Isolation and Purification of Xanthine Oxidase Inhibitor from Endophytic Fungi

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In

Chemistry

By

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CANDIDATE's DECLARATION

I hereby declare that the thesis entitled "**Isolation and Purification of Xanthine Oxidase Inhibitor from Endophytic Fungi**" is an authentic record of my work carried out as partial requirement for the award of the degree of **Master of Science** in **Chemistry** submitted in the **School of Chemistry and Biochemistry, Thapar University, Patiala, India** under the esteemed supervision of **Dr. Manmohan Chhibber** and **Dr. Sanjai Saxena** during January, 2017 to July, 2017. No part of the matter embodied in this report has been submitted to any other university or institute for the award of any other degree.

Date: July, 2017

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It is certified that the above statement made by the student is correct to the best of my/our knowledge and belief.

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Ankita Agarwal

"Hold fast to dreams

For if dreams die

Life is a broken-winged bird

That cannot fly"

- Langston Hughes.

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Abbreviations

PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
NBT	Nitroblue – tetrazolium
PCR	Polymerase Chain Reaction
DNA	Deoxyribonucleic acid
DPPH	2, 2-diphenyl-1-picrylhydrazyl
MHA	Mueller Hilton Agar
MSU	Mono Sodium Urate
NSAID	Non – Steroidal Antiinflammatory Drugs
RNA	Ribonucleic Acid
DNA	Deoxy Ribonucleic Acid
ATP	Adenosine triphosphate
GTP	Guanosine triphosphate
ULT	Uric Lowering Therapy
NADH	Nicotinamide adenine dinucleotide
XOD	Xanthine Oxidase
XOR	Xanthine oxidoreductase
XOH	Xanthine Dehydrogenase
MEA	Malt Extract Agar
SNA	Synthetic Nutrient Agar
WA	Water Agar
PLA	Pine leaf Agar
TLC	Thin Layer Chromatography

Abstract

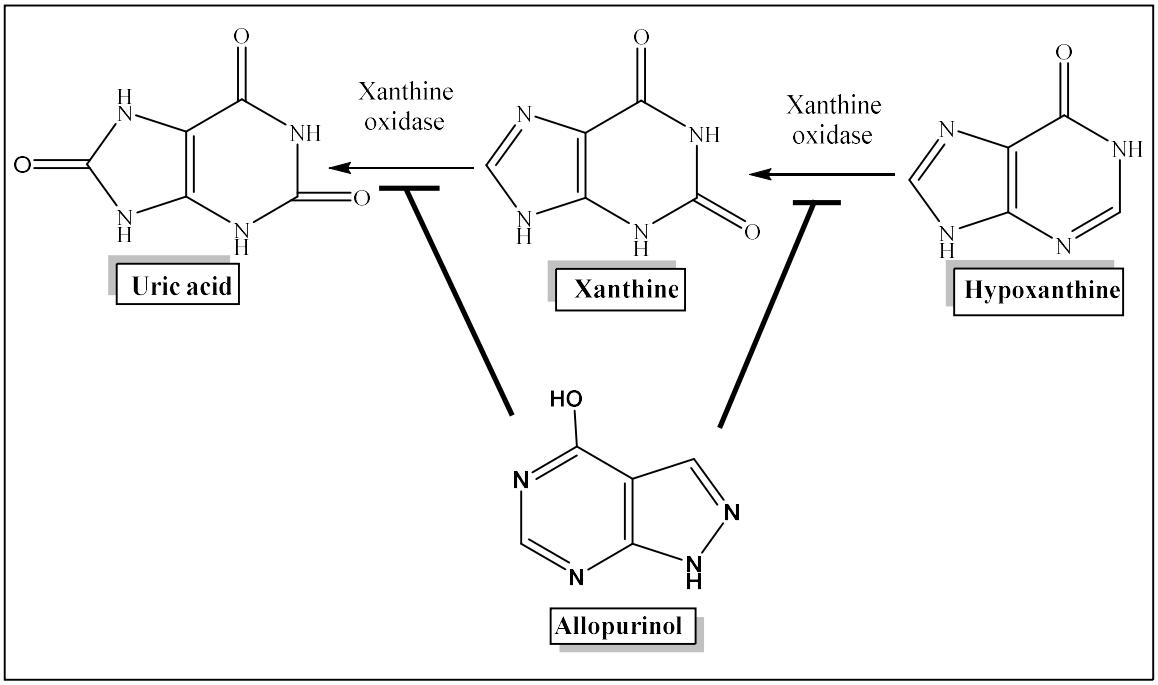
Endophytic fungi are a treasure of bioactive compounds with therapeutic properties due to their symbiotic association with host organisms. This research aims at isolating xanthine oxidase inhibitors (XOIs) for the treatment of gout from endophytic fungi. Extract of the fungal strains, isolated from Camellia sinensis, grown on PDA exhibited appreciable inhibitory activity against xanthine oxidase. The extract was purified using column chromatography to isolate five pure fractions. Two of the five pure fractions (Fraction -2 and Fraction - 4) gave best results when evaluated using NBT assay. Phytochemical testing was also carried out to identify secondary metabolites present. All the pure fractions obtained were also subjected to antioxidant assay and evaluated for their antibacterial activity. While antioxidant assay was done using DPPH radical scavenging activity, antimicrobial activity was carried out against various bacterial cultures using disk diffusion method on MHA plates. Genomic DNA of the fungus was also isolated and amplified using PCR and its products.

Keywords: Allopurinol, Febuxostat, Uric acid, Hypoxanthine, Nitroblue-tetrazolium assay, Ninhydrin test

Isolation and Purification of Xanthine Oxidase Inhibitors from Endophytic Fungi

Summary

Xanthine oxidase plays a part in purine metabolism where it converts xanthine to uric acid and also catalyzes the conversion of hypoxanthine to xanthine. During this process it generates Reactive Oxygen Species as side production which leads to oxidative stress, the building stone of many diseases. Whereas, the excess of uric acid in the body leads to a serious condition called gout.



The incidence of gout is increasing worldwide, therefore in order to find effective treatment for the same, the field of xanthine oxidase inhibitors is being explored. Allopurinol and febuxostat are the current XOI available in market although both have some side effects therefore novel XOI is the need of the hour. Besides various natural sources, endophytic fungi are comparatively a less exploited field and it holds a treasure of bioactive compounds with therapeutic properties as seen from the literature.

This research aims at finding XOI from an endophytic fungi isolated from *Camellia sinensis.* Pure fungal strain of fungus was grown on PDA which exhibited appreciable inhibitory activity against XO. The purification of XOI was carried out by first optimizing the

mobile phase for column chromatography and then collecting the pure compounds. Out of the five different fractions collected after column chromatography, fractions 2 & 4 exhibited the best activity evaluated using NBT assay. For identification of functional groups, ninhydrin test for amine and Charring test for carbohydrates using sulphuric acid was carried out along with phytochemical testing for the presence of secondary metabolites.

