SCREENING OF ENDOPHYTIC FUNGI FOR THE INHIBITION OF INDOLAMINE 2,3 DIOXYGENASE

Thesis submitted in partial fulfilment of the requirements for the award of degree of

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Submitted By

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CERTIFICATE

This is to certify that the thesis entitled, ***"Screening of endophytic fungi for the inhibition of indolamine 2,3 dioxygenase"***, being submitted by **Ms. Sarika Srivastava (601704006)** in partial fulfilment of the requirements for the award of degree of Master of Technology in Biotechnology, Thapar Institute of Engineering and Technology, Patiala, Punjab is a bonafide work carried out under the supervision of **Dr. Sanjai Saxena** and that no part of this thesis has been submitted for the award of any other degree.

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CANDIDATE'S DECLARATION

I hereby declare that the work being presented in the thesis entitled, ***"Screening of endophytic fungi for the inhibition of indolamine 2,3 dioxygenase"***, in partial fulfilment of the requirements for the award of degree of Master in Biotechnology, Department of Biotechnology, Thapar institute of Engineering and Technology, Patiala, Punjab is my own laboratory work during the period of July 2018 to July 2019, under the conception and supervision of **Prof. Sanjai Saxena**, Department of Biotechnology, Thapar institute of Engineering and Technology, Patiala, Punjab. I have not submitted the matter embodied in this thesis for the award of any other degree.

Place: Patiala

Date: 15/07/19

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

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Date: 15/07/19

Place: Patiala

Sarika Srivastava

DEDICATION

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

especially those who were very close to our heart.

My humble effort I dedicate to my sweet and loving

Father& Mother,

Whose affection, love, encouragement and prays of day and night make me able to get such success and honour,

Along with all hard working and respected

Teachers

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ABBREVIATIONS

S.NO.	ABBREVIATIONS	FULL FORM
1.	µg	Micro gram
2.	µl	Micro litre
3.	CMA	Corn meal agar
4.	DNA	Deoxyribonucleic acid
5.	EDTA	Ethylene diamine tetra acetic acid
6.	Et Br	Ethidium bromide`
7.	HA	Hydroxyanillic acid
8.	HK	Hydroxykynurenic acid
9.	Hr	Hours
10.	IDO	Indolamine 2,3 dioxygenase
11.	ITS	Internal transcribed spacer
12.	mg	Milli gram
13.	ml	Milli litre
14.	PCR	Polymerase chain reaction
15.	PDA	Potato dextrose agar
16.	PDB	Potato dextrose broth
17.	pH	Potential of hydrogen
18.	PWA	Pine leaf water agar
19.	QUIN	Quinolic acid
20.	RPM	Revolutions per minute
21.	SNA	Synthetic poor nutrient agar
22.	T_{reg}	T-regulatory
23.	UV	Ultra-violet
24.	WA	Water agar

ABSTRACT

Indolamine 2,3 dioxygenase (IDO) found inside the human body which is responsible for causing various chronic diseases such as Alzheimer, Parkinson, cancer etc. Endophytic fungi are microorganisms which resides inside the plant tissue which contains huge bioactive compound such as Taxol and is known as gold mines of enzyme inhibitor. Various enzyme inhibitor has been isolated and reported from endophytic fungi such as huperzine, Lovastatin, Peptide etc. Current studies deals with the production of bioactive compounds from endophytic fungi for the inhibition of IDO enzyme. The present approach deals with isolation and screening of endophytic fungi for the inhibition of IDO. 26 endophytic cultures were isolated from small segments of apple (*M. pumila*) plant. Out of which 10 fungal extract exhibited above 70% inhibition during preliminary test. Formation of intense yellow colour shows the presence of high kynurenine production with high OD value which signifies presence of poor inhibitor or low kynurenine production with low OD value which signifies presence of strong inhibitor at 492nm. Among them #77MPLSH fungal extract resulted above 80 % inhibition of IDO. Therefore, On the basis of preliminary data, #77MPLSH was found to be potent inhibitor of IDO. So, further molecular identification, extraction and morphological studies were done and the potential culture #77MPLSH was tentatively identified as *Botryosphaeria* sp. Hence, due to strongest inhibition activity against IDO, it was concluded that #77MPLSH could be considered as a potential inhibitor from endophytic fungi and further studies can be proceeded to isolate novel compounds.

Key words: Endophytic fungi, IDO enzyme, #77MPLSH, *Malus pumila, Botryosphaeria* sp.

CHAPTER 1

INTRODUCTION

1.0. Introduction

Tryptophan is an aromatic and critical amino acid which includes an indole-ring and is used for protein synthesis and production of neurotransmitters (Richard et al., 2009; Fatokun et al., 2013). Ninety five percentage of tryptophan is involved in kynurenine pathway whereas remaining 5% reported in serotonin pathway and protein formation inside the brain (Fujigaki et al., 2017; Torok et al., 2015). Anomalous tryptophan degradation has been determined in a many diseases, inclusive of most cancers (1 in each 6 deaths) (Siegel et al., 2019) and neurodegenerative illnesses such as Parkinson's disease (2%), Alzheimer's disease (10%) (Tariot et al., 2004). Indolamine 2,3 dioxygenase (IDO) is an intracellular, monomeric, heme-containing protein found in extrahepatic tissues which is located on 8p11.21 and highly expressed in brain, lung, spleen, and eye lens (Cui et al., 2018; Meininger et al., 2011) which utilises tryptophan as a substrate. Usually, IDO shows lesser activity but irritation, stress, melancholy, neurological disease etc. can aberrantly activate IDO and start overproduction of kynurenine metabolites through over degradation of L-tryptophan and formation of N-formyl kynurenine that is a remarkable limiting step by the enzyme IDO via oxidative cleavage of the two, three-double bond of L-tryptophan known as kynurenine pathway (Bilir et al., 2017; Chen et al., 2011). Similarly, formamidase enzyme hydrolyse N-Formyl kynurenine to kynurenine which further metabolized to a range of neuroactive products such as QUIN (Quinolic acid), kynurenic acid, HAA (3-hydroxyanthranilic acid) etc. (Bilir et al., 2017). One of the back of those illnesses may be kynurenine pathway and indolamine 2,3 dioxygenase (IDO) enzyme which suggests vital role in degradation of tryptophan. Thus, scientists are becoming a lot interested in researching novel and effective IDO inhibitors by using the numerous natural sources bioactive compound isolated from endophytic fungi. Ethylnaphthoquinone and its derivatives has been extracted from *Neofusicoccum austral* endophytic fungi which is isolated from Mangrove plant against cancer disease are the only IDO inhibitors reported till date (Cui et al., 2018). Endophytic fungi produces plenty of bioactive compounds that provides protection and persistence to the host without exhibiting symptoms to the host plant and also reported as ubiquitous in nature (Kamana et al., 2016; Kusari et al., 2013). It produces enormous bioactive compounds with high therapeutic potential having anti-microbial, anti-cancer, anti-fungal, anti-viral, anti-parasitic and cytotoxic as well as neuroprotective activities (Kusari et al., 2013; Aly et al., 2011; Gunatilaka et

al., 2006). Endophytic fungi is reported as gold mine of enzyme inhibitor as it is found as specific inhibitor of enzyme (Meshram et al., 2018). Numerous diseases are linked with anomalous enzyme activities ultimately causes serious metabolic disorders such as HIV, cardiovascular disease, Alzheimer's and Parkinson's disease, diabetes, and gout (Singh and Kaur 2015; Kapoor and Saxena 2014; Voet et al., 2013; Lehninger et al., 2005). At present, various enzyme inhibitors are extracted from endophytic fungi which are proved to be useful in treating life threatening disorders such as huperzine A is an inhibitor of the enzyme acetylcholinesterase isolated from *Shiraia sp.* which is found to be effective against neurological disorder and blindness (Meshram et al., 2018; Zhu et al., 2010). Apple (*Malus pumila*) was reported as vital source of various phenolic compounds and have possess high antioxidant properties (Sun et al., 2002; Khanizadeh et al., 2008). Scientists reported the diversity and abundance of endophytic fungi among young, mature and old leaves associated with apple plant (Liu et al., 2018).

So far, no compound has been extracted from endophytic fungi of apple leaves or stems which may inhibit indolamine 2,3 dioxygenase (IDO). Hence, the aim of current study is "screening of indolamine 2,3 dioxygenase inhibitors from endophytic fungi of *Malus pumila*..

CHAPTER 2

REVIEW OF LITERATURE

2.1. Indolamine 2,3 dioxygenase (IDO)

IDO is a monomeric protein found in cytoplasm but generally expressed in most mammalian organs including the intestine, placental trophoblast giant cells of foetal origin, respiratory organ, blood mono-nuclear phagocytes, epithelial duct, secretory organ and central nervous systems (Suzuki et al., 2001; Heyes and Morrison 1997). IDO is encoded with 403 amino acids having molecular weight of 45 kDa located on chromosome number 8 (8p11.21) where 8, p, 11.21 represents chromosome number, short arm, bands, respectively in *Homo sapiens*. Two types of enzymes are responsible for tryptophan degradation, first is IDO which is found to be present in every part of human body including CNS (central nervous system) regulated by interferon-γ (INF-γ) specifically and also defends foetus from allogenic rejection whereas next one is tryptophan 2,3 dioxygenase (TDO) which is highly tissue specific and continuously exhibited in liver and maintain tryptophan levels (Prendergast et al., 2018; Austin et al., 2004; Stone et al., 2002). IDO is encoded of two types of homologous gene, indolamine 2,3 dioxygenase 1 (IDO1) and indolamine 2,3 dioxygenase 2 (IDO 2) (Mellor et al., 2017). IDO1 is highly expressive whereas IDO2 is deficiently expressive. IDO is involved in degradation of tryptophan through a pathway known as "kynurenine pathway". This pathway results in formation of an intermediate first and rate-limiting step known as N-formyl kynurenine which ultimately results in formation of kynurenine and is reported in various diseases such as cancer, neurological disorders etc. (Bilir et al., 2017; Tariot et al., 2004). Hence, due to its dual role in immunity and the pathogenesis of many diseases it received considerable attention of scientists for medicinal uses (Austin et al., 2008).

