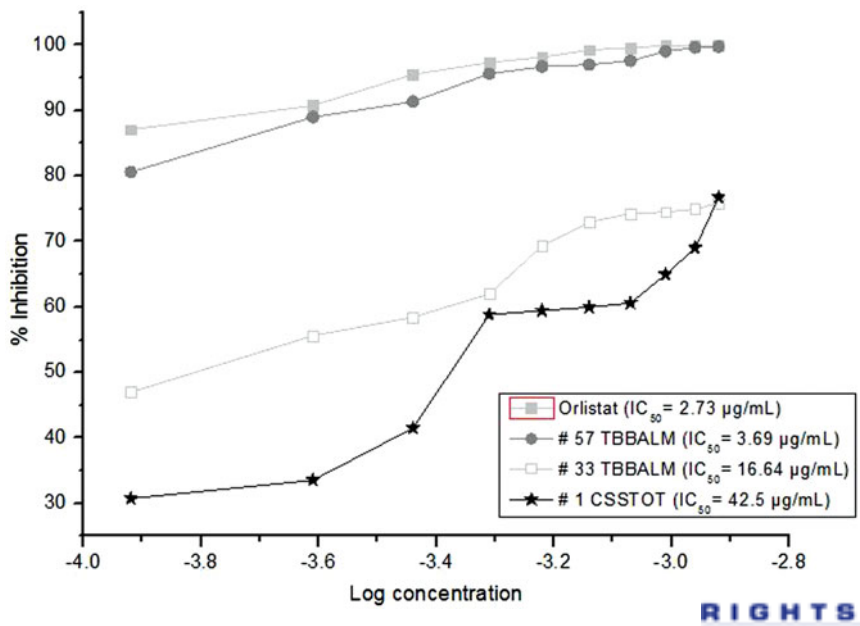

Figure 1. Dose–response curves for inhibition of porcine pancreatic lipase by ethyl acetate extracts of culture filtrates of the selected test fungi. Orlistat is used as a positive control.

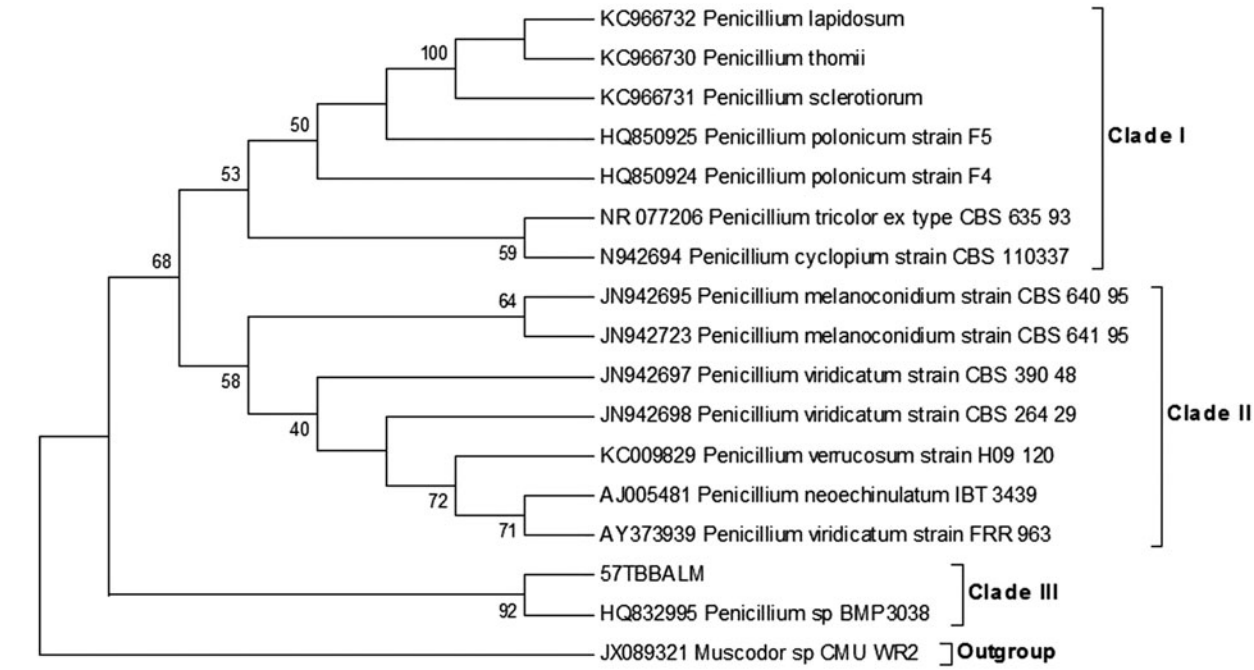

Figure 2. Phylogenetic tree showing ITS-based molecular taxonomy and phylogenetic analysis of #57 TBBALM.