The fractions thus obtained were subjected to antioxidant assay using DPPH radical scavenging system and antimicrobial test against various bacterial cultures using disk diffusion method on MHA plates.

Furthermore identification of fungus was carried out, the genomic DNA isolated was amplified using PCR and the PCR products have been sent for DNA sequencing.

Chapter 1

INTRODUCTION

1. Introduction

Gout is an arthritic disease caused by increased levels of uric acid in blood affecting 4-6 % of world's population. In this disease, excess of mono sodium urate (MSU) deposits in joints and surrounding tissues causing inflammation, severe pain and tenderness of the joints [1].Escalated serum uric acid (SUA) level leads to formation of uric acid crystals. Increased level of uric acid leads to a state called hyperurecemia which is connected to gout, having pathological threshold of 6.8 mg/dl. Although hyperurecemia is considered to be the primary reason for gout, not all people having hyperurecemia suffer from gout or develop uric acid crystals.

The probability of men suffering from gout is twice as much as that of women [2]. Gout is linked to many other co-morbidity like cardiovascular disease, chronic kidney disease, diabetes, obesity and other major diseases [3, 4]. Beside drug therapy, alteration in life style is recommended for the treatment of gout which includes therapeutic agents like non-steroidal anti-inflammatory drugs (NSAIDs), colchicine and corticosteroid. To prevent long term gout attacks urate lowering therapy (ULT) is used [4]. Urate lowering drugs are of 3 types:

(i) Xanthine Oxidoreductase (XOR) Inhibitors: Allopurinol, Febuxostat and Topiroxostat.

(ii) Uricosuric agents, the drugs which increases the excretion of uric acid, includes probenecid, sulfinpyrazone and benzbromarone.

(iii) Urate degrading enzymes like rasburicase and pegloticase degrade urate [5].

Uric acid in human beings is the end product of purine catabolism. These are heterocyclic aromatic ring compounds which form nucleotide bases (adenine and guanine) which make up DNA and RNA [6]. Purines are also constituents of various bio molecules like ATP, GTP, NADH, CoenzymeA. High amount of purine content is found in food products like dairy products, coffee, beer and meat. Other mammals and plants degrade purines to form allantoin and gyloxylate respectively [7].

Xanthine oxidoreductase (XOR), an enzyme found in various organisms from bacteria to humans, exists in inter convertible forms of Xanthine oxidase (XOD) and Xanthine dehydrogenase (XDH). However, there have been reports which show that increased level of Xanthine oxidase leads to excessive oxidative stress due to production of reactive oxygen species (ROS) [8].

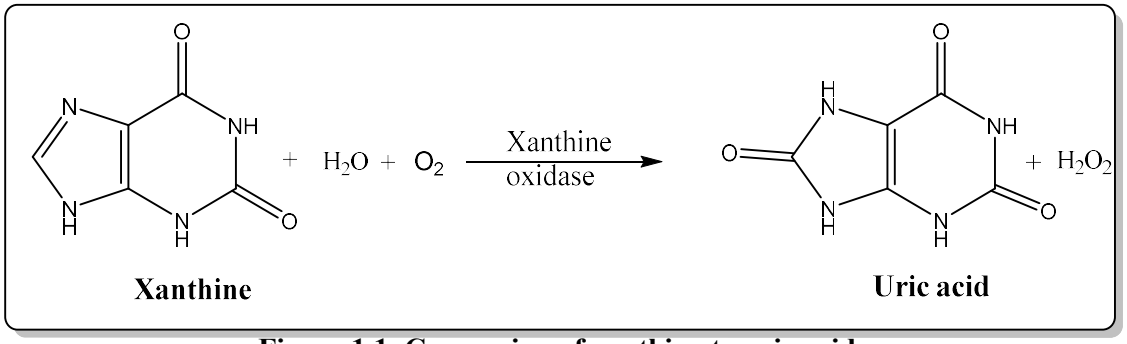


Figure 1.1: **Conversion of xanthine to uric acid.**

Therefore in order to treat long term hyperurecemia and gout, there is a growing need to explore new Xanthine oxidase inhibitors. Present drugs used to treat this medical condition have some side effects for example; Allopurinol has side effects like gastrointestinal distress, skin rashes and high fever. The other famous drug Febuxostat also suffers from side effects. Topiroxostat is used for patients that are intolerant to Allopurinol and Febuxostat [9].

Beside natural sources like plants, animals and marine organisms, endophytic fungi offer a rich source of present day drugs. Endophytic fungi reside in the interior of the host plant for a part or its complete lifetime. The endophytic fungi and the plants show mutualistic symbiosis where endophytes get nutrition and shelter from the host plant and produce susbstances which aids the survival and healthy growth of the host plant [10].The secondary metabolites facilitates increased resistant to herbivores, parasites and drought[11]. These secondary metabolites are found to show great potential use in medicine, agriculture and industry [12]. Various bioactive substances isolated from endophytic fungi includes anticancerous agent like Taxol, antimicrobial agent like Emodin, antioxidant agent like Pestacin, antifungal compound like cytochalasin D [13].

This research aims at isolation of a Xanthine oxidase inhibitor from endophytic fungi isolated from *Camellia sinensis* and its purification

Chapter 2

REVIEW OF LITERATURE

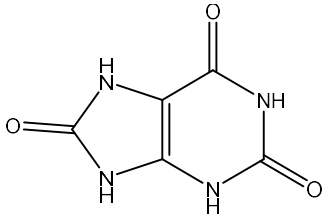
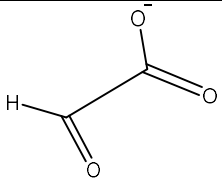
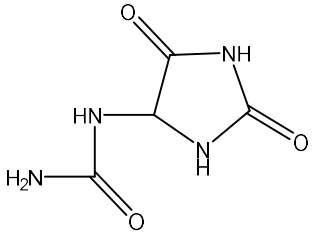
2. Review of Literature

Hyperuercemia is a condition caused by excess of uric acid in the body, which accumulates in joints, kidneys to cause severe pain and in some cases gout. Uric acid is the end product of purine catabolism in humans and the process of producing uric acid is catalyzed by an enzyme called xanthine oxidase. Inhibiting xanthine oxidase will lead to lower production of uric acid in the body and thus can help in treating hyperurecemia.

2.1 Purine metabolism

Purines perform an array of functions in the cell. Not only are they the precursor of DNA and RNA but they are also the structural component of higher nucleotides like ATP, GTP. Purines play an important role in energy metabolism, neurotransmission and in the physiology of platelet and muscles. Enzymes that carry out the purine metabolism maintain a balance between the synthesis and degradation of purines. The balanced amount of purines aids in the growth and survival of the cells [14].

