SCREENING ENDOPHYTIC FUNGI FOR THE PRODUCTION OF HISTONE DEACETYLASE INHIBITORS

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT OF THE DEGREE OF

Master of Science in Biochemistry

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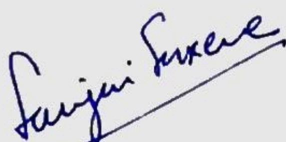
CERTIFICATE

This is to certify that the thesis **"Screening endophytic fungi for the production of histone deacetylase inhibitors"** being submitted by **Ms Disha Kapila (Roll No: 301707005)** in partial fulfilment of the requirements for the award of degree of Master of Science in Biochemistry, Thapar Institute of Engineering and Technology, Patiala, Punjab is a bonafide work carried out under the supervision and conception of Dr Sanjai Saxena and Dr Manmohan Chhibber and that no part-of this thesis has been submitted for the award of any other degree.



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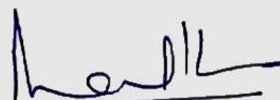

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CANDIDATE'S DECLARATION

I hereby declare that the work being presented in the thesis entitles **"Screening Endophytic Fungi for the production of histone deacetylase inhibitors"** in the partial fulfilment of the requirements for the award of degree of Master of Science in Biochemistry, School of chemistry and biochemistry, Thapar Institute of Engineering and Technology, Patiala is my own laboratory work during the period of January 2019 to July 2019, under the conception and supervision of Dr Sanjai Saxena and Dr Manmohan Chhibber. I have not submitted the matter embodied in this thesis for the award of any other degree.

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DEDICATION

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

especially those who were very close to our heart.

My humble effort I dedicate to my sweet and loving

Father & Mother,

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

able to get such success and honour,

Along with all hard working and respected

Teachers

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LIST OF ABBREVIATIONS

S.NO.	ABBREVIATIONS	FULL FORM
1.	β	Beta
2.	ε	Epsilon
3.	µl	Micro litre
4.	µM	Micro molar
5.	ACS	Acetyl CoA synthetase
6.	AMP	Adenosine-5'-monophosphate
7.	ATP	Adenosine-5'-triphosphate
8.	bp	Base pair
9.	CDA	Czapek dox agar
10.	CMA	Corn meal agar
11.	CS	Citrate synthase
12.	DNA	Deoxyribose nucleic acid
13.	DNMT	DNA methyltransferase
14.	dNTP	Deoxynucleotide triphosphate
15.	EA	Ethyl acetate
16.	EDTA	Ethylene diamine tetra acetic acid
17.	EtBr	Ethidium bromide
18.	FAD	Flavin adenine dinucleotide
19.	HAT	Histone acetyltransferase
20.	HDAC	Histone deacetylase
21.	HDACi	Histone deacetyalse inhibitior
22.	Hr	Hours
23.	ITS	Internal transcribed spacer
24.	L-MDH	L-malate dehydrogenase
25.	min	Minute
26.	mm	Millimetre
27.	NAD	Nicotinamide-adenine dinucleotide
28.	ng	Nano gram
29.	PCR	Polymerase chain reaction
30.	PDA	Potato dextrose agar
31.	PDB	Potato dextrose broth

32.	pH	Potential of hydrogen
33.	PLA	Pine leaf agar
34.	RPD3/HDA1	Reduced Potassium Dependency/ Histone deacetylase
35.	rpm	Rotations per minute
36.	SAHA	Suberoylanilide hydroxamic acid
37.	Sir	Sirtuins
38.	sp.	Species
39.	STD	Standard
40.	TAE	Tris acetate EDTA
41.	TE	Tris EDTA
42.	TSA	Trichostatin A
43.	USA	United states of America
44.	WA	Water agar

EXECUTIVE SUMMARY

HDAC inhibitors demonstrate anti-neoplastic effects by inhibiting cell migration, inducing growth arrest and programmed cell death. HDAC activity can be inhibited by certain natural dietary sources such as dietary fibres, vegetables (cruciferous vegetables), whole grains (parsley, celery), fruits (grapes, blueberries etc.) and certain micronutrients. Endophytic fungi are microorganisms which colonize inside plant tissue without causing apparent harm to the host. Besides upon colonization of host plant endophytes synthesize an array of secondary metabolite which may defend the host plant against survival and stress conditions and in turn the host plant supply nutrients and habitat for endophytic fungi. Therefore, in addition to interaction of endophytes with the host plant, endophyte acquires the property of their host plant and start producing analogue bioactive compounds which have medicinal value. The current study reports the exploration of endophytic fungi isolated from *R. serpentine, C. camphora, C. zeylanicum, C. roseus, Taxus baccata, A. marmelos, Vitis vinifera, C. malabaricum, M. pumila* for their potential to produce HDAC inhibitors. In the proposed study culture filtrate of endophytic fungi obtained from potato dextrose broth were screened for Biochemical assays namely: Acetic Acid Standard Test (K-ACET, Megazyme) was used for the identification, where out of 80 endophytic isolates, only 21 isolates were found to exhibit >75% activity among which 2 isolates were selected with >99% activity and subjected to mass production. These were subsequently extracted in four different solvents and again tested for presence of HDAC inhibitors. Hexane extract of #23CZSTITG and EA extract of #40CMBLRT were found to be potent producer of HDAC inhibitors. The potent producers of HDAC inhibitors from endophytic isolates #23CZSTITG and #40CMBLRT, were tentatively identified as *Botryosphaeria sp. and Penicillium sp.* respectively. The genomic DNA of the selected isolates were extracted, amplified and further send for sequencing for molecular identification.

Keywords: Endophytic Fungi, HDAC, Cinnamon, HDAC inhibitors, *Penicillium sp, Botryosphaeria sp.*

CHAPTER 1

INTRODUCTION

1.0. INTRODUCTION

Epigenetic regulation is essential for the gene expression and result in normal growth and development and maintains various cellular functions such as transcription, cell survival (Blewitt et al., 2013). Coppede (2014) described negative impacts on the health due to epigenetic dysregulation such as colorectal cancer. Histone modification associated with DNA which is responsible for regulation of its structure and functions, is a significant process for epigenetic regulation. Methylation, phosphorylation and acetylation are certain mechanisms that has an influence on histone modification. Acetylation is one of transient histone modifications in which hyperacetylation leads to an increased gene expression, and hypoacetylation has an opposite effect (Bassett et al., 2014). There are several large, multisubunit enzyme complexes that may target histone deacetylation.

Histone deacetylases are the enzymes that has the ability to effect the gene expression by removing the acetyl group from histones associated with DNA. histone deacetylase (HDAC) also functions on the non- histone proteins such as transcription factors, by removing acetyl group from the lysine residues. Thus influencing the cellular functions in a more complex way (Bassett et al., 2014). HDAC inhibitors are further responsible to regulate activity of histone deacetylases and thus are widely used in certain cancer and neurodegenerative diseases such as Alzheimer's disease (Simoes-Pires et al., 2013). Kim et al., (2010) described the association of HDAC genes with schizophrenia. HDAC2 seems to have a significant role in diabetes and also transform growth factor-β-1 that induce renal injury (Noh et al., 2009).

Kharwar et al., (2011) reported that endophytic fungi belong to the class of fungi which resides inside the plant tissue and maintain a symbiotic and modest relationship with their hosts, at least for a season of their life cycle. They produce a number of compounds that are biologically active such as secondary metabolites that maybe have therapeutic values obtained from plants for direct use as a drug (Dwibedi et al., 2019). These also include novel compounds reported from endophytic fungus, such as taxol and camptothecin. These reports described gene transfer events may occur between endophytic fungi and plants.

Fungi are considered to be capable of producing compounds that can be used in pharmaceutics, as demonstrated by penicillin, lovastatin etc. (Keller et al., 2005). The genomic sequences of the fungi revealed that there are significant numbers of genes that code for enzymes those results in production of secondary metabolites which not express artificially under the laboratory conditions. Thus, to attain novel bioactive compounds, one can focus on the

expression of dormant genes in the fungi (Chen et al., 2013). Recent studies revealed that dormant biosynthetic genes can be activated by HDAC and DNA methyltransferase (DNMT) inhibitors (Williams et al., 2008; Henrikson et al., 2009; Wang et al., 2010; Asai 2011). Gene expression or silencing is regulated by the chromatin modification due to histone deacetylation and DNA methylation. Hence, chromatin remodelling is induced by the epigenetic modifiers such as HDAC inhibitors (Netzker et al., 2015). Yang et al., (2013) reported two epigenetic modifiers isolated from *Pestalotiopsis acacia* i.e. Suberoylanilide hydroxamic acid as an HDAC inhibitor and 5-azacytidine as DNMT inhibitor that effect the production of 3 novel aromatic compounds. Further, Yang et al (2014b) reported another epigenetic modifier 5-Azacytidine for DNMT inhibition isolated from *Pestalotiopsis crassiuscula* that effects the production of 7 metabolites such as pestalotiopyrone G, scirpyrone A etc.

So far, no compound has been extracted from endophytic fungi of cinnamon leaves, stem or bark which may inhibit histone deacetylase. Hence, the aim of current study is "screening of endophytic fungi for the production of HDAC inhibitors".

CHAPTER 2

REVIEW OF LITERATURE

2.0. REVIEW OF LITERATURE

2.1 Overview of Histone

Around 1903, scientists examined that chromatin is composed of nucleic acid and a protein part. During that time, the protein part of the chromatin was not understood, but Levene (1903) suggested that chromatin might be a substance with highly complex protein. Moore et al., (1913) alluded histones a class of proteins that has basic properties and are essentially obtained from the sperm cells. Mirsky et al., (1946) reported that chromosin is a deoxyribose nucleoprotein complex that is composed of vast cell diversity. The report also affirmed that it was obtained from the nuclear fraction that enclosed deoxyribose nucleic acid along with the histone and non-histone proteins. The presence of histones in many animal cells, some plant cells and cells of bacteria was also observed. Histones' key role is to provide the protein framework for the DNA to wrap around. With the ongoing development in molecular biology techniques, it became more certain that histones play a major role in DNA function and regulation (Mellor 2006).

Different sub-fractions of the histones have been studied and ascertained that they were created by modifying the amino acid residues of the side chain, which occurred after protein translation. Gershey et al., (1968) described the process of histone acetylation with the characterized presence of an ε-N-acetyl derivative of lysine in histones. It was assumed that such histone modifications were everlasting and were a process for the formation of the various types of histones. However, these modifications are not permanent that grabbed the attention of the researchers and work in this area of research. Also, the systems that operate them have a level of control on the gene expression.

