Screening of endophytic fungi for lipase inhibitors as therapeutic modalities in obesity treatment

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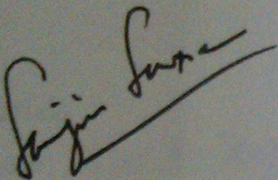
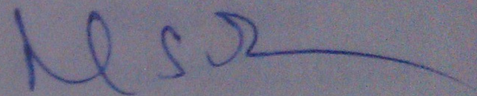
Rahul Rana

Certificate

This is to certify that the thesis entitled ***"Screening of endophytic fungi for lipase inhibitors as therapeutic modalities in obesity treatment"*** being submitted by **Rahul Rana (Roll No-301105014)** in partial fulfillment of the requirements for the award of degree of Master of Science in Microbiology, Thapar University, Patiala is a bonafide work carried out under the esteemed supervision and conception of Dr. Sanjai Saxena and that no part of this thesis has been submitted for the award of any other degree.





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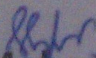

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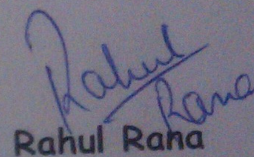
I hereby declare that the work being presented in the thesis entitled "***Screening of endophytic fungi for lipase inhibitors as therapeutic modalities in obesity treatment***" in partial fulfilment of the requirements for the award of degree of Masters in Microbiology, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala is my own laboratory work during the period of January 2013 to June 2013, under the conception and supervision of Dr. Sanjai Saxena, Associate Professor, Department of Biotechnology and Environmental Sciences (DBTES), Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree.



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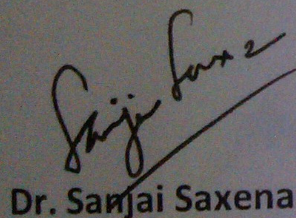

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This thesis is dedicated to one of the nature's masterpieces my parents and my guide

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ABBREVIATIONS

%	Percentage
µg/ml	Microgram per millilitre
µL	microlitre
µM	Micromole
°C	Degree Celsius
5-HT	5-hydroxytryptamine
BLA	Banana Leaf Agar
CHL	Chloroform
DCM	Dichloromethane
DEE	Diethyl ether
DIMS	Direct infusion mass spectra
DNA	Deoxy Ribonucleic Acid
EC_{50}	Effective concentration 50
et al	and others
EtOAc	Ethyl acetate
FFA	Free Fatty Acids
GCMS	Gas chromatography Mass spectroscopy
HFD	High fat diet
i.e.	That is
ITS	Internal transcribed spacer
Kg	Kilogram
mg	milligram
min	minutes
ml	millilitre
mM	millimole

nm	nanometer
PCR	Polymerase chain reaction
PDA	Potato Dextrose Agar
PE	Petroleum ether
PL	Pancreatic Lipase
PNPL	Para nitrophenyl laurate
rpm	Rotations per minute
SNA	Synthetischer nahrstoffarmer agar
THL	Tetrahydolipstatin
TLC	Thin Layer Chromatography
UV	Ultraviolet

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Abstract

In the current study, culture filtrates of 71 endophytic fungal isolates from different medicinal plants were screened for their lipase inhibitory activity. 8 fungal isolates including #1069 AMSTYEL, #1013 AMSTITYEL, #1058 AMSTITYEL, # 59 AMSTWLS showed inhibition of the pancreatic lipase during primary screening using phenol red and rhodamine B plate assay. Quantitative estimation via PNPL assay of the culture filtrate and ethyl acetate residue of #1069 AMSTYEL exhibited 100% and 84.3% inhibition of the given pancreatic lipase. The culture filtrate produced over Czapek Dox Broth (CDB) was extracted with different organic solved based on their polarity. Spectrophotometric analysis reveals that the ethyl acetate residue exhibited maximum inhibitory activity. The ethyl acetate residue was further partially purified using TLC. Out of three fractions obtained, the third fraction showed 84.6% inhibition of the provided porcine pancreatic lipase. The endophytic fungus producing lipase inhibitor molecule was taxonomically identified as *Fusarium* species based on morphotaxonomy and molecular tools.

Further studies on purification and characterization would open the possibilities for the production of a novel lipase inhibitor used in obesity treatement.

Chapter 1

INTRODUCTION

Lipases (EC 3.1.1.3, triacylglycerol hydrolases) are the hydrolysing enzymes that play an important role in fat and lipid metabolism. These enzymes catalyze the hydrolysis of glyceride ester bonds showing they are more active with insoluble ester substrates. According to their site of action lipases are of different types in humans like lingual lipases in mouth cavity, gastric lipases in stomach and pancreatic lipases in intestine. Pancreatic lipase is secreted into the duodenum through the duct system of the pancreas and it is responsible for the hydrolysis of 50 – 70% of total dietary fats (triglycerides) into monoglycerides and fatty acids (Birari *et al*., 2007). Hence, major amount of fats are metabolised and absorbed in intestine. This is critical since these particular lipids cannot be absorbed through the intestinal lining without first undergoing hydrolysis. Hence, pancreatic lipase plays an important role in the dietary fat absorption. The hydrolysis of triglycerides is also dependent on the adequate availability of bile salts provided by the liver. Most dietary fat is ingested as triglycerides (90-95%) and their hydrolysis starts in the mouth, then through the stomach by an acid stable gastric lipase, continues in the duodenum, through the synergistic actions of gastric and colipase-dependent pancreatic lipases (PL) leading to the formation of monoglycerides and free fatty acids (FFA). These FFA are absorbed by the enterocytes to synthesize new triglyceride molecules, which are transported to the different organs via lipoproteins, especially chylomicrons, after a meal. Hence, this enzyme has been widely used in determining the potential efficacy of natural products as antiobesity agents (Sugiyama *et al*., 2007).

Over the past 20 years obesity has become a worldwide concern of frightening proportion. The World Health Organization estimates that there are over 400 million obese and over 1.6 billion overweight adults, a figure which is projected to almost double by 2015. Obesity is characterized by an imbalance between energy intake and energy expenditure (Schrauwen *et al.,* 2000*;* Abete *et al.,* 2010). It leads to various diseases including Type II diabetes, coronary heart disease, hypertension, asthma, orthopedic disorders and certain forms of cancer (Barsh *et al*., 2000; Kopelman, 2000; Luchsinger, 2006). Humans are frequently exposed to fat rich foods, which are generally associated with high energy intake (Little *et al.,* 2007; Astrup *et al.,* 2001). Thus, those foods with a high energy and dietary fat content can be considered to promote body fat storage as well as weight gain in humans (Moreno *et al*., 2003). One explanation is that in commercially available food items, the percentage of energy derived from fat is highly correlated with energy density. Fat contains 9 kcal/g compared with 4 kcal/g for carbohydrates and proteins, foods rich in fat are often high

in energy density. Thus, when a similar volume of food is consumed, energy intake will be higher in high fat diets compared with low fat diets (Schrauwen *et al*., 2000). Furthermore, the consumption of a high fat diet has the ability to modulate the gastrointestinal responses to ingested fat and therefore, it may lead to impairments in appetite regulation which further favours the development of obesity. Physical activity might be helpful in the prevention of obesity by increasing average daily metabolic rate as well as by increasing energy expenditure. Unfortunately, this approach is not helpful for long period of time and weight regain is often seen. So, that's why drugs that prevent weight regain appear necessary in obesity treatment. Development of natural products for the treatment of obesity is a challenging task, which can be launched faster and cheaper than conventional single-entity pharmaceuticals. Different anti obesity drugs are available in the market which are having different modes of action. Currently, one of the most important strategies in the treatment of obesity includes development of inhibitors of nutrient digestion and absorption.

Orlistat is the only FDA approved drug which is clinically approved for obesity management in Europe that acts on peripheral nervous system. This drug acts by inhibiting the activity of PL (pancreatic lipase) thereby reducing the absorption of triglycerides in the intestine and its long-term administration accompaning energy restricted diet, results in weight loss (Neovius *et al*., 2008). Reduction on intestinal lipid digestion has been related to a decrease in the intra-abdominal fat content (Rubio *et al*., 2007). Thus, this drug is associated with a small, but statistically significant weight loss of about 3% more than diet alone in overweight and obese people (Drew *et al*., 2007). Orlistat leads to various adverse affects which includes a range of gastrointestinal side effects, including steathorrhea, bloating, oily spotting, faecal urgency and faecal incontinence, as well as hepatic adverse effects. These adverse effects are very much similar to those which are observed for other lipase inhibitors such as Cetilistat (Kopelman *et al*., 2007). In recent years, obesity has been considered to be a neuroendocrine and metabolic disease, which results from the interface between an obesogenic environment and an intrinsic genetic predisposition. Despite this, lifestyle changes proposed in the treatment of obesity are essential and may be effective, but the rates of long term success tend to be low (Halpern *et al*., 2010).

Other anti obesity drugs that act on central nervous system are sibutramine and rimonabant. Sibutramine is a serotonin-norepinephrine reuptake inhibitor that reduces appetite. Rimonabant is another drug which is a selective blocker of the cannabinoid receptor CB1 targets central as well as peripheral regulation of food intake, was approved in some

European countries until very recently (Luisa Isidro and Fernando Cordido, 2010). Sibutramine was withdrawn from the European market in 2009 due to its side effects leading to Sibutramine Cardiovascular Outcomes Trial (SCOUT) which results in increasing cardiovascular mortality in patients using the medication in the group with prior cardiovascular disease while Rimonabant has also some side effects like depression, anxiety and nausea. Although still these drug are available in other countries, but they are not recommended for those patients who are suffering from this disease. Therefore, at the same time new drugs are being tested, it seems attractive to use different strategies such as combination of drugs.

The medicinal plants were used for the treatment of many diseases. As, the population is increasing, it is not possible to fully depend and afford plant based medicine. So, this demand made many researchers to explore bioactive compounds from the endophytic species which can be used in the treatment of disease (Onifade, 2007). The term "endophytic fungi" refers to an organism that lives within photosynthetic plant tissue by forming symbiotic relationship with host (Arnold *et al.,* 2000; Shekhawat *et al.,* 2010) and it does not cause any harmful effect to the plant species (Saithong *et al.,* 2010; Wei *et al.,* 2007). Arnold *et al.,* (2003) and Selvanathan *et al.,* (2011) found that endophytic fungi provides defence mechanism to host plant against pathogen. The endophytic mycobiota are distributed based on the ecological and physiological factors in plants (Khan *et al.,* 2010) such as geographical location (Fisher *et al.,* 1994; Collado *et al.,* 1999), age and specificity of host tissue (Khan *et al.,* 2010; Sahashi *et al.,* 2000). Zhang *et al.,* (2006) and Strobel, (2000) reported that endophytic fungi harboured in medicinal plants makes the host to adapt extreme climatic conditions and with the passage of time these endophytic fungi resembles their host plant. The fungal communities reside inside the healthy tissue of medicinal plants which increases the absorption of soil nutrients thereby changing the nutrient cycle (Krishnamurthy *et al.,* 2008). Hence, the endophytic fungi from the plants are an important source for the production of various secondary metabolites and bioactive compounds which is useful for pharmaceutical industries (Strobel, 2000; Krishnamurthy *et al.,* 2008; Khan *et al.,* 2010)

Chapter 2

REVIEW OF LITERATURE

2.1 Obesity and Its Related Problems

'Obesity' is defined as an excess storage of fat in the body to such an extent that it causes health problems leading to excess mortality (Sorensen *et al.,* 2010). The pragmatic definition of obesity is based on the fact that an increase in body mass is predominantly driven by an increase in fat store. The lean (non fat) body mass contributes to the total body mass and may show considerable heterogeneity for a given size of fat mass, but lean mass is also correlated and etiologically linked to fat mass (Sorensen *et al.,* 2010).

Numerous diseases are caused or made worse by obesity. These include type II diabetes, hypertension, dyslipidemia, ischemic heart disease, stroke, obstructive sleep apnea, asthma, nonalcoholic steatohepatitis, gastroesophageal reflux disease, degenerative joint diseases, infertility and polycystic ovary syndrome, various malignancies and depression. Type II diabetes is perhaps the most visible obesity-related problem (Brien and Dixon, 2002).

2.2 Pancreatic Lipase

Pancreatic lipase (PL), the principal lipolytic enzyme synthesized and secreted by the pancreas plays a key role in the efficient digestion of triglycerides. The primary structure of the PL was established by analysis of cDNA clones isolated from a human pancreas cDNA library and found to be a single chain glycoprotein of 449 amino acids (Birari and Bhutani, 2007). It removes fatty acids from α and α' position of dietary triglycerides yielding β-monoglycerides and long chain fatty acids (saturated and polyunsaturated) as lipolytic products (Birari and Bhutani, 2007).

PL inhibition is one of the most widely studied mechanisms for the determination of the potential efficacy of natural products as anti-obesity agents. In recent years, several bacterial, fungal and marine species as well as extracts from different plant sources have been screened for finding new compounds with pancreatic lipase inhibitory activity.

2.3 Selective drug therapies for obesity

During 1990s some of the promising areas for the pharmacotherapy of obesity included central and/or peripheral neurochemical mechanisms which involve serotonin (5-hydroxytryptamine, 5-HT), catecholamines and certain neuropeptides. Serotonergic and catecholaminergic mechanisms were targeted by Fen –Phen i.e. by effective combination therapy, composed of the 5-HT releasers fenfluramine or dexfenfluramine combined with phentermine, a noradrenergic releaser (Sundaresan *et al.,* 1992). The combination therapy

was adopted due to the action of drugs on separate monoaminergic systems and therefore might synergize, allowing for reduced dosing and consequently leads to fewer side effects [Bray *et al.,* 2010]. These expectations were met and the effective dose of each of the combined drugs was lower than either of the drugs given alone (Bray *et al.,* 2010). Unfortunately, even the lower dose of fenfluramine was leading to serious side effect of cardiac vulvulopathy and this therapy was removed from the market (Connolly *et al.,* 1997). Another target of promise was the peripheral β-3 adrenergic receptor. This receptor was targeted using selective agonists to increase energy expenditure through the activation of brown fat but these compounds lacked efficacy and the less selective compounds produced adverse side effects in humans (Lipworth *et al.,* 1996).

2.4 Lipase inhibitors from plant source

Flavan dimmers which showed lipase-inhibiting effects were isolated from fruits of *Cassia nomame* (Leguminosae). Structures of two new compounds among them were determined to be (2S)-3^1, 4^1, 7-trihydroxyflavan-(4-8)-catechin. Four flavan dimers structurally related to these two compounds were also synthesized for spectral comparision. Among 10 flavan dimmers tested for lipase inhibitory activity, (2S)-3^1, 4^1, 7-trihydroxyflavan-(2S)-catechin showed the most potent inhibitory effect (Hatano *et al.,* 1997).

In China and Korea, the fresh roots of *Platycodin grandiflorum* are consumed as pickles for preventing obesity. The saponin-rich fraction of *Platycodin grandiflorum* radix has been screened for its antiobesity effect and these effects correlate with inhibition of PL (Han *et al*., 2000).