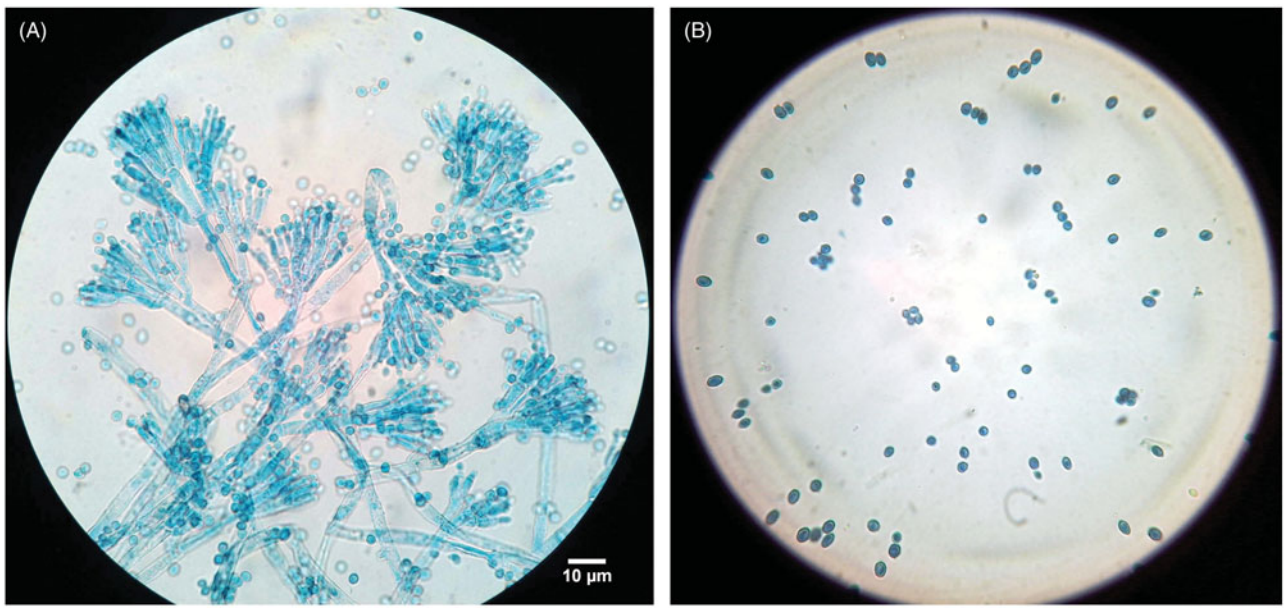

Figure 3. Light microscope micrographs of (A): Stipe, metulae and phialides of #57 TBBALM. (B) Conidia. Bar 10 µm.

compound is underway for possible development into a drug for obesity management.

Declaration of interest

The authors declare no conflicts of interests. The authors alone are responsible for the content and writing of this article. Mahiti Gupta thanks the University Grants Commission (UGC), New Delhi for providing UGC-BSR fellowship.

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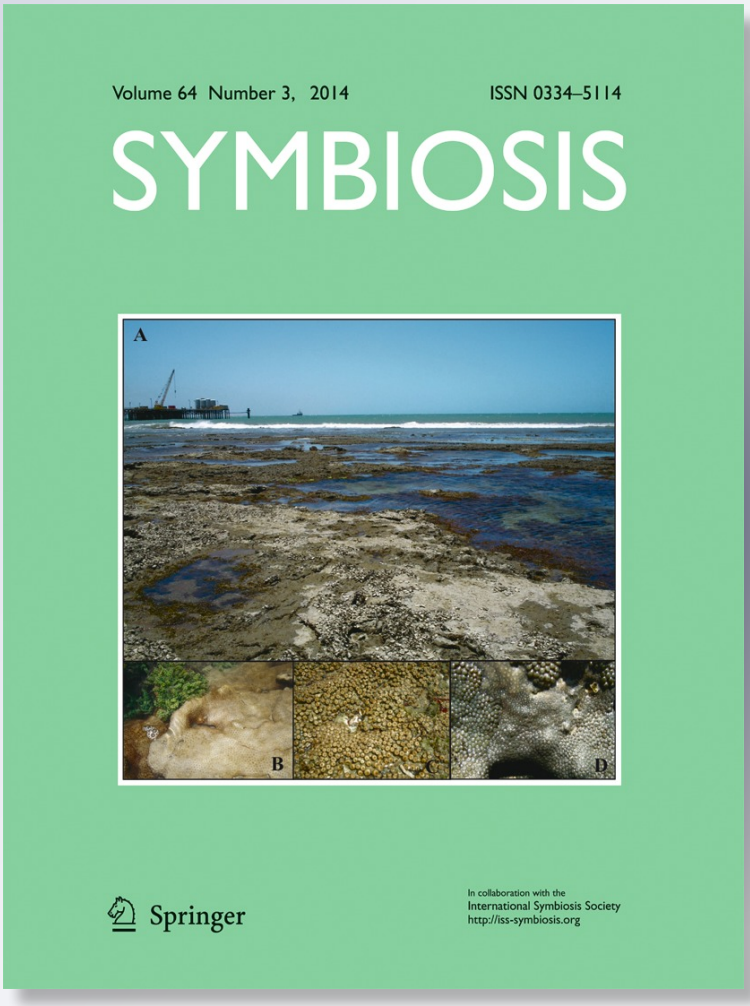

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Lipase inhibitory activity of endophytic fungal species of *Aegle marmelos*: a bioresource for potential pancreatic lipase inhibitors

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Abstract Obesity is the most prevailing disease spread all over the world. Targeting lipid metabolism for the development of anti-obesity drugs is best therapeutic option. Primary drug target is pancreatic lipase which causes degradation of 50–70 % of ingested fat. In the present study cell free extracts of 70 endophytic fungi isolated from medicinal plants of Western Ghats were primarily tested for their pancreatic lipase inhibitory activity using chromogenic olive plate assay. Three endophytic fungi #6 AMLWLS, #59 AMSTWLS and #1058 AMSTITYEL showed reduction in the halo formation indicating potential pancreatic lipase inhibition. Further quantitative assays for pancreatic activity of these fungi were estimated using p-nitrophenyl laurate as substrate. The IC_{50} of crude aqueous extract of #6 AMLWLS was 2.12 μg ml^{-1} which is better than orlistat exhibiting IC_{50} of 2.79 μg ml^{-1}. The crude compound was further purified by column chromatography. The pure compound was characterized to be an amino acid derivative. On the basis of classical and molecular taxonomy the bioactive fungus was identified as *Fusarium incarnatum*.

Keywords Obesity · Rhodamine · Olive oil · Phenol red · p-nitrophenyl red · Endophytic fungi · Aegle marmelos

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1 Introduction

Medicinal properties of plants have been extensively exploited in Ayurveda as well as in Modern Medicine. India is a rich source of medicinal plants and holds a special place amongst the 12 megabiodversity hotspots of the world (Mcneely et al. 1990). The humongous biodiversity of Western Ghats had earned it a status of 'biodiversity hot spot' (Myers et al. 2000; Ramesh et al. 1997). These medicinal plants hold a diverse assemblage of endophytic fungi whose relationship varies from pathogen to symbionts. Hence, there exists a possibility of these endophytic fungi acquiring the medicinal principles of the plants during the course of evolution. The production of billion dollar anticancer drug Paclitaxel by *Taxomyces andrenae*, an endophyte of *Taxus brevifolia* is an excellent example to authenticate this hypothesis (Strobel et al. 1996). Endophytic fungi are fountainheads of unexplored wealth for discovery of other pharmacophores.