Table 2. 1: End products of purine degradation in different in different organisms.

S. No.	Name of the organism	End product of Purine metabolism	Enzyme responsible	Structure
1)	Humans	Uric acid	Xanthine Oxidase	
2)	Plants	Glyoxylate	Ureidoglycolate hydrolase	
3)	Mammals (excluding humans)	Allantioin	Uricase	

In primates (counting humans), birds and reptiles, the final product of purine degradation is urate (or uric acid).Other mammals are capable of converting uric acid to allantoin using Uricase enzyme, which can be simply expelled through urine. Plants completely degrade purines to form glyoxylate and ammonia as end products, which can further be used to synthesize organic molecules that help to promote growth of the plants [15].

In humans, the process of purine degradation begins with conversion of the two nucleotides, adenosine monophosphate (AMP) to inosine and Guanine monophosphate (GMP) to guanosine with the help of nucleotidase. The nucleosides so formed, inosine and guanosine, are then converted to hypoxanthine and guanine respectively, with purine nucleoside phosphorylase (PNP). Guanine undergoes deamination to form xanthine via guanine deaminase and Hypoxanthine is oxidized to xanthine via xanthine oxidase. Xanthine is further oxidized by xanthine oxidase to form the end product, uric acid [14].

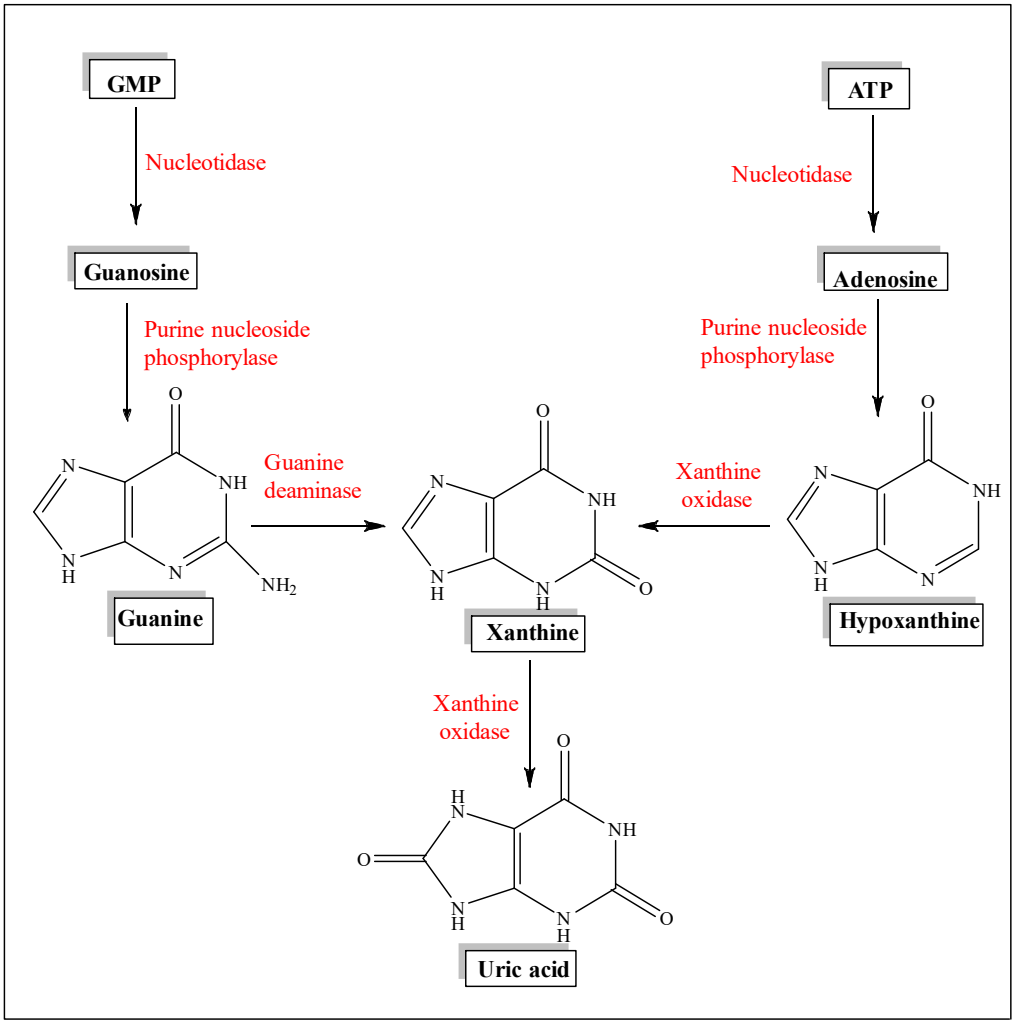


Figure 2.1: Purine degradation in humans

2.2 Xanthine Oxidase

Xanthine oxidase (or xanthine oxidoreductase) is a reactive oxygen species generating enzyme. Xanthine oxidase is formed when Xanthine dehydrogenase undergo proteolysis. It is mainly involved in purine catabolism. XOD uses hypoxanthine or xanthine as a substrate and produce superoxide and uric acid whereas XDH produce NADH instead. It metabolizes a pro drug (mercaptopurine) into the active form 6-Thioinosine 5'- triphosphate [16]. It also catalyzes the decomposition of S-Nitrosothiols (reactive nitrogen species) to nitric oxide (NO), which on aerobically reacting with a superoxide anion forms peroxynitrite [17]. It is found mainly in the gut and the liver, and also in plasma, lungs, heart, brain, kidneys. The gene which encodes this enzyme is found on the short arm of chromosome 2. Xanthine oxidase was isolated from the source, bovine milk serum.

Hypoxanthine + O_2 + H_2O → xanthine + H_2O_2

Xanthine + H_2O + O_2 → urate + H_2O_2

2.2.1 Crystallographic structure of Xanthine oxidase

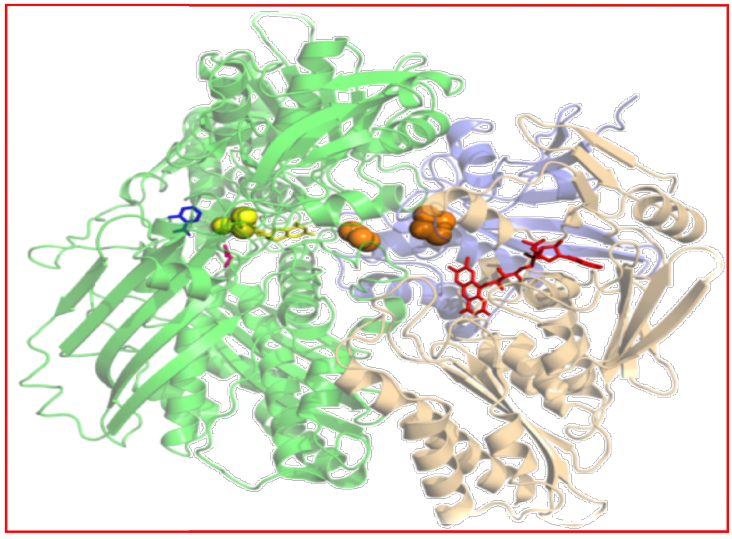


Figure 2.2: Crystallographic structure of a monomer of xanthine oxidase isolated from bovine milk.