2.2. Relationship between IDO and Kynurenine pathway

Tryptophan is an essential amino acid obtained from the diet that act as fuel required by the body to build proteins needed for cellular growth as well as immune functions (Richard et al., 2009). Its depletion in our body may be the reason for the occurrence of various diseases like cancer and neurological disorders (Richard et al., 2009). On the other hand, tryptophan is the main substrate in kynurenine pathway as shown in (Fig.1) which is degraded by the enzyme indolamine 2,3 dioxygenase (IDO) and tryptophan 2, 3 dioxygenase (TDO). It has been reported that TDO specifically express in liver is an

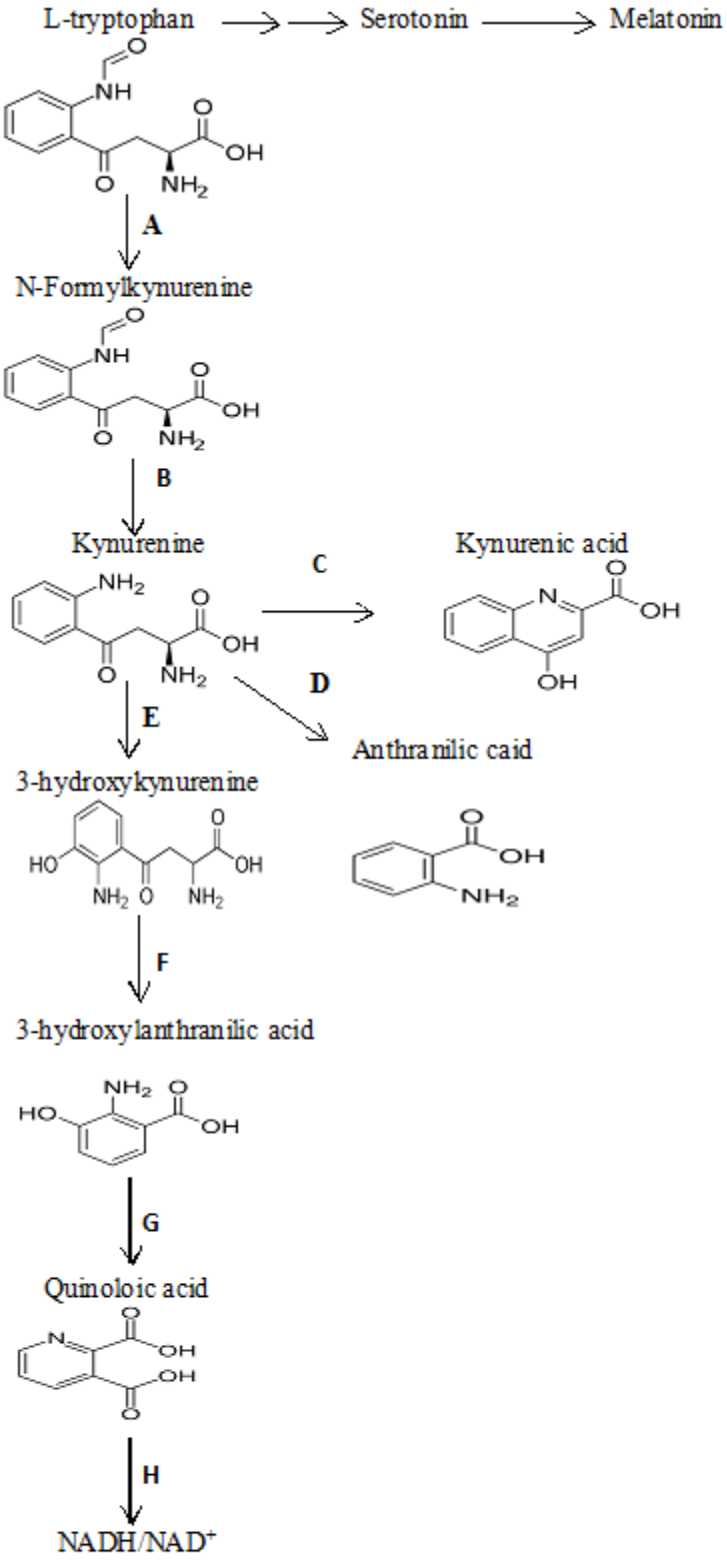

Fig.1: The kynurenine pathway; **A**-Indolamine 2,3 dioxygenase (IDO), **B**-N-formylkynurenine formamidase, **C**-Kynurenine aminotransferase, **D&F**-Kynureninase, **E**-kynurenine monooxygenase, **G**-3-Hydroxyanthranilic dioxygenase, **H**-Quinolic acid phosphoribosyltransferase (Cervenka et al., 2017).

alternative to IDO enzyme which expresses in most of the tissues including CNS (Stone et al., 2002). However, both of them has distinctive distribution of tissues and divergent physiological characteristics (Seeger et al., 2017). Cui et al., (2018), suggested that IDO first of all act on tryptophan thereby results in formation of N-formylkynurenine which further converted into kynurenine and leads to the formation of anthranilic acid, 3-HAA (3-hydroxyanthranilic acid), Quinolic acid (QUIN), kynurenic acid etc. In 2004, Robert Schwartz studied that 3-hydroxykynurenine and 3-hydroxyanthranilic acid are free radicals and QUIN and kynurenic acid are neuroactive compounds which acts on glutamate receptor. Free radicals and neuroactive compounds plays key role physiologically and pathologically. Upregulation in kynurenine pathway creates number of disorders due to production of various metabolites such as QUIN, kynurenic acid etc. which contribute to excitotoxicity throughout inflammation in brain (Bilir et al., 2017). QUIN, Anthranilic acid, 3-HAA, 3-HK (3-hydroxykynurenic acid) has been proven to generate free radicals leading to neuronal damage (Stone et al., 2002). So, the kynurenine pathway may lead to discovery of second generation novel therapeutic drugs to deal with various disorders as mentioned above.

2.3. Functions of IDO

IDO under regular or normal condition always express low activity unless there is stimulation of inflammation, cytokines, viral or bacterial infection (Matin et al., 2006). Most of the cells which express IDO comply with the kynurenine pathway like macrophage and dendritic cell which might be antigen presenting cells (APC's) (Meininger et al., 2011). Commonly, tryptophan continually present in excess which helps in healing of T-cell which shows the crucial activity of IDO towards T-cell existence cycle (Fatokun et al., 2013). IDO facilitates in cellular cycle progression of activated T-cells and does not allow cellular cycle to arrest in mid G1 segment which results in T-cellular proliferation (Zhang et al., 2018; Williams et al., 2012). IDO expresses and helps APC's to spark off T-cell to combat against invasion of tumour cell and other sicknesses. Chang et al., (2018) pointed out that IDO commends to maternal endurance to semi-allogeneic foetal tissues during pregnancy through immune suppression and regulation of foetal invasion and circulation via the depletion of tryptophan.

2.4. Factor affecting IDO over-expression

IDO elevated due to some important factors first, production of interferon-γ (IFN-γ) through effector T- cells (Seegers et al., 2014). Second, stimulation of inflammatory cytokines of innate immune cells (Zulfiqar et al., 2017). Third, stimulation of IL-10 (interleukin-10), IL-27(interleukin-27) (Carbotti et al., 2015). Fourth, dendritic cell expresses CTLA4 (cytotoxic T-lymphocyte associated protein 4) on T_{reg} cells which causes secretion of IDO1 anonymously (Belladona et al., 2009). Fifth, elevation of IDO occurs due to production of TGF-β (transforming growth factor), IL-10 (Interleukin-10) and adenosine from T_{reg} (T-regulatory) cell and other immunosuppressive cells (Hornyak et al., 2018). Sixth, several MAPK (mitogen-activated protein kinase) pathway leads to constitutive expression of IDO1 because most of the tumour cells carry P13K (phosphoinositide 3- kinase) and MAPK oncogenic mutation (Hennequart et al., 2017). Other factors responsible for over activation of IDO are tryptophan level and kynurenine and its metabolites such as QUIN, kynurenic acid etc. (high) (Stone at al., 2002).