The methanolic extract from the levels of *Salvia officinalis* L (sage) showed inhibitory effect on serum triglyceride elevation in olive oil fed mice (500 and 1000 mg/kg) and inhibitory activity (IC_{50} 94 μg/mL) against pancreatic lipase. Through bioassay-guided separation using inhibitory activity against pancreatic lipase activity, 4 abietan-type diterpenes (carnosic acid, carnosol, royleanonic acid, and 7-methoxyrosmanol) and a triterpene (oleanolic acid) were isolated from the active fraction. Among these compounds carnosic acid and carnosol substantially inhibited pancreatic lipase activity with IC_{50} values of 36 μM and 13 μM respectively. Carnosic acid significantly inhibited triglyceride elevation in olive oil fed mice at doses of 5-20 mg/kg. However, other constituents (carnosol, rorleanonic acid, oleanoic acid) did not show any effects even at a dose of 200 mg/kg. Furthermore, carnosic acid (20 mg/kg/day) reduced the gain of body weight and the

accumulation of epididymal fat weight in high fat diet-fed mice after 14 days (Ninomiya *et al.,* 2004).

Bioactive-guided fractionation of a saponin-rich fraction of the leaves of *Accanthopanax sessiliflorus* led to the isolation of the active lupane-type saponins, sessiloside and chiisanoside, both of which showed strong inhibition of PL *in vitro*. Further, it inhibited lipase activity in a dose-dependent manner and their IC_{50} values were 0.36 and 0.75 mg/ml respectively (Birari and Bhutani, 2007).

In 2009 Dougall *et al.,* tested polyphenol rich extracts from a range of berries for their ability to inhibit pancreatic lipase activity *in vitro*. Blackcurrant and rowan extracts showed no inhibitory effect, blueberry showed slight inhibition, while lingonberry, Arctic bramble, cloudberry, strawberry and raspberry were considerably more effective. Inhibition showed by the cloudberry extract has saturation effect with an apparent EC_{50} of around 5 µg phenols/ml. Further, the inhibitory components from cloudberry were retained in a tannin-rich fraction which was prepared by sorption to Sephadex LH-20. Comparison of the polyphenol composition of the active and inactive fractions using liquid chromatography–mass spectrometry (LC–MS) strongly suggested that the active components were ellagitannins. Similarly prepared fractions from raspberry and strawberry were also found to be effective inhibitors against pancreatic lipase. Direct infusion mass spectra (DIMS) of the raspberry tannin and cloudberry tannin fractions showed similarity with minor differences in the abundance of certain ellagitannin components whereas the strawberry tannin fraction was found to be enriched in a mixture of ellagitannin and proanthocyanidin components. The effective inhibition of lipase by the lingonberry extract may have been due to proanthocyandin components.

Tea is found to be the most commonly consumed beverage in the world other than water. Flavan-3-ols and flavonols are the major phenolics which are present in tea. The flavan-3-ols are characterized by epicatechin and its galloylated derivatives especially in green tea whereas these derivatives are present in lesser amount in back tea due to their oxidative conversion into theaflavins and thearubigins (Balentine, 1992; Finger *et al.,* 1992; Del Rio *et al.,* 2004; Mizukami *et al.,* 2007). The flavonols are mainly derivatives of quercetin and kaempferol (Del Rio *et al.,* 2004; Price *et al.,* 1998) but there are smaller amounts of tannins and hydroxycinnamate derivatives. Epidemiological studies have suggested correlations of tea intake with favourable outcomes with regard to cardiovascular disease, cancer incidence, inflammation, obesity and type 2 diabetes risk .Therefore, a range

of mechanisms have been proposed for the beneficial effects of tea and health, which largely focus on the polyphenol components, especially the flavan-3-ols.

In 2010, McDougall *et al.,* assayed green, white and black tea for inhibition of pancreatic lipase activity *in vitro*. White tea was found to be more effective than green tea with black tea showing little inhibition even at 200 µg GAE/ml. The EC_{50} values for inhibition were 22 µg/ml and 35µg/ml for white tea and for green tea respectively; both easily achievable from normal infusions of tea. Liquid chromatography-mass spectroscopy was performed. It showed that white and green teas had essentially equal amounts of flavan-3-ols but green tea had higher levels of flavonols. White tea had higher levels of 5-galloyl quinic acid, digalloyl glucose, trigalloyl glucose and the tannin, strictinin.

Activity guided fractionation of a methanolic extract of the leaves of *Eremochloa ophiuroides* (centipede grass) using a pancreatic lipase inhibitory assay led to the isolation and identification of a new *C*-glycosidic flavone, luteolin 6-*C*-β-D-boivinopyranoside, as well as eight known compounds. The known compounds were identified as orientin, isoorientin, derhamnosylmaysin, isoorientin 2-*O*- L-rhamnoside, luteolin, chlorogenic acid, methyl chlorogenate and caffeic acid. The structures of these compounds were determined on the basis of interpretation of their spectroscopic data. Among these isolates, the *C*-glycosidic flavones 1–5 showed potent inhibitory effects on pancreatic lipase, with IC_{50} values ranging from 18.5 ± 2.6 to 50.5 ± 3.9 µM (Lee *et al.,* 2010).

Crude extracts from 400 plants were tested for anti obesity activity using porcine pancreatic lipase assay. Furthermore, 44 plant extracts were investigated for their inhibition of lipid accumulation in 3T3-L1 cells. Among these 44 extracts examined, crude extracts from 4 natural plant species were active. *Salicis Radicis Cortex* had the highest fat inhibitory activity whereas *Rubi Fructus*, *Corni Fructus* and *Geranium nepalense* exhibited fat inhibitory capacity higher than 30% at concentration 100 µg/mL in 3T3-L1 adipocytes suggesting anti obesity activity (Roh C and Jung U, 2012).

Polyphenols have been known to have benefits in relation to health and importance in number of studies (John and Shahidi, 2010, Khanam *et al.,* 2012 and Torronen *et al.,* 2012) and even more in anti-cancer effects (de Mejia *et al*., 2010). They own potential to prevent obesity by inhibiting enzymes which are related to lipid metabolism including lipoprotein lipase, pancreatic lipase and glycerophosphate dehydrogenase (Birari and Bhutani, 2007). The anti-obesity effects of hot water extract from the leaves of *Acanthopanax sessiliflorus* and *Corchorus olitorius* L. are produced mainly via decreasing a pancreatic lipase activity (Wang *et al.,* 2011). Similar results were also observed in a study of Yang *et al.,* (2010)

which indicated that water extracts of longan flower (*Dimocarpus longan* Lour.) can restrain hypercaloric dietary rats partially from overweight and reduce body lipid accumulation by an inhibition of the lipase activity, downregulation of lipiogensis and increased energy expenditure.

Polyphenol-rich water extracts from litchi flower have anti-obesity and anti-inflammatory potentials. According to a report from Chen *et al.,* (2011) acetone, methanol and water extracts of litchi flower contains a large amount of phenols, flavonoids and condensed tannins. Thus, shows a strong antioxidative capacities and anti-inflammatory effect (Chang *et al.,* 2013). In 2013, Samuel *et al.,* assayed litchi flower and their previous report indicated that drinking litchi flower-water extracts (LFWEs) can lower serum lipid levels in high-fat/cholesterol diet fed hamsters. Hence, this assumes that litchi flower-water extracts (LFWEs) have anti-obesity effects. As, Litchi flower–water extract (LFWE) contains plenty of phenolic acids, flavonoids, condensed tannins, anthocyanins, and proanthocyanidins. Therefore, the result of this investigation match the anticipation, which LFWE indeed possesses a potential nutraceuticals for anti-obesity effects.

In 2013, Kato *et al.,* found inhibitory activity in the extracts of *E. polyantha* leaves against pancreatic lipase. Purification of the compounds resulted in isolation of hydroxychavicol, and two structurally new dimers. Inhibitory activity was showed by all the isolated compounds against porcine pancreatic lipase and high content of hydroxychavicol (1.83 wt. %) indicated this compound to be responsible for the majority of inhibitory activity of *E. polyantha* extract. Furthermore, hydroxychavicol is also reported to possess anti-carcinogenic, anti-microbial, anti-infammatory and anti-oxidant activity which is related to traditional usage of this plant. These results offer this plant as an attractive material for treating various health problems including obesity.

Dioscin isolated from methanol extract of *Dioscorea nipponica* powder was shown to inhibit PL with an IC_{50} of 20 mg/ml. It is a glycone diosgenin, which was also found to be active with an IC_{50} value of 28 mg/ml. Both of them suppressed the time-dependant increase of plasma triglyceride concentration in mice injected with corn oil. Other oleanan type of saponins isolated from the same plant viz. prosapogenin A (IC_{50} 1.8 mg/ml), prosapogenin C (IC_{50} 42.2 mg/ml), and gracillin (IC_{50} 28.9 mg/ml) also showed a strong inhibition of PL *in vitro* (Mukherjee and Sengupta 2013).

Aegle marmelos (bael), known is a medicinally important plant belonging to rutaceae family. It is amongst one of the important plants in the Indian pharmacopeia; Ayurveda.

Various parts of this plant such as leaves, roots, seed, bark and fruit possess different medicinal properties (Maity *et al.,* 2009). Extracts of beal possess anti-diabetic property. In 2013, Birari and Bhutani have studied dichloromethane (DCM), ethyl acetate (EtOAc) and *n*-butanol extracts of *A. marmelos* leaves for their lipolytic effect. Lipolysis was measured by determining the amount of glycerol released at 12 h and 24 h at 50 µg/ml and 100 µg/ml concentrations. Phytochemical investigation of the most active DCM extract yielded 14 compounds. After this, the isolated compounds were evaluated for their lipolytic effects at 50 µM and 100 µM. Umbelliferone and esculetin i.e. most active compounds were further screened for their antiobesity effects *in vivo* in the high fat diet (HFD) induced obese rat model.

2.5 Lipase inhibitors from microbial sources

Ebelactone A and B, natural products from *Streptomyces aburaviensis* are potent inhibitors of pancreatic lipase. Ebelactone B inhibited, in a dose dependent manner, the intestinal absorption of fat in animals. The most effective inhibition was observed when the inhibitor was administered 60 min prior to fat feeding. When ebelactone B was administered at 10mg/kg, the serum level of TG and cholesterol were decreased by 58 and 35 % respectively. Since ebelactone B is effective inhibitor for fat absorption, it may be a promising molecule for therapy of hyperlipidemia and obesity (Umezawa, 1980).

Lipstatin, a novel and very potent inhibitor of pancreatic lipase has been isolated from *Streptomyces toxytricini.* Its hydrogenated analogue, tetrahydolipstatin (THL) ((s)-1-{[(1s, 2s, 3s)-3-hexyl-4-oxo-2-oxetanyl] methyl} dodecyl-(s)-1-fomamido-4-methylvalerate) has selective inhibitory action for pancreatic lipase, where as phospholipase A2, amylase and trypsin activity was not altered by THL. In addition to pancreatic lipase, other lipases, such as carboxylester lipase, gastric lipase and the bile salt stimulated lipase of human milk are also inhibited by THL. The inhibition is due to covalent binding of THL to ser 152, which is one of the residues in the catalytic trial of this enzyme (Hadvary *et al.,* 1991). Lipstatin is closely related to the esterase inhibitor esterastin, which contains an n-acetyl asparagine side chain instead of N-formyl leucine. Lipoprotein lipase was rapidly inactivated by low concentration of the inhibitor tetrahydrolipstatin (Weibel *et al.*, 1987).

Panclicins A, B, C, D and E are novel pancreatic lipase analogues of tetrahydrolipstatin, which contain a β - lactone and a N- formyl leucine ester, isolated from *Streptomyces* sp. The potency of the inhibitory activity of each compound is attributed to the amino acid moiety of each structure. The IC_{50} of panclicins A, B, C, D and E for porcine

pancreatic lipase are 2.9, 2.6, 0.62, 0.66 and 0.89 micron, respectively. The panclicins are either glycine- type compounds such as Panclicin A, C, D and E which are two or three fold more potent than THL or alanine type compounds such as Panclicin A and B, which are less potent than the glycine type compounds. They irreversibly inhibit pancreatic lipase. However, the compounds do not irreversibly inhibit the enzyme as strongly as THL (Mutoh *et al.*, 1994).

The presence of an inhibitor of pancreatic lipase (triacylglycerol acylhydrolase) was screened in 54 marine algae. An active inhibitor, caulerpenyne, was purified from an extract *Caulerpa taxifolia* using ethyl acetate extraction. Caulerpenyne competitively inhibited lipase activities using emulsified tirolein and dispersed 4-methylumbelliferyl oleate (4-MU oleate) as substrates. The concentrations producing 50% inhibition against triolein and 4-MU oleate hydrolysis were 2 mM and 13 µM respectively (Bitou *et al.*, 1999).

A total of 120 methanol and dichloromethane extracts from 60 species of wood-damaging fungi and 50 methanol/water extracts from macrofungi were screened for inhibition of pancreatic lipase using the chromogenic substrate *p*-nitrophenyl palmitate. Out of the extracts screened, *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).

Vibralactone, an unusual fused β-lactone type metabolite produced by cultures of the Basidiomycete *Boreostereum vibrans*, was found to inhibit pancreatic lipase with an IC_{50} of 0.4µg/mL (Liu *et al.,* 2006).

A S*treptomyces sp*. MTCC 5219 isolated from a soil sample of a cow barnyard in India was found to produce a new lipase inhibitor. This bioactive compound was produced under optimum fermentation conditions and extracted by solvent extraction followed by chromatographic separation. The pure compound was found to have a molecular weight of 176 Daltons belonging to the class of enol acetate of p-amino phenyl acetaldehyde. This compound inhibited the hydrolysis of trioleate by porcine pancreatic lipase dose dependently with IC_{50} of 7.46µM. The IC_{50} value of this molecule is high as compared to commercial pancreatic lipase inhibitor orlistat (Tokdar *et al.,* 2011).

A *Streptomyces* species isolated from the western ghat soil samples of Agumbe, Karnataka, India was reported to show pancreatic lipase inhibition. Pancreatic lipase inhibitory (Anti-obesity) activity of butanol extract was tested against chicken pancreatic lipase using olive oil as the substrate. The inhibition of lipase by the extract was

concentration dependent and an inhibition of >60% was observed at extract concentration 50mg/ml. The LC_{50} of solvent extract was found to be 42.11 μg/ml (Kekuda *et al.,* 2011).