Aegle marmelos also known as bael or bilva has been used in Ayurvedic and unani medicinal preparations to treat diabetes (Dhankar et al. 2011). *Aegle marmelos* possesses active principles which help in reduction of obesity due to inhibition of pancreatic lipase (Karmase et al. 2013).

Pancreatic lipase (PL) is the key enzyme that is mainly targeted in lipid metabolism to restrict triglycerides formation. FDA approved pancreatic lipase inhibitor Orlistat that has been isolated from actinobacterium *Streptomyces toxytricini* suffers from many side effects like oily stools, flatulence, faecal urgency, abdominal cramps. The efficacy of orlistat and its side effects has motivated researchers to explore new sources for pancreatic lipase inhibitors with least unpleasant side effects.

Based upon the above hypothesis of symbiosis and the anti obesity properties of *Aegle marmelos* the present study explores the pancreatic lipase inhibitory activity of 52 culture filtrates of endophytic fungi isolated from *Aegle marmelos*.

The IC_{50} of #6 AMLWLS was also found to be better than that of orlistat. Hence, this is the first report of a bioactive crude compound showing better inhibitory results than orlistat. This fungus producing the bioactive metabolites was identified as *Fusarium incarnatum* based on morphological and molecular taxonomy.

2 Materials and methods

2.1 Plant sample collection and isolation of endophytic fungi from Aegle marmelos

Healthy and mature twigs of *Aegle marmelos* was collected from the conserved rain forests areas, geographically located between 11°59′–11°99′ N, 77°8′–77°14′E (BRT wildlife sanctuary, Karnataka); 11°35′–11°51′ N, 76°14 02′–76°27′ E (Muthanga Wildlife Sanctuary, Wayanad, Kerala) and 11°59 38 N, 77°8 26 E (Yelandur, Karnataka) during early rainy season of 2009. Plant samples were kept in sterile bags and stored at 4 °C till further use. The isolation was done following a modified procedure (Schulz et al. 1993). Briefly plant samples were cut into segments and surface sterilized with 75 % ethanol for 30 s, followed by 2 % sodium hypochlorite for 3 min and 95 % ethanol under aseptic conditions. Using a sterile blade, surface sterilized samples were sliced into small pieces of 1–3 mm and placed over PDA plates supplemented with streptomycin (50 mg/l). The plates were incubated at 26±2 °C for 10 day with 12 h light/dark cycles. The germinating fungal mass was picked from the fine tipped sterile needle and sub-cultured over PDA and were maintained as pure cultures.

2.2 Production of culture filtrates

The culture filtrates of 52 different endophytic fungi were produced as per procedure of Raviraja et al. (2006). 5 mm plug of actively growing endophytic fungal cultures were inoculated into Erlenmeyer flasks containing 100 ml of presterilized Richard's Broth (composition: Sucrose- 5 g; Potassium Nitrate-1 g; Potassium dihydrogen phosphate- 800 mg, Magnesium Sulphate- 250 mg; Ferric chloride- 2 mg; pH 4.5). The flasks were incubated at 28 °C for 15 days at 160 g. Subsequently the fungal cultures were filtered through Whatman filter paper no.4. The filtrate thus obtained was centrifuged at 12,000 g for 10 min at room temperature and supernatant was collected in a separate flask.

2.3 Qualitative screening using olive oil plate assays

Two chromogenic olive oil plate assays were performed to test the porcine PL inhibitory activity of the culture filtrates produced by the endophytic fungi. The decrease in halo formation showed the porcine PL inhibitory potential.

Rhodamine olive oil plate assay The assay was carried as per the method of Sheikh et al. (2003) which involved preparation of 4 mm thick olive-agarose plate consisting of 2.5 % olive oil, 1.3 % agarose and 0.3 % rhodamine as indicator. After solidification, 5 mm wells were punched using a sterile cork borer. Subsequently, 35 μl of the master-mix containing pre-incubated 15 μl of porcine PL (Stock = 40 U/ml) and 20 μl of culture filtrates was dispensed into 5 mm wells and incubated at 37 °C for 24 h. The control comprised of 15 μl of porcine PL and 20 μ of sterile saline. Appearance of orange coloured halo under ultra violet (UV) rays indicated the PL activity in the control while reduction the diameter of halo as compared to control indicated PL inhibition. All the tests were performed in triplicates and their mean and SD was calculated. Orlistat was used as a positive control.

Phenol red olive oil plate assay This assay access the change in colour due change in pH from neutral to acidic thereby forming a yellow halo on a red background (Singh et al. 2006). 4 mm thick olive oil-agar plates where prepared containing 2 % agar with 2.5 % olive oil and 0.01 % phenol red as indicator. The assay conditions were similar to those described in rhodamine olive oil plate assay. All the tests were performed in triplicates and their mean and SD was calculated.

2.4 Bioactivity guided isolation of lipase inhibitor

The cell free filtrate was extracted thrice with ethyl acetate in the ratio 2:1. The organic layer so obtained was pooled and dehydrated using anhydrous sodium sulphate. The remaining aqueous layer was then extracted sequentially with chloroform, petroleum ether and finally hexane. The solvent was evaporated using nitrogen blowout to obtain ethyl acetate, chloroform, petroleum ether and hexane extract residue at room temperature. The residue so obtained was weighed and a stock solution was prepared using methanol/DMSO.

2.5 Quantitative estimation of pancreatic lipase inhibition

The ethyl acetate, chloroform, petroleum ether, hexane and aqueous (after solvent extraction) residues of the selected endophytic fungi were then screened quantitatively for pancreatic lipase inhibitory activity using p-nitrophenyl laurate (PNPL) as substrate. Action of lipase on the substrate releases a yellow coloured product p-nitrophenol measured spectrophotometrically at 410 nm. The reduction in intensity of yellow colour confirms PL inhibition. Solvent extracted residues of different fractions were pre-incubated with porcine pancreatic lipase (prepared in potassium phosphate buffer, pH 7.4) at

37 °C for 1 h prior to assaying the activity. The reaction was started by adding 100 μl of PNPL (2 mM) as a substrate and 20 μL of enzyme (40 U) and rest making the volume up to 250 μL using buffer. After incubation at 37 °C for 3 h, the amount of *p*-nitrophenol released in the reaction was measured at 410 nm using a Bioteck Powerwave 340 plate reader. The control comprised of the enzyme and substrate without any inhibitor and orlistat was used as a positive control in the assay. A negative control containing DMSO/methanol and substrate was used. All the tests were performed in triplicates. The percentage inhibition (I) was calculated by the formula;

$$\textit{Inhibitory activity}\ (I) = 100 - ((B-b)/(A-a) \times 100)$$

Where, 'A' is the activity without inhibitor; 'a' is the negative control without inhibitor; 'B' is the activity with inhibitor; and 'b' is the negative control with inhibitor (Kim et al. 2010).