In Mammalian xanthine oxidoreductases, each molecule is made of a 20-kDa N-terminal having two iron sulphur centres , a 40-kDa central flavin adenine dinucleotide (FAD), and a 85-kDa C-terminal molybdopterin-binding centre with four redox centres aligned linearly. The active enzyme is in the form of a homodimer (290 kDa), where each monomer acts

independently for catalysis. Each subunit consists of one molybdopterin cofactor, one FAD cofactor and two [2Fe-2S] centres that are spectroscopically different. The following figure depicts bounded FAD (in red), FeS-cluster (in orange), the molybdopterin cofactor with molybdenum (in yellow) and salicylate (in blue) [18].

2.2.2 Mechanism of action

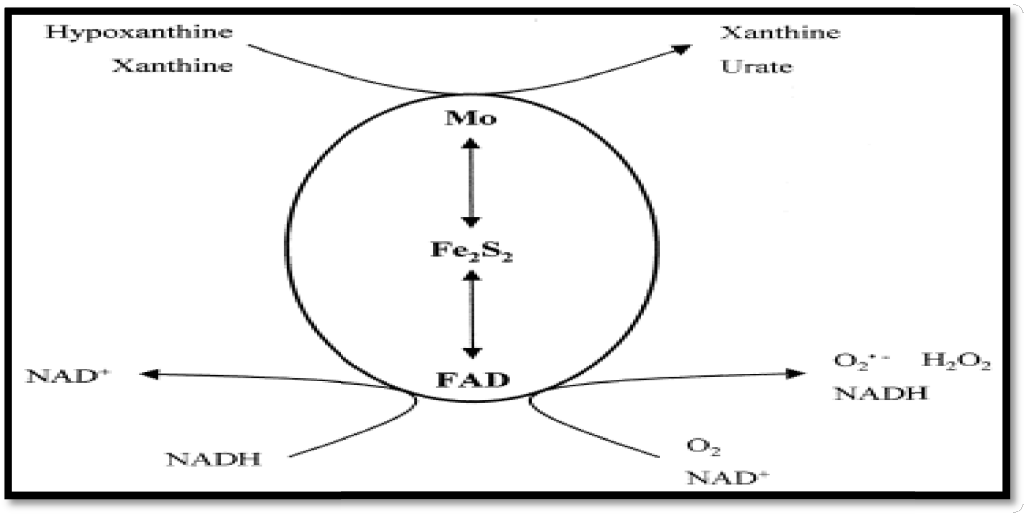


Figure 2.3: Xanthine oxidase catalyzed oxidation of hypoxanthine and xanthine

In the reaction where xanthine is oxidised to form uric acid, an oxygen atom is transferred from molybdenum centre (Mo-pt) to xanthine. The reactive molybdenum centre is reformed by the addition of water. Like other molybdenum-containing oxidoreductases, the oxygen atom added to the substrate by Xanthine oxidase originates from water and not from dioxygen (O_2). The electrons produced are immediately circulated to the FAD centre, either to NAD^+ or to molecular oxygen by the process of intra-molecular electron transfer [19].

Reactive Oxygen Species generated during metabolic processes are responsible for various physiological and pathophysiological responses. Therefore increased level of Xanthine oxidase leads to excessive oxidative stress which is the underlying reason for various cardiovascular diseases, inflammatory diseases, and tissue injuries [12]. XO also might be linked with blood pressure management [20].

2.3 Gout, Hyperurecemia and other diseases

Uric acid exists in the body mainly as urate, the salt form of uric acid. As the concentration of urate increases in blood, higher amount of uric acid crystals are formed. Hyperurecemia is characterized by increased level of uric acid in blood. The normal level of uric acid in human blood is found to be 1.5 to 6.0 mg/dL in women and 2.5 to 7.0 mg/dL in men [14]. When the level of uric acid increases from 6.8 mg/dL, crystals of uric acid arrange to form monosodium urate (MSU) deposits in joints and kidneys. Individuals suffering from gout should avoid consuming food items like organ meats, sweet breads, sweetened beverages and alcohol. Low-fat dairy products and vegetables should be inculcated in their diet. [21]

In Humans, uric acid and urate accumulates in the form of calculi in the joints or connective tissues leading to arthritis and rheumatic pain. It may also deposit in kidneys leading to kidney disease/failure. Diabetes mellitus (DM) is also a noteworthy risk factor responsible for hyperuriceamia and gout [2] Recent epidemiologic studies suggest that hyperuricaemia may alone lead to renal failure [4].

Occurrences of gout are uneven around the globe, being highly prevalent in Pacific countries. Developed countries suffer from higher incidents of gout rather than developing countries. Gout is more prevalent in men than women. Generally, gout affects men older than 40 and women after their menopausal stage [22].

2.4 Therapeutic treatment of Gout

2.4.1 Treatment of acute gout

Acute gout is a condition that lasts for few days, and the treatment ensures pain relief and also speeds up the recovery process. Therapeutic agents used to treat the condition of acute gout consist of (i) nonsteroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen, steroids like corticosteroid; (ii) the anti-inflammatory drugs like colchicine or glucocorticoid. Corticosteroids may interfere with blood pressure or regulation of glucose levels. Therefore, Colchicine is given to patients who cannot be treated using NSAIDs or corticosteroids.

None of these drugs are universally effective or completely safe. As a result, new therapies are being developed to manage acute gout, an open-labeled study showed that anakinra, an IL-1 receptor antagonist, resulted in rapid and complete pain relief without

causing any side-effects in patients who failed conventional treatments such as NSAIDs, colchicine or corticosteroids [23].