SOURCE	**ACTIVE COMPONENT**	**MAJOR ACTIVITY**	**REFERENCES**
Juniperus communis(bark)	Crude ethanol extract	IC_{50} = 20.4µg/ml	Kim and Kang, (2005)
Illicium religiosum (wood)	Water extract	IC_{50} = 21.9µg/ml	Kim and Kang, (2005)
Panax japonicas (rhizomes)	Chikusetsusaponins	22% decrease in body weight gain	Han *et al*., (2005)
Platycodi radix	Platycodin saponins	13% decrease in body weight gain	Zhao *et al*., (2005), Zhao and Kim, (2004), Han *et al*., (2002)
Platycodi radix	Crude aqueous/ethanolic extract (saponins)	12% decrease in body weight gain	Han *et al*., (2000)
Acanthopanax senticosus	10.6% ellagic acid	54% decrease in body weight gain	Lei *et al*., (2007), Han *et al*., (2000)
Thea sinensis (Oolong Tea)	Crude aqueous extract (caffeine)	10% decrease in body weight gain	Han *et al*., (1999)
Thea sinensis (Leaf)	Saponins	17% decrease in body weight gain	Han *et al*., (1999,2001)
Nelumbo nucifera (Leaf)	Crude ethanolic extract	28% decrease in body weight gain	Ono *et al*., (2006)
Salvia officinalis	Methanolic extract	IC_{50} = 36µg/ml	Ninomiga *et al*., (2004)
Dioscorea nipponica	Crude methanolic extract	IC_{50} = 5-10µg/ml, 37% decrease in body weight gain	Kwon *et al*., (2003)
Laetiporus sulphureus	Mycelia extract	83% inhibitory activity on lipase activity	Slanc *et al*., (2004)
Tylopilus felleus	Mycelia extract	96% inhibition	Slanc *et al*., (2004)
Hygrocybe conica	Mycelia extract	97% inhibitory activity on lipase activity	Slanc *et al*., (2004)
Boreostereum vibrans	Vibralactone	IC_{50} = 0.4µg/ml	Liu *et al*., (2006)
Streptomyces toxytricini	Lipstatin	IC_{50} = 0.14µM	Weibel *et al*., (1987), Hochuli *et al*., (1987)
Streptomyces Sp.NR0619	Panclicins	IC_{50} = 0.89µM with Panclicin D	Mutoh *et al*.,(1994), Yoshinari *et al.,*

			(1994)
Actinomycetes Sp.MG147-CF2	Valilactone Esterastin Ebelactone B Ebelactone A	IC_{50} = 0.00014µg/ml IC_{50} = 0.2µg/ml IC_{50} = 0.001µg/ml IC_{50} = 0.003 µg/ml	Kitahara *et al.*, (1987) Umezawa *et al.*, (1978) Umezawa *et al.*, (1980) Umezawa *et al.*, (1980)
Citrus unshiu	Hesperidin	IC_{50} = 32 µg/ml	Kawaguchi *et al.*, (1997)
Marine algae (*Caulerpa taxifolia*)	Caulerpenyne	IC_{50} = 2mM	Tomoda *et al.*, (2002)

Table No.1 Anti-Obesity compounds showing inhibition of Pancreatic Lipase from different microbial sources

2.6 Endophytic fungi: Nature's green gold

Endophytic fungi are the rich source for novel organic compounds with biological activities. They represent an ecological source and their secondary metabolites are active because of their interaction with host. For example, the endophytic fungus *Taxomyces andreanae* produced taxol with anticancer activity. Other reports showed that certain endophytic fungi produced more than twelve metabolites similar to those produced by host plants with therapeutic function, including alkaloids, steroids, terpenoids, isocoumarin derivatives, flavinoids, quinines, phenylpropanoids, phenylpropanoids and ligans, peptides, phenol and phenolic acid, aliphatic compounds and chlorinated metabolites (Chung *et al.,* 2012).

Since, no report exists on lipase inhibitor production from endophytic fungi. Thus, endophytic fungi can represent a biological resource for production of lipase inhibitor, which can be used as therapeutic agent in obesity.

2.7 Assays for estimating lipase inhibition

2.7.1 Rhodamine B Assay Rhodamine B is used to observe the zone of lipolysis as an orange fluorescence under UV light at 350 nm. Olive oil added to the medium will act as a substrate for lipases which on breakdown results in the formation of fatty acids and

monoglycerides. Rhodamine forms a fluorescent complex with these free fatty acids thus giving orange fluorescent halo visible under UV light (Gupta *et al.,* 2006).

2.7.2 Phenol Red Assay Phenol red is a pH indicator dye showing difference in colour at various pH ranges differing from acidic to basic (Yellow to Orange- Below pH 7, Blood red- pH 7, Magenta to Pink – Above pH 8). Olive oil added to the medium will act as a substrate for lipases which on breakdown results in the formation of fatty acids and monoglycerides. As fatty acids are formed the pH of media drops to acidic showing yellow coloured halos (Singh *et al.,* 2005).

2.7.3 Quantitative Estimation (Spectrophotometric assay)

p-Nitrophenyl esters of various-chain-length fatty acids are generally used as substrates and release of *p* -nitrophenol is measured spectrophotometrically at 410 nm. Short-chain esters are water-soluble and therefore their hydrolysis provides a measure of esterase, rather than lipase, activity. However, *p* –nitrophenyl palmitate is used to measure lipase activity. A major limitation of this assay is that the enzymic reactions cannot be performed at acidic pH owing to the lack of absorbance of *p*-nitrophenol at acidic pH. Thus enzyme activity at only neutral and alkaline pH values can be ascertained by this procedure. For acidic enzymes, this assay has been used by raising the pH of the reaction mixture after culmination of the reaction (Gupta *et al., 2003*). This assay was also used during screening of crude plant extracts by Roh C and Jung U (2012).

2.7.4 Partial Purification

All extracts, dissolved in ethyl acetate, were evaluated by TLC with sclerotiorin as standard. Elution was carried out with hexane:ethyl acetate 2:1 (v/v). The TLC adsorbent used was 60 G silica gel (Merck, article 7731), with a layer thickness of 0.25 mm on glass and activated at 100 °C for 30 min. TLC plates were viewed on UV-Vis., iodine fumes and Godin solution (comprised of 1 % vanillin in methanol and 3 % perchloric acid in water, 1:1). This method was used during the production of pharmacologically-active sclerotiorin by *Penicillium sclerotiorum* (Lucas *et al.,* 2010).

2.7.5 Identification of Endophytic Fungi

The identification of endophytic fungi based on morphological and molecular technique. Morphological taxonomy has to be carried by examining colony morphology by naked eye and/or characteristics of asexual spores and/or sexual spores under a compound microscope (Chung *et al.*, 2012). Some methods have been used to promote sporulation of isolates in order to overcome the shortcomings of isolates failing to sporulate in culture (Taylor *et al.,* 1999; Guo *et al.,* 1998, 2000, 2008). Various media such as Banana Leaf Agar (BLA), Water Agar (WA), and Synthetischer nahrstoffarmer agar (SNA) have been used in which fungi sporulate (Guo *et al*., 2000).

In order to overcome the potential technical bias, cultivation-independent approaches, eg., molecular techniques, to analyze endophytic fungal communities of plants are needed. DNA was extracted using a DNeasy Plant Mini kit according to the manufacturer's protocols (Qiagen Co., Valencia, CA, USA). PCR was then conducted to amplify the internal transcribed spacer (ITS) region of the extracted DNA, including the 5.8s rDNA, using the primers ITS-1 and ITS-4 (Yoo and Eom, 2012).

Chapter 3

AIM OF THE STUDY

3.1 Aim of the study

The current study aimed at 'Screening of endophytic fungi for lipase inhibitors as therapeutic modalities in obesity treatment'.

The objectives of the current study were:

1. Screening of endophytic fungi for lipase inhibitory activity
2. Quantitative/spectrophotometric estimation of lipase inhibitory activity
3. Identification of the endophytic fungus producing lipase inhibitor

Chapter 4

MATERIALS AND METHODS

4.1 Media preparation and Maintenance of pure culture

4.1.1 Preparation of potato dextrose agar (PDA) plates

PDA plates were prepared by dissolving 39.0 g/L of potato dextrose agar (Hi-Media) in lukewarm double distilled water and stirred thoroughly and was autoclaved at 121°C for 15 minutes. Around 25 ml of the autoclaved PDA was dispensed in sterile 90 mm petri plates under sterile conditions and was allowed to solidify at room temperature. The PDA plates were incubated at 28°C for further use.

4.1.2 Sub culturing and Maintenance of Endophytic Fungal cultures

71 endophytic fungal cultures isolated previously in the laboratory from some of the medicinal plants were aseptically sub cultured on petri plates containing Potato Dextrose Agar and incubated at 28°C for 7-10 days. The pure isolates thus obtained were transferred and maintained on PDA slants at 28°C. Pure isolates of endophytic fungal cultures were maintained on PDA slants containing 10% glycerol for long term use at 28°C (Macarthur and McGee, 2000).

4.2 Production of Culture Filtrate

Czapek Dox Broth (CDB) was used for the production of culture filtrate of 71 endophytic fungal cultures. 5 mm plug of actively growing endophytic fungal cultures were inoculated into Erlenmeyer flasks containing 30 ml Czapek Dox Broth. The flasks were incubated over orbital shaker (New Brunswick) at 28°C for 10days at 120 rpm. After incubation, the fungal cultures were filtered through Whatman filter paper no.4. The filtrate thus obtained was centrifuged at 10000 rpm for 10 mins at room temperature. Supernatant was collected in a separate flask and the cell free culture filtrate- obtained were then stored at -80°C for further use (Setyawat, 2003).

S.No.	Ingredients	g/l
1.	Sucrose	30
2.	Sodium nitrate	3
3.	Dipotassium phosphate	1
4.	Magnesium sulphate	0.5
5.	Potassium chloride	0.5
6.	Ferrous sulphate	0.01

Table No.2 Composition of Czapek Dox Broth

4.3 Primary Screening- Qualitative screening using olive oil plate assay

4.3.1 Rhodamine Plate Assay

Rhodamine B is a fluorescent dye used to observe the zone of lipolysis as an orange fluorescence under UV light at 350 nm. This assay includes 1.3% agarose, 2.5% olive oil, 0.3% rhodamine (2.5 mg/ml), tween 80, 2.5 mM phosphate buffer (pH 7.4), Porcine pancreatic lipase (Sigma). Medium containing 1.3% agarose was autoclaved at 121°C for 15 minutes. After autoclaving 2.5% olive oil was added to the medium with proper mixing. Few drops of Tween 80 to emulsify oil and 0.3% of rhodamine were subsequently added. This media was further poured in petri plates and was allowed to solidify. Wells were scooped in petri plates with the help of 5 mm core borer. Further, reaction mixture of culture filtrate of different endophytic fungi and lipase (100 mg/ml in 2.5 mM phosphate buffer) was prepared by adding 110 µl of culture filtrate and 60 µl of lipase in eppendorf tubes. This reaction mixture was further incubated at 37°C for 30 minutes. After incubation, 35 µl of above reaction mixture was added in each well along with 30 µl of lipase as a control in separate well in the petri plate. Petri plates were kept for incubation at 37°C for 24-48 hours. Similarly, same procedure was followed for 70 culture filtrate of endophytic fungi.

4.3.2 Phenol Red Plate Assay

Phenol red is a pH indicator dye showing difference in colour at various pH ranges differing from acidic to basic (yellow to orange- below pH 7, blood red- pH 7, magenta to pink – Above pH 8). This assay includes 2% agar, 1% olive oil, 0.01% phenol red (10 mg/ml), few drops of tween 80, water, 2.5 mM phosphate buffer (pH 7.4), Porcine pancreatic lipase. Media was prepared by adding 2% agar in distilled water which was further autoclaved at 121°C for 15 minutes. After autoclaving, the media was allowed to cool till 60°C. Then 1% olive oil, few drops of tween 80, 0.01% phenol red were added in flask containing 2% agar. The pH of the media was brought upto neutral by adding few drops of 1 N NaOH giving media blood red colouration. This media was further poured in petri plates and wells were made in petri plates with the help of presterlised 5 mm core borer. Further, reaction mixture of culture filtrate of different endophytic fungi and lipase (100 mg/ml in 2.5 mM phosphate buffer) was prepared by adding 110 µl of culture filtrate and 60 µl of lipase in eppendorf tubes. This reaction mixture was further incubated at 37°C for 30 minutes. After this, 35 µl of above reaction mixture was added in each well along with 30 µl of lipase as a control in

separate well in the petri plate. Further, petri plates were kept for incubation at 37°C for 24-48 hours. Similarly, same procedure was followed for 70 culture filtrate of endophytic fungi.

4.4 Quantitative estimation

4.4.1 Enzyme Kinetics

This included 2 mM para nitrophenyl laurate (PNPL), sodium deoxycolate, 0.5 M monosodium orthophosphate buffer, 2.5 mM phosphate buffer, isopropanol, autoclaved water, Porcine pancreatic lipase (1mg/ml). Stock of 2 mM PNPL (dissolved in isopropanol) was prepared and further diluted to different concentrations (180 µM, 220 µM, 260 µM, 300 µM, 340 µM, 400 µM, 430 µM, 500 µM). 180 µl of different concentrations of PNPL, 20 µl of Phosphate buffer, 20 µl of lipase were added to micro titre plate. Reading at 405 nm was taken for different concentrations for 0 minute. Similarly, at the regular intervals of 10 minutes readings of different concentrations were taken till 3 hrs. K_m and V_{max} values were calculated by plotting lineweaver burk graph using Graph pad prism 5.

S.No.	Concentrations of PNPL	Preparation of PNPL Concentrations
1.	180 µM	36 µl of PNPL+364 µl of water
2.	220 µM	44 µl of PNPL+356 µl of water
3.	260 µM	50 µl of PNPL+350 µl of water
4.	300 µM	60 µl of PNPL+340 µl of water
5.	340 µM	68 µl of PNPL+332 µl of water
6.	400 µM	80 µl of PNPL+320 µl of water
7.	430 µM	86 µl of PNPL+314 µl of water
8.	500 µM	100 µl of PNPL+300 µl of water

Table No. 3 Represents preparation of different concentrations of PNPL

	1	2	3	4	5	6	7	8	9	10	11	12
A	C1	C1	C									
B	C2	C2	C									
C	C3	C3	C									
D	C4	C4	C									
E	C5	C5	C									
F	C6	C6	C									
G	C7	C7	C									
H	C8	C8	C									

Table No.4 Template for different concentrations of PNPL Where 'C1' to 'C8' represents concentrations from 180 µM to 500 µM. 'C' represents control (PNPL+Phosphate buffer)

4.4.2 PNPL (para nitrophenyl laurate) Assay

Endophytic fungi cultures which showed positive results during primary screening were further screened by quantitative assay. This assay was done by using 2 mM PNPL (para nitrophenol laurate), sodium deoxycolate, 0.5 M monosodium orthophosphate buffer, isopropanol, autoclaved water, porcine pancreatic lipase (1mg/ml in 2.5 mM phosphate buffer).

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	C	C`	C	C7	C7	C7						
B	Csb	Csb	Csb	Co	Co	Co						
C	C1	C1	C1									
D	C2	C2	C2									
E	C3	C3	C3									
F	C4	C4	C4									
G	C5	C5	C5									
H	C6	C6	C6									

Table No.5 Template for inhibition assay

C1-C7= Culture filtrate+ Phosphate buffer+Enzyme+Substrate

C (enzyme control) = Phosphate buffer+Enzyme+Substrate

Csb (substrate control) = Phosphate buffer+Substrate

Co (positive control) = Orlistat+Phosphate buffer+Enzyme+Substrate

In this assay, 30 µl of phosphate buffer was added to microtitre plate to which 100 µl of culture filtrate and 20 µl of lipase enzyme was added. Reaction mixture was further set up for one hour at 37°C. Along with above reaction mixture substrate control, enzymatic control and positive control (orlistat) were also added to micro titre plate. After this, 100 µl of substrate (PNPL) was added to each well in micro titre plate containing above reaction mixture. Microtitre plate was incubated at 37°C for 3 hours. Release of *p*-nitrophenol was measured at 405 nm and inhibition % was calculated.

$$\text{Inhibition } \% = \frac{\text{Enzyme activity} - \text{Inhibitory activity}}{\text{Enzyme activity}} * 100$$

4.5 Solvent Extraction

Liquid-liquid extraction procedure was adopted to extract the culture filtrate. Culture filtrate of the endophytic fungus that has shown maximum inhibitory activity during screening process was subjected to extraction process using different solvents based upon their polarities ranging from polar to non polar (Ethyl acetate, Chloroform, Dichloroform, Diethyl ether, Toluene, petroleum ether and Hexane) with the help of separating funnel. Aqueous layer was extracted three times with each solvent and the solvent layers were pooled. Then organic layer containing compounds of interest was dehydrated with anhydrous sodium sulphate. The organic layer is then collected in a pre-weighed crucible and the solvent is removed. Organic compound obtained was further reconstituted in 1ml DMSO/Methanol. Similarly, same procedure was followed during extraction process by different solvents.