2.6 Column chromatography of the bioactive fraction

The aqueous extract showing potential inhibitory activity was purified by silica gel column chromatography with chloroform-methanol gradient elution in a stepwise manner starting from 95:5. These fractions were further tested for pancreatic lipase inhibitory activity using the quantitative method described above. The active fraction so obtained was further tested for its purity using preparative TLC on silica gel plates using chloroform: methanol (35:65) as mobile phase.

2.7 Phytochemical characterization

The crude aqueous extract and the pure fraction were tested for presence of carbohydrates, amines, lipids, phenolic compounds, saponins, flavanoids and anthraquinones. Phytochemical characterization of the crude aqueous extract and pure fraction was carried out according to the standard methods (Harborne 1998).

2.8 Identification of endophytic fungi

Three endophytic fungal isolates #6 AMLWLS, #1058 AMST ITYEL and #59 AMSTWLS that were exhibiting inhibitory activity against pancreatic lipase was identified using microscopy and molecular methods.

2.8.1 Microscopic identification

The endophytic fungal isolates#6 AMLWLS and #1058 AMSTITYEL were grown over PDA and Synthetischer nahrstoffarmer agar (SNA) for 8–10 days at 26°±2 °C with 12 h of photoperiod whereas #59 AMSTWLS was grown over PDA and Pine Leaf Agar (PLA) for 3–4 weeks in dark. The fungi were identified based upon morphological characteristics like colony size, texture, colour and microscopic characters like the hyphae, conidiophores and conidia. The microscopic characters were studied using a Nikon eclipse 50i microscope coupled with CCD camera and measurements carried out using NIS element software. At least 30 observations were made per structure (Booth 1971; Seifert 1996).

2.8.2 Molecular taxonomy and phylogenetic basis

Genomic DNA extraction of bioactive fungal isolates #6 AMLWLS, #1058 AMSTITYEL and #59 AMSTWLS was carried out by scrapping off the cultured mycelia from a 3–4 days old culture with pre-sterilized inoculation loop and grounded to very fine powder with liquid nitrogen in pestle and mortar. Further DNA extraction was performed by Wizard® Genomic DNA purification kit (Promega, USA) following manufacturer's instructions.

PCR reaction was carried out by using universal ITS1 and ITS4 primers amplifying ITS-5.8S rDNA region. Amplification was performed in 25 μl reaction mixture containing 1 μl of extracted genomic DNA, 10 μmol of each primer, 2.5 mmol of dNTP, 1.5 U of Taq DNA Polymerase in 10 X Taq buffer containing 25 mmol $MgCl_2$. The PCR cycling conditions consisted of initial denaturation at 96 °C for 5 min followed by 39 cycles of 95 °C for 45 s, 60 °C for 45 s, 72 °C for 45 s followed by final extension at 72 °C for 5 min. PCR products (700 bp) were sequenced at Chromous Biotech Labs, Bangalore.

2.9 Sequence assembly, alignment and phylogenetic analysis

The obtained sequences of #6 AMLWLS, #1058 AMST ITYEL and #59 AMSTWLS were aligned using Sequencher, Version 5 (Gene Codes, Ann, Arbor, MI) and deposited in the GenBank with accession number KC 960885, KJ816862 and KJ816863 respectively. The sequence was subjected to similarity search using BLAST against the database maintained by NCBI to ascertain the homology with closely related organisms. The sequences under study along with their respective selected reference taxa were aligned using CLUSTAL W in MEGA5.2. The alignment was edited manually wherever necessary. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter=2). All ambiguous positions were removed for each sequence pair. Bootstrap analysis (1000 replicates) was carried out to infer the phylogenetic relationship.

3 Results and discussion

3.1 In vitro plate assays

Present study consists of 52 endophytic fungal isolates from *Aegle marmelos* out of which most of the fungi were identified as *Fusarium* followed by *Laiodiplodia/Botryosphaerea* (Table 1). In rhodamine and phenol red plate assay, complete inhibition of pancreatic lipase has been observed by the culture filtrate of #6 AMLWLS (Table 2). Excellent inhibition of PL was also shown by #59 AMSTWLS and #1058 AMST ITYEL.

3.2 Quantitative screening

To verify the qualitative results, spectrophotometric analysis was done by using p-nitrophenyl laurate as the substrate. Quantitative testing of culture filtrates of #6AMLWLS, #59 AMSTWLS and #1058 AMSTITYEL confirmed the qualitative results showing 96.7 %, 85.07 % and 80.14 % inhibition respectively. Chloroform, petroleum ether and hexane extracts of #6 AMLWLS did not exhibit any PL inhibitory activity. Strong PL inhibitory activity was exhibited by aqueous extract of #6 AMLWLS left after the sequential extraction. The aqueous extract exhibited an IC_{50} of 2.12 μg ml^{-1}which was better than the positive control orlistat exhibiting IC_{50} of 2.73 μg ml^{-1} (Fig. 1). Similarly the crude ethyl acetate extracts of #59 AMSTWLS and #1058 AMSTITYEL showed IC_{50} of 14.48 μg ml^{-1} and 28.18 μg ml^{-1} respectively.

3.3 Purification and activity testing of the fractions

The eluted fractions from the silica gel column were then tested for their pancreatic lipase inhibitory activity. Fraction 2 exhibited 100 % inhibition of the porcine pancreatic lipase (40U) whereas fraction 1 and 3 showed no inhibition of pancreatic lipase.

3.4 Physical properties

The isolated compound from second fraction was white solid powder having melting point greater than 300 °C. It is highly polar only miscible in water.

3.5 Phytochemical characterization

Phytochemical testing for the presence of 13 different chemical classes (carbohydrates, amino acids, lipids, steroids, alkaloids, terpenoids, resins, tannins, phlobatannins, esters, saponins, flavonoids and anthroquinones) was done in crude aqueous extract and the pure compound. Out of 13 tests performed for the presence or absence of the above chemical compounds (Table 3), crude compound showed positive results for amino acids, sugars and terpenoids whereas the pure compound gave purple coloration with ninhydrin thereby confirming the bioactive compound to be an amino acid derivative.