2.5. Disease caused by IDO

Over-activation of IDO leads to overproduction of kynurenine metabolites which creates many psychoneurotical disorders adding Huntington's disease (HD), Alzheimer's disease (AD), Parkinson's disease (PD) and the acquired immunodeficiency syndrome (AIDS)- dementia complex (Braidy et al., 2009; Stone et al., 2002). Mechanism of action of IDO is yet not clearly understood ; however increase in kynurenine level is known to inhibit the function of NK cells (Wang at al., 2012), T_{reg} cell (T-regulatory cell) (Mellor et al., 2002), dendritic cell (Li et al., 2016) which leads to cause inflammation and causes various types of diseases (Hornyak et al., 2018). T cells must divide for activation of IDO, but it is very much sensitive towards shortage of tryptophan. This affects proliferation of T cell and gets arrested in cell cycle in mid-G1 phase which leads to inhibition of activated and proliferated T-cell (Cui et al., 2018). Accordingly, all these prevent immunological rejection of the tumour which causes cancer in human (Williams et al., 2012). In similar ways, it was found that patients having Alzheimer disease have low levels of serum tryptophan and high levels of serum kynurenine (Seegers et al., 2014). It was mentioned by author that 95% of tryptophan takes part in kynurenine pathway and synthesize nicotinamide (NAD^+) in human brain

(Torok et al., 2015). Two types of QUIN production enzymatic pathway takes place within central nervous system (CNS) where one shows neuroprotective activity and another one has been reported for neurotoxic activity (Fujigaki et al., 2017). Interestingly, CNS astrocytes (star-shaped glial cells in the brain and spinal cord) and oligodendrocytes lack kynurenine 3-monooxygenase and IDO. Hence, they do not produce QUIN and act as neuroprotective. Similarly, within CNS when infiltrating macrophage, activated microglia, neurons, astrocytes present helps in overexpression of IDO and complete pathway which produces kynurenine metabolites and QUIN in large concentration leading to neurotoxicity in brain and development of the symptoms to Parkinson's disease (Fujigaki et al., 2017). Austin et al., (2004) have concluded that IDO over-expression is the major cause for blindness. It is implicated in age-related nuclear cataract which is caused by kynurenine metabolites. Metabolites reacts with lens protein (crystalline) which results in tanned or yellow products which resembles nuclear cataract material which is an indication of blindness in human eye.

2.6. Need and Exploration of IDO inhibitor

It has been proved that inhibiting IDO is a holistic approach for developing various drug for various types of diseases like cancer (breast cancer, lung cancer, etc.), neural diseases (Alzheimer, Parkinson, Huntington etc.) and blindness (Cui et al., 2018; William et al., 2012; Stone et al., 2002). Scientists established that IDO, after metabolizing the pathway and producing kynurenine metabolites, may suppress the effector T-cellular and enhance the suppressor activity of T_{reg} cell (Hornyak et al., 2018; Bilir et al., 2017). Consequently, it has been proved that IDO1 controls and amends both innate and adaptive immune responses under an expansion of situations, such as pregnancy, transplantation, contamination, chronic irritation, autoimmunity, neoplasm and depression (Yamamoto et al., 2014; Hornyak et al., 2018). As we can recognize from the above studies that tryptophan degrades fast which leads to enhancement of IDO expression and formation of kynurenine whenever there is occurrence of diseases, so inhibitor is required to decrease the activity of enzyme which might be achieved by using manner of inhibition of IDO and also no drug target has been approved by the US Food and Drug Administration to IDO inhibitor, although, there are three IDO inhibitors such as 1-methyl-L- tryptophan, epacadostat (INCB024360), indoximod (NLG8189 or 1- methyl-D- tryptophan) which already had entered advanced phase of

clinical trials (Cui et al., 2018; Hornyak et al., 2018; Vacchelli et al., 2014). It has been reported that one of the potent competitive inhibitor of IDO are 1-methyl-L-tryptophan reported as strongest inhibitor shows to elicit immune rejection of allogenic conceptive during pregnancy and also act against cancer like breast cancer (Cady et al., 1991;Cui et al., 2018). In the similar way, epacadostat was revealed to show potential inhibitor of IDO1 which was reported as beneficial in metastasis cancer treatment (Zhang et al., 2018). Accordingly, Indoximod was also reported as strong IDO1 inhibitor for the treatment against cancer diseases as shown in (Table 2.1). But all these inhibitors have some adverse side effects such as stomach pain, dizziness, vomiting, constipation, diarrhoea, cough and cold, backbone pain, anaemia, infection, nausea, hyperglycaemia etc. (Hornyak et al., 2018). So, there is need of exploration of IDO inhibitor from natural sources i.e. endophytic fungi to reduce the side effects of drugs.

Table 2.1: Several inhibitors of indolamine 2,3 dioxygenase (IDO) are as follows.

S.NO.	INHIBITOR	METHODS	CLINICAL TRIAL	REFERENCES
1.	1-methyl-L-Tryptophan	Recombinant human IDO1	Advanced clinical trial	Cady et al.,1991
4.	Menadione	T-RE-derived cell assay	Yes	Kumar et al., 2008
5.	INCB024360 (Epacadostat)	HeLa cell assay	Advanced clinical trial	Liu et al., 2010
6.	4-aryl-1H-1,2,3-Triazole	Recombinant human IDO1	Yes	Huang et al., 2011
7.	Pyranonaphthoquinone derivatives	Recombinant human IDO1	Yes	Bridewell et al., 2013
8.	Galanal	Recombinanthuman IDO1	Yes	Yamamoto et al., 2014
9.	Indoximod	Recombinant human IDO1	Advanced clinical trial	Horyak et al., 2018
10.	β-carboline, canthinones	Virtual screening	No	Wang et al., 2019

Benzomalvin derivatives has been reported as an inhibitor in opposition to NK1(Natural killer) receptor antagonists have long been proposed to have potential therapeutic activity against pathological situation including ache, tension, arthritis, bronchial asthma, expulsion, migraine, malignant neoplastic disease and dementia praecox (Jang et al., 2012). Menadione is an organic compound with the molecular formula of $C_6H_4(CO)_2C_2H(CH_3)$. It is an analogue of one, four-naphthoquinone with the group of methyl at the 2-position derived as effective inhibitor of IDO enzyme which exhibits its anticancer activity (Kumar et al., 2008).

2.7. Natural sources of IDO inhibitor

Cui et al., (2018) has reported three new ethylnaphthoquinone derivatives from Mangrove endophytic fungi as plant mutualists, developing in the intertidal region of marine coastal environments, are broadly diagnosed as prolific assets of biologically active and structurally specific herbal merchandise. It is the first time whilst enzyme inhibitor of IDO from endophytic fungi have been reported and published. The compounds that are reported are named as neofusnaphthoquinoneA, 6-(1-methoxylethy1)-2, 7-dimethoxyjuglone, (3R, 4R)-3-methoxyl-botryosphaerone D as inhibitors of indoleamine-2, three-dioxygenase from the mangrove endophytic fungus *Neofusicoccum austral* SYSU-SKS024 (Cui et al., 2018). Sun et al., (2017) extracted anthraquinone derivatives such as aloin A, aloe emodin etc. as the inhibitor of IDO from *Aloe vera* plant which are reported for treatment of most cancers and immunopathological disorders. Due to presence of phytochemicals such as anthraquinone and its derivatives, *Aloe Vera* leaf extract behaves as primary affect in alleviating symptoms associated with prevention of cardiovascular diseases, cancer, neurodegenerative and diabetes. Galanal is a singular phytochemical has been reported as competitive inhibitor of the enzyme IDO which has been isolated from the extract of Myoga flower buds (Yamamoto et al., 2014). Williams et al., (2012) observed two new merohexaprenoids, halicloic acids A and B isolated from the Marine Sponge *Haliclona sp*. collected in the Philippines which shows functionality of Inhibiting indolamine 2,3-Dioxygenase. Brastianos et al., (2006) proved Exiguamine A as a robust inhibitor reported from the marine Sponge *Neopetrosia exigua* for the treatment of cancer. In spite of all, till date only one author Cui et al., (2018) has reported endophytic fungus isolated compound as a source of inhibitor of indolamine 2,3 dioxygenase.

2.8. Endophytic fungi

Endophytes are microorganism found inside the plant tissues and are found plentiful in rain-forest flora (Strobel 2006). They are ubiquitous in nature and consist of an extremely diverse assemblage of microbes which shows symptomless and unremarkable combination with their hosts for at least a period of their life cycle (Naik, 2019; Saxena et al., 2015; Stone et al., 2000). Endophytic fungi emerged as basic purveyor of similar and non-similar bioactive compounds with high medicinal value as shown in (Table 2.2) (Gouda et al., 2016; Wang and Dai., 2011; Zhou et al., 2009; Wei et al., 2007; Shankar Naik et al., 2006) . It was expected that above 1.5 million species of endophytic fungi may possibly exist in the biodiversity (Arnold et al., 2001). Hence, there is developing call for the invention of novel compound from the isolates of varieties of endophytic fungus (Mane et al., 2018). Numerous bioactive compounds are isolated from endophytic fungus as shown in (Table.2.2) exhibits various pharmacological properties such as anti-cancerous, anti-fungal, antioxidant, antiviral, antimicrobial, antibiotics, anti-malarial activities. (Elsebai et al., 2014; Zhao et al., 2011; Firakova et al., 2007) Taxol is one of the novel, widely well-known compound which acts as anti-cancerous agent and work against breast cancer, uterine cancer and others most cancers too. It is isolated from bark, root and branches of western yew, *Taxus Brevifolia* in the year of 1960 (Chandra 2012). Endophytic fungi is reported as gold of enzyme inhibitor and also found to inhibit specific enzymes recently in 2018 (Meshram et al., 2018). It was studied that the enzyme inhibitors binds to the active sites of the specific enzyme, thus blocks the reaction which is one of the symptoms of numerous diseases showing anomalous enzyme activities. Presently, a number of enzyme inhibitors like etoposides, lovastatins, Lasdiplacton, peptide, allopurinol, febuxostat and orlistat are accessible in the marketplace (Roy 2017; Gupta et al., 2015; Kapoor and Saxena 2014; Baikar and Malpathak 2010). Some of the enzyme inhibitor isolated from endophytic fungi are shown in (Table 2.3).

Table 2.2: Bioactive compounds isolated from endophytic fungi.