2.4.2 Management of long-term hyperurecemia

Urate lowering therapy is recommended for patients with long term hyperurecemia. Two classes of drugs which lower serum urate levels in patients are: (i) *Uricostatic* drugs, which reduce uric acid production via competitive inhibition of xanthine oxidase like allopurinol, febuxostat and; (ii) U*ricosuric* drugs, which increase the excretion of uric acid by preventing renal tubular re-absorption of urate such as sulphinpyrazone, probeneceid and benzbromarone. Febuxostat is a non-purine based xanthine oxidase inhibitor with efficacy at 80 to 120 mg/day. This lower dosage in comparison to allopurinol (300 mg/day) might be due to its selectivity to xanthine oxidase in the purine metabolism [23]. It has been found recently that vitamin C supplementation is also useful for patients suffering from gout as it lowers serum urate via a uricosuric effect.

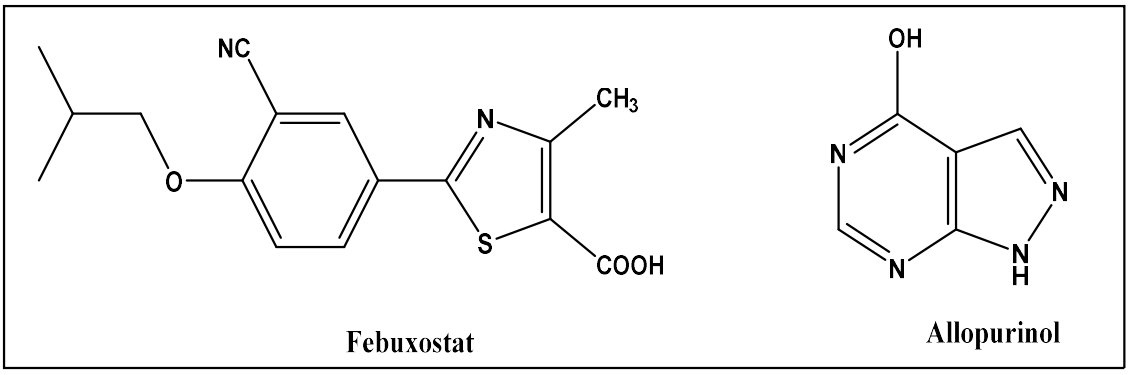


Figure 2.4: Chemical structure of some common xanthine oxidase inhibitor

2.5 Need for novel XOI

Allopurinol is normally a safe drug, but around 2% of patients exhibit hypersensitivity towards it. Common drug reactions include gastrointestinal distress, headache, liver function abnormalities, nausea, rash and worsening of renal function. In the recent years, new drugs have become available: pegloticase, a recombinant uricase and febuxostat, a novel xanthine oxidase inhibitor.

Febuxostat is less toxic than allopurinol and is therefore used in patients hypersensitive to allopurinol. Uricosuric drugs can also be given to such patients for example probenecid and the URAT1 inhibitor. Febuxostat is usually well tolerated, but some patients (~1%) may suffer from side effects like nausea, diarrhoea and headache.

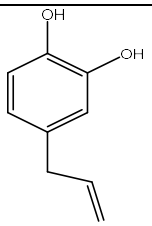
Side effects caused by allopurinol are somewhat related to its structural framework which is based on the purine and pyrimidine motifs. The rashes are a result of metabolic conversion of the drugs to corresponding nucleotides with the help of phosphoribosyl transferase. This encouraged a search for novel xanthine oxidase inhibitors that are non purine based [24].

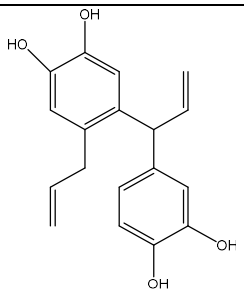
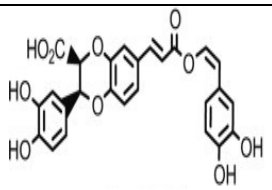
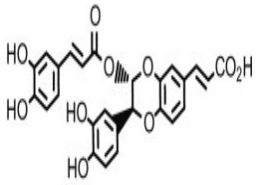
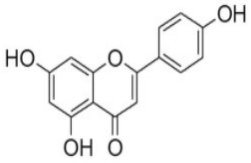
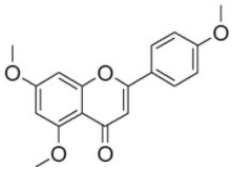
Other drugs in clinical development are primarily URAT1 inhibitors such as levotofisopam, lesinurad, arhalofenate and RDEA3170 and 3,4-dihydroxy-5-nitrobenzaldehyde (DHNB),which is a potential xanthine oxidase inhibitor.

2.6 Plants as a source of XOI

The use of plant materials is gaining renewed interest as the budding source of new drugs in the search for new the treatment of different diseases. Natural products isolated from medicinal plants propose an opportunity for the advancement of new therapeutic agents. Table 2.1 provides an insight to Xanthine oxidase inhibitors that have been isolated from plants.

Table 2.2 Some XOI isolated from plants

S. No.	Source	Name	Structure	Reference
1.	*Piper nudibaccatum*	Hydroxychavicol		[25]

2.	*Piper nudibaccatum*	Neotaiwanensol B		[25]
3.	*Hyptis rhomboids*	Hyprhombin C		[26]
4.	*Hyptis rhomboids*	Epihyprhombin B		[26]
5.	*Perilla frutescens*	Apigenin		[27]
6.	*Perilla frutescens*	4′,5,7-Trimethoxyflavone		[27]

2.7 Microorganisms as a source of XOI

Endophytes are an endosymbiotic set of microorganisms. Bacteria or fungi colonizes the intercellular and/or intracellular parts of plants, therefore they have been demonstrated to be a rich source of biological active and novel compounds that may exhibit great medicinal or agricultural potential. Various XOI from microorganisms (fungi, bacteria and algae) have been listed in the following tables.

Table 2.3 Some XOI isolated from bacteria

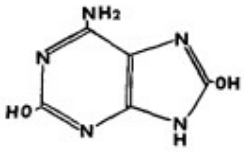
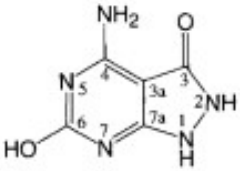
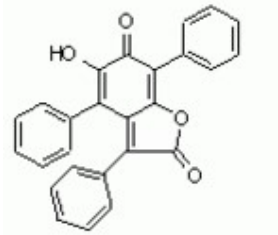
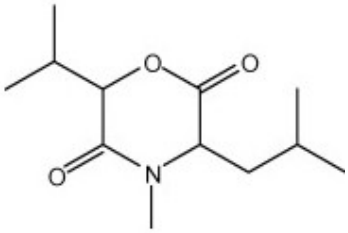
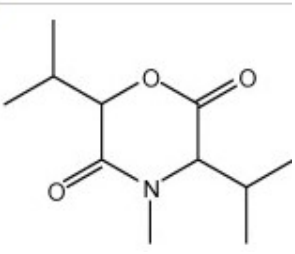
S. No.	Source	Name	Structure	Reference
1.	*Alcaligenes aquamarines*	2, 8-dihydroxyadenine.		[28]
2.	*Agrobacterium aurantiacum*	Hydroxyakalone		[29]