3.6 Identification of the bioactive endophytic fungi

3.6.1 Morphotaxonomy

#6AMLWLS Over PDA medium, colonies moderately growing (55.67±2.08), aerial mycelium floccose, initially white later becoming light pink to avellaneous, reverse is orange to peach coloured with rough margins (Plate 1a and b). Hypahe (1.96(−2.57±0.65-) 4.32 μm), thick, septate, long and branched. Conidiophores present in the aerial mycelium (Plate 1). Conidia present in sporodichia straight to fusiform. Conidia developed over aerial conidiophores are generally borne singly on scattered denticle, fusiform to falcate in shape, usually 3–7 septate (15.93(−23.5±3.69-) 27.67) μm × (2.63(−3.26±0.3-) 3.97 μm) (Plate 1d–e). Microconidia absent. Intercalary chlamydospores are rare and present in chain (Plate 1f).

Over SNA medium, colonies moderate to rapidly growing, white in color, downy to floccose (Plate 1c). Hyphae (1.69(−2.56±0.48-) 3.35 μm) septate and thick. Macroconidia (22.62(−26.18±2.47-) 32.74 μm × 1.97(−2.78 ±0.39-) 3.21 μm) fusiform to slightly curved with foot cell, mostly 3–4 septate (Plate 1g). Microconidia absent. Based on the following characters the fungus was identified as *Fusarium incarnatum*.

#1058 AMSTITYEL When grown on PDA at 26 °C for 7 days, fungal colonies were floccose, moderate to fast growing, initially white later turning to orange yellow color due to production of abundant sporulation and covering 90 mm plate in 8–10 days. On SNA medium the colony appeared floccose, white colored, fast growing covering 90 mm plate in 10 days. Microscopic examination of the isolate #1058 AMSTITYEL on PDA and SNA media exhibited long branched, slender and septate hyphae. Macroconidia were observed whereas microconidia were absent. Macroconidia born on loose conidiophores. Over SNA medium microconidia (31.93–48.37 × 3.14–5.25 μm) were 3–6 septate, falcate to straight. The apical cell was beak shaped and the basal cell was blunt.

#59 AMSTWLS On PDA colonies floccose, grayish green colored from front and dark green or black colored from reverse, fast growing with formation of some aerial mycelium. On PLA (Pine Leaf Agar) fungal colonies downy, dark green to black colored, fast growing. Black to brown ascomata formed. Hyphae septate, broad, thick walled. Conidiogenous cells (10–14 × 3–4 μm) simple, smooth, cylindrical, indeterminate and holoblastic. Paraphyses septate. Conidia (13.5-) 21.94± 6.38 (−31.07) × (11.49-) 13±0.95 (−14.3) μm aseptate, oblong

Table 1 Endophytic fungal isolates from different parts of *Aegle marmelos*

S.no.	Culture Code	Plant Part	Tentative identification	Sampling Place
1	#1006 AMLBRT	Leaf	*F. commune*	BRT Wildlife Sanctuary, Karnataka
2	#1007 AMLBRT	Leaf	*F. commune*	BRT Wildlife Sanctuary, Karnataka
3	#1016 AMLBRT	Leaf	*F. chlamydosporum*	BRT Wildlife Sanctuary, Karnataka
4	#9 AMLBRT	Leaf	*F. culmorum*	BRT Wildlife Sanctuary, Karnataka
5	#7 AMSTYEL	Stem	*F. equiseti*	Yelandur, Karnataka
6	#9(B) AMSTYEL	Stem	*F. equiseti*	Yelandur, Karnataka
7	#1069 AMSTITYEL	Stem	*F. equiseti*	Yelandur, Karnataka
8	#1070 AMSTITYEL	Stem	*F. equiseti*	Yelandur, Karnataka
9	#22 AMSTYEL	Stem	*F. incarntum*	Yelandur, Karnataka
10	#6 AMLWLS	Leaf	*F. incarntum*	Yelandur, Karnataka
11	#1022 AMSTITYEL	Stem	*F. lateritium*	Yelandur, Karnataka
12	#2 AMSTYEL	Stem	*F. moniliforme*	Yelandur, Karnataka
13	#4 AMSTYEL	Stem	*F. moniliforme*	Yelandur, Karnataka
14	#6 AMSTYEL	Stem	*F. moniliforme*	Yelandur, Karnataka
15	#4 AMLBRT	Leaf	*F. oxysporum*	BRT Wildlife Sanctuary, Karnataka
16	#1010 AMSTITYEL	Stem	*F. oxysporum*	Yelandur, Karnataka
17	#24 AMBAWLS	Bark	*F. solani*	Wayanad, Kerala
18	##7 AMSTYEL	Stem	*F. solani*	Yelandur, Karnataka
19	#1017 AMLBRT	Leaf	*F. semitectum*	BRT Wildlife Sanctuary, Karnataka
20	#1058 AMSTITYEL	Stem	*F. semitectum*	Yelandur, Karnataka
21	#15 AMSTYEL	Stem	*Botryosphaeria* sp.	Yelandur, Karnataka
22	#23 AMSTITYEL	Stem	*Botryosphaeria* sp.	Yelandur, Karnataka
23	#32 AMSTYEL	Stem	*Botryosphaeria* sp.	Yelandur, Karnataka
24	#28 AMSTWLS	Stem	*Botryosphaeria* sp.	Wayanad, Kerala
25	#39 AMSTWLS	Stem	*Botryosphaeria* sp.	Wayanad, Kerala
26	#59 AMSTWLS	Stem	*B. stevensii*	Wayanad, Kerala
27	#1111 AMSTITWLS	Stem	*Barriopsis iraniana*	Wayanad, Kerala
28	#1003 AMSTITYEL	Stem	*Diplodia* sp.	
29	#1048 AMSTITYEL	Stem	*Lasiodiplodia pseudotheobromae*	Wayanad, Kerala
30	#1088 AMSTITWLS	Stem	*Lasiodiplodia pseudotheobromae*	Wayanad, Kerala
31	#1079 AMSTITWLS	Stem	*L. theobromae*	Wayanad, Kerala
32	#1082 AMSTITWLS	Stem	*L. theobromae*	Wayanad, Kerala
33	#1104 AMSTITWLS	Stem	*L. gonubiensis*	Wayanad, Kerala
34	#23(B) AMSTITYEL	Stem	*L. gonubiensis*	Yelandur, Karnataka
35	#1013 AMSTITYEL	Stem	*Sphaeropsis sapinea*	Yelandur, Karnataka
36	#1032 AMSTITYEL	Stem	*Sphaeropsis sapinea*	Yelandur, Karnataka
37	#18 AMSTYEL	Stem	*A. alternata*	Yelandur, Karnataka
38	#29 AMSTWLS	Stem	*A. alternata*	Wayanad, Kerala
39	#24 AMSTWLS	Stem	*A. solani*	Wayanad, Kerala
40	#1005 AMLBRT	Leaf	*A. marmelos*	BRT Wildlife Sanctuary, Karnataka
41	#22 AMBAWLS	Bark	*A. niger*	Wayanad, Kerala
42	#23 AMBAWLS	Bark	*P. chrysogenum*	Wayanad, Kerala
43	#1011 AMSTITYEL	Stem	*P. chrysogenum*	Yelandur, Karnataka
44	#61 AMLWLS	Leaf	*T. viride*	Wayanad, Kerala
45	#29 AMBAWLS	Leaf	*T. viride*	Wayanad, Kerala
46	#16 AMLWLS	Leaf	*M. kashayum*	Wayanad, Kerala
47	#9 AMSTYEL	Stem	*P. microspora*	Yelandur, Karnataka
48	#33 AMSTWLS	Stem	*P. rubrigenum*	Wayanad, Kerala
49	#37(a) AMSTWLS	Stem	*Pn rubrigenum*	Wayanad, Kerala