4.5.1 PNPL Assay

Organic compound obtained by different solvents through extraction process was further screened for its inhibitory activity as described above.

	1	2	3	4	5	6	7	8	9	10	11	12
A	C1	C1	C1	C5	C5	C5	Csb	Csb	Csb			
B	C1aq	C1aq	C1aq	C5aq	C5aq	C5aq						
C	C2	C2	C2	C6	C6	C6						
D	C2aq	C2aq	C2aq	C6aq	C6aq	C6aq						
E	C3	C3	C3	C7	C7	C7						
F	C3aq	C3aq	C3aq	C7aq	C7aq	C7aq						
G	C4	C4	C4	C	C	C						
H	C4aq	C4aq	C4aq	Csbm	Csbm	Csbm						

Table No.6 Template for Inhibition Assay where,

C1-C7= Extract + Phosphate buffer + Enzyme+Substrate

C1aq-C7aq= Aqueous fraction+ Phosphate buffer+Enzyme+Substrate

Csbm=Phosphate buffer + Methanol +Substrate

C (enzyme control) = Phosphate buffer +Enzyme+Methanol+Substrate

Csb (substrate control) = Phosphate buffer +Substrate

4.6 Partial Purification

The crude residue showing maximum inhibitory activity was partially purified to obtain the pure compound. Silica gel was prepared in water and was spread as a thin layer (approximately 250 µm) on a 75 X 20 mm glass plates. Plates were dried in oven for 3-4 hours. Chloroform: Methanol was prepared in the ratio of 93:7 as mobile phase in TLC chamber. Small amount of sample was applied about 1 cm above from the lower end of the TLC plate. After the spot had dried completely, the adsorbent-coated plate was propped more or less vertically in a closed container, with the edge to which the spot was applied being down. The solvent, which was in the bottom of the container, creeps up the layer of adsorbent, passed over the spot, and, as it continues up, effects a separation of the materials in the spot ("develops" the chromatogram). When the solvent front had nearly reached the top of the adsorbent, the thin layer plate was removed from the container. Spots were visualized with ultraviolet light or the plate was placed in an iodine chamber. The retention factor (R_f) of a component was measured.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	C1	C1	C1	C3	C3	C3						
B	C1	C1	C1	Csb	Csb	Csb						
C	C1	C1	C1	C	C	C						
D	C2	C2	C2									
E	C2	C2	C2									
F	C2	C2	C2									
G	C3	C3	C3									
H	C3	C3	C3									

Table No.7 Template for inhibition assay where,

C1-C3= compound +phosphate buffer+enzyme+substrate

C (enzyme control) = phosphate control+enzyme+substrate

Csb (substrate control) = phosphate buffer+substrate

$$\mathbf{R_f} = \frac{\text{Distance travelled by the sample}}{\text{Distance travelled by the solvent}}$$

The separated compounds were recovered by scraping the adsorbent off the plate (or cutting out the spots if the supporting material can be cut) and then by washing three to four times with methanol.

4.6.1 PNPL Assay

Partially purified compounds were further subjected for their inhibitory activity by PNPL assay. This assay was done by using 2 mM PNPL (para nitrophenol laurate), sodium deoxycolate, 0.5 M monosodium orthophosphate buffer, 2.5 mM phosphate buffer, isopropanol, autoclaved water, porcine pancreatic lipase (1 mg/ml).

In this assay, 80 µl of phosphate buffer was added to micro titre plate followed by 50 µl of pure compound and 20 µl of lipase enzyme. Reaction mixture was further set up for one hour at 37°C. Along with above reaction mixture substrate control and enzyme control were also added to micro titre plate. After this, 100 µl of substrate (PNPL) was added to each well in micro titre plate containing above reaction mixture. Micro titre plate was incubated at 37°C for 3 hours. Release of *p*-nitrophenol was measured at 405 nm and inhibition % was calculated.

4.6.2 GCMS Analysis

The purified compound was injected in the Shimadzu QP 2010 plus Gas chromatograph with thermal desorption system TD 20. A RTX column (diphenyl 95%, dimethyl polysiloxane 5%) with 30 m × 0.25 mm ID and 0.25 mm DF was used for separating of the fungal volatiles. The column was programmed at 100°C for 2 minutes followed by an increase in the temperature to 250°C for 2 minutes and finally to 300°C for 13 minutes. The carrier gas was Helium and the initial column head pressure was 94.4 KPa. Data acquisition and processing was done on GCMS solution software. The obtained compounds were then tentatively identified based on their high quality matching with database of National Institute of Standard and Technology (NIST) compounds (NIST05).

4.7 Identification of Endophytic Fungi

Endophytic fungal isolate that was showing inhibitory activity against pancreatic lipase was identified using microscopy and molecular technique.

4.7.1 Microscopy

The endophytic fungal isolate which was showing pancreatic lipase inhibitory acitivty was examined under the microscope to characterize it on the basis of its microscopic characters and morphology. The culture was grown on different media namely Potato dextrose Agar (PDA), Corn meal agar (CMA), Water agar (WA), Synthetischer nahrstoffarmer agar (SNA), Banana leaf agar (BLA). Briefly describing, the glass slide was cleaned with ethanol and dried. A drop of water was put on glass slide, upon which the mycelial mass that was taken from the tip of the colony using a fine tipped needle and was placed along and teased properly. It was then stained with Lactophenol cotton blue (Hi Media). The slide was covered with 18 X 10 mm coverslip avoiding the formation of air bubble and was mounted with DPX. The slide was microscopically observed at 10X, 40X and 100X using Nikon binocular microscope. The fungi were identified based upon their spore structure and other morphological characteristics.

4.7.2 DNA Isolation

0.5-1 g wet mycelium was taken from 3-4 days grown old fungal culture on PDA plate and was crushed into very fine powder using liquid nitrogen with the help of mortar pestle. 600-750 µl of the extraction buffer was added and the biomass was crushed again. The contents were transferred to a 1.5 ml microcentrifuge tube followed by addition of 10 µl of β-mercaptoethanol and 4 µl of Proteinase K. The contents were vortexed and were incubated at 65°C in water bath for 1 hour. After the incubation was over, the microcentrifuge tubes were centrifuged at 10,000 rpm for 15 minutes in order to remove cell debris. After this, 6 µl of RNase was added to each tube and was incubated at 37°C for 30 minutes. For the removal of protein contents, equal volume of phenol:chloroform (1:1) solution was added to each tube and was mixed properly for 15 mins and centrifuged at 12,000 rpm for 10 minutes, this was done three to four times. Aqueous layer was transferred carefully to the fresh microcentrifuge tube containing DNA avoiding the inclusion of debris and other impurities along with it. Further, 20 µl of 3 M sodium acetate was added and the content of each microcentrifuge tube

was top up with absolute ethanol and was incubated at -4°C overnight. The contents were mixed by inverting the tubes so as to observe the white threads of precipitating DNA. On the next day, the microcentifuge tubes were centrifuged at 12,000 rpm for 10 minutes; the pellet was washed again with 70% ethanol and centrifuged at 12,000 rpm for 5 minutes. The pellet was air dried and was dissolved in 30 µL of Tris EDTA buffer (pH 8). The qualitative estimation of the DNA isolated was done by agarose gel electrophoresis.

4.7.2.1 Agarose gel electrophoresis

0.8% agarose gel was casted in the electrophoretic apparatus along with 8 chambered comb. The gel was allowed to solidify and after solidification, the comb was carefully removed. Electrophoretic running buffer (1 X TAE) was poured into the tank. The DNA samples were mixed with the 5 X loading dye and loaded into wells and allowed to run at 50 volts. The gel was observed under UV transilluminator for the presence of DNA. Gel imaging was performed under UV light in Bio- Rad Gel documentation System using Quantity-1-D analysis software.

4.7.3 PCR Amplification, Sequencing and BLAST Analysis

DNA sample was amplified using ITS-1 and ITS-4 primers synthesized by Integrated DNA Technologies (IDT), USA. PCR reaction was carried out by using the primers ITS-1 (5' TCC GTA GGT GAA CCT GCG G 3') and ITS-4 (5' TCC TCC GCT TAT TGA TAT GC 3') (White *et al*., 1990). Amplification was performed in 25 µl reaction mixture containing 3 µl of extracted fungal DNA, 10 µM of each primer (ITS1 and ITS4), 2.5 mM of dNTP (Bangalore GeNei), 1.5 U of Taq DNA Polymerase (Bangalore GeNei) in 10 X Taq buffer (Bangalore GeNei). The PCR cycling conditions consisted of initial denaturation at 96ºC for 5 min followed by 39 cycles of 95ºC for 45 sec, 60ºC for 45 sec, 72ºC for 45 sec followed by final extension at 72ºC for 5 mins. The PCR products were examined using gel electrophoresis in a 1.5% agarose gel dissolved in 1X TAE buffer at 40 V for 1:30 hr. Gel imaging was performed under UV light in Bio- Rad Gel documentation System using Quantity-1-D analysis software. Purified rDNA amplicons were send for direct PCR sequencing to Chromus Biotech lab Bangalore. The obtained sequence was subjected to sequence similarity search using BLAST software at NCBI server.

S.No.	Reagent	Stock conc.	Quantity	Final conc.
1	Sterile double distilled water		14 µl	
2	PCR buffer	10x	2.5 µl	1x
3	dNTPs	2.5 Mm	2.5 µl	0.25 mM
4	Forward primer	5 µM	1.5 µl	0.3 µM
5	Reverse primer	5 µM	1.5 µl	0.3 µM
6	DNA		3 µl	
7	Taq polymerase	3 U/µL	0.5 µl	1 U

Table No.8 Different reagents used during PCR reaction

Steps	Temperature	Time
Step1:Initial Denaturation	96°C	5 min
Step2:Denaturation	95°C	45 sec
Step3:Annealing	60°C	45 sec
Step4:Extension	72°C	45 sec
Step5: step2 to step 4 are repeated 39 times		
Step6:Final extension	72°C	5 min
Step7:Store	4°C	∞

Table No.9 Temperature profile for PCR reaction

Chapter 5

RESULTS AND DISCUSSIONS

5.1 Sub culturing and Maintenance

71 fungal isolates were grown on potato dextrose agar and were maintained in pure form by reculturing at regular interval of time on PDA at 28ºC. The cultures were preserved in the form of PDA slants with 15% glycerol at 28ºC. In present study, fungal isolates were obtained from different medicinal plants like *Cinnamomum* sp., *Aegle marmelos, Cimellia sinensis, Piper nigrum, Jatropha* sp., *Rauwolfia serpentina, Tabernaemontana divaricata; Wild ginger* belonging to *Lauraceae, Rutaceae, Theaceae, Piperaceae, Euphorbiaceae, Apocynaceae, Taxaceae, Apocynaceae, Zingiberaceae* family was used. Thus, these medicinal plants are expected to have some endophytic isolates which have a great potential to produce a novel lipase inhibitor which can be further used in the development of new drug.

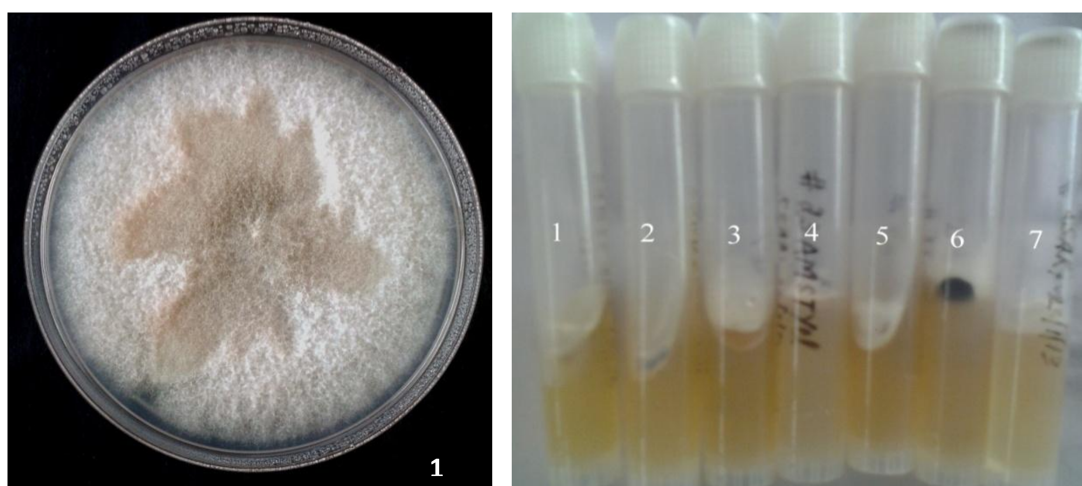


Fig.1 PDA slants of different endophytic fungal isolate: (1) # 7 AMSTYEL, (2) #22 AMSTYEL, (3) #1069 AMSTYEL, (4) #23 AMSTYEL, (5) #1016 AMLBRT, (6) #6 AMLWLS, (7) #1007 AMLBRT

5.2 Primary screening

All 71 endophytic fungal isolates, 29 obtained from *Aegle marmelos* out of which 15 were from stem, 7 from stem internal tissue, 6 from leaf and 1 from bark. 21 were from *Cinnamomum malabaricum* out of which 5 were from bark, 8 from leaf, 3 from stem and 5 from stem internal tissue. 3 were from stem internal tissue of *Cinnamomum camphora*. 1 was from stem of *Cimellia sinensis*. 4 were from *Cinnamomum zeylnicum* out of which 3 were from stem internal tissue and 1 was from stem. 1 was from leaf of *piper nigrum*. 4 were from *Rauwolfia serpentina* out of which 2 were from bark and rest 2 were from leaf. 4 were from bark of *Taxus baccata* 1 was from stem of *Tabernaemontana divaricata.* 1 from stem of *Wild ginger* as well as 1 from stem of *Jatropha* sp. All these 71 fungal isolates were subjected for

primary screening i.e. phenol red and rhodamine assay for checking their potential in the inhibition of pancreatic lipase.