S.NO.	ENDOPHYTIC FUNGI	HOST PLANT	BIOACTIVE COMPOUND	ACTIVITY	REFERENCES
1.	*Pestalotiopsis microspora*	*Taxus brevifolia* Nutt. (Pacific yew)	Pestalotiopsis A and B	Anti-carcinogenic	Pulici et al., 1996
2.	*Taxomyces andreanae*	*Taxus brevifolia* Nutt. (Pacific yew)	Taxol	Anti-carcinogenic	Rodrigues 1996
3.	*Pestalotiopsis microspora*	*Taxus wallichiana* (Nepalese yew)	Taxol	Anti-cancerous	Strobel et al., 1996
4.	*Acremonium* sp.	*Taxus baccata* L.	Leucinostatin A	Anti-oomycetes and anticancerous	Strobel et al., 1998
5.	*Colletotrichum gloeosporioides*	*Artemisiamongolica Fisch*. ex Bess	Colletotric acid	Antibacterial and antifungal	Zou et al., 2000
6.	*Cryptosporiopsis quercina*	*Tripterygium wilfordii* Hook. f.	Cryptocin	Antifungal	Li et al., 2000
7.	*Muscodor albus*	*Cinnamomum zeylanicum Nees* (cinnamon tree)	Volatile antimicrobials	Antimicrobial	Strobel et al., 2001
8.	*Pestalotiopsis microspora*	*Terminalia morobensis* Coode	Pestacin	Antioxidant, antifungal	Harper et al., 2003
10	*Guignardia* sp.	Mangrove plant	Anthracenedione derivatives	Potential anticancer activity	Zhang et al., 2010
11.	*Neofusicoccum austral*	Mangrove plant	Ethylnaphthoquinone derivatives	Anticancer	Cui et al., 2018
12.	*Acropilus aureus*	*Vitis vinifera*	Reservatrol	Neurological, cardio vascular disease	Dwibedi et al., 2019

Table 2.3: Inhibitors of enzyme isolated from endophytic fungi:

S. No.	ENZYME	ENZYME INHIBITOR	ENDOPHYTIC FUNGI	DISEASE TARGETED	REFERENCES
1.	Angiotensin converting enzyme	Cytosporin A	*Cytospora* sp.	Hypertension	Steven-Miles et al., 1995
2.	Protease	Cytonic acids A–B	*Cytonaema* sp..	Viral infections	Guo et al., 2000
3.	DNA polymerase	Solanapyrone A	*Alternaria solani*	Cancer, viral Infections	Mizushina et al., 2001
4.	Xanthine oxidase	Aurasperone A, rubrofusarin B	*Aspergillus niger*	Gout	Song et al., 2004
5.	Monoamine oxidase	Nectriapyrone	*Erythrina crista-galli*	Neurological, Psychiatric disorders	Weber et al., 2005
6.	Heat shock protein 90 kD	Radicicol	*Chaetomium chi Versii*	Cancer	Turbyville et al., 2006
7.	Topoisomerases I and II	Fusaristatin A	*Fusarium* sp.	Cancer	Shiono et al., 2007
8.	Trypanothione reductase	Altenusin	*Alternaria* sp.	Trypanosomiasis	Cota et al., 2008
9.	Aromatase	Corynesidone A	*Corynespora cassiicola*	Breast cancer	Chomcheon et al., 2009

10.	Acetylcholinesterase	Huperzine A	*Shiraia* sp.	Alzheimer's , Parkinson's disease, Glaucoma	Zhu et al., 2010
11.	Histone Deacetylases	Epicocconigrones A	*Epicoccum nigrum*	Cancer	Amrani et al., 2014
12.	ATPase	Aurovertin B–D	*Calcarisporium arbuscular*	Cardiovascular disorders, Ulcers	Mao et al., 2015
13.	Urease	Bipolarisenol	*Bipolaris sorokiniana*	Rheumatoid arthritis	Khan et al., 2015
14.	α-Amylase	Peptide	*Aspergillus awamori*	Diabetes	Singh and Kaur 2015
15.	α-glucosidase	Peptide	*Aspergillus awamori*	Diabetes	Singh and Kaur 2015
16.	HMG-CoA reductase	Lovastatin	*Phomopsis aexans*	Cholesterol inhibitor	Parthasarathy and Sathiyabama 2015
17.	Lipase	Fustat	*Fusarium incarnatum*	Obesity	Gupta et al., 2015
18.	Xanthine oxidase	Lasdiplacton and lasdiploic acid	*Lasodiplodia pseudotheobromae*	Oxidative stress	Kumar et al., 2019

2.9. *Malus pumila*

Malus pumila is a scientific name of apple plant. It is cultivated throughout the world in industrial level. Leaves of apple contains wide range of polyphenols such as flavonoids example phloretin and its glycosides, quercetin and its glycosides etc., which are beneficial for health (Sowa et al., 2016; Khanizadeh et al., 2008; Sun et al., 2002). Apple was reported as favourable effect in reducing high risk diseases such as cancer, cardiovascular diseases, diabetes, asthma, neurodegenerative disease (Alzheimer's), osteoporosis etc. (Dianne A. Hyson.2012).Very often we can find A*lternaria*, *Xyleria* ,*Botryosphaeria* and *Colletotrichum* and which was experimental prove provided by the author (Sowa et al., 2016). Scientists reported the diversity and abundance of endophytic fungi among young, mature and old leaves associated with apple plant (Liu et al., 2018). So, we are studying *Malus pumila* for the isolation novel fungus and also for the foundation of novel compound to inhibit IDO.

CHAPTER 3

OBJECTIVES OF THE STUDY

3.0. Aim and objectives

- Screening of endophytic fungi for indolamine 2,3 dioxygenase inhibitor.
- Identification and production of selected endophytic fungi.
- Characterization of IDO from potential endophytic fungus.

CHAPTER 4

MATERIALS AND METHODS

4.1. Preparation of Potato Dextrose Agar (PDA) plates

PDA (Hi-Media, India) was mixed in double distilled water (39 g of PDA/L) into Erlenmeyer flask and was covered with cotton plug and paper with the help of elastic rubber. Flask with media was then autoclaved at 121℃ at 15 psi for 15 min and little warm autoclaved PDA (22.5 ml) was poured onto 90mm diameter petri-plates under sterile conditions inside the Thermodyne Bio safety Cabinet Class I and allowed to solidify at room temperature. The plates were stored at 26 ± 2 °C until further use. Always use ethanol to clean hand, flask for media preparation and Biosafety cabinet.

4.2. Isolation of endophytic fungi

Healthy and mature twigs were collected from *Malus pumila* growing in the nature of Shimla (longitude **77.00"** and **78.19"** east and latitude **30.45"** and **31.44"** north), India during the month of October 2018 and kept in zip pouches at 4℃ until isolation which was further isolated in the lab of Prof. Sanjai Saxena. Fungi were selectively isolated using the standard protocol of isolation of endophytic fungi with some modifications given by Strobel 2006. Initially, surface of twigs (1–2 cm cut pieces aseptically) were sterilized 1% sodium hypochlorite for 1 min, 70% ethanol for 45 min, and 30% ethanol for 30 s and air dried under a laminar airflow respectively. The sterilized twigs were cut into very small parts of 5–10 mm then, placed into a potato dextrose agar (PDA) plate. The plates were incubated at 28 ± 2 °C for a fortnight with a 12 h photo-period and periodically observed for mycelial development or growth of the culture. The fungi growth observed, were aseptically picked from the tip, out of the plant tissue and transferred to fresh PDA plates to acquire pure culture of isolates. Then isolates were preserved and maintained in PDA slants and vials which is known as purification step of isolates. Further, morphological characters (e.g., colony color, texture, growth pattern, pigment formation, diameter and odour) and microscopic structures (e.g., hyphal characteristics, spores, cellular bodies etc.) were observed under Nikon binocular microscope (Eclipse E 100) (Dwibedi et al., 2019).

4.2.1. Isolation rate of *Malus pumila* -producing endophytes

Diversity of *Malus pumila* culture was calculated by finding the isolation rate. The percentage of isolation rate (IR) were calculated by the method adopted by Dwibedi et

al., (2019). Samples were incubated and growth was examined daily during 6 weeks and further isolation rate was calculated by the following formula.

$$\text{Isolation rate (\%)} = \frac{\text{Total no. endophytic fungi recovered}}{\text{Total number of segment}} \text{x}100 \quad \text{—(1)}$$

4.3. Storage and sustenance of endophytic fungi

Pure cultures were obtained from the isolation of endophytes from *Malus pumila.* The procured cultures which were inoculated on PDA plates and incubated at 28˚C for 10 days for uniform growth were then aseptically transferred to PDA slants containing 10% glycerol for long-term storage which has already been autoclaved at 121℃ at 15 psi for 15 min and cooled down.

4.4. Production of isolated Culture

Aseptically inoculating mycelial disk (5mm) of 7-10 day old culture for the production with the help of autoclaved borer was kept in pre-sterilized Potato Dextrose Broth (24g / L) medium made in Erlenmeyer flasks and incubated at 28 ± 2 °C, 120 rpm in an incubator shaker for 10-15 days. Spent broth was separated from mycelia mass by filtration through Whattman filter paper no.4 after the incubation period is over which was then centrifuged at 12,000 rpm for 10 minutes. The filtrate, free of cell, was kept at -20 °C until further test done (Rodrigues et al., 2000; Vicente et al., 2001).