Histone residues experience a wide range of modifications. There are eight distinct ways, each with different characterized enzymes for histone modification (Table 2.1). Kouzarides (2007) reported that sixty different modified residues on histones were identified by the techniques such as modification-specific antibodies or mass spectroscopy. The complexity level increased when it was studied that there is the existence of mono-or di-methylated arginine residues and mono-, di-or tri-methylated lysine residues. It later turned out that epigenetic mechanisms regulate histone modifications such that DNA methylation influence the expression of the gene (Andreu-Vieyra et al., 2013).

2.2 Histone Modifications

A number of enzymes are responsible for the histone modifications and it is considered as a reversible change. These enzymes are responsible to either enhance or eliminate covalent modifications. Further, these modifications control the level of contact between the DNA and histone which further has an effect on DNA transcription. There are different types of histone modifications (Table 2.1) that are responsible for the alterations at the DNA level by the processes such as transcription, repair, replication and condensation (Bassett et al., 2014).

Table 2.1: Types of Histone modifications

TYPE	AMINO ACID MODIFICATION	MODIFYING ENZYMES	EXAMPLES	REFERENCES
Acetylation	Lysine	Histone Acetyltransferases (HATs) Histone Deacetylases(HDACs)	HAT1 HDAC1	Gershey et al., 1968 Glozak et al., 2007
Methylation	Arginine Lysine	Arginine Methyltransferase Arginine Demethyltransferase Lysine Methyltransferase Lysine Demethyltransferase	PRMT4 JMJD6 SUV39H1 LSD1 /BHC110	Bannister et al., 2005 Chang et al., 2007 Nottke et al., 2009
Phosphorylation	Threonine Serine	Dephosphorylated by phosphatases Serine/Threonine Kinases	PP4 WEE1	Nakada et al., 2008 Rossetto et al., 2012
Ubiquitination	Lysine	Ubiquinases (Ubiquitin Ligases) Deubiquitinating Enzymes	RING1B USP22	Cao et al., 2005 Wang et al., 2006 Cao et al., 2012
SUMOylation	Lysine	Small Ubiquitin-like Modifier proteins De-SUMOylating Enzymes: Sentrin-Specific proteases	SUMO-1 SENP1	Liu et al., 2012 Drag et al., 2008
ADP ribosylation	Glutamate	ADP-Ribosyltransferases	ARTD1	Hassa et al., 2006 Kassner et al., 2013
Deimination	Arginine (to citrulline)	Peptidylarginine Deiminases	PADI4	Cuthbert et al., 2004 Denis et al., 2009
Proline Isomerisation	Proline	P-cis > P-trans	Pin1	Nelson et al., 2006 Raghuram et al., 2013

2.2.1 Mechanism of histone modification

Acetylation is an important histone modification process that is connected with transcriptionally active regions (i.e. euchromatin region) and is responsible for gene expression (Barnett et al., 2016). Histone acetyltransferases (HATs) are responsible for the acetyl group transfer from acetyl coenzyme A to lysine residues. On the contrary, Histone deacetylases (HDACs) play a role in removing the acetyl groups. A large variety of non-histone proteins such as transcription factors, chaperone proteins are modified by both HATs and HDACs (Barnett et al., 2016; Haberland et al., 2009; Ho et al., 2010). Changes in acetylation at different level alter the activity of non-histone proteins such as protein stability, protein-protein interactions, and protein-DNA interactions.

Figure 2.1 depicts some of the histone modifications that influence the expression of the gene. The left side of the figure depicts closed chromatin, in which the transcriptional machinery could not be recruited on the DNA and therefore, transcription is repressed. The right side of the figure represents an acetyl group transfer to an ε-lysine residue via HAT that leads to the unfolding of the chromatin. This further helps to recruit the transcriptional machinery to the DNA and thus, activates the process of activation. On the contrary, HDAC removes the acetyl group and thus, alters the configuration of histone, the chromatin re-return to the closed form. In histone methylation, confirmation of chromatin depends on the methylation of a specific lysine residue.

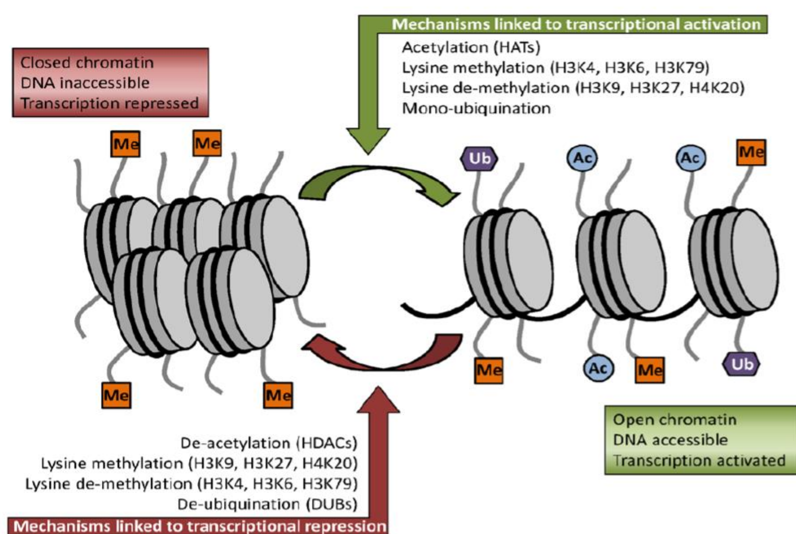

Figure 2.1: Gene expression is controlled by various histone modifications (Bassett et al., 2014)

2.3 Histone deacetylases (HDACs)

HDACs are responsible for transcriptional repression due to a reversal of acetylation. HDACs control various functions of the cells, such as cell cycle progression and proliferation (Glozak et al., 2007; Xu et al., 2007). The HDACs are classified into two families, the histone deacetylase family and the Sir2 regulator family. They further belong to arginase/deacetylase superfamily and deoxyhypusine synthase-like NAD/FAD-binding domain superfamily, respectively. HDACs are further categorised to four classes and comprised of 18 different proteins. Classes, I, II, and IV have sequence similarity to the RPD3/HDA1 family of *Saccharomyces cerevisiae*. HDACs 1–3 and HDAC8 belong to Class I. They are universally expressed and play a major role in regulating cell proliferation and survival. Tissue-specific functions are regulated by Class II HDACs and they are further subdivided into class IIa and class IIb. HDAC activity of class IIa is weaker. They can transport between the cytosol and the nucleus. Class IIb, on the other hand, prefer to act on non-histone proteins and are mostly found in the cytosol. HDAC11 belongs to class IV. Classes, I, II and IV belong to histone deacetylase family as they are all zinc-dependent hydrolases whereas NAD+ dependent sirtuins are yeast sir2 homologs that belong to class III which is categorised under sir2 regulator family (Table 2.2). Huang et al., 2010; Verdin et al., 2010 described SIRTs 3–5 play a significant role to regulate the mitochondrial enzymes responsible for energy production, metabolism, apoptosis, and intracellular signalling. Range of cellular functions such as transcription, apoptosis, inflammation are regulated by sirtuins (Blander et al., 2004; Howitz et al., 2003; Porcu et al., 2005; Shoba et al.,2009).

The Class I, II, and IV HDACs are categorised according to their sequential order of discovery. For example, Taunton et al., (1996) first reported HDAC1, followed by the discovery of HDAC2 (Yang et al. 1996). HDAC3 was reported in the subsequent year (Yang et al., 1997). Taunton et al., 1996 described HDAC4, 5, and 6. HDAC7 and HDAC8 were discovered in early 2000 (Kao et al., 2000; Hu et al., 2000). Frye 1999 reported Sirtuins 1-5.

Table 2.2: Classification of Histone deacetylases

FAMILY	CLASS	SUBCLASS	PROTEIN	LOCALIZATION IN CELL
Histone deacetylase family	Class I		HDAC1	Nucleus
			HDAC2	Nucleus
			HDAC3	Nucleus
			HDAC8	Mainly in Nucleus
	Class II	Class IIa	HDAC4	Nucleocytoplasmic traffic
			HDAC5	Nucleocytoplasmic traffic
			HDAC7	Nucleocytoplasmic traffic
			HDAC9	Nucleus
		Class IIb	HDAC6	Mainly in cytoplasm
			HDAC10	Nucleus and cytoplasm
	Class IV		HDAC11	Nucleus
Sir2 regulator family	Class III	I	SIRT1	Mainly in nucleus
			SIRT2	Nucleus and cytoplasm
			SIRT3	Mitochondria
		II	SIRT4	Mitochondria
		III	SIRT5	Mitochondria
		IV	SIRT6	Nucleus
			SIRT7	Mainly in the nucleus

2.3.1 Mechanism of histone deacetylases

The mechanism of action is such that HDAC enzymes remove acetyl group from the histones associated with the nucleosome. The space between the nucleosome and the DNA is effected due hypoacetylation. When DNA is tightly wrapped around the histones, its accessibility for the transcription factors decreases followed by transcriptional repression (Ruijter et al., 2003). HDAC can be explained on the basis of two different mechanisms, namely, zinc-dependent hydrolases dependent for the HDAC classes I, II and IV and nicotinamide adenine dinucleotide (NAD^+)- dependent for HDAC class III.

2.3.1.1 Mechanism zinc-dependent hydrolases

HDAC classes I, II and IV follow the mechanism of zinc-dependent hydrolases for their activity. The left side of the figure 2.2 consists of a catalytic core with zinc-binding site in the tubular pocket along with an active site that consists of tyrosine residue and two histidine residues that undergo H-bonding with two residues of aspartate. Carbonyl substrate coordinated with the zinc ion gets nucleophilic attack by one of the histidine residues by catalytic water molecule. This results in the formation of an oxyanion tetrahedral intermediate which is balanced out by a tyrosine residue in the catalytic core. At that point, there is an acceptance of proton due to the transfer between aspartate and histidine residues to the acetate bonded with the N-terminal of the lysine residue. Henceforth, carbon-nitrogen breaks and results in deacetylation of the substrate and release acetate (Marmorstein and Zhou 2014).