S. No.	Culture code	Host plant	Plant part
1	#1069AMSTYEL	*Aegle marmelos*	Stem
2	#7AMSTYEL	*Aegle marmelos*	Stem
3	#22AMSTYEL	*Aegle marmelos*	Stem
4	#1003AMSTYEL	*Aegle marmelos*	Stem
5	#23AMSTYEL	*Aegle marmelos*	Stem
6	#1032AMSTYEL	*Aegle marmelos*	Stem
7	#23(b)AMSTYEL	*Aegle marmelos*	Stem
8	#9(b)AMSTYEL	*Aegle marmelos*	Stem
9	#1048AMSTYEL	*Aegle marmelos*	Stem
10	#1013AMSTITYEL	*Aegle marmelos*	Stem internal tissue
11	#1010AMSTITYEL	*Aegle marmelos*	Stem internal tissue
12	#1058AMSTITYEL	*Aegle marmelos*	Stem internal tissue
13	#1070AMSTITYEL	*Aegle marmelos*	Stem internal tissue
14	#1007AMLBRT	*Aegle marmelos*	Leaf
15	1016AMLBRT	*Aegle marmelos*	Leaf
16	#9AMLBRT	*Aegle marmelos*	Leaf
17	#1006AMLBRT	*Aegle marmelos*	Leaf
18	#59AMSTWLS	*Aegle marmelos*	Stem
19	#28AMSTWLS	*Aegle marmelos*	Stem
20	#33AMSTWLS	*Aegle marmelos*	Stem
21	#25AMSTWLS	*Aegle marmelos*	Stem
22	#20AMSTWLS	*Aegle marmelos*	Stem
23	#37(a)AMSTWLS	*Aegle marmelos*	Stem
24	#1104AMSTITWLS	*Aegle marmelos*	Stem internal tissue
25	#1088AMSTITWLS	*Aegle marmelos*	Stem internal tissue
26	#42AMSTITWLS	*Aegle marmelos*	Stem internal tissue
27	#61AMLWLS	*Aegle marmelos*	Leaf
28	#6AMLWLS	*Aegle marmelos*	Leaf
29	#11AMBAWLS	*Aegle marmelos*	Bark
30	#3BJSS	*Jatropha* sp	Stem
31	#18CMBANEY	*Cinnamomum malabaricum*	Bark
32	#2CMBANEY	*Cinnamomum malabaricum*	Bark
33	#14CMBANEY	*Cinnamomum malabaricum*	Bark
34	#12CMBANEY	*Cinnamomum malabaricum*	Bark
35	#12CMBABRT	*Cinnamomum malabaricum*	Bark

36	#1CCSTITD	*Cinnamomum camphora*	Stem internal tissue
37	#2CCSTITD	*Cinnamomum camphora*	Stem internal tissue
38	#36CCSTITD	*Cinnamomum camphora*	Stem internal tissue
39	#2CMLNEY	*Cinnamomum malabaricum*	Leaf
40	#37CMLNEY	*Cinnamomum malabaricum*	Leaf
41	#1CMLNEY	*Cinnamomum malabaricum*	Leaf
42	#31CMLNEY	*Cinnamomum malabaricum*	Leaf
43	#29CMLNEY	*Cinnamomum malabaricum*	Leaf
44	#4CMLBRT	*Cinnamomum malabaricum*	Leaf
45	#40CMLBRT	*Cinnamomum malabaricum*	Leaf
46	#27CMLBRT	*Cinnamomum malabaricum*	Leaf
47	#44CMSTNEY	*Cinnamomum malabaricum*	Stem
48	#4CMSTNEY	*Cinnamomum malabaricum*	Stem
49	#1622CMSTITNEY	*Cinnamomum malabaricum*	Stem internal tissue
50	#54(b)CMSTITNEY	*Cinnamomum malabaricum*	Stem
51	#96CMSTITNEY	*Cinnamomum malabaricum*	Stem internal tissue
52	#21CMSTITNEY	*Cinnamomum malabaricum*	Stem internal tissue
53	#79CMSTITNEY	*Cinnamomum malabaricum*	Stem internal tissue
54	#1CMSTITBRT	*Cinnamomum malabaricum*	Stem internal tissue
55	#4CSSTOT	*Cimellia sinensis*	Stem
56	#4CZSTSTE	*Cinnamomum zeylnicum*	Stem
57	#2164CZSTITG	*Cinnamomum zeylnicum*	Stem internal tissue
58	#2131CZSTITG	*Cinnamomum zeylnicum*	Stem internal tissue
59	#2106CZSTITG	*Cinnamomum zeylnicum*	Stem internal tissue
60	#2PNLNEY	*Piper nigrum*	Leaf
61	#1RSBANEY	*Rauwolfia serpentina*	Bark
62	#16RSBANEY	*Rauwolfia serpentina*	Bark
63	#15RSLBRT	*Rauwolfia serpentina*	Leaf
64	#16RSLBRT	*Rauwolfia serpentina*	Leaf
65	#14TBBALM	*Taxus baccata*	Bark
66	#20TBBALM	*Taxus baccata*	Bark
68	#97TBBALM	*Taxus baccata*	Bark
69	#42TBBALM	*Taxus baccata*	Bark
70	#4TMDSTYEL	*Tabernaemontana divaricata*	Stem
71	#15(a)WGSTNEY	*Wild ginger*	Stem

Table No.10 Endophytic fungal isolates used for the screening studies

5.2.1 Phenol Red Plate Assay

The culture filtrates of 71 endophytic fungal isolates were subjected to phenol red plate assay in which phenol red was added at a concentration of 0.01%. A slight drop in pH resulted in yellow colour formation due to release of fatty acids (Singh *et al.,* 2005). Out of 71 endophytic fungal isolates, only 7 cultures have shown reduction in yellow zone formation.

S.No.	Culture Code	Zone of Inhibition(mm)			
		A	B	Mean	Control-mean
1	**#1069 AMSTYEL**	**0**	**0**	**0**	**18.2**
2	**#1013 AMSTITYEL**	**0**	**0**	**0**	**18.2**
3	#1058 AMSTITYEL	9.86	13.14	11.5	6.7
4	**#59 AMSTWLS**	**0**	**0**	**0**	**18.2**
5	#4 CZSTSTE	9.02	9.98	9.5	8.7
6	**#42 TBBALM**	**0**	**0**	**0**	**18.2**
7	#97 TBBALM	10.16	10.75	10.5	7.7
8	#28 AMSTWLS	20.67	20.14	20.4	- 2.2
9	#61 AMLWLS	23.78	21.46	22.6	- 4.4
10	#36 CCSTITD	27.86	30.46	29.2	- 11
11	Control (lipase)	18.1	18.4	18.2	

Table No.11 Represents zone of inhibition (mm) after 24 hours

During qualitative estimation of lipase inhibition only 7 cultures have shown best possible results as compared to rest 64 endophytic fungal isolates in which either very less or no reduction in yellow colour formation take place. Out of 7 positive cultures, 4 fungal cultures (#1069 AMSTYEL, #1013 AMSTYEL, #59 AMSTWLS and #42 TBBALM) have shown maximum inhibitory potential by completely reducing yellow coloured halos. These 7 positive endophytic fungal isolates have been selected for further screening by PNPL assay which is a quantitative estimation (Gupta *et al.,* 2003). Figure 2 shows reduction in yellow colour formation in phenol red agar plates and Graph 1 shows the comparison of zone of inhibition (mm) for 10 cultures.

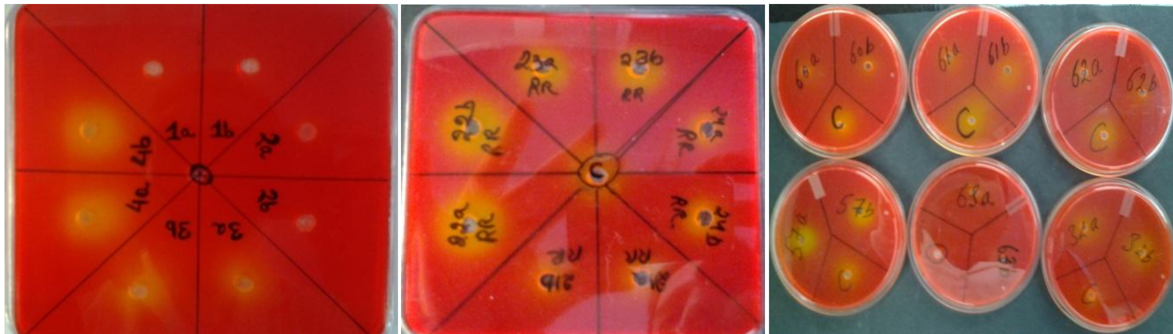
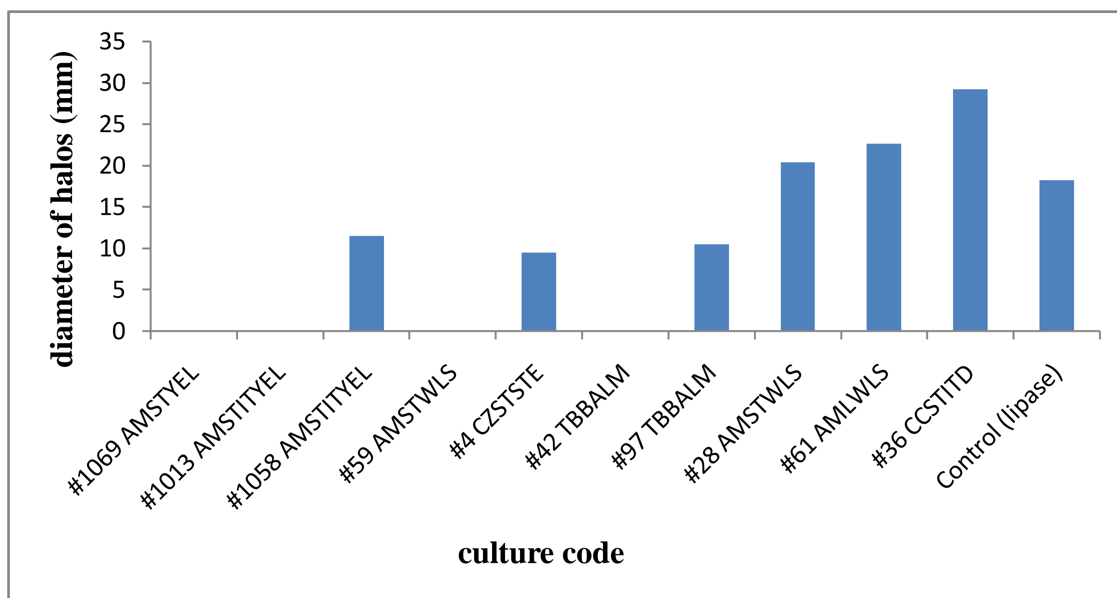


Fig.2 Shows halo formation (mm) where 1a and 1b stands for #1069 AMSTYEL, 2a and 2b stands #59 AMSTWLS, 3a and 3b stands for #1013 AMSTITYEL, 4a and 4b stands for #1007 AMLBRT, 24a and 24b stands for #1058 AMSTITYEL, ‘C’ stands for control, ‘O’ stands for positive control (orlistat).



Graph 1 Represents diameter of halos (mm) of endophytic fungal isolates

5.2.2 Rhodamine B plate assay

The lipase inhibitory activity of 71 endophytic fungal isolates was observed by reduction in halo formation under UV light at 350 nm which was due to hydrolysis of olive oil by the action of lipase enzyme. Out of 71 fungal isolates, only 7 fungal cultures had shown reduction in halo formation thereby, representing maximum inhibitory potential for lipase. Table no.12 shows the data recorded during rhodamine B plate assay, figure 3 shows zone of inhibition and graph 2 represents the percentage inhibition of different endophytic fungal cultures.

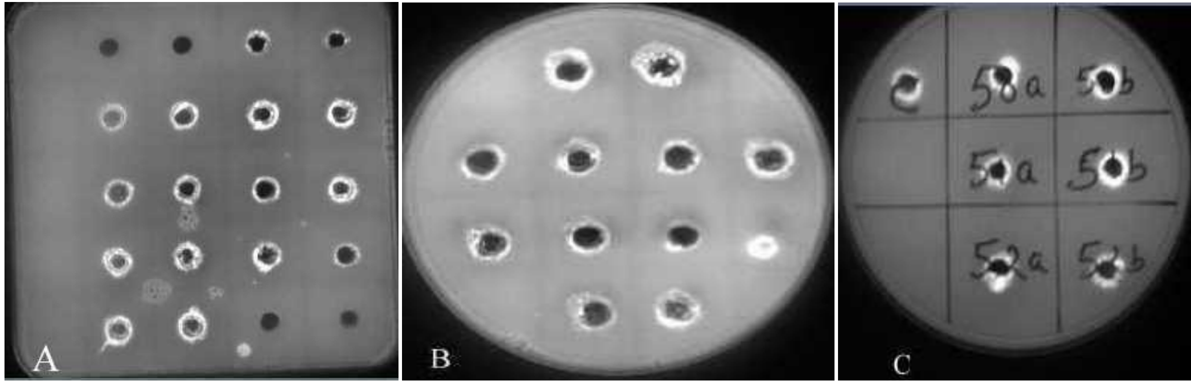
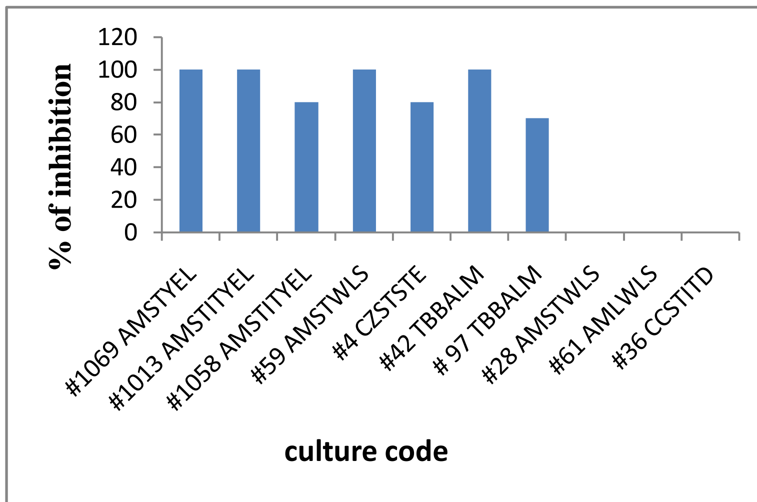


Fig.3 Represents zone of inhibition where Fig. 4A: 1 and 2 well denotes #1069AMSTYEL, 3 and 4 well denotes #59AMSTWLS, 19 and 20 well denotes positive control (orlistat). Fig. 4B: 1 and 2 well denotes control (enzyme), 8 and 9 well denotes #1058AMSTITYEL. Fig 4C: 4 and 5 well denotes #36CCSTITD, 6 and 7 denotes #15RSLBRT.

S.No.	Culture Code	Reduction in halos formation
1	#1069 AMSTYEL	+++
2	#1013 AMSTITYEL	+++
3	#1058 AMSTITYEL	++
4	#59 AMSTWLS	+++
5	#4 CZSTSTE	++
6	#42 TBBALM	+++
7	# 97 TBBALM	+
8	#28 AMSTWLS	-
9	#61 AMLWLS	-
10	#36 CCSTITD	-

Table No. 12 Shows reduction in halos formation where '+++' denotes 100% inhibition, '++' denotes 80% inhibition, '+' denotes 70% inhibition and '–' denotes no inhibition.



Graph 2 Represents zone of inhibition in percentage (%)

5.3 Secondary Screening

5.3.1 Enzyme kinetics

Kinetics of the enzymatic hydrolysis of *p*-nitrophenyl laurate using lipase has been investigated. The initial rate of reaction was determined experimentally at different substrate concentration by measuring the rate of *p*-nitrophenol produced.