Table 1 (continued)

S.no.	Culture Code	Plant Part	Tentative identification	Sampling Place
50	#42 AMSTWLS	Stem	*Phomopsis* sp	Wayanad, Kerala
51	#11 AMBAWLS	Bark	*Aureobasidium* sp.	Wayanad, Kerala
52	#20 AMSTWLS	Leaf	unidentified	Wayanad, Kerala

Table 2 Inhibition of procine Pancreatic Lipase by culture filtrates of endophytic fungi in qualitative plate assay

Culture code	Diameter of halo(mm) Rhodamine assay	% inhibition (Rhodamine)	Diameter of halo(mm) Phenol red assay	% inhibition (Phenol red)
Control	5.64±0.01	0	17.80±0.01	
Orlistat (positive control)	0.21±0.01	96.34	0.64±0.04	96.40
#6 AMLWLS	0.19±0.00	96.57	0.62±0.01	96.52
#59 AMSTWLS	0.83±0.05	85.34	2.42±0.04	86.43
#1058AMSTITYEL	1.11±0.02	80.38	2.89±0.01	83.79
#1013AMSTITYEL	1.79±0.03	68.20	5.33±0.01	70.06
# 9 (B) AMSTYEL	1.97±0.05	65.20	6.04±0.01	66.23
#1104AMSTITWLS	2.07±0.05	63.36	6.15±0.01	65.48
#7 AMSTYEL	2.14±0.08	62.73	5.38±0.02	69.80
#42AMSTITWLS	2.17±0.02	61.58	6.81±0.02	61.77
#1048 AMSTITYEL	2.21±0.03	60.76	5.87±0.0	67.02
#1070AMSTITYEL	2.25±0.04	60.11	6.56±0.16	63.15
#2AMSTYEL	2.31±0.09	57.29	8.13±0.02	54.99
#29AMBAWLS	2.49±0.08	55.91	8.77±0.01	50.76
#1082 AMSTITWLS	2.88±0.05	48.94	10.47±0.64	41.21
#22AMSTYEL	2.91±0.03	48.35	10.57±0.03	40.62
#1005 AMLBRT	2.98±0.05	47.67	9.91±0.04	44.52
#32 AMSTYEL	3.03±0.02	46.34	10.19±0.03	42.75
#1003AMSTYEL	3.05±0.03	45.92	10.01±0.01	43.79
1016AMLBRT	3.17±0.03	43.85	10.33±0.02	41.99
#1006AMLBRT	3.18±0.02	43.68	10.58±0.01	40.59
#28AMSTWLS	3.21±0.03	43.03	10.90±0.01	38.76
#7AMSTYEL	3.45±0.06	38.89	11.92±0.06	33.03
#1010AMSTITYEL	3.56±0.03	36.94	11.76±0.04	33.93
#1022 AMSTITYEL	3.71±0.07	34.28	11.76±0.01	33.96
#1069AMSTITYEL	3.74±0.04	33.63	12.46±0.08	30.03
#20AMSTWLS	3.85±0.06	31.80	11.94±0.01	32.95
#1007AMLBRT	3.90±0.07	30.79	11.94±0.04	32.92
#23(B)AMSTYEL	3.94±0.01	30.08	12.12±0.02	31.94
#37(a)AMSTWLS	3.95±0.06	29.91	14.13±0.02	20.65
#61AMLWLS	4.15±0.04	26.48	13.37±0.04	24.92
#11AMBAWLS	4.44±0.08	21.28	13.71±0.02	23.01
#1088AMSTITWLS	4.48±0.09	20.57	13.72±0.09	22.95
#33AMSTWLS	4.73±0.03	16.08	15.90±0.01	10.67
#9AMLBRT	4.74±0.04	16.02	16.01±0.02	10.08
#1032AMSTYEL	5.39±0.09	4.49	–	–