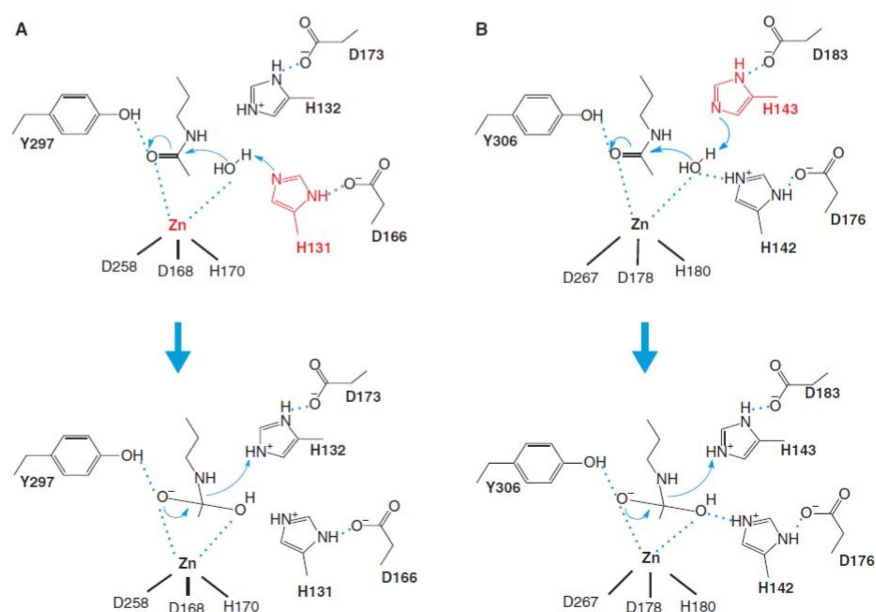

Figure 2.2: Zinc-dependent hydrolases (A) A model proposed from the HDLP structure. B) A model proposed from the HDAC8 structure (Seto and Yoshida 2014)

2.3.1.2 Nicotinamide Adenine Dinucleotide (NAD^+)- dependent mechanism

HDAC Class III depends on the mechanism of nicotinamide adenine dinucleotide (NAD^+) for their reactions. In Figure 2.3, the nucleophilic attack is at the C1′ position of the nicotinamide ribose by oxygen of acetyl group that results in the formation of C1′-O-alkylamidate intermediate and free nicotinamide. Later, histidine residue in the active site results in the activation of the 2′-hydroxy group of the NAD+ ribose and it further attacks the C1′-O-alkylamidate to form a cyclic intermediate. Catalytic water molecule further attacks on the

1′,2′-cyclic intermediate and results in the deacetylation of lysine residue and formation of 2′-O-acetyl-ADP ribose and it can be freely converted to 3′-O-acetyl-ADP ribose by non-enzymatic intramolecular transesterification in an aqueous solution. Thus, the final reaction products are nicotinamide, the deacetylated peptide, and a mixture of 2′- and 3′-O-acetyl-ADP ribose (Du et al., 2011).

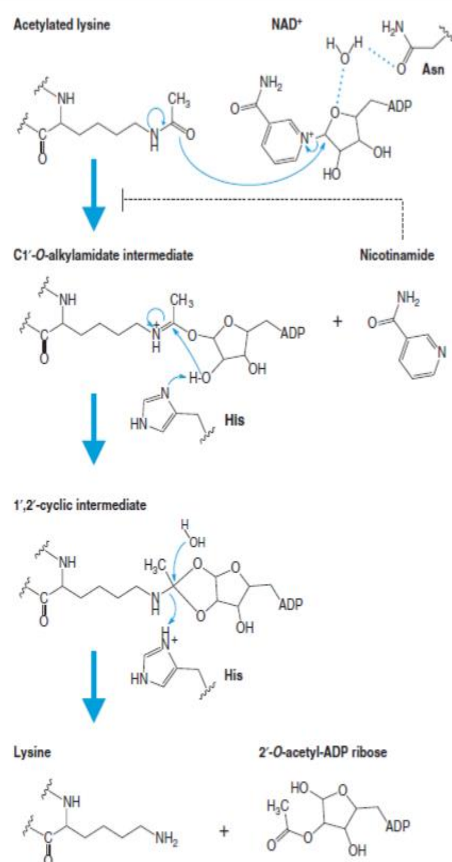

Figure 2.3: Catalytic mechanism of HDAC Class III (Sirtuins) (Seto and Yoshida 2014).

2.3.2 Expression of HDACs on various diseases

HDACs regulate a number of cellular functions that are accountable for carcinogenesis, growth of the cell and homologous recombination and thus, are considered a promising therapeutic target for cancer (Glozak et al., 2007; Adimoolam et al., 2007; Claus et al., 2003; Khan et al., 2008). A variety of human diseases are linked to the dysregulation of the HDAC enzyme. Newkirk et al., (2009) reported that the class I enzymes are associated with the survival and cancer cells proliferation, while class II (HDAC8) may be responsible for tumorigenesis. Gui

et al., (2004) described that silencing of tumour suppressor genes results in cancer are associated with HDACs. e.g., HDACi can reverse the hypoacetylation status of the p21WAF1 promoter and its corresponding gene inactivation. Table 2.3 briefly describes the expression of each class of HDAC in a number of diseases.

Table 2.3: Diseases due to HDAC dysfunction

Type of HDAC	Effect	Disease	Reference
HDAC1	HDAC1 overexpression	Prostate cancer, Ovarian cancer	Song et al., 2011; Halkidou et al. 2004; Jin et al., 2008
	Reduced expression of HDAC1	Colorectal cancer	Sudo et al., 2011
HDAC2	HDAC2 overexpression	Ovarian cancer	Jin et al., 2008
	Reduced expression of HDAC2	Chronic obstructive pulmonary disease	Barnes et al., 2005
HDAC3	HDAC3 overexpression	Ovarian cancer	Jin et al., 2008
	HDAC3 protein overexpression	Colon cancer	Wilson et al., 2006
HDAC4	HDAC4 overexpression	Endocrine cancer, Colon cancer, Lung cancercinoma, Breast cancer	Halkidou et al., 2004; LLeonart et al., 2006; Ozdag et al., 2006
	Reduced HDAC4 expression	Huntington's disease	Mielcarek et al., 2013
HDAC5	HDAC5 overexpression	Colorectal cancer	Ozdag et al., 2006
	Reduced HDAC5 expression	Major depression	Iga et al., 2007
HDAC6	HDAC6 protein overexpression	Polycystic liver disease	Gradilone et al., 2014
	Reduced HDAC6 expression	Hodgkin's lymphoma	Gloghini et al., 2009
HDAC7	Overexpression	Colorectal cancer, Pancreatic cancer	Ozdag et al., 2006; Ouaissi et al., 2008
HDAC8	Mutations	Cornelia de Lange disease	Deardorff et al., 2012
HDAC9	Gene variants	Multiple sclerosis	Inkster et al., 2013
	Gene disruption	Peter's anomaly	David et al., 2003
HDAC10	Overexpression	Chronic lymphocytic leukaemia	Wang et al., 2011
HDAC11	Gene variants	Multiple sclerosis	Inkster et al., 2013

SIRT1	Overexpression Reduced expression	Breast, colorectal and prostate cancer Colorectal cancer	Kuzmichev et al., 2005 Ozdag et al., 2006
SIRT2	Polymorphism	Alzheimer's disease	Wei et al., 2014
SIRT3	Reduced expression of mRNA and protein	Gastric cancer	Yang et al., 2014
SIRT4	Gene variants	Multiple sclerosis	Inkster et al., 2013
SIRT5	Overexpression Gene variant	Alzheimer's disease Multiple sclerosis	Inkster et al., 2013 Lutz et al., 2014
SIRT6	SIRT6 underexpression	Liver cirrhosis	Marquardt et al., 2013
SIRT7	Overexpression	Breast cancer	Ashraf et al., 2006

2.4 HDAC inhibitors

Epigenetic regulations, rather than mutations, are reversible and are thus, considered as the remedial targets. Most of such compounds bind to the catalytic zinc site, a tail (linker) that mimics the side chain of lysine and a cap that obstructs the entrance to the active site. HDACi is classified into five categories on the basis of their chemical structure: short-chain fatty acids, hydroxamic acids, cyclic peptides, benzamides and depsipeptide (Bieliauskas and Pflum 2008; Khan et al., 2008). Other than these 5 types of HDACi, there are many molecules with a potent inhibitory activity have been identified with different chemical structures such as flavonoids, isothiocyanates, organosulfur (Seidel et al., 2012). HDACi plays a major role in blocking the access to the reversible or irreversible active site of HDAC. HDACs dysregulation may result in cancer thus HDAC inhibitors (HDACi) are being formulated for cancer treatments. Many HDAC inhibitors have been reported to date (Table 2.4), but Trichostatin A (TSA) is considered as the most potent inhibitor discovered so far. It is a fermentation product of *Streptomyces*. TSA has anti-fungal properties as well and was discovered before, but later it was found that it is inhibiting the proliferation of cancer cells. Yoshida et al., (1990) reported the ability of TSA to inhibit HDAC. A considerable number of these compounds are mentioned in table 2.4 that were isolated from natural sources.

Table 2.4: List of natural sources of HDAC inhibitors.