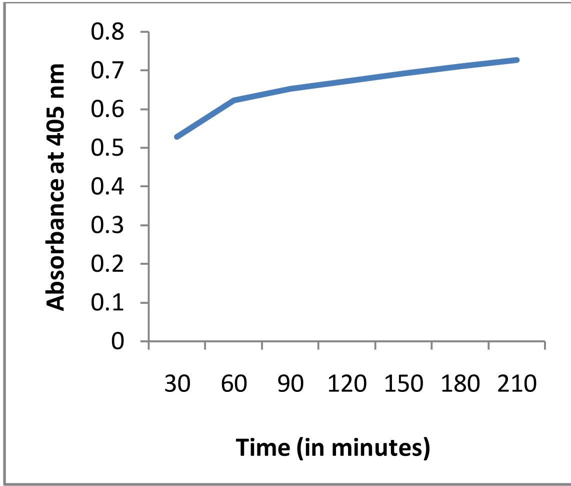
For 0 minutes					
Conc.	**Absorbance at 405 nm**			**Control**	**Conc. –Control**
	A	**B**	**Mean**		
C1=180µM	0.229	0.206	0.217	0.056	0.161
C2=220µM	0.236	0.240	0.238	0.067	0.171
C3=260µM	0.256	0.267	0.261	0.081	0.180
C4=300µM	0.279	0.268	0.273	0.106	0.167
C5=340µM	0.316	0.297	0.306	0.111	0.195
C6=400µM	0.352	0.343	0.347	0.144	0.203
C7=430µM	0.349	0.373	0.361	0.135	0.226
C8=500µM	0.427	0.423	0.425	0.186	0.239
For 30 minutes					
C1=180µM	0.601	0.569	0.585	0.057	0.528
C2=220µM	0.655	0.666	0.660	0.071	0.589
C3=260µM	0.722	0.759	0.740	0.082	0.658
C4=300µM	0.771	0.792	0.784	0.112	0.672
C5=340µM	0.875	0.812	0.843	0.115	0.728
C6=400µM	0.905	0.916	0.910	0.148	0.762
C7=430µM	0.913	0.977	0.945	0.137	0.808
C8=500µM	1.059	1.049	1.054	0.186	0.868
For 60 minutes					
C1=180µM	0.691	0.668	0.679	0.058	0.621
C2=220µM	0.828	0.829	0.828	0.074	0.754
C3=260µM	0.932	0.981	0.956	0.086	0.870
C4=300µM	1.042	1.054	1.048	0.118	0.930
C5=340µM	1.161	1.104	1.132	0.119	1.013
C6=400µM	1.222	1.264	1.243	0.148	1.095
C7=430µM	1.251	1.328	1.289	0.138	1.151
C8=500µM	1.436	1.461	1.448	0.191	1.257
For 90 minutes					
C1=180µM	0.719	0.703	0.711	0.059	0.652
C2=220µM	0.884	0.881	0.882	0.074	0.808
C3=260µM	1.004	1.044	1.024	0.088	0.936
C4=300µM	1.141	1.154	1.147	0.117	1.030
C5=340µM	1.272	1.272	1.272	0.124	1.148
C6=400µM	1.414	1.470	1.442	0.154	1.288
C7=430µM	1.456	1.557	1.506	0.145	1.361
C8=500µM	1.686	1.743	1.714	0.199	1.515
For 120 minutes					
C1=180µM	0.740	0.722	0.731	0.059	0.672
C2=220µM	0.909	0.905	0.907	0.075	0.832
C3=260µM	1.035	1.073	1.054	0.092	0.962
C4=300µM	1.185	1.196	1.190	0.117	1.073
C5=340µM	1.324	1.347	1.335	0.125	1.210
C6=400µM	1.519	1.577	1.548	0.160	1.388
C7=430µM	1.570	1.684	1.627	0.150	1.477
C8=500µM	1.833	1.919	1.876	0.206	1.670
For 150 minutes					

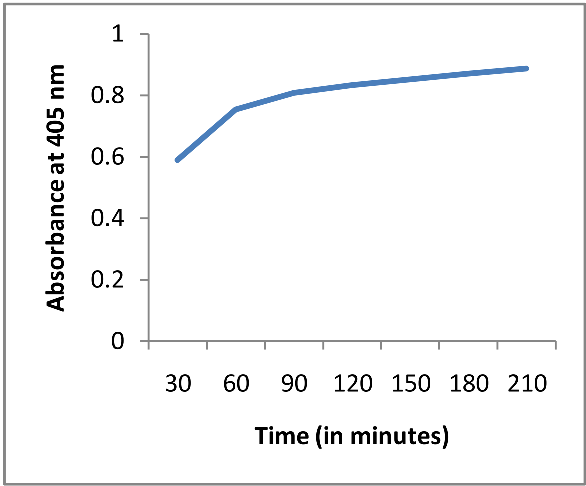
C1=180µM	0.761	0.741	0.751	0.06	0.691
C2=220µM	0.936	0.924	0.930	0.079	0.851
C3=260µM	1.061	1.097	1.079	0.095	0.984
C4=300µM	1.213	1.223	1.218	0.116	1.102
C5=340µM	1.356	1.387	1.371	0.128	1.243
C6=400µM	1.577	1.630	1.603	0.161	1.442
C7=430µM	1.633	1.752	1.692	0.160	1.532
C8=500µM	1.938	2.029	1.983	0.202	1.781
For 180 minutes					
C1=180µM	0.779	0.756	0.767	0.057	0.710
C2=220µM	0.956	0.942	0.949	0.078	0.871
C3=260µM	1.082	1.113	1.097	0.095	1.002
C4=300µM	1.239	1.247	1.243	0.117	1.126
C5=340µM	1.388	1.419	1.403	0.130	1.273
C6=400µM	1.621	1.668	1.644	0.160	1.484
C7=430µM	1.684	1.797	1.740	0.160	1.580
C8=500µM	2.014	2.099	2.056	0.200	1.856
For 210 minutes					
C1=180µM	0.796	0.769	0.782	0.056	0.726
C2=220µM	0.977	0.959	0.968	0.081	0.887
C3=260µM	1.105	1.132	1.118	0.097	1.021
C4=300µM	1.264	1.267	1.265	0.119	1.146
C5=340µM	1.416	1.444	1.430	0.131	1.299
C6=400µM	1.656	1.698	1.677	0.159	1.518
C7=430µM	1.723	1.831	1.777	0.164	1.613
C8=500µM	2.060	2.152	2.106	0.203	1.903

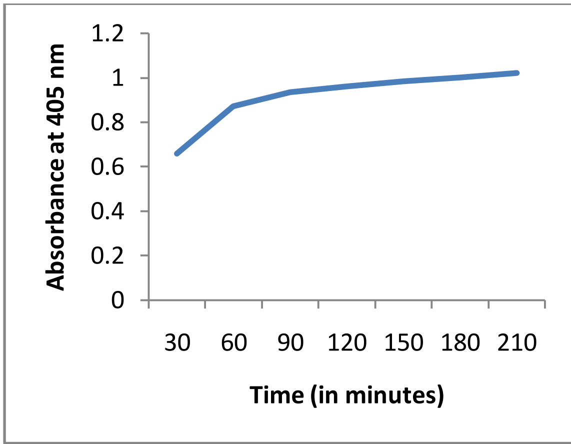
Table No.13 Represents the data recorded during enzyme kinetics where, C1-C8 denotes different concentrations of substrate (PNPL) and A, B denotes absorbance at 405 nm when lipase acts on different concentrations of substrate

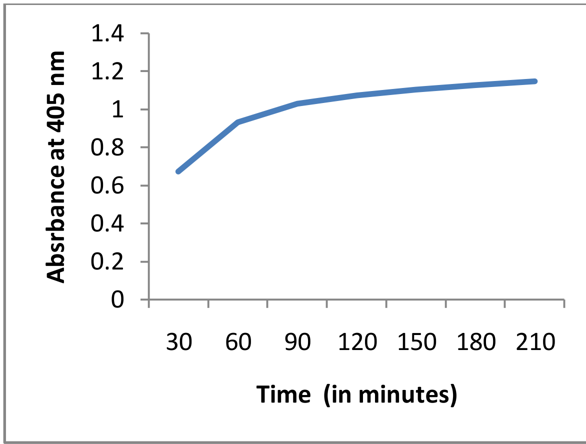
	Time (Minutes)							
Conc. (µM)	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**Initial velocity**
180	0.528	0.621	0.652	0.672	0.691	0.710	0.726	0.00096
220	0.589	0.754	0.808	0.832	0.851	0.871	0.887	0.00139
260	0.658	0.870	0.936	0.962	0.984	1.002	1.021	0.00166
300	0.672	0.930	1.030	1.073	1.102	1.126	1.146	0.00224
340	0.728	1.013	1.148	1.210	1.243	1.273	1.299	0.00227
400	0.762	1.095	1.288	1.388	1.442	1.484	1.518	0.00381
430	0.808	1.151	1.361	1.477	1.532	1.580	1.613	0.00410
500	0.868	1.257	1.515	1.670	1.781	1.856	1.903	0.00543

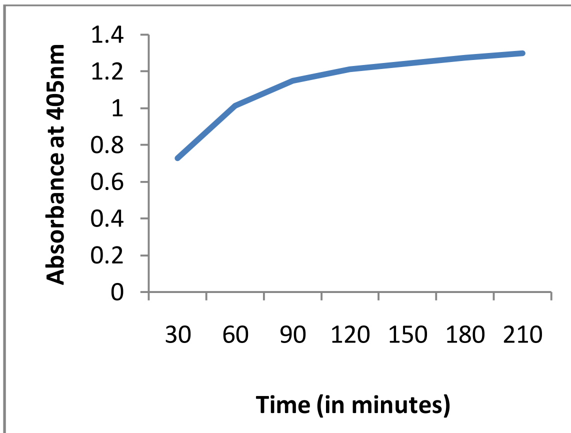
Table No.14 Represents initial rate of reaction at different substrate concentration

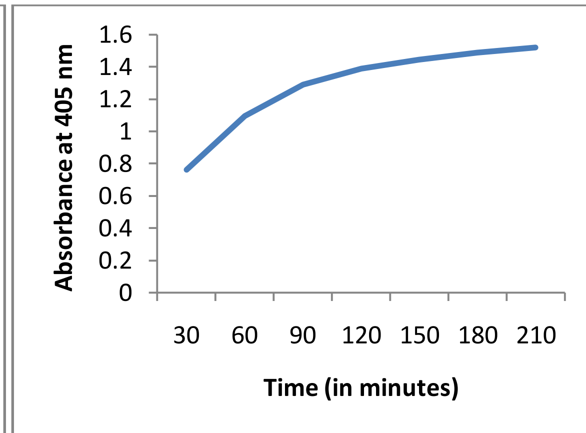

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Absorbance at 405 nm
30 60 90 120 150 180 210
Time (in minutes)

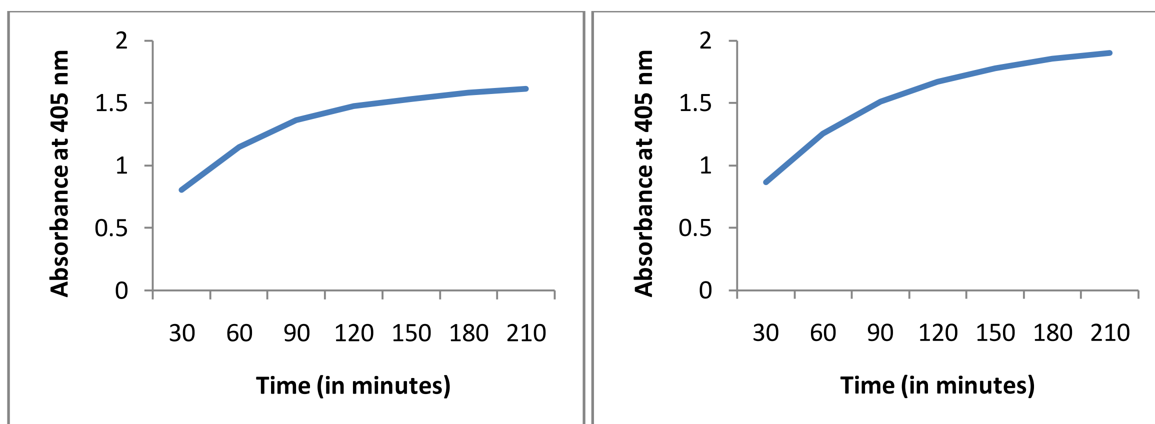

1
0.8
0.6
0.4
0.2
0
Absorbance at 405 nm
30 60 90 120 150 180 210
Time (in minutes)


1.2
1
0.8
0.6
0.4
0.2
0
Absorbance at 405 nm
30 60 90 120 150 180 210
Time (in minutes)


1.4
1.2
1
0.8
0.6
0.4
0.2
0
Absrbance at 405 nm
30 60 90 120 150 180 210
Time (in minutes)


1.4
1.2
1
0.8
0.6
0.4
0.2
0
Absorbance at 405nm
30 60 90 120 150 180 210
Time (in minutes)


1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Absorbance at 405 nm
30 60 90 120 150 180 210
Time (in minutes)



Graph 3 Represents the rate of reaction at different substrate concentration with respect to time

Hence from this data, slope of each graph was calculated giving initial velocity (V_0) of the reaction at different substrate concentrations. Using this initial velocity and substrate concentration, Lineweaver-Burk plot was made using graph pad prism 5. The values of Km and Vmax were calculated as 800 µM and 0.03 µM/min/mg respectively.

5.3.2 PNPL (para nitrophenyl laurate) Assay

7 endophytic fungal isolates which have shown maximum inhibition for pancreatic lipase were further screened by PNPL assay. The inhibitory activity shown by culture filtrates of different endophytic fungal isolates was measured as the rate of *p*- nitrophenol production when lipase and PNPL were incubated along with culture filtrates of endophytic fungal isolates.