*Rest culture filtrates were not showing lipase inhibitory activity

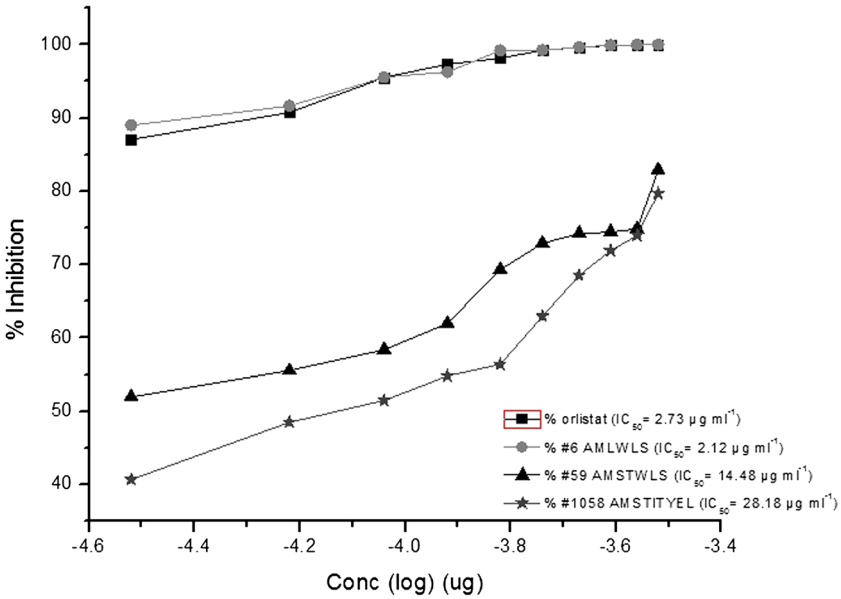

Fig. 1 Dose–response curves for inhibition of porcine pancreatic lipase by aqueous residue of #6 AMLWLS and ethyl acetate extracts of culture filtrates of the selected test fungi. Orlistat is used as a positive control

to ovoid double walled with broad rounded ends (Alves et al. 2004).

3.6.2 Molecular taxonomy and phylogenetic analysis

The combined phylogenetic tree of three selected isolates viz. #6AMLWLS, #1058AMSTITYEL, #59AMSTWLS based on ITS-rDNA sequences results into formation of 5 clusters: *Fusarium incarnatum-equiseti* complex which clustered *F. incarnatum, F. equiseti* and #6AMLWLS; *Fusarium oxysporum* complex; *Fusarium solani* complex; *Fusarium lateritum* complex clustering #1058AMSTITYEL along with reference *Fusarium lateritium* species thereby confirming it to be *Fusarium lateritium*; and *Botryosphaeria stevensii* complex in which #59AMSTWLS was clustered confirming that it belongs to *B. stevensii* species (Fig. 2).

Table 3 Phytochemical characterization of the crude extract and pure compound

Possible compound	Crude extract	Pure compound
Alkaloids	–	–
Steroids	–	–
Terpenoids	++	–
Saponins	–	–
Flavonoids	–	–
Resins	–	–
Tannin	–	–
Anthraquinone	–	–
Phlobatannins	–	–
Esters	–	–
Carbohydrates	+++	–
Lipids	–	–
Amino Acids	+++	+++

*+ sign denotes the severity of the presence of compound

4 Discussion

Anti- obesity drugs in the present era are no doubt effective but they are bound with some unacceptable side effects leading to serious problems like heart attack and other life threatening diseases. After 13 years of success of a pancreatic lipase inhibitor orlistat, FDA has approved another anti- obesity drug Qsymia with a different mode of action but the salts contained in Qsymia have been prescribed by the doctors for weight loss years ago. So, despite of good performance of Qsymia in clinical trials, it is not a step forward for treating obesity and hence it shows the slow pace of researchers for anti obesity drugs. A new pancreatic and gastric lipase inhibitor Cetilistat by Alizyme therapeutics is under phase III clinical trials and is found to have lesser side effects than orlistat but is purely synthetic drug (Kopelman et al. 2007). As a result there is great need of exploiting natural resources for safe and effective anti obesity drugs.

The above results clearly exhibits that #6 AMLWLS isolated from the leaves of *Aegle marmelos* has potential in vitro pancreatic inhibitory activity. The cultures #1058AMSTITYEL, #59AMSTWLS isolated from stem of *Aegle marmelos* also posses the inhibitory activity but less than #6 AMLWLS. #6 AMLWLS (IC_{50}=2.12 µg/ml) posseses the best pancreatic