INHIBITORY COMPONENT	NATURAL SOURCES	REFERENCES
Apicidin D	*Fusarium sp.*	Han et al., 2000
Azumamide E	*Mycale izuensis*	Maulucci et al., 2007
Chlamydocin	*Diheterospora chlamydosporia*	De schepper et al., 2003
FR235222	*Acremonium sp.*	Petrella et al., 2008
Trapoxin A and B	*Corollospora intermedia*	Furumai et al., 2001
FK228 (Romidepsin)	*Chromobacterium violaceum*	Furumai et al., 2002
Largazole	*Symploca sp.*	Ying et al., 2008
Spiruchostatin A	*Pseudomonas*	Crabb et al., 2008
Butein	*Rhus verniciflua*	Yang et al., 2009
Daidzein	Soybean	Rasbach and Schnellmann (2008)
Flavone	*Feijoa sellowiana*	Bontempo et al., 2007
Genistein	Soy	Basak et al., 2008
Luteolin	Sweet pepper, celery, parsley	Attoub et al., 2011
Pomiferin	*Maclura pomifera*	Son et al., 2007
Quercetin	Citrus fruits, buckwheat	Link et al., 2010
Silibilin	Milk thistle	Zhou et al., 2006
Amamistatin	*Nocardia asteroides*	Fennell and Miller (2007)
TSA	*Streptomyces hygroscopius*	Woo et al., 2007
PEITC	Cruciferous vegetables	Wang et al., 2008
Sulforaphane	Cruciferous vegetables	Myzak et al., 2004
AM	Garlic (*Allium sativum*)	Nian et al., 2008
Bis (4-hydroxybenzyl) sulphide	Knotweed (*Pleuropterus ciliinervis*)	Son et al., 2007
DADS	Garlic (*Allium sativum*)	Druesne et al., 2004
Sodium butyrate	Fermentation of dietary fibres	Davie (2003)
Sodium propionate	Fermentation of dietary fibres	Aoyama et al., 2010
Piceatannol	Blueberries	Wang et al., 2008
Resveratrol	*Vitis Vinifera*	Dwibedi et al., 2018
Caffeine	*Coffea arabica*	Mukwevho et al., 2008
Cyclostelletamines	Marine sponges	Oku et al., 2004
Dihydrocoumarin	*Melilotous officinalis*	Olaharski et al., 2005

DIM	Cauliflower and broccoli	Bhatnagar et al., 2009
Depudecin	*Alternaria brassicicola*	Kwon et al., 1998
MCP30	Bitter melon seeds	Xiong et al., 2009
Nicotinamide	Vitamin B3 metabolite	Zhang et al., 2011
Psammaplin A	*Aplysinella rhax*	Kim et al., 2007

2.5 HDAC inhibitors from Endophytic fungi

In the distal regions of the chromosomes, there are fungal biosynthetic gene clusters, that generally exist in a heterochromatin state. Histone deacetylation and DNA methylation transcriptionally control these genes by epigenetic regulation (Pettit, 2010). Gene expression or silencing in fungi and other organisms are regulated by the chromatin modification via histone deacetylation and DNA methylation. Hence, Brakhage and Schroeckh (2011) proposed a strategy for unlocking cryptic biosynthetic gene clusters by using epigenetic modifiers like DNA methyltransferase (DNMT) and histone deacetylase (HDAC) inhibitors to induce chromatin remodelling. Cichewicz (2010) described that DNMT inhibitors include 5-azacytidine and 5-aza-20-deoxycytidine, while HDAC inhibitors include hydroxamic acid containing compounds or cyclic peptides such as trichostatin A and trapoxin B.

Chen et al. (2013) reported suberoylanilide hydroxamic acid (SBHA) as the potent HDAC inhibitor from endophytic *Fusarium oxysporum* strain R1 that is responsible for the production of novel fusaric acid derivatives 5-Butyl-6-oxo-1,6-dihydropyridine-2-carboxylic acid and 5-(But-9-enyl)-6-oxo-1,6-dihydropyridine-2-carboxylic acid. Asai et al., (2013) reported nicotinamide (an NAD+-dependent HDAC inhibitor) isolated *Graphiopsis chlorocephala* that produced 6 novel benzophenones, namely, cephalanones A–F and a known metabolite: 2-(2,6-dihydroxy-4-methyl benzoyl)-6-hydroxybenzoic acid. Beau et al., (2012) reported another HDAC inhibitor, sodium butyrate isolated from *Leucostoma persoonii* that produced cytosporone R as the novel compound and also enhanced production of known cytosporones B (360%), C (580%) and E (890%). Sun et al., (2012) further described 5-Azacytidine (DNMT inhibitor) and/or suberoylanilide hydroxamic acid (HDAC inhibitor) isolated from endophytic *Alternaria sp.* that produced mycotoxins including alternariol, alternariol-5-O-methyl ether, 3′-hydroxyalternariol-5-O-methyl ether, altenusin, tenuazonic acid, and altertoxin

CHAPTER 3

OBJECTIVES OF THE STUDY

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3.0. OBJECTIVES OF THE STUDY

- Screening of endophytic fungi for the production HDAC inhibitors.
- Quantitative analysis of HDAC inhibitors.
- Morphological and molecular identification of selected endophytic fungus.

CHAPTER 4

MATERIALS AND METHODS

4.0. MATERIALS AND METHODS

4.1. Procurement, maintenance and preservation of endophytic fungi

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

4.2. Production of culture filtrates

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

4.3. Screening of HDACi producing endophytic fungi

The endophytic fungi culture filtrates were screened for HDACi production using a biochemical 2-step assay that well suited for HDAC activity measurements (Wenger et al., 2003). It is based on the quantification of the acetate moiety that is released during enzymatic activity due to the activity from non-labelled substrates using an Acetic Acid Standard Test (K-ACET, Megazyme). HDAC results in repression of transcription by deacetylating the histone. The decrease in HDAC activity results in hyperacetylation of the histone and thus HDACi presence can be analysed.

Table 4.1: Reagents used in Acetic Acid Assay

Pipette into microplate	Blank	Sample
Distilled water (at ~25° C)	184.8 µl	176 µl

<table>
<tr><td>Sample

Solution 1 (buffer)

Solution 2 (NAD^+/ATP/PVP/CoA)</td><td>-

44 µl

17.6 µl</td><td>8.8 µl

44 µl

17.6 µl</td></tr>
<tr><td colspan="3">Mix well and read the absorbances of the solutions(A_0) after approx. 3 minutes and start the reactions by addition of:</td></tr>
<tr><td>Suspension 3 (L-MDH/CS)</td><td>1.76 µl</td><td>1.76 µl</td></tr>
<tr><td colspan="3">Mix well and read the absorbance of the solutions (A_1) after approx. 4 minutes and start the reaction by addition of:</td></tr>
<tr><td>Suspension 4 (ACS)</td><td>1.76 µl</td><td>1.76 µl</td></tr>
<tr><td colspan="3">Mix and read the absorbance of the solutions (A_2) at the end of the reaction, after approx. 12 minutes.</td></tr>
</table>

The following formula, which should generally be used for preceding indicator reactions, serves to calculate the ΔAacetic acid:

$$\Delta A_{\text{acetic acid}} = [(A_2-A_0)_{\text{sample}} - \frac{(A_1-A_0)^2{}_{\text{sample}}}{(A_2-A_0)_{\text{sample}}}] - [(A_2-A_0)_{\text{blank}} - \frac{(A_1-A_0)^2{}_{\text{blank}}}{(A_2-A_0)_{\text{blank}}}]$$

According to the general equation for calculating the concentration:

$$c = \frac{V \times MW}{\varepsilon \times d \times v \times 1000} \times \Delta A \text{ [g/l]}$$

V = Final volume [ml]

v = Sample volume [ml]

MW = Molecular weight of the substance to be assayed [g/ml]

d = Light path [cm]

ε = Extinction coefficient of the NADH at 340nm = 6.3 [l x $mmol^{-1}$ x cm^{-1}]

4.4. Solvent extraction

The cultures exhibiting HDAC inhibition activities were subjected to Liquid-liquid extraction using four solvents, namely, hexane, chloroform, dichloromethane, ethyl acetate. The culture filtrate and solvents were taken up in a ratio of 1:3 and extracted three times (Figure 4.1). The organic layer was pooled and dehydrated by the addition of anhydrous sodium sulfate. Subsequently, the solvent fraction was evaporated overnight in an incubator at 37°C to dryness to get the crude fraction (Kjer et al., 2010). The fraction so obtained was weighed, reconstituted in methanol, and again screened for the presence of HDACi by HDAC assay as described previously. Crude fractions which exhibited negative results to HDAC assay were subjected to TLC separation to confirm the presence of HDAC inhibitor.

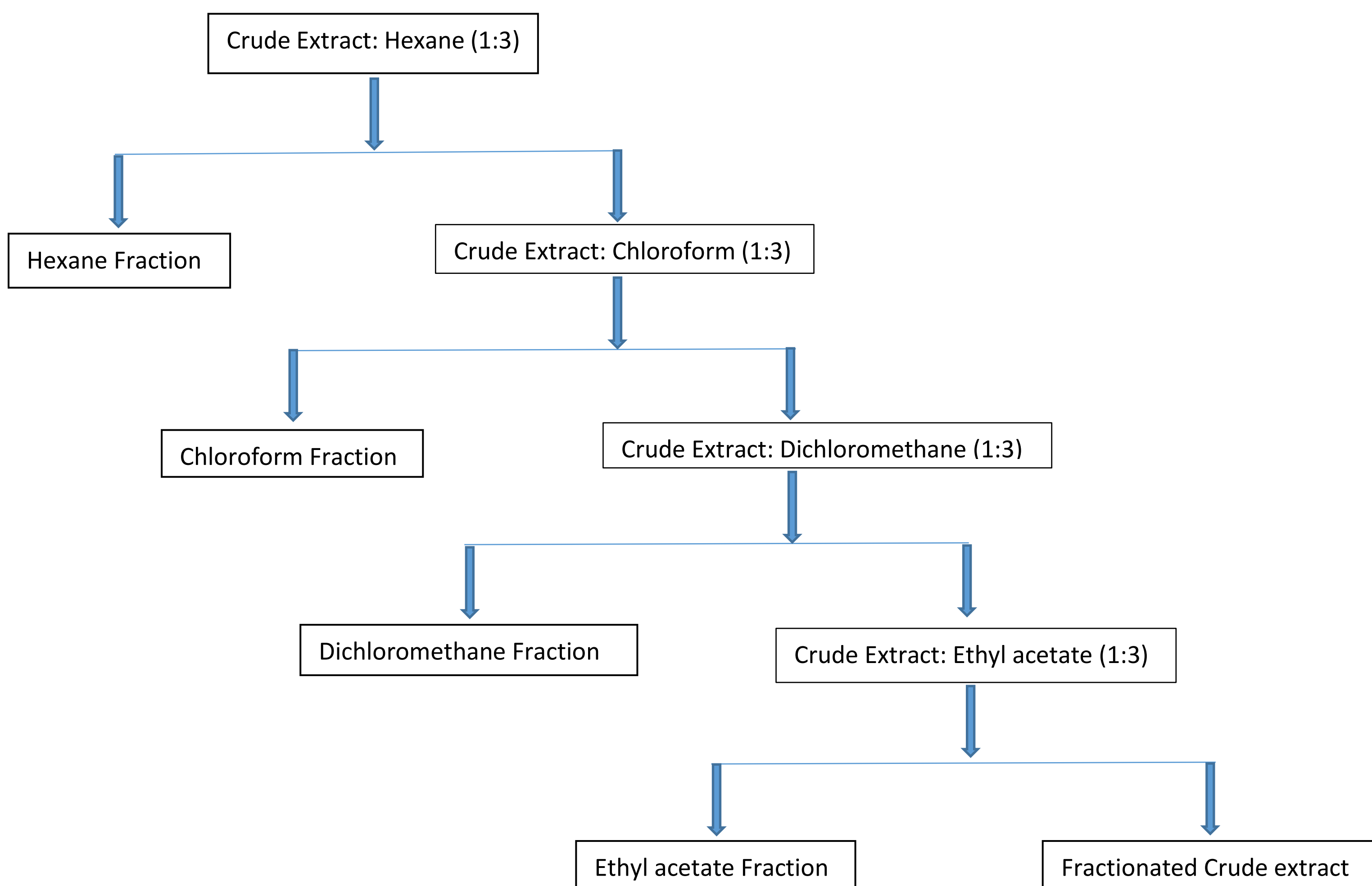

Figure 4.1: Solvent extraction of crude fungal extract in four different solvents.