4.5. Screening of endophytic fungi for inhibitor of IDO

With some modification screening was done (Sun et al., 2017; Nokano et al., 2012). recombinant human IDO (rhIDO) as already discussed in the above chapter is an enzyme which consume tryptophan as substrate and in turn produces kynurenine as product which was measured by absorbance assay (λ_{max} 492 nm) using 96-well microtiter plate reader. It is extremely temperature sensitive so, kept under -80℃ and also pH sensitive which must be maintained at 6.5. The absorbance method measures kynurenine indirectly. It depends on the production of imine group when aromatic amino group reacts with *p*-dimethylaminobenzaldehyde (Ehrlich's reagent). At first, rhIDO was incubated with the extract of different endophytic fungi having Potassium phosphate assay buffer (pH 6.5) which maintain pH condition, ascorbic acid (pH 7 neutralized with 1M NaOH), methylene blue, catalase for 1 hour at 37℃. Then, L-tryptophan (substrate) was added and again incubate for 1 hour at 37℃. Aqueous tri-

chloro acetic acid (TCA) (stops reaction) was added to the solution and heated for 15 min at 50℃, and spinned at 11500 rpm for 10 min. Ehrlich's reagent was added the supernatant. The produced kynurenine was measured at 492nm with microtiter plate reader (Biotek-Monochromator based UV/Vis absorbance). The results were calculated from the following formula shown below. Details of concentration and volume taken of the reagent for the test is shown in (Table 4.1).

$$\%\text{Inhibition} = \frac{\text{Absorbance(control)} - \text{absorbance(sample)}}{\text{Absorbance(control)}} \times 100 \quad \text{—(2)}$$

Table 4.1: Materials required

S.NO.	MATERIALS REQUIRED	CONCENTRATION	VOLUME TAKEN
1.	rhIDO	20µg/ml	10µl
2.	Potassium phosphate buffer	50Mm	102µl
3.	L-ascorbic acid	20mM	20µl
4.	Catalase	100µg/ml	4µl
5.	Methylene blue	10mM	4µl
6.	L-Tryptophan	200µM	20µl
7.	TCA	30% (v/v)	40µl
8.	Ehrlich's reagent	2%(w/v)	75µl
9.	Test compound	----	40µl
10.	PDB(control)	----	40µl
11.	Methanol(control)	----	40µl

4.6. Extraction process of bioactive crude compounds

Liquid-liquid extraction is the process of separation of different biomolecules from the extract of endophytic fungi on the basis of their polarity from highly non-polar to highly polar such as Hexane<Chloroform<Ethyl acetate<Acetone<Methanol<Water (Altemimi et al., 2017). Multiple solvents, were used for the isolation of fraction of cell free filtrate (50 ml) of bio-active crude, like hexane, chloroform, DCM (di-chloromethane), ethyl acetate etc. in the ratio 3:1 in the lab of Prof. Sanjai Saxena. By extracting with appropriate solvents (3 × 100 ml), the organic layer of solvent was then kept into anhydrous sodium sulphate (HiMedia, Mumbai, India) for dehydration (Kjer

et al., 2010; Altemimi et al., 2017). Then, kept for evaporation of the organic solvent which ultimately gives crude product which was then weighed and reconstituted in methanol. The fractions were stored at -20 °C until further use. The fraction so obtained was weighed, reconstituted in methanol, and again screened to confirm the presence of inhibitory compound through screening assay.

4.7. Identification of Endophytic fungi

The selected isolate showing inhibition of indolamine 2,3 dioxygenase was identified by classical and molecular taxonomy.

4.7.1. Morphotaxonomy

The isolate was examined under the microscope for identification on the basis of its morphological characteristics. The culture was grown in different media plates namely PDA (Potato Dextrose Agar), SNA (Synthetic Nutrient Poor Agar), PLA (Pine Leaf Water Agar) , CMA (Cornmeal Agar) and Water Agar for 4 weeks at 28 ± 2 °C for growth optimization. For microscopic features, water droplet on the glass slide were placed and small amount of mycelia was taken from plate and kept on it, spreaded with a fine tip needle and stained with Lacto phenol Cotton Blue dye .The strained mycelia was covered with cover slip (18 x 10 mm) avoiding the formation of air bubbles and mounted with DPX (Barnett et al., 1998). Morphological characters such as colony colour, appearance and characteristics like hypha features, conidia formation, and another cellular bodies such as fruiting bodies (asexual or sexual spores) or structures, were observed at 10X, 40X and 100X magnifications using microscope and observations were noted down.

4.8. Molecular Identification of endophytic fungi

4.8.1. DNA Isolation

The fungal genomic DNA isolation was basically executed on 5-7 day old culture using Wizard® Genomic DNA purification kit (Promega, USA), mycelial mass (70 mg) was solidified using liquid nitrogen and crushed in a sterile mortar and pestle. Nuclei lysis buffer (700 µL) was added to the crushed mass and the contents were transferred to a micro centrifuge tube, vortexed followed by incubation at 65 °C in water bath for 15 minutes. After the incubation, the micro centrifuge tubes were centrifuged at 12,000 rpm for 5 min to remove the cell debris. This was followed by addition of RNase (5

µL) into the tube and incubation at 37°C for 15 min and addition of protein precipitation solution (220 µL). The micro centrifuge tubes were then centrifuged at 12,000 rpm for 3 min to remove protein contamination. The aqueous phase containing DNA was transferred to another micro centrifuge tube containing isopropanol and centrifuged at 13,000 rpm for 1 min. The pellet thus formed was air dried and dissolved in DNA Dehydration buffer (50 µL). The qualitative estimation of DNA isolation was done by agarose gel electrophoresis (Dwibedi et al., 2018; Guo et al., 2000).

4.8.2. Gel Electrophoresis

For gel electrophoresis, Agarose gel (0.8 %) added with ethidium bromide (0.5 µg/ml) was casted in the electrophoretic apparatus with running buffer (1X TAE) poured into the electrophoretic tank. Then, DNA samples were mixed with 6X loading dye and loaded into the agarose gel at 50 V voltage for 1h and after completion of running of DNA towards positive direction as it is negatively charged. Further, after completion DNA fragments were visualized under UV Transilluminator and gel imaging with the help of UV light in Bio-Rad Gel documentation system using Quantity-1-D analysis software was done. Spectrophotometric analysis of the sample is the way of estimating quantitative analysis of genomic DNA. The absorbance of the one sample was observed at 260 nm and 280 nm to estimate the concentration and purity of the DNA. 1 OD is equivalent to 50µg/ml DNA sample. The purity of the DNA sample was given by using the ratio of absorbance at 260nm and at 280 nm. If the ratio is less than 1.6, it shows RNA contamination and if the ratio lies between 1.6-1.8, then it shows pure form of DNA sample. If the ratio is higher than 1.8, then might be there is DNA contaminated with protein. The concentration of the DNA sample was measured from the following formula (Dalawai et al., 2017).

Formula: Concentration (µg/ml) = OD 260nm x 50µg/ml x Dilution factor —(3)

4.8.3. PCR Amplification

ITS primer is the gene sequences of ribosomal RNA of 18s and 28s. ITS primer is used as standard primer to identify endophytic fungus are ITS F (internal spacer region forward primer) having sequence 5’ TCC GTA GGT GAA CCT GCG G 3’ and ITS R (internal spacer region reverse primer) having sequence 5’ TCC TCC GCT TAT TGA TAT GC 3’ of 18s ribosomal RNA (Gardes and Bruns 1996). 25 µL reaction mixture

as shown in (Table 4.2) which contain extracted fungal DNA, primers (ITS 1 and ITS 4), dNTP, Taq DNA Polymerase in 10 X Taq buffer was carried out for the amplification of isolated DNA. The required conditions of thermal cycles are described in (Table 4.3). Amplified DNA was run and examined with gel electrophoresis in agarose gel (1.5 %) and gel electrophoresis was done as represented in the above theory.

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

5.1. Isolation of endophytic fungi from apple leaf (*Malus pumila*)

Isolation of endophytic fungus from apple (*Malus pumila)* plant leaves and stems, collected from hot spot biodiversity of Shimla region, a capital city of Himachal Pradesh, India of which 26 culture were obtained as pure culture which was then screened for the inhibition of indolamine 2,3 dioxygenase in search of novel compound from endophytic fungi. 25 from leaves and 1 from stem were named as MPLSH and MPSSH where MP for *Malus pumila*, L and S for leaf and stem respectively, SH for Shimla, Himachal Pradesh for example #77MPLSH, #201MPSSH etc. (Fig.5.1) describes the isolation of endophytic fungi from leaves to pure culture of endophytic fungi. Picture (a) is representing the leaf and stem of apple plant. Picture (b) is showing growth of first isolation from leaves or stem on PDA medium plate and picture (c) shows pure culture growth of fungal endophytes selected from (b). Pure cultures of MPLSH and its morphological pictures are shown in (Fig.5.2 and Fig.2.3) respectively. Also, list of all 26 pure cultures with their tentative identifications are shown in (Table. 5.2) Calculated isolation rate of MPLSH is recorded in (Table.5.1). Isolation rate percentage is found to be 28.73% which signifies rate at which fungal endophyte can be isolated from apple plant.

Table 5.1: Calculation of isolation rate (%) of endophytic fungi.

NO. OF PLATES SHOWS ENDOPHYTIC FUNGUS GROWTH	**NO. OF SEGMENTS OF PLANT SAMPLE USED FOR ISOLATION**	**NO. OF ENDOPHYTIC FUNGAL ISOLATES RECOVERED**	**ISOLATION RATE (%)**
43	348	100	28.73

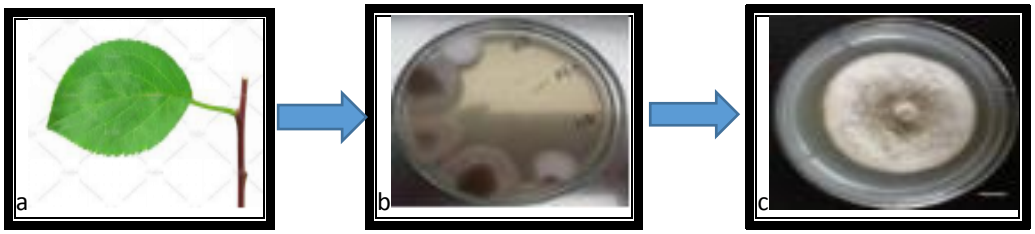

Fig.5.1: Apple *(Malus pumila)* leaf with stem next shows Numbering of plates and twigs infected (after isolation) and next is pure culture.