Table 2.4 Some XOI isolated from Fungi

S.No.	Source	Name	Structure	Reference
1.	Peniophora sanguine	5-Hydroxy-3,4,7-triphenyl-2,6-benzofurandione,		[30]
2.	*Fusarium sporotrichioides*	3-(2-methylpropyl)-6-(propan-2-yl)-4-methyl-morpholine-2,5-dione		[31]
3.	*Fusarium sporotrichioides*	3,6-di(propan-2-yl)-4-methyl-morpholine-2,5-dione		[31]

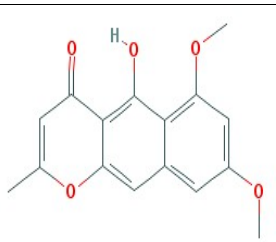
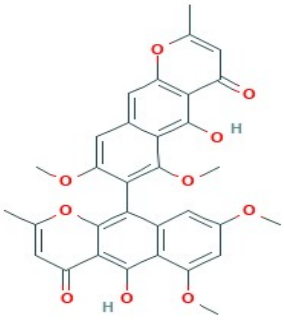
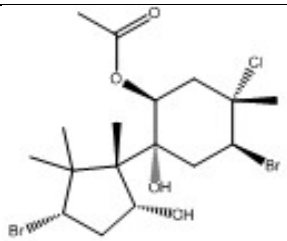
4.	*Aspergillus niger*	Rubrofusarin B		[32]
5.	*Aspergillus niger*	Aurasperone A		[32]

Table 2.5 Some XOI isolated from algae

S. No.	Source	Name	Structure	Reference
1.	*Laurencia natalensis*.	Algoane		[33]

2.8 Endophytic fungi as a novel source of bioactive metabolites

Endophytic fungi colonize the healthy tissue of the host plant and they do so in an array of relationships from symbiotic to somewhat pathogenic. There is mutualism interaction between the plant and the endophytes and therefore both the host and the fungi get benefitted. Endophytic fungi acquire energy, nutrition and shelter from the host plant and in return help to promote the growth of the plant by providing them with resistance to abiotic stress conditions like drought, light and biotic stress like herbivore attack, insects, and invasion of pathogens [11].They do so by producing a plethora of chemical substances called as secondary metabolites. These bioactive secondary metabolites have a potential use in safety and human health issues. Endophytes provide a variety of secondary metabolites with distinctive structure, like alkaloids, flavonoids, benzopyranones, chinones, quinones, steroids,

terpenoids, phenolic acids, xanthones, and others [10]. Such bioactive metabolites find wide-ranging application as agrochemicals, antibiotics, immunosuppressants, antiparasitics, antioxidants, and anticancer agents [12].

Important anticancer compounds include Taxol ($C_{47}H_{51}NO_{14}$), Camptothecin ($C_{20}H_{16}N_2O_4$), Ergoflavin" ($C_{30}H_{26}O_{14}$) and Secalonic acid D ($C_{32}H_{30}O_{14}$) [34, 35, 36]. Pestacin" ($C_{15}H_{14}O_4$) and "isopestacin", 1,3-dihydro isobenzofurans, isolated from the endophytic fungus *Pestalotiopsis microspora* displayed potential antioxidant activity[37, 38].

The bioactive compound 7-amino-4-methylcoumarin, isolated from the endophytic fungus *Xylaria* sp. YX-28 offered broad-spectrum inhibitory activity against microorganisms counting *S. aureus, E. coli, A. hydrophila, Yersinia sp., S. typhia, S. typhimurium, P. expansum, S. enteritidis, V. parahaemolyticus, C. albicans,* and *A. niger,* and was therefore recommended to be used as a food preservative [39].

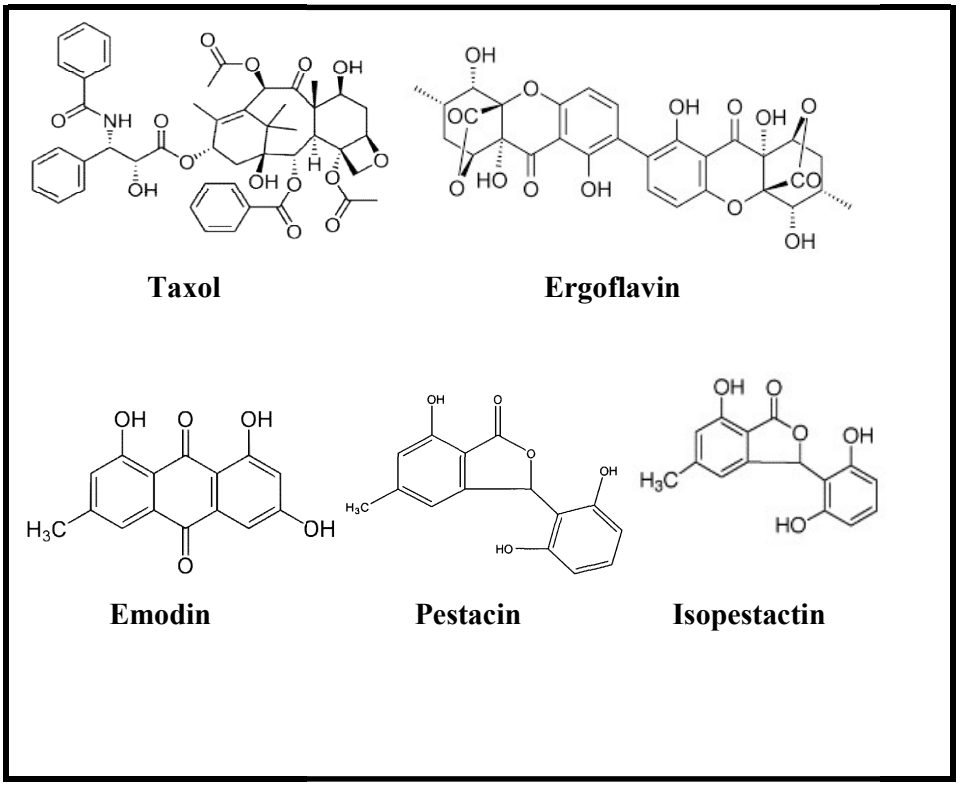


Figure 2.5: Structure of some compounds isolated from endophytic fungi

Henceforth, we aim to isolate xanthine oxidase inhibitors from endophytic fungi isolated from *Camellia sinensis*, which is a store house of antioxidants. Some herbal plant extracts possess antioxidant activity which can eliminate the oxidative and inflammatory response produced by xanthine oxidase.