4.5. Identification of Endophytic Fungi

The selected isolate showing HDAC inhibition was identified by classical and molecular taxonomy.

4.5.1. Morphotaxonomy of HDAC inhibitor Endophytic Fungus

For morphotaxonomic studies, the endophytic fungus showing HDAC inhibition activity has been inoculated on different nutrient media viz. Potato dextrose agar (PDA), Pine leaf agar (PLA), Czapex dox agar (CDA), Water agar (WA), Cornmeal agar (CMA) 10 days at 26 ± 2°C including 12 h of photoperiod. Growth rate and features, including its microscopic characteristics like hypha features, conidia formation, and other cellular bodies such as fruiting bodies (asexual or sexual spores) or structures, were critically observed under a Nikon stereo zoom microscope. All the micrometric observations were carried out using the Nikon NIS element software by taking at least 30-40 observations per structure (Shi et al., 2012; Wang et al., 2016).

4.5.2. Molecular identification of endophytic fungi

4.5.2.1. DNA isolation

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

4.5.2.2. Agarose gel electrophoresis

1X TAE (Tris Acetate EDTA) buffer was used to develop 0.8% agarose gel including 0.5 µg/ml of ethidium bromide (EtBr) and then the gel was cast in the electrophoretic apparatus. The gel was left to harden and the comb was carefully taken out. The running buffer (1X TAE) was poured in the electrophoretic tank. The DNA samples and the 6X loading dye were loaded into wells and allowed to run at 80V for 1 hr. The DNA fragments were visualized under UV transilluminator. Gel imaging was done using UV light in a Bio-Rad Gel documentation system using Quantity-1-D analysis software.

4.5.2.3. PCR amplification

Amplification of ITS1-5.8S-ITS2 rDNA sequence was done using universal primer pair i.e. ITS 1 and ITS 4, synthesized by Integrated DNA Technologies (IDT), USA, in a Thermocycler (My Cycler, Bio-Rad Laboratories, Inc.). The amplification reaction was carried out by using the primers ITS1 (5' TCC GTA GGT GAA CCT GCG G 3') AND ITS 4 (5' TCC TCC GCT TAT TGA TAT GC 3') (White et al., 1990). Amplification was achieved in the 25µl reaction mixture including 25 ng of fungal DNA, 0.8 µM of both ITS1 and ITS4 primers, 2.5mM of dNTP (Bangalore GeNei), 1.5 U of Taq DNA Polymerase (Bangalore GeNei), 1.5 mM MgCl2 (Bangalore GeNei) into 10 X Taq buffer (Bangalore GeNei). The Thermal cycling conditions include primary denaturation for 5 minutes at 96ºC supported by 30 cycles of 95ºC for 1 minute, 58ºC for 1.30 minutes, 72ºC for 1 minute supported by the final extension at 72ºC for 5 minutes. The PCR amplicons were examined by utilizing gel electrophoresis i.e. 1.5 % agarose gel at 80 V for 1 hr. Gel imaging was done using UV light inside the Bio-Rad Gel Documentation System. An estimated 550- 600bp PCR amplicon was purified by using the Wizard® SV Gel and PCR clean-up system kit (Promega, USA) (Dwibedi et al., 2018; Shearin et al., 2018). The purified amplicon was sent for sequencing to Genei Laboratories, Bangalore.

Table 4.2: Reagents used in PCR reaction

S. No.	Reagent	Stock Conc.	Quantity (25 µL)	Final Conc.
1.	Sterile double distilled water	-	14.5 µL	-
2.	Taq buffer	10X	2.5 µL	1X
3.	Forward primer (ITS 1)	10 µM	2.0 µL	0.8 µM
4.	Reverse Primer (ITS 4)	10 µM	2.0 µL	0.8 µM
5.	dNTPs	2.5 Mm	2.0 µL	0.2 mM
6.	Taq DNA Polymerase	3 U /µL	1.0 µL	2.5 U
7.	DNA Template	25 ng /µL	1.0 µL	25 ng

Table 4.3: Temperature profile for PCR reaction

Step No.	Name	Temperature	Time
I	Initial denaturation	96 °C	5 min
II	Denaturation	95 °C°	45 sec
III	Annealing	60 °C	45 sec
IV	Extension	72 °C	45 sec
V	Step II to IV repeated 39 times	-	-
VI	Final extension	72 °C	5 mins
VII	Store	4 °C	-

CHAPTER 5

RESULTS

5.0. RESULTS

5.1. Sub-culturing of endophytic isolates

In the present study a total of 80 endophytic fungi were procured from existing repository of Dr. Sanjai Saxena, DBT, TIET Patiala, and screened for the production of HDAC inhibitors. All of the isolates of endophytic fungi were preserved in PDA-glycerol slants for long-term preservation. The endophytic fungi isolates in this investigation were isolated from different plants, namely, *R. serpentine, C. camphora, C. zeylanicum, C. roseus, Taxus baccata, A. marmelos, Vitis vinifera, C. malabaricum, M. pumila* obtained from the biodiversity hotspots of India. During the centuries, humans have constantly relied on the plants for the treatment of numerous disorders such as diabetes, cancer etc. During the earlier two decades, several important unique bioactive compounds including anti-aging, anti-microbial, and anticancer characteristics have been discovered from the endophytic fungi. Throughout the prolonged duration of co-evolution, a symbiotic relationship was formed within endophyte and its host plant. Few of the endophytes have the capability to mimic the character of the host plants and are able to produce the identical or related bioactive compounds as those obtained from their host plants. Therefore, the existing investigation involves the exploitation of endophytic fungi isolated from the therapeutic plant for the production of novel bioactive compounds. All of the 80 endophytic fungi were isolated from different parts of the plants mentioned above.

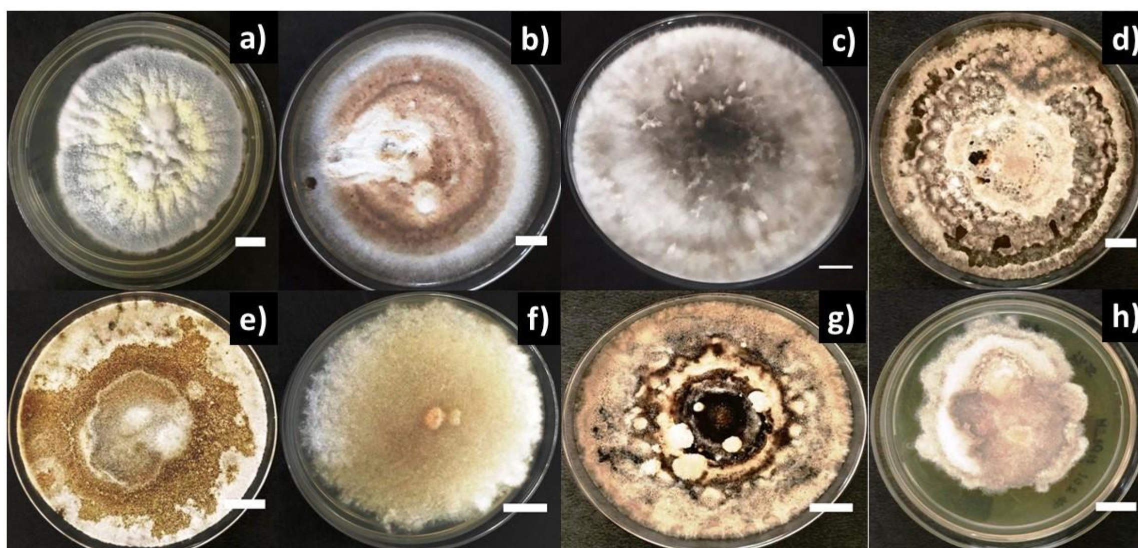

Figure 5.1: Pure cultures of different endophytic fungi procured from lab a) *Penicillium sp.*; b) *Alternaria sp.*; c) Unidentified; d) *Alternaria sp.*; e) *Botryosphaeria sp.*; f) *Botryosphaeria sp.*; g) Unidentified.; h) *Alternaria sp.* (Bar : 10mm).

5.2. Preservation of endophytic fungi

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

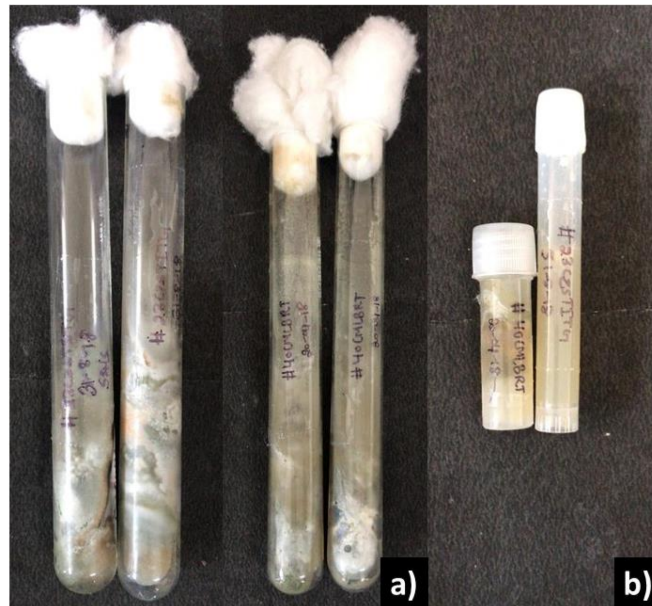

Figure 5.2: Endophytic isolates preserved for long term storage by different methods, a) PDA slants of #23CZSTITG and #40CMBLRT b) PDA vials.