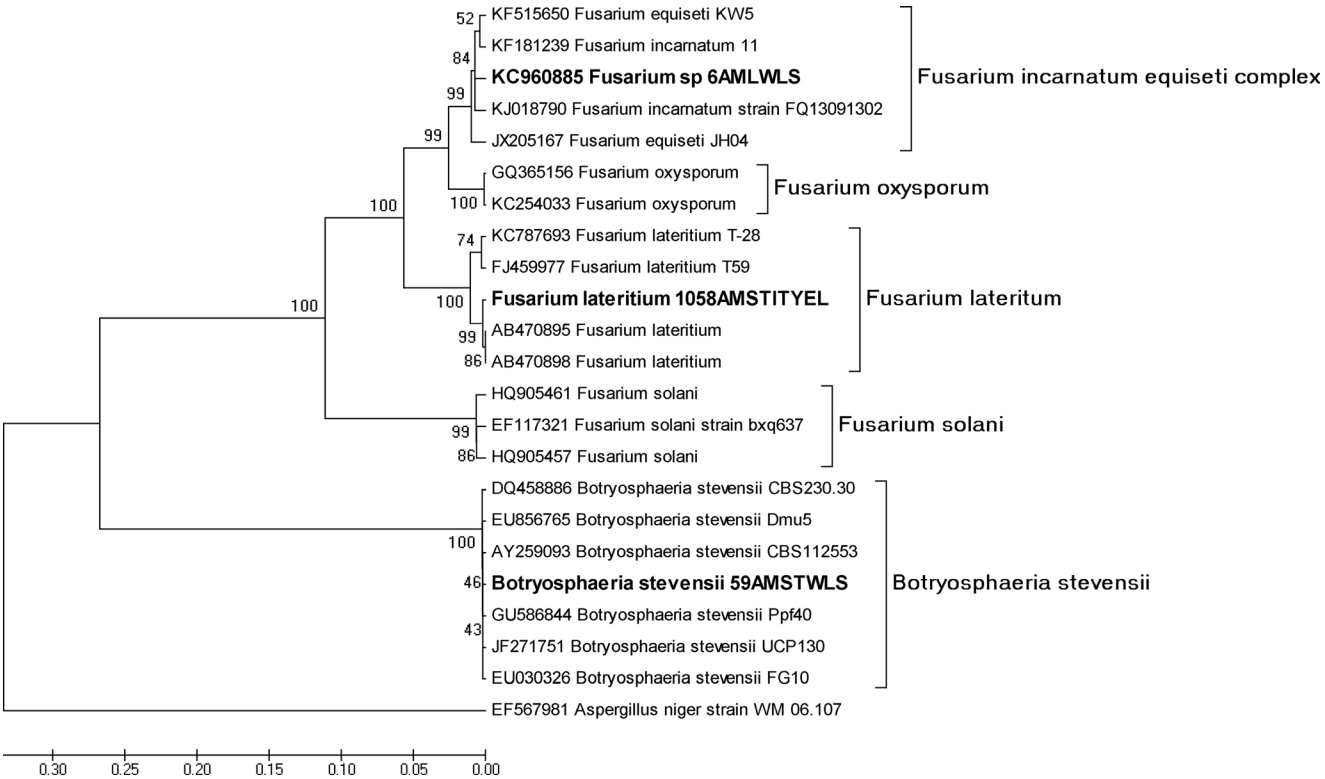

Plate 1 **a** Morphological and Microscopic features of #6 ALMWLS. **a** Colony morphology over PDA, Front view, **b** Colony reverse side, **c** Colony over SNA, 1**d**–**e** Macroconidia over PDA, 1**f** Intercalary chlamydospores, 1**g** Macroconidia over SNA medium

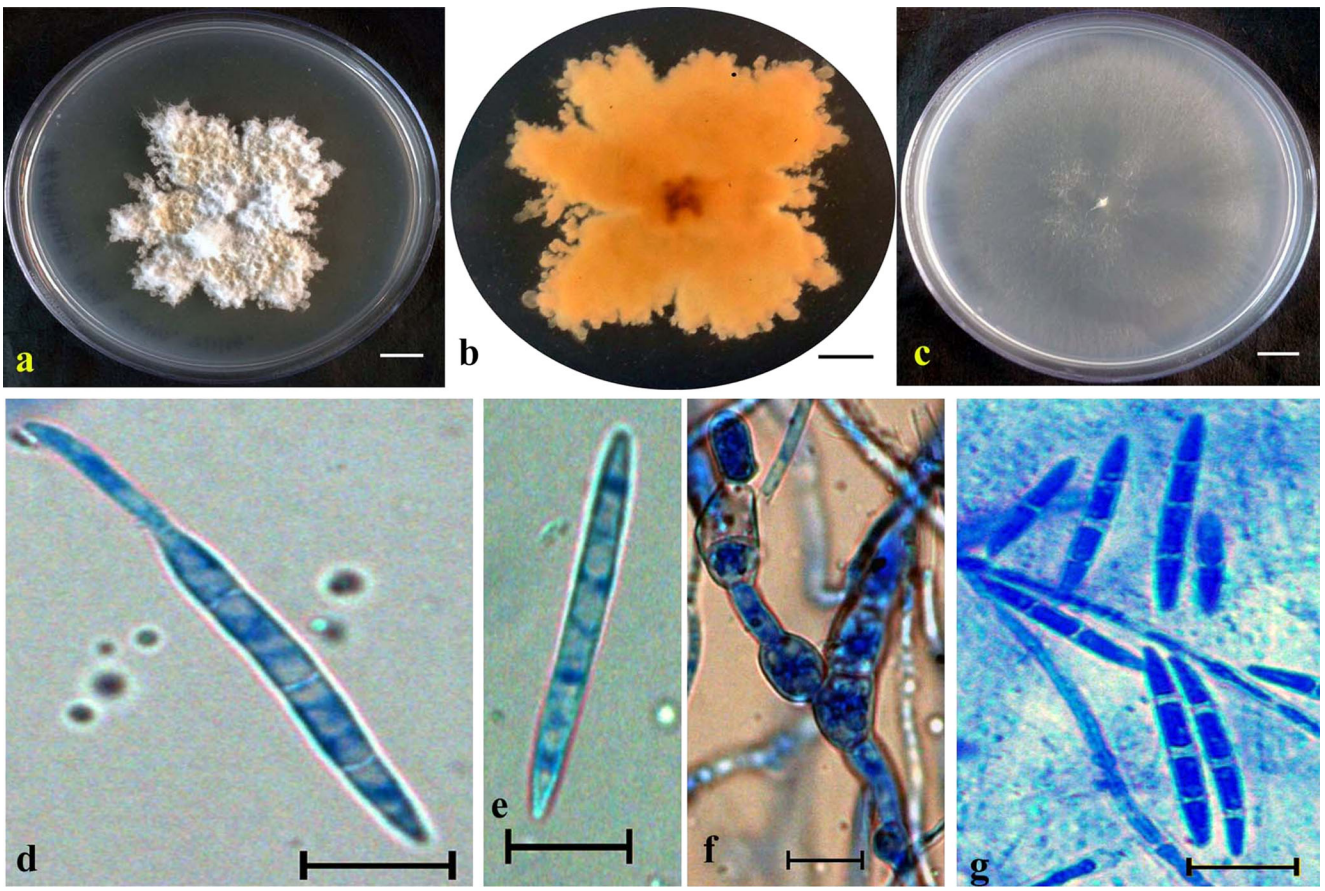

Fig. 2 Phylogenetic tree showing ef-1α gene based molecular taxonomy and phylogenetic analysis of #6 AMLWLS

inhibitory activity amongst the only reported endophytic *Penicillium* species (IC_{50}=3.69 μg/ml) from *Taxus baccata* (Gupta et al. 2014) and the only FDA approved drug Orlistat (IC_{50}=2.79 μg/ml). After column purification, the activity shown by fraction 2 was better than that shown by pure c-glycosidic flavones of *Eremochloaophiuroides* (Lee et al. 2012).

Thus, these results clearly support the hypothesis of mimicking the anti-obesity properties of the host plant *Aegle marmelos* (Karmase et al. 2013) by endophytic *Fusarium* and *Botyrosphareia* species. Hence there exits a symbiotic relationship between host and endophytic fungi. Moreover *Aegle marmelos* is reported to posses *Fusarium* (Gond et al. 2007) and *Botyrosphareia* (Kapoor and Saxena 2014) as endophytic species.

Therefore #6AMLWLS possesses a lead compound that can be further exploited to produce a better anti-obesity drug. This is a first report for exploiting endophytic *Fusarium* species as potent PL inhibitor possessing better inhibitory potential than orlistat.

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