Chapter 3

AIM OF THE STUDY

3. Aim of the study

1. Isolation of endophytic fungi from *Camellia sinensis*.

2. Screening of Xanthine Oxidase Inhibitors from the endophytic fungi.

3. Purification of Xanthine Oxidase inhibitor from endophytic fungi.

4. Identification of the endophytic fungi.

Chapter 4

MATERIALS AND METHODS

4. Materials and Methods

The double distilled water purified using Merck/Millipore water purification system was used for all media preparations. All the apparatus and media were autoclaved 121°C, 15 psi for 15 minutes and the discard was autoclaved at 121°C, 15 psi for 30 minutes before use or disposal. All culture handling like isolation, inoculation were done in Thermodyne Bio safety Cabinet Class I after disinfecting it with spirit and exposure of UV light for at least 20 minutes.. All chemicals were procured from Himedia. Xanthine oxidase was procured from Sigma Aldrich (source: Bovine milk). TLC Aluminium sheets Silica gel 60 from SDFCL were used for purification of compound and Whatman filter paper used was procured from GE healthcare, USA. The absorbance was observed at using Biotek throughput reader, Powerwave 340. The fungal genomic DNA isolation was carried using Wizard Genomic DNA purification kit (Promega, USA). Gel imaging was performed under UV light in Bio-Rad Gel documentation system using Quantity-1-D analysis software. Nikon binocular microscope (Eclipse E 100) was used for morphological study of the fungus. All necessary safety precautions were taken while working in the lab.

I. Isolation of Xanthine Oxidase Inhibitors

Maintenance of Pure Culture of Endophytic Fungi

Preparation of Potato Dextrose Agar plates

PDA prepared in double distilled water (39 g of PDA/L) was dispensed into Erlenmeyer flask covered with cotton plug and was autoclaved. Autoclaved PDA (22.5 ml) was dispensed onto 90mm petriplates under sterile conditions and allowed to solidify at room temperature. The plates were stored at 26 ± 2 °C until further use [40].

Sub-culturing of Endophytic Fungi

Culture from the stock was inoculated aseptically onto the sterile PDA plates using an inoculation loop which were then incubated at 26 ± 2 °C for 5-7 days till fungal mycelium growth was observed (Figure 5.1).

Production of Culture Filtrates

Endophytic culture filtrate production was carried out by aseptically inoculating mycelial disk (5mm) of 7-10 day old culture to pre-sterilized Potato Dextrose Broth (24g / L) medium kept in Erlenmeyer flasks and incubated in an incubator shaker at 26 ± 2 °C, 120 rpm for 10-

15 days. After the incubation period is over (Figure 5.2), spent broth was separated from mycelia mass by filtration through Whatman filter paper no.4 followed by centrifugation at 12,000 rpm for 10 minutes. The cell free filtrate was kept at -20 ℃ until further use [41].

Isolation of Bioactive fractions

The cell free filtrate (50 ml) was subjected to liquid-liquid extraction for isolation of bioactive fraction [42] by extracting with appropriate solvents (3 × 100 ml) .The organic layer was pooled together and dried over anhydrous sodium sulphate. Evaporation of the organic solvent gave crude product which was weighed and reconstituted in DMSO. The fractions were stored at - 20 °C until further use (Table 5.1)

Qualitative Screening of Xanthine Oxidase Inhibitors

Qualitative screening of Xanthine oxidase inhibitors was done using Xanthine-Nitroblue tetrazolium (NBT) agar plate assay. The plates were prepared with Agar (0.8% w/v), xanthine (1.5 mg/ml) and NBT (0.11 mg/ml), 5mm wells were made using a sterile cork borer. In each well tris-HCl buffer (5 µL of 10 mmol/L) was suspended followed by addition of xanthine oxidase (10 µL of 0.04U) and culture filtrate (30 µL) while blank was used as standard and allopurinol (50 mmol/L) as control. The plate was kept at incubation 37 °C for 24h. After the incubation period, the plates were observed for reduction of blue colour halo formation indicating the presence of Xanthine oxidase inhibitors. The halo diameter was recorded in mm [43].

II. Purification of Xanthine Oxidase Inhibitors and its Bioactive Assays

TLC Analysis

Reconstituted chloroform extract was analysed using TLC with different solvent systems (Figure 5.4), toluene was used as the optimized solvent system.

Crude compound dissolved in methanol was spotted on the TLC plate and allowed to move in toluene, the mobile phase to reach ¾ th of the TLC plate's height. It was then air dried and TLC chromatogram was observed under short UV light (254 nm) in the TLC UV Cabinet.

Column Chromatography

Column chromatography was performed in a glass column (55 cm × 1.1 cm) with silica gel (60-120 mesh) as stationary phase. The sample was loaded on the column bed (height 45 cm) and eluted with toluene as mobile phase. Different fractions were collected and observed on TLC with crude as standard. Fractions appearing at same height were pooled and solvent evaporated to get pure compound.

Phytochemical Screening

Phytochemical screening of various secondary metabolites was carried out with stock solutions of 1 mg/ml as per the test performed in Table 4.1.

Table 4.1 : Phytochemical screening of various secondary metabolites

Secondary metabolite	Test	Methodology	Result(s)	References
1.Alkaloids	a)Wagner Test	1ml of extract was mixed with 2ml of HCl, heated on steam bath. Few drops of Wagner Reagent was added to it.	Creamy white Precipitate	[44]
2. Flavonoids	b) NaOH Test	To the extract dilute NaOH was added followed with addition of dilute HCl.	Yellow in NaOH, Colorless in HCl	[45]
3.Phenol	a) Ferric Chloride Test	1ml of extract was mixed with 2 ml solution of (2%) Ferric Chloride.	Blue-green, Red color.	[46]
4.Terpenoids	a)Liebermann – Burchardt Test	In 1ml of extract 2ml of Chloroform, 2ml of Acetic anhydride and 2 ml Concentrated sulphuric acid was added from the sides of the test tube.	Pink or Red Color	[47]

5.Tannin	a)Braemer's Test	A few drops of 0.1% Ferric chloride was added to the 1 ml extract.	Dark blue or Greenish-grey color	[48]
6.Steroids	a)Liebermann – Burchardt Test	In 1ml of extract 2ml of Chloroform , 2ml of Acetic anhydride and 2 ml Concentrated sulphuric acid was added from the sides of the test tube.	Dark green Color	[47]

Functional group identification

(i) Amine: Fractions (2 & 4) were spotted on a TLC plate along with standard amine and dipped in Ninhydrin solution (1% w/v). The plate was air dried and observed for purple spot to confirm the presence of an amine group.

(ii) Carbohydrates: Fractions (2 & 4) were spotted on a TLC plate with sucrose as reference and dipped in sulphuric acid solution (1ml in 10ml methanol). The plate was air dried and heated to 100 °C, black spot indicates the presence of carbohydrates.