5.3. Production of culture filtrate

The isolates following existing research were constrained to secondary metabolites production in the PDB medium. Secondary metabolites produced by 80 cultures were filtered out and the filtered extracts were subjected to biochemical screening. Two cultures, namely, #23CZSTITG and #40CMBLRT showing maximum inhibition were subjected for mass production in the conical flask.

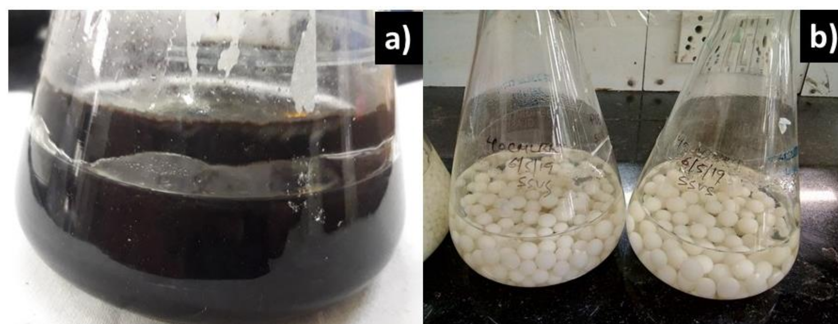

Figure 5.3: Production cultures of #23CZSTITG and #40CMBLRT.

5.4. Biochemical screening assay for the HDAC inhibitors from endophytic fungi

A biochemical 2-step assay for the HDAC activity measurement, namely Acetic Acid Standard Test (K-ACET, Megazyme) was performed for the probable identification of HDAC inhibitors in filter extracts of each endophytic fungi. Out of 80 endophytic fungi only 21 isolates were found to be affirmative inhibitors in screening assay and out of 21 isolates, 2 isolates namely #23CZSTITG and #40CMBLRT were found potent producers of HDAC inhibitors. In acetic acid assay, Acetyl-CoA synthetase (ACS) in the presence of adenosine-5'-triphosphate (ATP) and coenzyme A (CoA) converts acetic acid (acetate) into Acetyl-CoA with the formation of adenosine-5'-monophosphate (AMP) and pyrophosphate. Citrate synthase (CS) in the presence of Acetyl-CoA, converts oxaloacetate into citrate. The oxaloacetate required in the reaction is formed from L-malate and nicotinamide-adenine dinucleotide (NAD^+) in the presence of L-malate dehydrogenase (L-MDH), here NAD^+ is reduced to NADH. The determination is based on the formation of NADH which is measured by increase in absorbance at 340 nm. NADH production depends upon the number of acetyl groups, as the concentration increases, there is an increase in NADH production. Further concentration of acetate ions is calculated, determining the presence of HDAC inhibitors. In the screening assay, 21 isolates were showing >75% HDAC inhibition activity and further 7 out of 21 isolates were showing >99% inhibition. However, #23CZSTITG and #40CMBLRT showed the highest HDAC inhibition among all 6 showing greater than 99% activity and therefore could be recognized as a possible yielder of fungal HDAC inhibitor (Table 5.1). Accordingly, 2 cultures were further subjected to liquid-liquid extraction (Table 5.2).

$$\% \text{ Inhibition} = \frac{\text{Control concentration} - \text{Sample concentration}}{\text{Control concentration}} \text{X } 100$$

Table 5.1: Biochemical assays for screening HDAC inhibitors

CULTURE CODE	PLANT NAME	PLANT PART	LOCATION	TENTATIVE IDENTIFICATION	BIOCHEMICAL SCREENING
4RSLBRT	*R. serpentine*	Leaves	BRT Wildlife Sanctuary, Karnataka	*Fusarium solani*	+++
1CCBD	*C. camphora*	Bark	Darjeeling, West Bengal	*Fusarium* sp.	++
23CZSTITG	*C. zeylanicum*	Stem	Guwahati, Assam	*Botryosphaeria* sp.	+++
2164CZSTITG	*C. zeylanicum*	Stem	Guwahati, Assam	Unidentified	++
2CZSTITG	*C. zeylanicum*	Stem	Guwahati, Assam	Unidentified	+
41CZSTITG	*C. zeylanicum*	Stem	Guwahati, Assam	Unidentified	+
2107CZSTITBRT	*C. zeylanicum*	Stem	BRT Wildlife Sanctuary, Karnataka	Unidentified	+
34CZSTITBRT	*C. zeylanicum*	Stem	BRT Wildlife Sanctuary, Karnataka	Unidentified	+
2CZBAWLS	*C. zeylanicum*	Bark	Wayand, Kerala	Unidentified	No inhibition
16CRLPAL	*C. roseus*	Leaves	Palampur	*Fusarium* sp.	+
37CRLPAL	*C. roseus*	Leaves	Palampur	*Schizophyllum* sp.	+
15CRSTBRT	*C. roseus*	Stem	BRT Wildlife Sanctuary, Karnataka	*Fusarium* sp.	++
37(b)CRSTBRT	*C. roseus*	Stem	BRT Wildlife Sanctuary, Karnataka	*Xylaria* sp.	+++
40(b)TBBALM	*Taxus baccata*	Bark	Almora, Uttarakhand	Unidentified	+++
91TBBALM	*Taxus baccata*	Bark	Almora, Uttarakhand	Unidentified	No inhibition
93TBBALM	*Taxus baccata*	Bark	Almora, Uttarakhand	Unidentified	+
43TBBALM	*Taxus baccata*	Bark	Almora, Uttarakhand	*Phomopsis* sp.	+
99TBBALM	*Taxus baccata*	Bark	Almora, Uttarakhand	Unidentified	+
44TBBALM	*Taxus baccata*	Bark	Almora, Uttarakhand	*Alternaria* sp.	+
57TBBALM	*Taxus baccata*	Bark	Almora, Uttarakhand	*Penicillium* sp.	+
23AMSTYEL	*A. marmelos*	Stem	Yelandur, Karnataka	*Aureobasidium* sp.	No inhibition
9AMLBRT	*A. marmelos*	Leaves	BRT Wildlife Sanctuary, Karnataka	*Fusarium* sp.	No inhibition
91AMLBRT	*A. marmelos*	Bark	BRT Wildlife Sanctuary, Karnataka	Unidentified	No inhibition
42VVLPM	*Vitis vinifera*	Leaves	Pune Maharashtra	*Fusarium* sp.	+++
90(c)VVLPM	*Vitis vinifera*	Leaves	Pune Maharashtra	Unidentified	+
50(d)VVLLK	*Vitis vinifera*	Leaves	Lucknow	Unidentified	++
18CMBANEY	*C. malabaricum*	Bark	Neyyar, Kerala	*Alternaria* sp.	+++

26CMSTNEY	*C. malabaricum*	Stem	Neyyar, Kerala	*Curvularia* sp.	++
43CMSTNEY	*C. malabaricum*	Stem	Neyyar, Kerala	*Acremonium* sp.	+
10CMLNEY	*C. malabaricum*	Leaves	Neyyar, Kerala	Unidentified	+
4CMBANEY	*C. malabaricum*	Bark	Neyyar, Kerala	*Botryosphaeria* sp.	++
54CMSTNEY	*C. malabaricum*	Stem	Neyyar, Kerala	*Pestalotiopsis* sp	No inhibition
17CMLNEY	*C. malabaricum*	Leaves	Neyyar, Kerala	*Chaetomium* sp.	+
1622CMSTNEY	*C. malabaricum*	Stem	Neyyar, Kerala	Unidentified	+
55CMSTNEY	*C. malabaricum*	Stem	Neyyar, Kerala	Unidentified	No inhibition
36CMSTNEY	*C. malabaricum*	Stem	Neyyar, Kerala	Mycelia sterilia	+
4CMSTNEY	*C. malabaricum*	Stem	Neyyar, Kerala	*Arthrinium phaeospermum*	+
11CMSTNEY	*C. malabaricum*	Stem	Neyyar, Kerala	*Pestalotiopsis sp.*	+
2CMLNEY	*C. malabaricum*	Leaves	Neyyar, Kerala	*Nigrospora* sp.	++
13CMSTNEY	*C. malabaricum*	Stem	Neyyar, Kerala	*Pestalotiopsis sp.*	+++
18CMLNEY	*C. malabaricum*	Leaves	Neyyar, Kerala	*Fusarium* sp.	++
49CMSTNEY	*C. malabaricum*	Stem	Neyyar, Kerala	Unidentified	+
31CMSTNEY	*C. malabaricum*	Stem	Neyyar, Kerala	*Curvularia* sp.	++
37CMLNEY	*C. malabaricum*	Leaves	Neyyar, Kerala	*Chaetomium* sp.	+++
12CMBNEY	*C. malabaricum*	Bark	Neyyar, Kerala	*Alternaria* sp.	No inhibition
79CMSTNEY	*C. malabaricum*	Stem	Neyyar, Kerala	Unidentified	+
35CMSTNEY	*C. malabaricum*	Stem	Neyyar, Kerala	*Phaeoacremonium* sp.	++
5CMSTNEY	*C. malabaricum*	Stem	Neyyar, Kerala	*Arthrinium phaeospermum*	No inhibition
27CMLBRT	*C. malabaricum*	Leaves	BRT Wildlife sanctuary, Karnataka	*Aspergillus* sp.	+
40CMLBRT	*C. malabaricum*	Leaves	BRT Wildlife sanctuary, Karnataka	*Penicillium* sp.	+++
1CMSTITBRT	*C. malabaricum*	Stem	BRT Wildlife sanctuary, Karnataka	*Pestalotiopsis* sp.	+
40CMBART	*C. malabaricum*	Bark	BRT Wildlife sanctuary, Karnataka	*Alternaria* sp.	+
18CMBART	*C. malabaricum*	Bark	BRT Wildlife sanctuary, Karnataka	*Bionectria* sp.	+
23CMBART	*C. malabaricum*	Bark	BRT Wildlife sanctuary, Karnataka	Unidentified	+
201MPLSH	*M. pumila*	Leaves	Shimla, Himachal pradesh	*Alternaria* sp.	+
168MPLSH	*M. pumila*	Leaves	Shimla, Himachal pradesh	*Alternaria* sp.	+
11MPLSH	*M. pumila*	Leaves	Shimla, Himachal pradesh	Unidentified	No inhibition
207MPSSH	*M. pumila*	Stem	Shimla, Himachal pradesh	*Negroshora sp.*	+