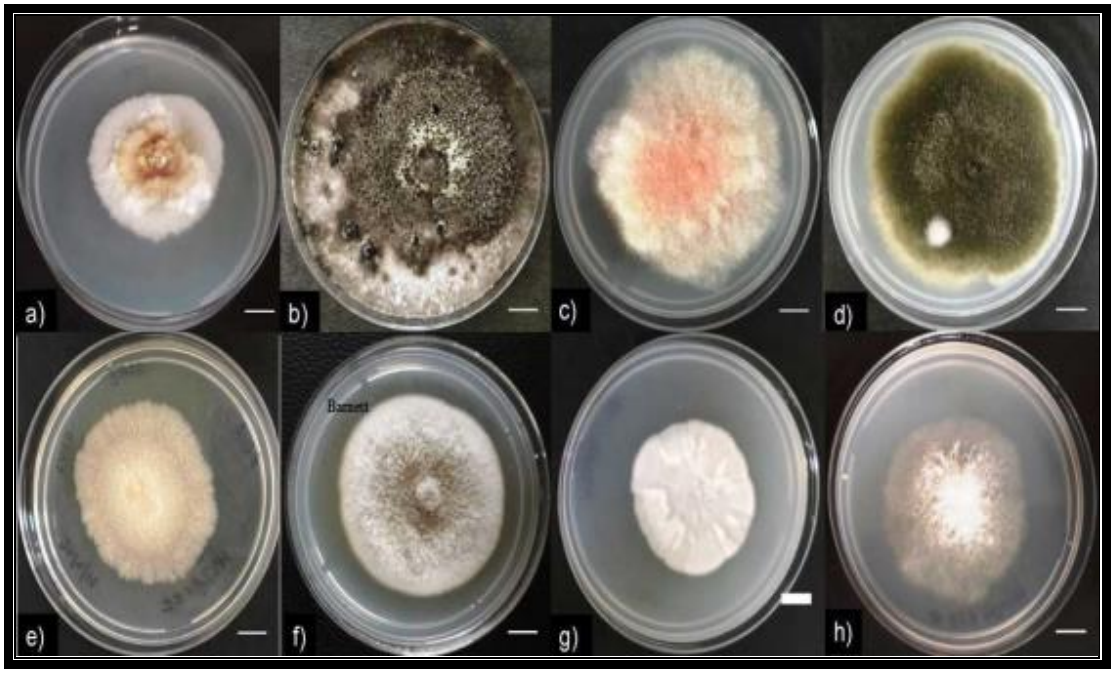

Fig.5.2: Pure cultures of different endophytic fungi isolated in lab a) Unidentified; b) *Fusarium sp*.; c) C*haetomium sp*. ; d) *Alternaria sp.*; e) B*otryosphaeria sp*.; f) Unidentified; g) Unidentified; h) *Nigrospora sp.* (Bar : 10mm)

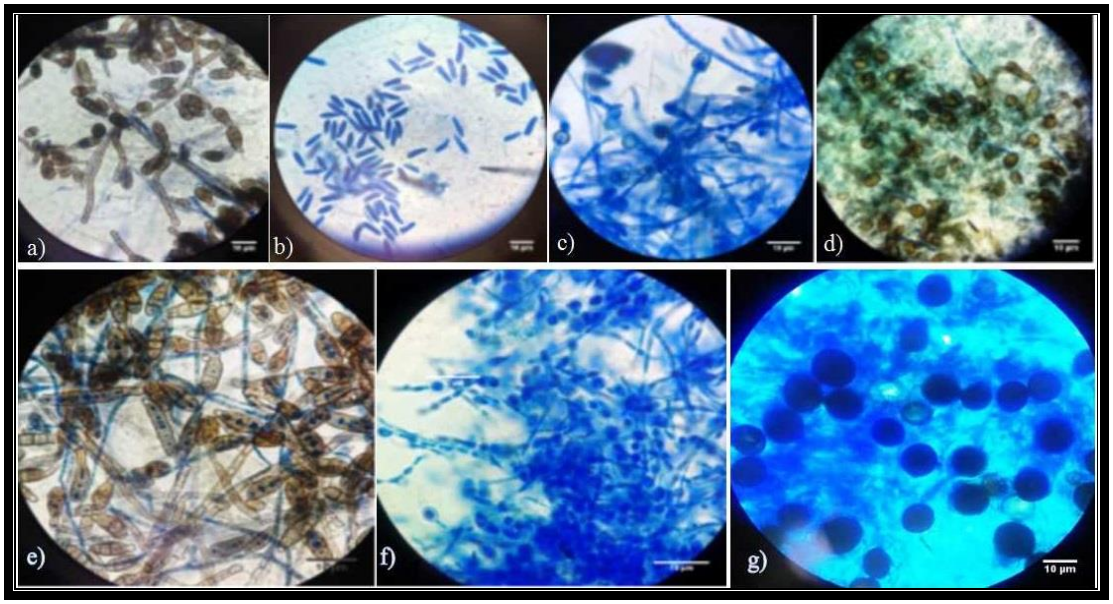

Fig.5.3: Microscopic feature of endophytic fungi observed during study a) *Alternaria sp*.; b) *Fusarium sp.*; c) *Botryosphaeria sp.*; d) *Cheatomium sp*.; e) *Curvularia sp.*; f) Unknown; g) *Nigrospora sp.*

Table 5.2: List of 26 identified endophytic fungus screened during study.

S.NO.	CULTURE CODE	SPECIES	PLANT PARTS	LOCATION	CULTURE TENTATIVE IDENTIFICATION	BIOCHEMICAL SCREENING
1.	201MPLSH	*M. pumila*	Leaves	Shimla, Himachal Pradesh	*Alternaria* sp.	++
2.	168MPLSH	*M. pumila*	Leaves	Shimla, Himachal Pradesh	*Alternaria* sp.	+++
3.	11MPLSH	*M. pumila*	Leaves	Shimla, Himachal Pradesh	Unidentified	++
4.	207MPSSH	*M. pumila*	Stem	Shimla, Himachal Pradesh	*Nigrospora* sp.	++
5.	114MPLSH	*M. pumila*	Leaves	Shimla, Himachal Pradesh	Unidentified	++
6.	96MPLSH	*M. pumila*	Leaves	Shimla, Himachal Pradesh	*Alternaria* sp.	+++
7.	62MPLSH	*M. pumila*	Leaves	Shimla, Himachal Pradesh	Unidentified	++
8.	180MPLSH	*M. pumila*	Leaves	Shimla, Himachal Pradesh	*Botryosphaeria* sp.	+++
9.	77MPLSH	*M. pumila*	Leaves	Shimla, Himachal Pradesh	*Botryosphaeria* sp.	+++
10.	117MPLSH	*M. pumila*	Leaves	Shimla, Himachal Pradesh	*Alternaria* sp.	+
11.	139MPLSH	*M. pumila*	Leaves	Shimla, Himachal Pradesh	Unidentified	+++
12.	53MPLSH	*M. pumila*	Leaves	Shimla, Himachal Pradesh	*Alternaria* sp.	+++

13.	93MPLSH	*M. pumila*	Leaves	Shimla, Himachal Pradesh	*Alternaria* sp.	+++
14.	179MPLSH	*M. pumila*	Leaves	Shimla, Himachal Pradesh	*Alternaria* sp.	+
15.	188MPLSH	*M. pumila*	Leaves	Shimla, Himachal Pradesh	Unidentified	+++
16.	32MPLSH	*M. pumila*	Leaves	Shimla, Himachal Pradesh	*Fusarium* sp.	+++
17.	159MPLSH	*M. pumila*	Leaves	Shimla, Himachal Pradesh	*Alternaria* sp.	+
18.	122MPLSH	*M. pumila*	Leaves	Shimla, Himachal Pradesh	Unidentified	++
19.	73MPLSH	*M. pumila*	Leaves	Shimla, Himachal Pradesh	*Alternaria* sp.	++
20.	82MPLSH	*M. pumila*	Leaves	Shimla, Himachal Pradesh	*Alternaria* sp.	++
21.	140MPLSH	*M. pumila*	Leaves	Shimla, Himachal Pradesh	Unidentified	++
22.	67MPLSH	*M. pumila*	Leaves	Shimla, Himachal Pradesh	*Alternaria* sp.	++
23.	3MPLSH	*M. pumila*	Leaves	Shimla, Himachal Pradesh	Unidentified	+++
24.	4MPLSH	*M. pumila*	Leaves	Shimla, Himachal Pradesh	Unidentified	++
25.	98MPLSH	*M. pumila*	Leaves	Shimla, Himachal Pradesh	Unidentified	++
26.	50MPLSH	*M. pumila*	Leaves	Shimla, Himachal Pradesh	*Chaetomium* sp..	++

[+++ (70-80%), ++ (50-70%), + (30-40%)]*

5.2. Preservation of endophytic fungi

Preservation is an important technique to store cultures for a long-term duration at -20℃ in 10% glycerol. 10% glycerol maintains stability and growth of culture for eternity. The isolated endophytic fungi were aseptically kept by using different methods like the PDA slants and vials as shown in (Fig.5.4).