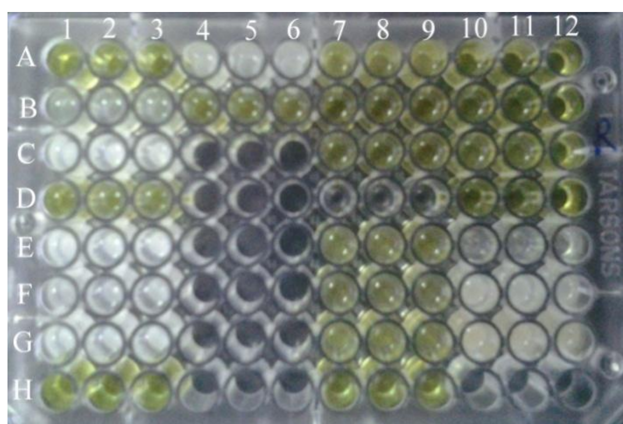


Fig.4 Represents micro titre plate where, C1- H3 culture filtrates of different fungal isolates (in triplicates), A1-A3 enzyme control, B1-B3 substrate control

$$\text{Inhibition \%} = \frac{\text{Enzyme activity} - \text{Inhibitory activity}}{\text{Enzyme activity}} * 100$$

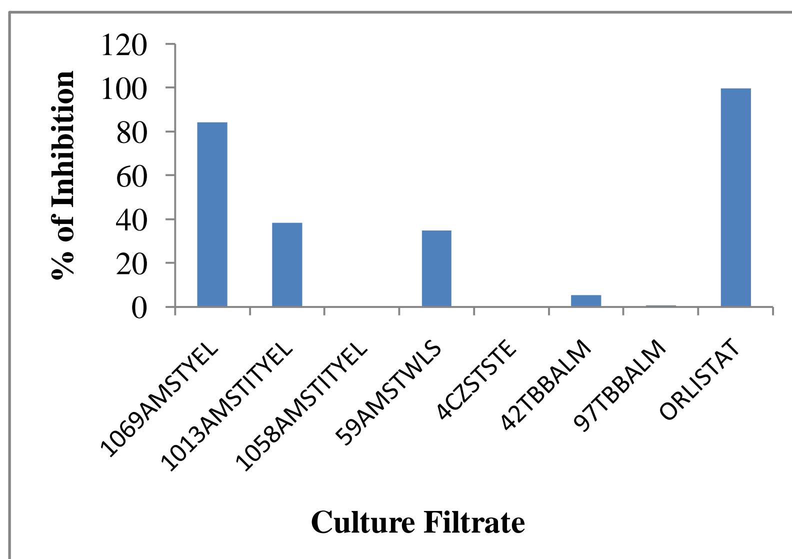
Enzyme control= (1.796+1.75+1.662)/3 = 1.736

Substrate control= (0.623+0.609+0.596)/3 = 0.6093

Enzyme activity= 1.736-0.6093 = 1.1267

Culture filtrate	Absorbance at 405 nm				Culture filtrate-Substrate control	Inhibition%
	A	B	C	Mean		
C1(#1069AMSTYEL)	**0.756**	**0.794**	**0.808**	**0.786**	**0.176**	**84.31%**
C2(#1013AMSTITYEL)	1.134	1.315	1.255	1.305	0.695	38.25%
C3(#1058AMSTITYEL)	1.929	1.788	1.760	1.825	1.216	0%
C4(#59AMSTWLS)	1.377	1.424	1.232	1.344	0.733	34.76%
C5(#4CZSTSTE)	1.781	1.658	1.790	1.743	1.133	0%
C6(#42TBBALM)	1.754	1.676	1.595	1.675	1.065	5.41%
C7(#97TBBALM)	1.781	1.634	1.771	1.728	1.119	0.65%
Control (orlistat)	**0.643**	**0.594**	**0.593**	**0.610**	**0.0011**	**99.9%**

Table No.15 Represents inhibition% where C1-C7 denotes culture filtrate of endophytic fungal isolates, orlistat denotes positive control



Graph 4 Represents % of inhibition of culture filtrates of different endophytic fungal isolates

The quantitative estimation for lipase inhibitory activity of different culture filtrates of endophytic fungi confirms their inhibitory potential against pancreatic lipase enzyme. #1069AMSTYEL showed maximum inhibitory potential of 84.3% for pancreatic lipase which almost comparable to that of positive control orlistat. Thus, a quantitative estimation of lipase inhibitory activity was accurately done by this method.

5.4 Mass production of culture filtrate

Culture filtrate of #1069 AMSTYEL was obtained using CZD (Czapek dox broth). CZD contains Sucrose, Sodium nitrate, Dipotassium phosphate, Magnesium sulphate, Potassium chloride, ferrous sulphate. Czapek Dox Broth is a semisynthetic medium used for the cultivation of fungi, containing sodium nitrate as the sole source of nitrogen. Sucrose serves as the sole source of carbon. Dipotassium phosphate buffers the medium. Magnesium sulphate, potassium chloride, ferrous sulphate serves as sources of essential ions. #1069 AMSTYEL was selected for the mass production of culture filtrate as it has shown maximum potential for lipase inhibition as compared to other fungal isolates.

Fig.5 Mass production of culture filtrate (#1069 AMSTYEL)

5.5 Solvent extraction

Culture filtrate of #1069 AMSTYEL was further extracted by different solvents ranging from polar to non polar with the help of separatory funnel. Compounds obtained by different solvents were further screened for their inhibitory activity against pancreatic lipase. These compounds were then reconstituted in methanol giving concentration of 1 mg/ml.

S.No.	Culture code	Initial wt.(g)	Final wt.(g)	Amount of obtained (mg)
1	#1069AMSTYEL(EA)	59.55379	59.56371	9.9
2	#1069AMSTYEL(Chloroform)	69.36816	69.3734	5.3
3	#1069AMSTYEL(DCM)	60.9426	60.9434	0.8
4	#1069AMSTYEL(DEE)	50.98404	50.99580	1.7
5	#1069AMSTYEL(Toluene)	50.411098	50.4128	1.8
6	#1069AMSTYEL(PE)	65.764	65.76841	4
7	#1069AMSTYEL(Hexane)	52.226	52.22892	2

Table No.16 Represents amount of compound obtained after solvent extraction

5.5.1 PNPL Assay

Extracts obtained from different solvents after solvent extraction were further screened for their inhibitory activity against pancreatic lipase using PNPL assay. The inhibitory activity of compounds obtained was measured by the rate of production of *p*-nitrophenol.

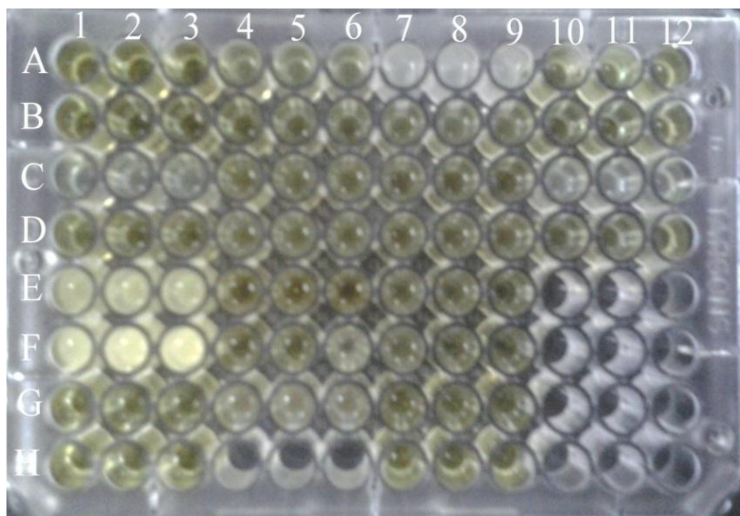


Fig.6 Microtitre plate representing the inhibition assay for solvent extract where A1-B3 PE fraction, C1-D3 EA fraction, E1-F3 CHL fraction, G1-H3 DEE fraction, A4-B6 DCM fraction, C4-D6 Toluene fraction, E4-F6 Hexane fraction

Culture code	Absorbance at 405 nm				Test-Substrate control	Inhibition%
	A	B	C	Mean		
#1069AMSTYEL(E.A)	**1.307**	**1.188**	**1.174**	**1.223**	**0.023**	**97.9%**
#1069AMSTYEL (E.A aq.)	2.856	3.312	2.664	2.994	1.616	22%
#1069AMSTYEL(Chloroform)	1.575	1.36	1.018	1.317	0.032	97%
#1069AMSTYEL (Chloroform aq.)	1.767	1.628	1.664	1.686	0.358	82.6%
#1069AMSTYEL(DCM)	1.752	1.840	1.760	1.784	0.499	54.63%
#1069AMSTYEL (DCM aq.)	2.172	1.983	2.071	2.075	0.747	63.9%
#1069AMSTYEL (D.E.E)	1.983	2.783	2.043	2.269	0.984	10.4%
#1069AMSTYEL (D.E.E aq.)	1.241	1.700	1.813	1.584	0.257	87.5%
#1069AMSTYEL (Toluene)	1.608	1.494	1.864	1.655	0.370	66.3%
#1069 AMSTYEL (Toluene aq.)	2.679	2.700	2.468	2.615	1.288	37.8%
#1069 AMSTYEL (P.E)	1.346	1.416	1.335	1.365	0.080	92.6%
#1069AMSTYEL (P.E aq.)	2.131	2.273	2.270	2.224	0.897	56.7%
#1069AMSTYEL (Hexane)	1.673	1.846	1.907	1.808	0.523	52.3%
#1069AMSTYEL (Hexane aq.)	1.580	2.168	2.328	2.025	0.697	66.3%

Table No.17 Represents inhibition percentage for extracts of different solvents

Enzyme control= (2.255+2.365+2.535)/3 = 2.385

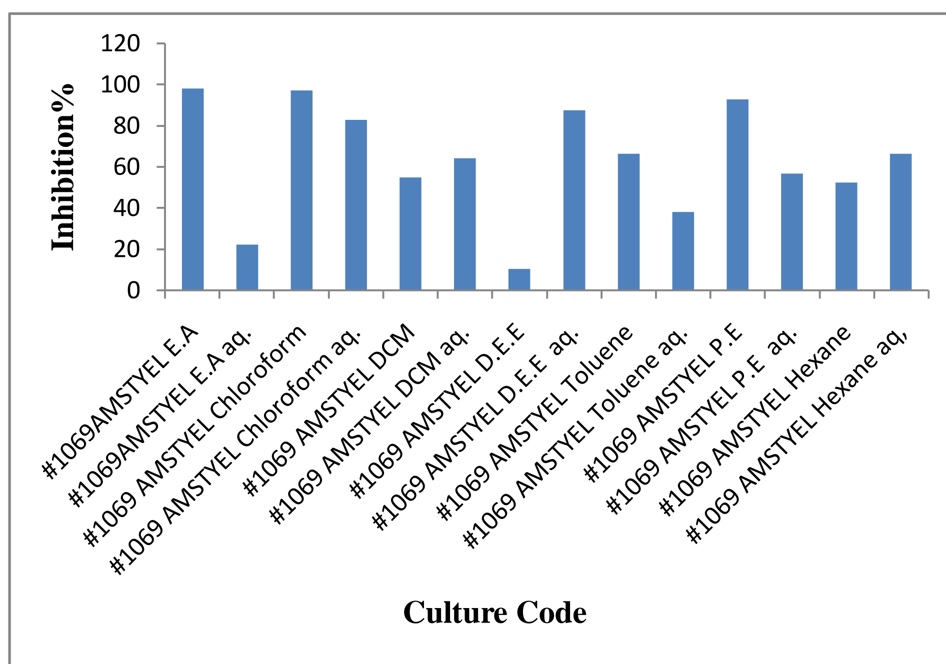
Substrate control= (1.303+1.284+1.268)/3 = 1.285

Enzyme activity= Enzyme control-Substrate control = 1.1

Enzyme control for aqueous= (3.551+3.367+3.282)/3 = 3.4

Substrate control for aqueous= (1.345+1.313+1.325)/3 = 3.4

Enzyme activity= Enzyme control-Substrate control = 2.07



Graph 5 Represents inhibition percentage for extracts

After quantitative estimation of extracts for inhibition against pancreatic lipase, ethyl acetate fraction has shown 97.9% inhibition which was maximum as compared to other solvent fractions. Thus, ethyl acetate fraction was further partially purified by TLC.

5.6 Partial purification

Ethyl acetate fraction which has shown maximum inhibitory potential against pancreatic lipase was partially purified by TLC as it is a method for analysing mixtures by separating the compounds in the mixture. R_f for different spots obtained during TLC of ethyl acetate fraction was calculated.

S.No.	Spot	R_f Value
1.	Spot 1	0.24
2.	Spot 2	0.64
3.	Spot 3	0.67

Table No.18 Shows R_f value for three different spot

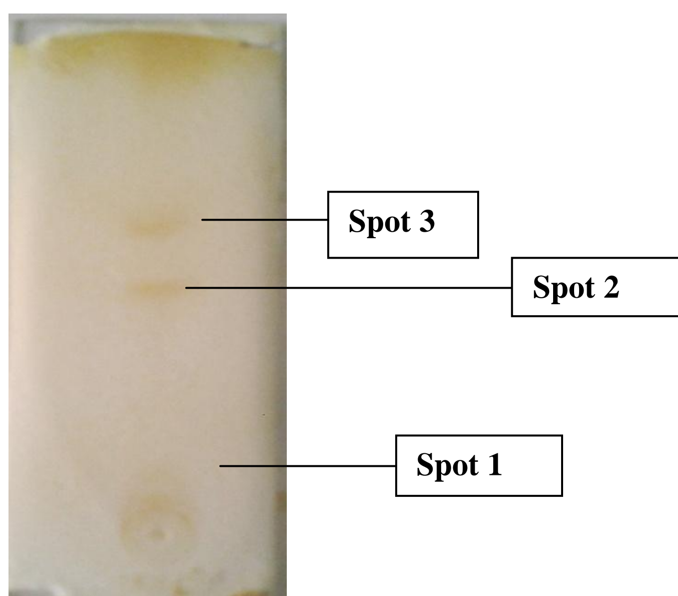


Fig.7 Represents TLC of ethyl acetate fraction

3 different spots were obtained after TLC of ethyl acetate fraction with R_f value of 4.13, 1.55 and 1.47 respectively. These spots were visualized when kept in iodine chamber. These spots were further scraped and screened for their inhibitory activity against pancreatic lipase by PNPL assay.

5.6.1 PNPL Assay

Spots obtained during TLC of ethyl acetate fraction were assayed for inhibition activity. The inhibitory activity was measured as the rate of production of *p*-nitrophenol at 405 nm.

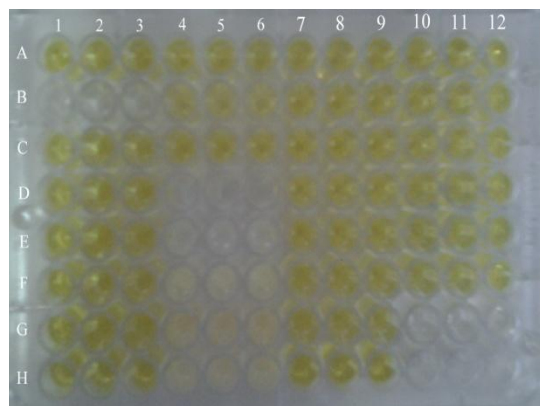


Fig.8 Micro titre plate showing inhibition assay for spots obtained during TLC Where, A1-C3spot1, D1-F3 spot 2, G1-A6 spot 3, B4-B6 substrate, Control, C4-C6 enzyme control.

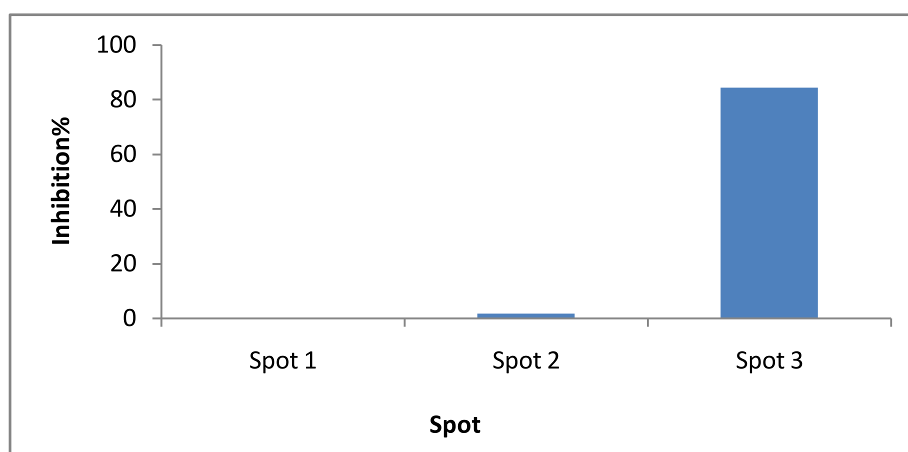
Enzyme Control= (3.048+3.182+3.075)/3 = 3.10

Substrate Control= (2.693+2.742+2.770)/3 = 2.73

Enzyme Activity= Enzyme control – Substrate control = 0.36

Spot	Absorbance at 405 nm				Test-Substrate control	Inhibition%
	A	B	C	Mean		
Spot 1	**3.277**	**3.289**	**3.051**	**3.205**	**0.470**	**0%**
	2.935	**3.229**	**3.356**	**3.173**	**0.438**	**0%**
Spot 2	3.102	3.179	3.13	3.137	0.402	0%
	3.210	2.842	3.234	3.095	0.360	1.7%
Spot 3	2.923	2.85	2.868	2.88	0.145	60.3%
	3.113	2.557	2.706	2.792	0.057	84.4%

Table No.19 Represents inhibition percentage for spots obtained during TLC



Graph 6 Represents inhibition percentage for spots obtained during TLC

Fig.9 Represents partially purified spot 3

During quantitative estimation of three different spots obtained during TLC of ethyl acetate fraction spot 3 has shown maximum inhibitory percentage as compared to two spots. Spot 3 has shown 84.4% inhibition against pancreatic lipase. Figure 10 represents the single spot which has shown maximum inhibitory activity. Hence, estimation done by this method is more accurate for screening inhibitory activity against pancreatic lipase.