114MPLSH	*M. pumila*	Leaves	Shimla, Himachal pradesh	Unidentified	+
96MPLSH	*M. pumila*	Leaves	Shimla, Himachal pradesh	*Alternaria sp.*	+
62MPLSH	*M. pumila*	Leaves	Shimla, Himachal pradesh		+
180MPLSH	*M. pumila*	Leaves	Shimla, Himachal pradesh	*Botryosphaeria sp.*	+
77MPLSH	*M. pumila*	Leaves	Shimla, Himachal pradesh	*Botryosphaeria sp.*	+
66MPLSH	*M. pumila*	Leaves	Shimla, Himachal pradesh	*Alternaria sp.*	+
139MPLSH	*M. pumila*	Leaves	Shimla, Himachal pradesh	Unidentified	+
53MPLSH	*M. pumila*	Leaves	Shimla, Himachal pradesh	*Alternaria sp.*	+
93MPLSH	*M. pumila*	Leaves	Shimla, Himachal pradesh	*Alternaria sp.*	++
179MPLSH	*M. pumila*	Leaves	Shimla, Himachal pradesh	*Alternaria sp.*	+
188MPLSH	*M. pumila*	Leaves	Shimla, Himachal pradesh	Unidentified	+
32MPLSH	*M. pumila*	Leaves	Shimla, Himachal pradesh	*Fusarium sp.*	+
159MPLSH	*M. pumila*	Leaves	Shimla, Himachal pradesh	*Alternaria sp.*	+
122MPLSH	*M. pumila*	Leaves	Shimla, Himachal pradesh	Unidentified	+
73MPLSH	*M. pumila*	Leaves	Shimla, Himachal pradesh	*Alternaria sp.*	+
82MPLSH	*M. pumila*	Leaves	Shimla, Himachal pradesh	*Alternaria sp.*	++
140MPLSH	*M. pumila*	Leaves	Shimla, Himachal pradesh	Unidentified	+
67MPLSH	*M. pumila*	Leaves	Shimla, Himachal pradesh	*Alternaria sp.*	+
3MPLSH	*M. pumila*	Leaves	Shimla, Himachal pradesh	Unidentified	+
4MPLSH	*M. pumila*	Leaves	Shimla, Himachal pradesh	Unidentified	No inhibition
98MPLSH	*M. pumila*	Leaves	Shimla, Himachal pradesh	Unidentified	+
50MPLSH	*M. pumila*	Leaves	Shimla, Himachal pradesh	*Chaetomium sp.*	+

Note: (+) moderate (<75%); (++) good (>75%); (+++) very high (>99%)

Table 5.2: Calculation of percentage inhibition.

S. NO.	CULTURE CODE	PERCENTAGE INHIBITION (%)
1.	40(b)TBBALM	98.91
2.	42VVLMP	119.01
3.	23CZSTITG	127.73
4.	4RSLBRT	82.92
5.	37(b)CRSTBRT	103.11
6.	1CCBD	75.78
7.	2164CZSTITG	79.07
8.	15CRSTCRT	76.96
9.	50(d)VVLLK	88.79
10.	93MPLSH	88.17
11.	82MPLSH	80.43
12.	18CMBANEY	101.16
13.	26CMSTNEY	80.75
14.	4CMBANEY	77.80
15.	40CMBLRT	120.43
16.	2CMLNEY	80.25
17.	13CMSTNEY	99.50
18.	18CMLNEY	75.78
19.	31CMSTNEY	96.89
20.	37CMLNEY	99.16
21.	35CMSTNEY	96.65

5.5. Solvent extraction

The liquid-liquid extraction of cell-free filtrates of 2 cultures namely: #23CZSTITG and #40CMBLRT were carried out using four solvent hexane, chloroform, dichloromethane and ethyl acetate. The bioactive residues were reconstituted in methanol. All the four solvent extracts of both the fungal cultures were subjected to biochemical screening assay. In the screening assay, hexane extract of #23CZSTITG and ethyl acetate extract of #40CMBLRT exhibited activity greater than 90%, so it was chosen for further investigation.

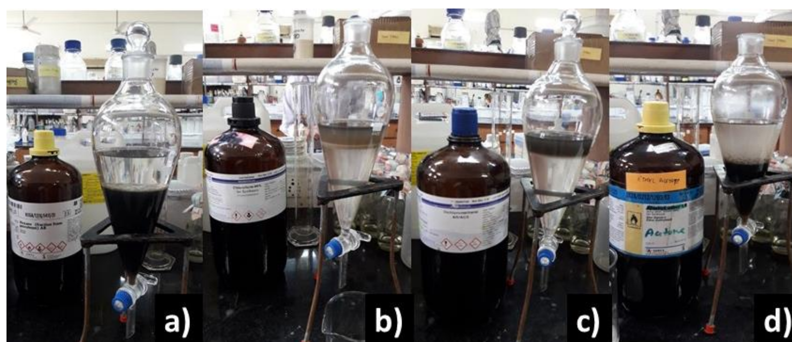

Figure 5.4: Extraction of cultures #23CZSTITG and #40CMBLRT from four different solvents a) Hexane b) Chloroform c) Dichloromethane d) Ethyl acetate

5.6. Identification of the selected HDAC inhibitor producing endophytic fungus

The potential HDAC inhibitor producing endophytic fungus #23CZSTITG and #40CMBLRT were identified using morphotaxonomic and molecular taxonomic methods.

5.6.1. Morphotaxomony

The endophytic fungus #23CZSTITG produced white, fast growing (90± 0), readily produced fluffy, white aerial mycelium on PDA, CMA and CDA media after 10 days of incubation with 12h photoperiod (Fig.5.5). Fungus start growing with white in color and later turned to tan color in PDA with flat margins over PDA and CMA (Fig.5.5). On CDA medium fungus was white in color initially with wooly appearance and later become Ivory color with smooth margin (Fig. 5.5). On PLA medium it was white in color with smooth margins and no growth in Water Agar (WA) (Fig.5.5 & Table 5.3). Whereas, endophytic fungus #40CMBLRT produced green, fast growing (90± 0), densely packed and near the margins arranged in radial lines, less floccose mycelium on PDA, CMA and CDA media after 10 days of incubation with 12h photoperiod (Fig.5.6). Fungus start growing with white in color and later becomes tan color with smooth margin over PDA and brown over CMA (Fig.5.6). On CDA medium fungus was

green in color with smooth margin (Fig. 5.6). On PLA medium it was brown in color with smooth margins and no growth in Water Agar (WA) (Fig.5.6 & Table 5.4).

Table 5.3: Colony morphology of #23CZSTITG on different medium after 20 days, 28˚C, 12h dark

Medium	Colony Color		Colony	Margin	Odour
	Front	Back	Diameter (mm)		
PDA	Tan-White	White	80 ± 0.5	Flat	No odour
CMA	White	Tan-white	63± 1	Flat	No odour
CDA	Ivory	Yellowish- white	75 ± 1	Smooth	No odour
PLA	White	Brown	65 ± 1	Smooth	No odour
WA	No growth	No growth	-	-	-

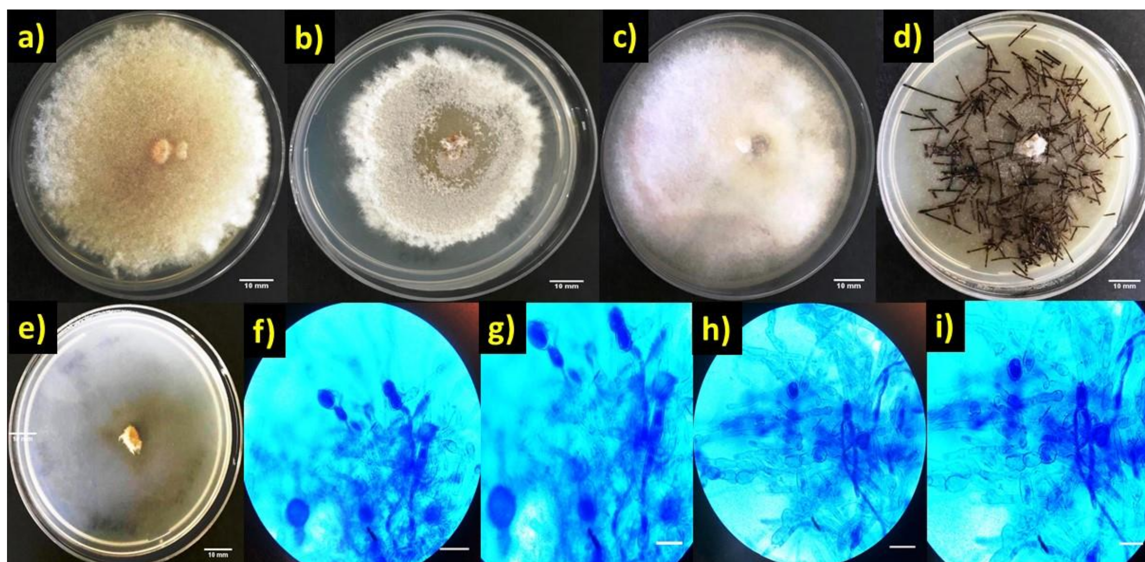

Figure 5.5: #23CZSTITG culture grown on different media plate a) PDA b) CMA c) CDA d) PLA e) WA. (f-i) *Botryosphaeria sp.* under microscope (10x-100X) with lactophenol cotton blue stain Conidiophores were hyaline, cylindrical, smooth, aseptate(immature), uni- to biseptate (mature) conidia.