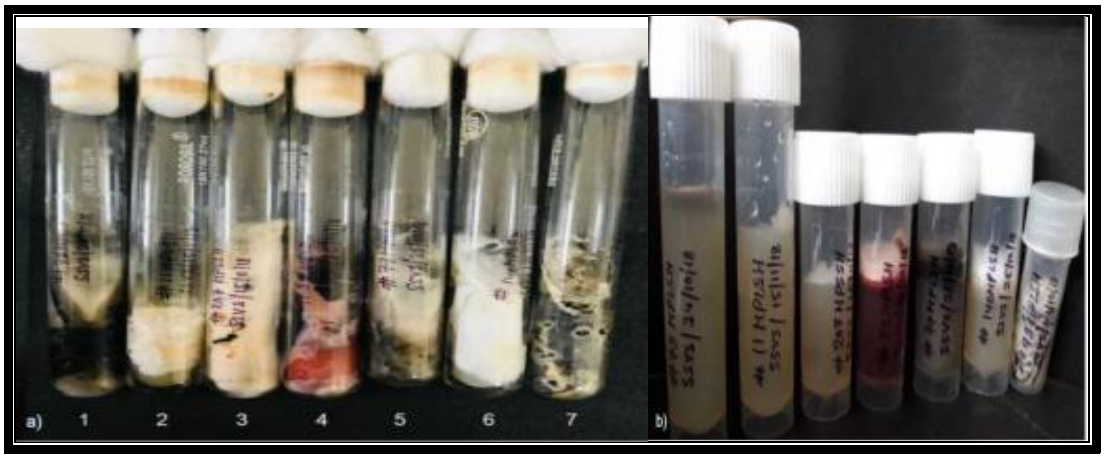

Fig.5.4: Endophytic isolates preserved for long term storage by different methods a) PDA slants b) PDA vials 1. #73MPLSH, 2. #11MPLSH, 3. #207MPLSH, 4. #50MPLSH, 5. #77MPLSH, 6. #140MPLSH, 7. #93MPLSH

5.3. Production of culture filtrate

The isolates following existing research were constrained to secondary metabolites production in the PDB medium as shown in (Fig.5.5) and the Biomass produced in the broth shows the growth rate of fungal culture in PDB in 19- 20 days. Data of biomass production of some of the fungal cultures from lowest to highest is shown in (Table 5.3). #32MPLSH is showing highest biomass production whereas #93MPLSH is showing lowest biomass production.

Table 5.3: 26 pure culture after isolation and biomass production (for 30 ml).

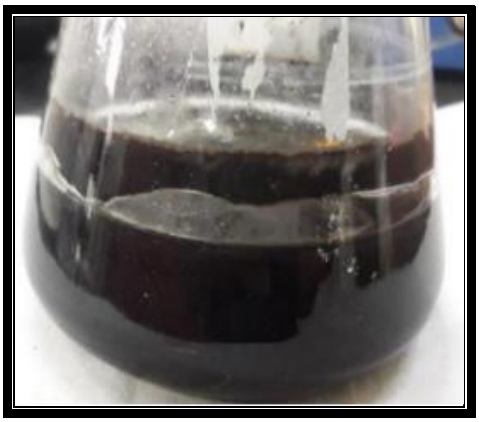

Fig.5.5: #77MPLSH production flask

ENDOPHYTIC FUNGUS	BIOMASS (mg)
#93MPLSH	111
#179MPLSH	160
#53MPLSH	177
#77MPLSH	219
#139MPLSH	288
#11MPLSH	298
#62MPLSH	324
#73MPLSH	330
#180MPLSH	357
#66MPLSH	369
#168MPLSH	371
#188MPLSH	483
#3MPLSH	490
#96MPLSH	531
#32MPLSH	595

5.4. Inhibition of IDO activity using crude filtrate

#77MPLSH is shows approx. 82% IDO inhibition against indolamine 2,3 dioxygenase which is highest among all other extract of endophytic fungi in preliminary test in which PDB is used as control through absorbance assay. Most of them are showing inhibition activity more than 50% and very less are exhibiting less than 40%. So, further extraction was done for #77MPLSH in ethyl acetate for its best inhibition activity against indolamine 2, 3 dioxygenase in which methanol is used as control and then final absorbance was done for 1mg/ml. Inhibition activity more than 70% with PDB as control is shown in (Table 5.4) and final test result in ethyl acetate with methanol as control is shown in (Table 5.5).

Table 5.4: Preliminary data of Inhibition activity against IDO.

S.NO.	CRUDE FILTRATE SAMPLE NO.	% INHIBITION
1.	**77MPLSH**	81.76
2.	139MPLSH	77.06
3.	32MPLSH	74.71
4.	3MPLSH	74.12
5.	96MPLSH	73.53

6.	93MPLSH	73.53
7.	180MPLSH	72.94
8.	168MPLSH	70.78
9.	53MPLSH	70.59
10.	188MPLSH	70.59
11.	PDB(CONTROL)	-

Table 5.5: Final results after extraction in ethyl acetate (1mg/ml)

S.NO.	CRUDE COMPOUND SAMPLE	% INHIBITION
1.	#77MPLSH	**72.120**
2.	METHANOL(CONTROL)	**--**

5.5. Solvent extraction

The liquid-liquid extraction of cell-free filtrates of one cultures namely: #77MPLSH was carried out using Hexane, Chloroform, Dichloromethane, ethyl acetate. The bioactive residue reconstituted in methanol. The highest yield of the bioactive residue collected from #77MPLSH (30.0 mg) in Ethyl acetate.as shown in (Table.5.5) and (Fig.5.6).

Fig.5.6: Extraction process from Hexane to ethyl acetate and 1mg/ml extraction of #77MPLSH in ethyl acetate.

5.6. Identification of the selected endophytic fungus

The potential indolamine 2,3 dioxygenase inhibitor producing endophytic fungus #77MPLSH was identified using morphotaxonomic and molecular taxonomic methods as shown in (Table 5.5) .

5.6.1. Morphotaxomony

The endophytic fungus #77MPLSH produced light creamish, fast growing (49± 1), floccose aerial mycelium on PDA media after 10 days of incubation with 12 h photoperiod (Fig.5.7). Fungus start growing with light creamish color in PDA, CMA (Cornmeal agar) and dark brown over WA (Water agar) and PLA (Pine leaf agar) (Fig.5.7). On SNA (Synthetic nutrient poor agar) medium fungus was off-white in colour initially with creamy appearance (Fig. 5.7). On PLA Water Agar (WA) medium it was brown in colour (Fig.5.7 & Table 5.6). Over Pine Leaf Agar (PLA) and Water Agar (WA) the margins were smooth and flat respectively molecular identification.

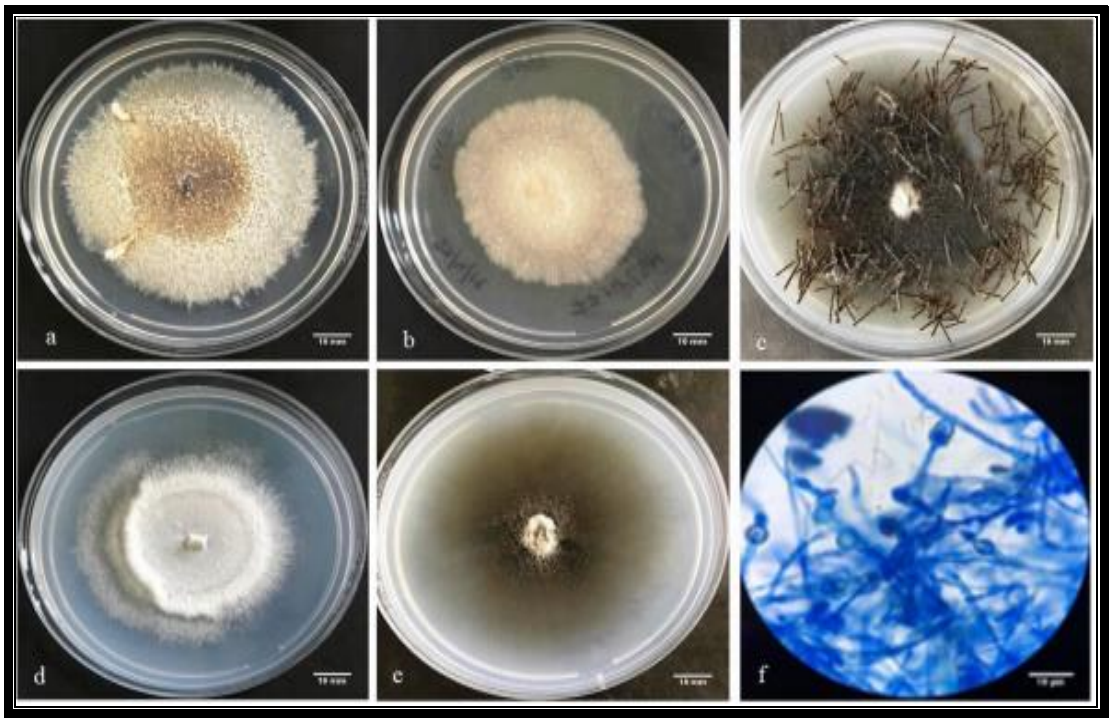

Fig.5.7: Morphological feature of #77MPLSH; colony morphology on a) WA medium; b) CMA medium; c) SNA medium; d) PDA medium; e) PLA medium; (Ba : 10mm); f) *Botryosphaeria sp*.