5.6.2 GCMS Analysis

The chromatograms of GCMS were well analysed showing 5 different compounds of different molecular weights. From these 5 compounds, 4 compounds were of low molecular mass while 1 compound was of high molecular weight. This high molecular weight compound might be the potential lipase inhibitor as secondary metabolites are generally of high molecular weight.

RT	Compound	Similarity	Area	Molecular weight	Molecular formula
11.968	Phenol, 2,4-bis(1,1-dimethylethyl)	96	5.55	206	$C_{14}H_{22}O$
16.141	Nonane, 5-methyl-5-propyl-	88	1.20	184	$C_{13}H_{28}$
20.628	hexadecanoic acid, methyl ester, Palmatic acid	95	9.85	270	$C_{17}H_{34}O_2$
20.993	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy	82	1.31	1177	$C_{73}H_{108}O_{12}$
24.383	Octadecanoic acid, methyl ester	93	7.88	298	$C_{19}H_{38}O_2$

Table No.20 Represents the IUPAC name of the compounds analysed from GCMS

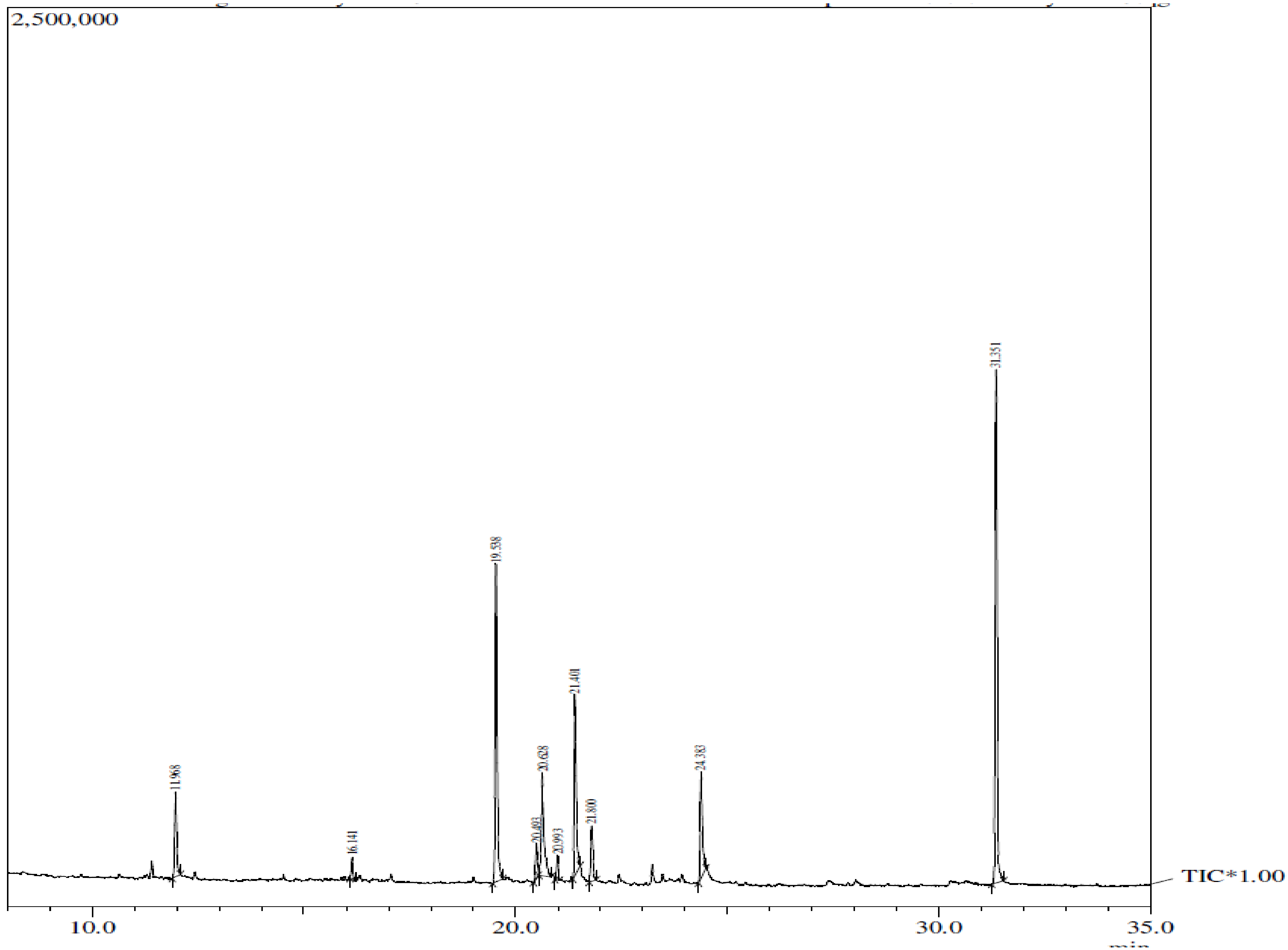


Fig. 10 Chromatogram showing GCMS analysis

5.7 Identification of Endophytic Fungi

The Endophytic fungi which has shown maximum inhibitory potential against pancreatic lipase was grown on different media such as PDA, CMA, SNA so that its taxonomy can be explained on the basis of microscopy and molecular characters.

5.7.1 Microscopy

(a) On PDA

Colonies were white in colour from front and tan coloured from back, fast growing and elevated. Hyphae were septate. Macroconidia were slightly sickle-shaped, thin-walled and were 2 to 5 septate. Microconidia were non-septate and cylindrical in shape.

(b) On SNA

Colonies were white in colour from front as well as white in colour from back, fast growing with a diameter of 50 mm and flat. Two types of conidia were present microconidia and macroconidia. Macroconidia were slightly sickle-shaped, larger in size, thin-walled, with a hooked apical cell and a barely notched foot cell. Microconidia were non-septate, smaller in size cylindrical in shape, slightly curved or straight.

Fig. 11 Growth of #1069 on SNA

(c) On CZD

Colonies were cottony in appearance, white in colour from front and tan coloured from the back, fast growing with a diameter of 75 mm and elevated. Macroconidia were slightly sickle shaped and were 3-5 septate. Microconidia were non septate.

Fig. 12 Growth of #1069 on CZD

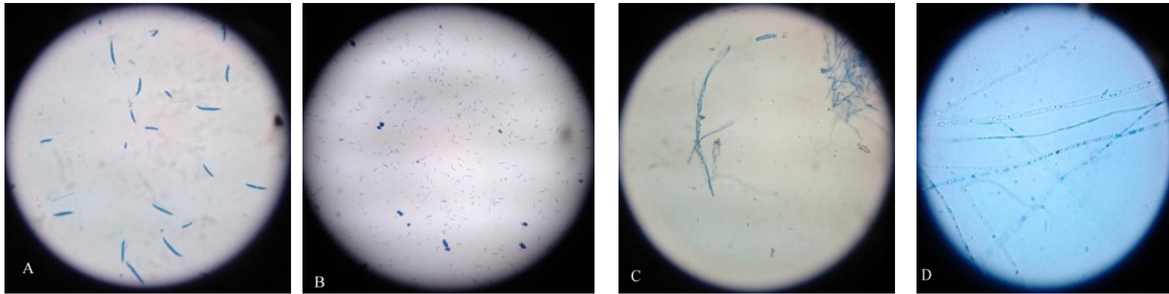


Fig.13 (A) and (B) sickle- shaped macroconidia with hooked apical cell and microconidia produced on SNA. (C) Microconidia produced on CZD. (D) Hyphae are septate on PDA.

Based on morphological characteristics #1069AMSTYEL was identified as ***Fusarium* sp**.

5.7.2 DNA Isolation and PCR Amplification

The genomic DNA isolation of #1069AMSTYEL showing the highest inhibitory potential against pancreatic lipase was done. The DNA was qualitatively estimated using 0.8% agarose gel electrophoresis and the size of the genomic DNA was deciphered by comparing its mobility in the gel with the 1 kilo base pair ladder which ranges from 1 kb to 10 kb. There was no RNA bands seen, hence the RNase treatment was successful. The size of the genomic DNA was found to be approximately 10 kb.

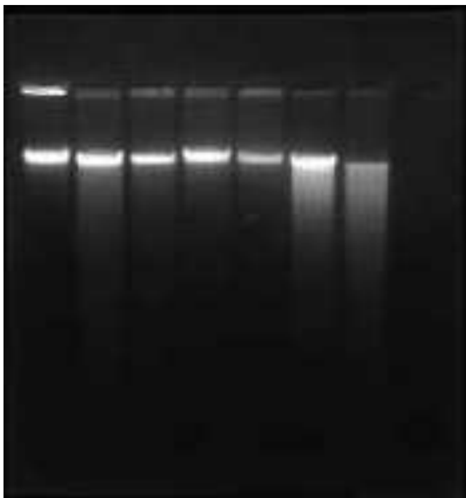


Fig. 14 Shows genomic DNA band of #1069 AMSTYEL in lane 1

The ITS1-ITS4 PCR amplication of the genomic DNA was carried out, the amplicon obtained was resolved on 1.5% Agarose in order to check the size on the basis of the mobility and comparison with the 500 bp ladder. The size of the amplicon was found to be approximately 550 bp. This size can be easily compared to the ITS region, which was amplified in order to characterize the fungi at molecular level.

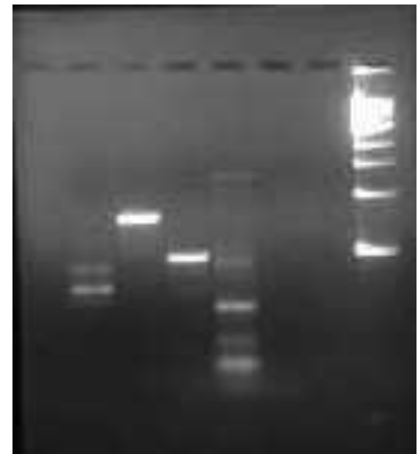


Fig.15 Shows 500 bp ladder in lane 8 and PCR Product of # 1069 AMSTYEL in lane 4

5.7.3 Sequencing and Blast Analysis

The sequences for ITS-1 and ITS-4 received from Chromus Biotech Bangalore were made into a final sequence of 546 bp using online software tools BLAST (http://blast.ncbi.nlm.nih.gov/) and Multalin (http://multalin.toulouse.inra.fr/multalin/). The FASTA format of the final nucleotide sequence of #1069 AMSTYEL obtained after sequencing from Chromus Biotech Bangalore.

```
>1069
TTCTGTAGGTGACTGGGAGGGATCATACCGAGTTTACAACTCCCAAANCNAANTAAAAATACTTAAACGC
CGGACGGATCACGCCCGGCGCCCCGTAAAACGGGACGGCCCGCCAGAGGACCCCTAAACTCTGTTTTTAG
TGGAACTTCTGAGTAAAACAAACAAATAAATCAAAACTTTCAACAACGGATCTCTTGGTACGGGCATCGA
TGAAGAACGCAGCAGAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG
CACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCAGCTT
GGTGTTGGGACTCGCGGTAACCCGCGTTCCCCAAATCGATTGGCGGCCAAGTCGAGCGTCCATAGCGTAG
TAATCATACACCACGAGACTAGTAATCAGCGCGGCCACGCNGTAAAACCCCAACTGCGGAAGCAACAACT
AGCANAAGGCAGGAATACCCGCTGAACTTAAGCATATCAGTAAGCGGAGGGGCGTC
```

The obtained sequence was then subjected to sequence similarity search in the database by using BLAST software at NCBI (http://blast.ncbi.nlm.nih.gov/).

S.No.	Description	Query cover	E value	Max identity	Accession No.
1	*Fusarium equiseti* isolate T34 18S ribosomal RNA gene	98%	0.0	91%	FJ459976.1
2	*Fusarium equiseti* isolate Mehandi SLB 18S ribosomal RNA gene	98%	0.0	91%	HQ589227.1
3	*Fusarium oxysporum f.* sp. ciceris isolate Foc156 18S ribosomal RNA gene	98%	0.0	91%	JN400714.1
4	*Fusarium* sp. 91DZ/F 18S ribosomal RNA gene	96%	0.0	91%	GQ407102.1
5	*Fusarium* sp. 141GP/S internal transcribed spacer 1	98%	0.0	91%	GQ352488.1
6	*Fusarium equiseti* genes for 18S rRNA	98%	0.0	91%	AB425996.1
7	*Fusarium equiseti* isolate T31 18S ribosomal RNA gene	98%	0.0	91%	FJ459975.1
8	*Fusarium* sp. 15 YS-2008 18S ribosomal RNA gene	98%	0.0	91%	EU594570.1
9	*Aspergillus flavus* strain AFM1 internal transcribed spacer 1	98%	0.0	91%	DQ198161.1
10	*Fusarium* sp. P37E2 18S ribosomal RNA gene	96%	0.0	91%	JN207334.1

Table No. 21 Shows first 10 results of BLAST analysis with Database

Hence, From this data we analysed that as the obtained sequence shows 98% identity with *Fusarium equiseti*, #1069 AMSTYEL was confirmed to be *Fusarium equiseti*.

CHAPTER 6

CONCLUSION

CONCLUSION

In the present study, we have screened a pancreatic lipase inhibitor that can be used as antiobesity drug in future. So, from 71 endophytic fungal isolates some isolates showed good inhibitory activity against lipase in qualitative screening by phenol red and Rhodamine B olive oil agar well diffusion assays. The 7 fungal isolates showing positive response in qualitative screening were further screened by a quantitative PNPL spectrophotometric assay. Fungal isolate #1069 AMSTYEL from medicinal plant *Aegle marmelos* showed maximum inhibitory potential of 84.3% against pancreatic lipase during quantitative analysis using PNPL assay.

Partial purification of crude culture filtrate increased its inhibitory potential from 84.3% to 97.7% in ethyl acetate fraction which is almost comparable to orlistat- the FDA approved only lipase inhibitor commercially available in market as an anti-obesity drug. Further the GC-MS of extract revealed the structure of compound to be Benzenepropanoic acid, 3, 5-bis (1,1-dimethylethyl)-4-hydroxy.

Morphotaxanomy of the culture was quite similar to that of *Fusarium* which was confirmed by molecular approaches and online softwares like BLAST to be *Fusarium equistei.* Further studies on purification and characterization of compound can lead to possibilities of using this lipase inhibitor as therapeutic modality in obesity treatement.

Chapter 7

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