Table 5.4: Colony morphology of #40CMBLRT on different medium after 20 days, 28˚C, 12h dark

Medium	Colony Color		Colony	Margin	Odour
	Front	Back	Diameter (mm)		
PDA	Tan-green	Tan-white	7.4 ± 0.3	Smooth	No odour
CMA	Brownish-green	Brown	69 ± 4	Smooth	No odour
CDA	Green	Whitish-pink	69 ± 2	Smooth	No odour
PLA	Brown	Pale brown	65 ± 0	Smooth	No odour
WA	No Growth	No Growth	-	-	-

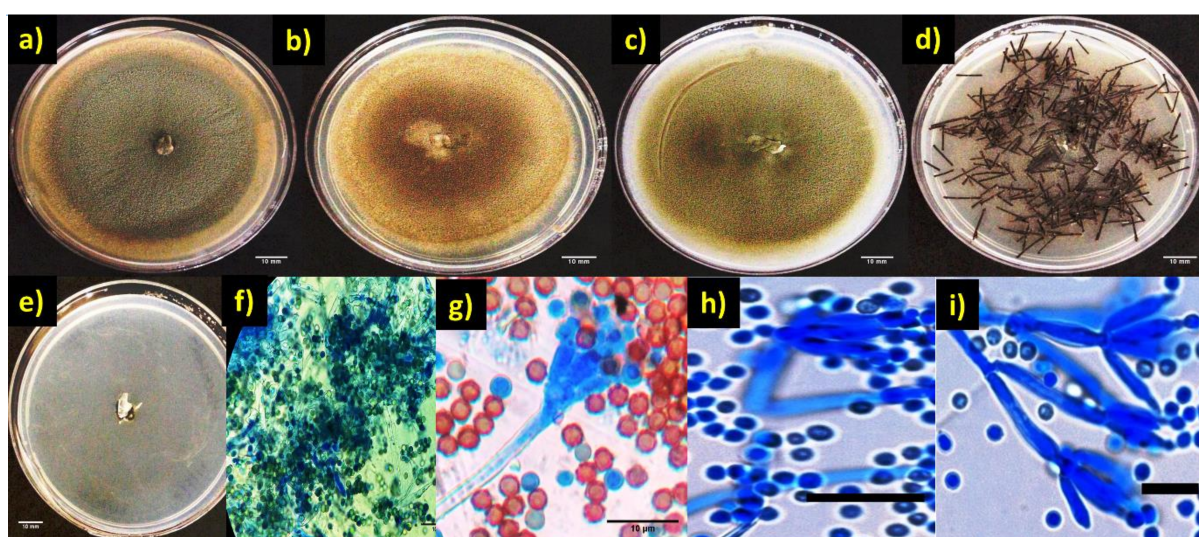

Figure 5.6: #40CMBLRT culture grown on different media plate a) CDA b) PDA c) CMA d) PLA e) WA (f- i) *Penicillium sp*. under light microscopy (10 x-100X) with lactophenol cotton blue stain conidiophores branches terminate in a cluster of conidiogenous cells known as the phialides that give off chains of conidia at their apex.

5.6.2. Molecular identification

5.6.2.1. Genomic DNA isolation and PCR amplification

The genomic DNA isolation of the potent HDAC inhibitor producing endophytic fungi, #23CZSTITG and #40CMBLRT, was done and the size of the genomic DNA was found to be approximately more than 10 kb (Fig. 5.7). The PCR amplicon was resolved on 1.5% agarose gel in order to check the size on the basis of the mobility and comparison with the 100bp ladder.

The size of the amplicon was found to be approximately 550 bp to 600 bp (Fig.5.7). This size can be easily compared to the ITS region, which was amplified in order to characterize the fungi at molecular level.

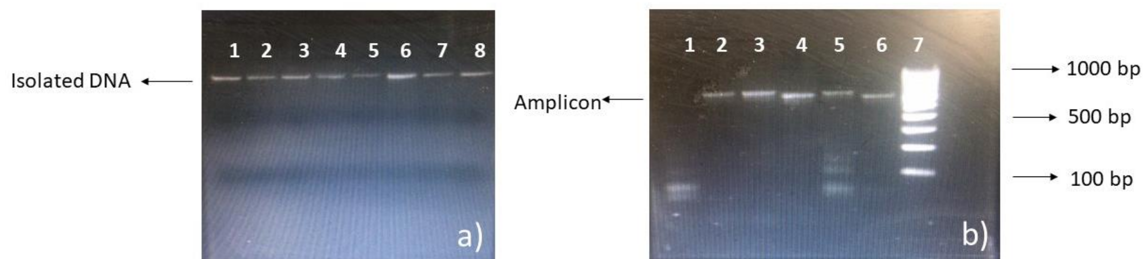

Figure 5.7: a) Genomic DNA isolation of #23CZSTITG and #40CMBLRT. b) PCR amplicon of ITS region of #23CZSTITG and #40CMBLRT, Lane 7: 100bp ladder, Lane 2-6: ITS amplicon of #23CZSTITG and #40CMBLRT.

CHAPTER 6

DISCUSSION

6.0. DISCUSSION

Histone deacetylase (HDAC) is responsible to regulate diverse cellular functions such as progression of cell cycle, survival and proliferation of the cell. Histone deacetylation or hypoacetylation results from overexpression of HDACs, mutation or silencing of HAT genes, or amplification or translocation of HAT or HDAC genes (Huang 2006). Song et al., (2005) observed that gastric adenocarcinoma tumours is due to overexpression of HDAC2. Some disorders in neurology are also associated with aberrant HDAC function such as Alzheimer's. Therefore, HDACs act to be appropriate targets for the treatment of cancer and neurological disorders. Inhibition of HDAC activity has proved to be very effective since the main role of HDAC inhibitors is hyperacetylation followed by gene expression. Rajendran et al., (2011) described the influence of HDACi on chromatin stability, mitosis and DNA repair mechanisms.

The endophytes are the micro-symbionts which reside within plant without causing any harm to plant. Endophytic fungi are considered to be ware houses of plethora of bioactive compounds which exhibits antimicrobial, antifungal and immunosuppressive activities (Dwibedi et al., 2019). It has been already proven that endophytic fungi have the capacity to producing same functional compounds as their host (Strobel et al., 2003; Suryanarayanan et al., 2009).

Until now, various compounds such as sodium butyrate (Davie 2003), Phenylacetate and phenylbutyrate (Marks et al., 2004) belongs to the chemical class of short chain fatty acids has been reported as the HDAC inhibitors that can be produced from polyphenols in fruits and vegetables. On the other hand, cinnamon is one of the most significant spices consumed on daily basis all over the world and it mostly contains derivatives of short chain fatty acids such as cinnamaldehyde, cinnamic acid, and cinnamate (Rao and Gan 2014). Thus, we may conclude that some likewise compound present in *C. camphora* and *C. zeylanicum* is responsible for HDAC inhibition.

Potent HDACi have been isolated from epigenetically modified endophytic fungus. Chen et al., (2013) reported suberoylanilide hydroxamic acid (SBHA) as the potent HDACi from endophytic *Fusarium oxysporum* strain R1. Yang et al., (2014b) reported 5-Azacytidine epigenetically modified *Pestalotiopsis crassiuscula.* An NAD^+ dependent HDAC inhibitor was isolated from *Graphiopsis chlorocephala* that produced 6 novel benzophenones (Asai et al., 2013). Sodium butyrate, another potent HDACi, was isolated from *Leucostoma persoonii* that produced cytosporone R as the novel compound (Beau et al., 2012).

In the current research, the study was performed for the production of HDAC inhibitors through free fermentation conditions by using different endophytic fungi previously isolated from different climatic regions. The endophytic fungi culture filtrates were screened for HDACi production using a biochemical 2-step assay that well suited for HDAC activity measurements (Wenger et al., 2003), where out of 80 endophytic fungi culture filtrates, only 21 isolates (26%) were observed to show >75% inhibition activity.

Out of 21 isolates, two were found to be potent producer of HDACi, #23CZSTITG and #40CMBLRT were showing maximum activity (>99%) and thus chosen for the study. Then the selected isolates were identified by classical and molecular taxonomy. Both the cultures were grown on different media plate namely, CDA, PDA, CMA, PLA and WA and later after 20-25 days of growing period, they were observed under light microscopy (10 x 100X) with lactophenol cotton blue stain. The tentative identification of the cultures showed that #23CZSTITG is *Botryosphaeria sp.* and #40CMBLRT is *Penicillium sp.*

In the present study endophytic fungus isolated from cinnamon species are identified as *Curvularia sp., Acremonium sp., Botryosphaeria sp., Pestalotiopsis sp., Chaetomium sp.,* Mycelia sterilia*, Arthrinium phaeospermum, Nigrospora sp., Fusarium sp., Alternaria sp., Phaeoacremonium sp., Aspergillus sp., Penicillium sp., Bionectria sp.* Whereas Marcellano et al., (2017) reported different endophytic species such as *Cunninghamella sp., Rhizoctonia sp., Rhizoctonia sp., Colletotrichum sp., Mucor sp., Phomopsis sp.,* alongwith the similar ones such as *Aspergillus sp., Penicillium sp., Pestalotiopsis sp., Fusarium sp.,* Mycelia sterilia.

The size of the genomic DNA was found to be approximately more than 10 kb after the genomic DNA isolation of the potent HDAC inhibitor producing endophytic fungi, #23CZSTITG and #40CMBLRT. The PCR amplicon was resolved and the size of the amplicon was found to be approximately 550 bp to 600 bp. This size can be easily compared to the ITS region, which was amplified in order to characterize the fungi at molecular level.

CHAPTER 7

CONCLUSION

7.0. CONCLUSION

In the current study we conclude that out of 80 cultures isolates of endophytic fungus, 26% isolates show HDAC inhibition. Out of 21 isolates, two isolates namely #23CZSTITG and #40CMBLRT prove as the potent inhibitors by showing >99% inhibition activity against HDAC. These two isolates are morphologically identified as *Botryosphaeria sp.* and *Penicillium sp.* respectively.

Thus, further work on purification and structure elucidation of the pure compound(s) could be performed in search of novel source of HDAC inhibitor.

CHAPTER 8

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8.0. REFERENCES

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APPENDIX

Media

1. Potato dextrose agar

Potato dextrose agar – 39gm

Distilled water – 1L

Final pH (at 26°C)- 5.6±0.2

Autoclave at 121°C for 15 min

2. Corn meal agar

Corn meal, infusion form- 50gm

Dextrose- 2gm

Agar- 15gm

Distilled water- 1L

Autoclave at 121°C for 15 min

3. Czapek Dox agar

Czapek Dox agar-49.01

Distilled water- 1L

Autoclave at 121°C for 15 min

4. Water agar

Agar – 15gm

Distilled water – 1L

Autoclave at 121°C for 15 min

5. Pine leaf agar

Pine leaves

Agar – 15gm

Distilled water – 1L

Autoclave at 121°C for 15 min

Buffers

1. 50XTAE

Tris base – 242g

Glacial acetic acid – 57.1ml

0.5M EDTA – 10ml

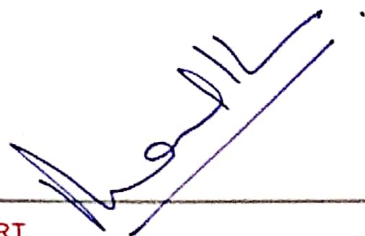
Distilled water – 1L

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

10 mM EDTA - 0.1 mM

Distilled water - 100ml

Thesis



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