Table 5.6: Colony morphology of #77MPLSH on different medium after 20 days, 28℃, and 12h dark**.**

S.NO.	MEDIUM	COLONY COLOR FRONT	BACK	COLONY DIAMETR (mm)	MARGIN	ODOUR
A.	**PDA**	Light Cream	Light Creamish	49±1	Circular	Fruity smell
B.	**SNA**	Pure White	Light Creamish	58±6	Circular	No odour
C.	**CMA**	Off-White	Pale Brown	60±7	Circular	Fruity smell
D.	**WA**	Pale Brown	Brown filament	62±3	Circular and smooth	No odour
E.	**PLA**	Pale Brown	Dark brown filament	65±0	Circular and smooth	No odour

5.6.2. Genomic DNA isolation and PCR amplification

The genomic DNA isolation of the potent inhibitor of IDO producing endophytic fungi, #77MPLSH, was done and the size of the genomic DNA was found to be approximately more than 10 kb (Fig. 5.8). The concentration of DNA was estimated by taking the absorbance at 260 nm and the amount was 25 ng/µl by using formula concentration (µg/ml) = O.D 260nm x 50 µg/ml x dilution factor. The PCR amplicon was resolved on 1.5% agarose gel in order to check the size on the basis of the mobility and comparison with the 100bp ladder. The size of the amplicon was found to be approximately 550 bp to 600 bp (Fig.5.8). This size can be easily compared to the ITS region, which was amplified in order to characterize the fungi at molecular level. Hence, the result represents #77MPLSH as fungal endophyte.

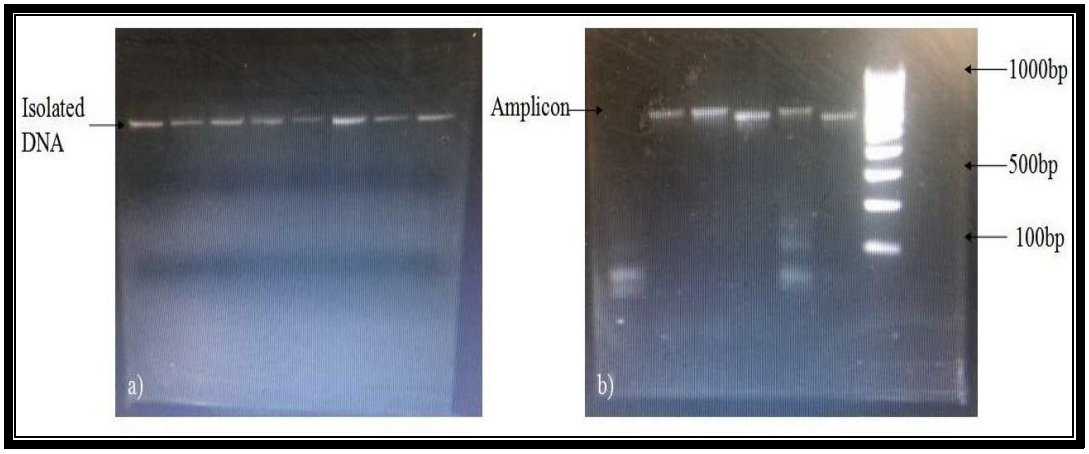

Fig.5.8: a) Genomic DNA isolation of #77MPLSH and b) PCR of isolated DNA

CHAPTER 6

DISCUSSION

6.0. DISCUSSION

IDO was reported as first rate -limiting step in tryptophan degradation in the pathway known as kynurenine pathway (Bilir et al., 2017; Chen et al., 2011). Inflammation, production INF-γ, cytokines, stress, activation of T_{reg} cell elevates IDO expression which ultimately leads to degradation of large amount of tryptophan (Zhang et al., 2018; Matin et al., 2006). Overexpressed IDO produces kynurenine and its metabolites such as QUIN, kynurenic acid, 3-HAA, 3-HK, etc. which causes various diseases and disorders in human for example Alzheimer, Parkinson, Huntington diseases, AIDS, aging problem, obesity etc (Cui et al., 2018; Stone et al., 2002).

Several inhibitors of IDO as drug target has been synthesized till date among them three drugs are under advanced clinical trials are known as 1-methyl-L-tryptophan, epacodsatat, indoximod (Hornyak et al., 2018). But these drugs shows some adverse effect inside the body such as vomiting, fatiguenss, stomach pain etc. (Hornyak et al., 2018). Hence, there is an urgent need of drug discovery by which these adverse side effects can be reduced which may be possible by developing therapeutic drug from natural sources.

The anthraquinone derivatives from *Aloe vera* extract were reported for the inhibition of indolamine 2, 3 dioxygenase (Sun et al., 2017). Galanal compound was isolated from Myoga flower bud (Yamamoto et al., 2014). Halicloic Acids A and B has been isolated from Marine Sponge *Haliclona sp.* (Williams et al., 2012) etc. was also reported for the inhibition of IDO.

Fungal endophytes are known as “Gold mine of inhibitor of enzyme” and mimic the properties of host plant and produces enormous bioactive compounds which was found to be reported in therapeutic potential treatment of several high risk diseases such as neurological disorder, obesity, cancer, viral infection etc. (Charis et al. 2017; Gouda et al. 2016; Strobel 2006). Taxol is one of the best example of novel drug discovered from endophytic fungi for the treatmenent of cancer diseses (Stierle et al. 1993).

Some examples of enzyme inhibitor isolated from fungal endophytes are solanapyrone A from *Alternaria solani*, Fusaristatin A from *Fusarium sp*., huperzine A from *shiraia sp*. etc (Meshram et al., 2018). Hence, fungal endophytes came into existence which can be isolated from plants and could be produced in pilot amount by the means of

fermentation. Cui et al., (2018) reported 10 varieties of mangrove plant fungal extract for inhibition of IDO.

Till date there is very scanty data present for IDO inhibitors which are isolated from endophytic fungi. It was Cui et al., (2018) who reported ethylnapthoquinone derivatives as IDO inhibitor from endophytic fungi *Neofusicoccum austral* and further no other compounds has been isolated by now. The data limitation shows its new aspects in finding novel compound as inhibitor of IDO.

It has been reported that polyphenols such as anthraquinone derivatives, ethylnapthoquinone derivatives etc. are potent inhibitor of IDO. *Malus pumila* is one the plant species which contains huge amount of polyphenols such as quercetin, flavonoids etc. (Sowa et al., 2016) and also shows promising effect against various diaseases (Dianne A.Hyson. 2012). Thus, current study was targeting particularly *Malus pumila* from Indian biodiversity in terms of colonization frequency and screening for the novel bioactive compounds as an inhibitor of IDO enzyme.

In this study, we reported screening of 26 culture *Malus pumila* named as #77MPLSH, #139MPLSH, #32MPLSH, #3MPLSH, #96MPLSH, #93MPLSH, #180MPLSH, #168MPLSH, #53MPLSH, #188MPLSH etc. were tentatively identified as *Botryosphaeria sp., Alternaria sp., fusarium sp., curvularia sp., Nigrospora sp.*. While Malus pumila is reported as A*lternaria sp., Botryosphaeria sp., Colletrichum sp.* and *Xyleria sp.* which is commonly found (Sowa et al., 2016)

Till date, inhibitor of IDO was reported from endophytic fungi *Neofusicoccum austral* named as SYSU-SKS024 from mangrove plant (Cui et al., 2018). While we reported *Botryosphaeria sp.* from endophytic fungus named as #77MPLSH from apple plant as a strongest inhibitor of IDO.

Among 26 culture, #77MPLSH shows maximum inhibition i.e.82% against IDO in preliminary test which was performed by using crude fungal extract whereas ethyl acetate #77MPLSH extract (1mg/ml) reconstituted in methanol exhibit over 70% inhibition. Further the culture #77MPLSH tentatively identified as *Botryosphaeria sp*. As ITS region is biomarker of fungus so, amplification of ITS region confirmed the sample is fungus (Dwibedi et al., 2019). Sun et al., (2017) reported 100µm of 7 compound showed above 60% inhibition of human IDO from *Aloe* extract. Aloin A, aloin B, desoxyaloin, feroxidin, aloeresin,7-hydroxy-5-(hydroymethyl)-2-

methylchromone etc. are some examples of compounds which shows inhibition against IDO.

Hence, we may conclude that #77MPLSH may be be further studied for the production of novel and potential IDO inhibitor.

CHAPTER 7

CONCLUSION

7.0. CONCLUSION

In the current study we isolated 26 endophytic fungi from *Malus pumila*. Out of 26 cultures, 50% cultures shows positive results against inhibition of IDO enzyme. Among all #77MPLSH shows the best inhibitory against indolamine 2,3 dioxygenase (IDO) i.e. 80%. The isolate are morphologically identified as *Botryosphaeria sp.* Hence, we conclude that #77MPLSH could be considered as potent inhibitor from Endophytic fungi.

Thus, furher work on optimization and purification of the compound(s) could be proceeded in search of novel source of IDO inhibitor.

CHAPTER 8

REFERENCES

8.0. REFERENCES

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APPENDIX

Media

1. Potato dextrose agar

Potato dextrose agar – 39gm

Distilled water – 1L

Final pH (at 26°C)- 5.6±0.2

Autoclave at 121ºC for 15 min

2. Corn meal agar

Corn meal, infusion form- 50gm

Dextrose- 2gm

Agar- 15gm

Distilled water- 1L

Autoclave at 121°C for 15 min

3. Synthetic nutrient poor agar

KN_2PO_4- 1gm

KNO_3- 1gm

$MGSO_4$.7H2O- 0.5gm

KCL-0.5gm

Glucose- 0.2gm

Sucrose-0.5gm

Agar- 2%

Autoclave at 121ºC for 15 min

4. Water agar

Agar – 15gm

Distilled water – 1L

Autoclave at 121ºC for 15 min

5. Pine leaf agar

Pine leaves

Agar – 15gm

Distilled water – 1L

Autoclave at 121ºC for 15 min

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

Screening of endophytic fungi for the inhibition of indole amine 2,3 deoxygenase

by Sarika Srivastava (601704006)

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