Quantitative Screening of Xanthine Oxidase Inhibitors

The bioactive residue was screened for xanthine oxidase inhibition using microtitre plate based NBT assay in triplicates as mentioned by Agarwal et al., 2009 [49] with slight changes. Briefly bioactive residue (25 µL) was incubated with XO (15 µL of 0.2 U) for 1hr at 37 °C. The reaction started with the addition of Xanthine (134 µL of 2mM) and NBT (25 µL of 2mM) and kept for 30 min incubation at 37 °C. Positive control comprised of Allopurinol (6.25 mM) whereas blank consists of Tris-HCl buffer (6.58 mM), XO, Xanthine and NBT. The absorbance was observed (at 575 nm) using microplate spectrophotometer, to estimate the amount of formazan formed which was calculated for the activity of the compound.

Antioxidant assay

Antioxidant assay of the compounds were carried out as the procedure of Ho et al. (2012) [50] with some modifications. In the microtitre plates, reaction mixture of sample extracts (30 µl) was mixed with DPPH solution (220 µL). The control comprised of methanol and DPPH solution and methanol was used as blank. The titre plate was then incubated at 25 °C for 30 min in dark. The test was performed in triplicates and data were represented as mean ± SD [51].

Antimicrobial activity

Antibacterial activities of the five fractions were evaluated using disk diffusion method on Mueller-Hinton Agar (MHA) plates, *Staphylococcus aureus* (MTCC 737, MTCC 96) *Bacillus subtilis* and *Escherichia coli* were used for the antibacterial assay. The test organisms were spread aseptically across the MHA plates using a cotton swab. Disk were suspended with the test samples (20 µL) and incubated at 37°C for 24 hours. After the incubation period, the bacterial growth inhibition zones were observed and the diameters were recorded in millimetre. The data was represented as mean ± SD [52].

III. Identification of Endophytic fungi

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, WA, PLA, SNA and MEA for 4 weeks at 26 ± 2 °C for growth optimization. For microscopic features, mycelia was placed in a water droplet on the glass slide, teased with a fine tip needle and stained with Lactophenol 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 [53]. Morphological characters such as colony colour, appearance were observed at 10X, 40X and 100X magnifications using microscope and observations were noted (Figure 5.10).

Molecular Identification of endophytic fungi

DNA Isolation

The fungal genomic DNA isolation was carried on 5-7 day old culture, 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 of DNA Dehydration buffer (50 µL). The qualitative estimation of DNA isolation was done by agarose gel electrophoresis.

Gel Electrophoresis

Agarose gel (0.8 %) wells containing ethidium bromide (0.5 µg/ml) was casted in the electrophoresis apparatus with running buffer (1X TAE) and the DNA samples mixed with 6X dye were loaded. A voltage of 50 V for 1h was applied and the DNA fragments were visualized under UV Transilluminator and gel imaging was done.

PCR Amplification

PCR reaction was carried out using primers ITS 1 (5’ TCC GTA GGT GAA CCT GCG G 3’) and ITS 4 (5’ TCC TCC GCT TAT TGA TAT GC 3’) [54]. Amplification of isolated DNA was performed in a 25 µL reaction mixture (Table 4.2) consisting of extracted fungal DNA, primers (ITS 1 and ITS 4), dNTP, Taq DNA Polymerase in 10 X Taq buffer. The thermal cycling conditions are described in Table 4.3. The PCR products were examined with gel electrophoresis in agarose gel (1.5 %) and gel imaging (Figure 5.11) was done as described earlier.

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 AND DISCUSSIONS

5. Results and Discussions

Endophytic fungi and plants show mutualistic symbiosis. Secondary metabolites generated by the fungi have been found to show great potential for the development of therapeutic agents. The work presented here aims to exploit the secondary metabolites of a fungus, #1CSSTOT, isolated from medicinally important plant *Camellia sinensis* for xanthine oxidase inhibition activity. The fungus was collected from Ooty, Tamil Nadu in India. Thus the work presented here has been divided into three parts.

(i) Isolation and identification of active metabolites
(ii) Purification and identification of pure compounds
(iii) Molecular and morphological identification of fungus

(i) Isolation and Identification of Active Metabolites

Pure culturing and long term storage of the endophytic fungi

Glycerol stock of the fungal strain (#1CSSTOT), maintained at 4 °C on PDA slants, was sub-cultured in PDA plates which were kept at 26 ± 2 °C (Figure 5.1).

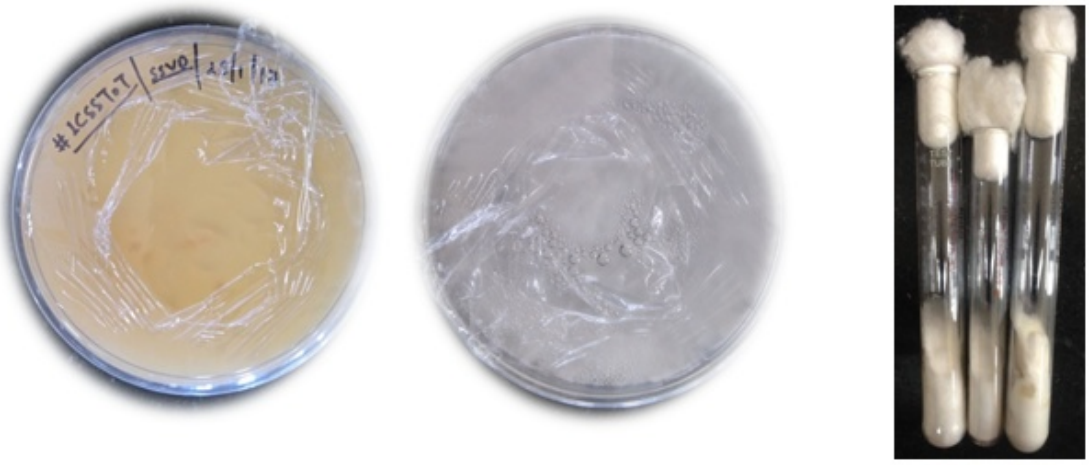


Figure 5.1: Culture maintained on (i) PDA plates and (ii) PDA slants

Production using submerged fermentation

Sub-cultured mycelia was inoculated into PDB under sterile conditions in the laminar flow and subjected to incubation at 26 ± 2 °C, 120 rpm for 8-10 days. After complete growth (Figure 5.2(i)) and separation of the mycelial mass using whatman filter paper, pH of the

filtrate was found to be acidic (5.2) and weight of thee biomass is equal to 0.69 g. The volume of the filtrate was found to be 54.5 ml starting with 100ml of PDB.

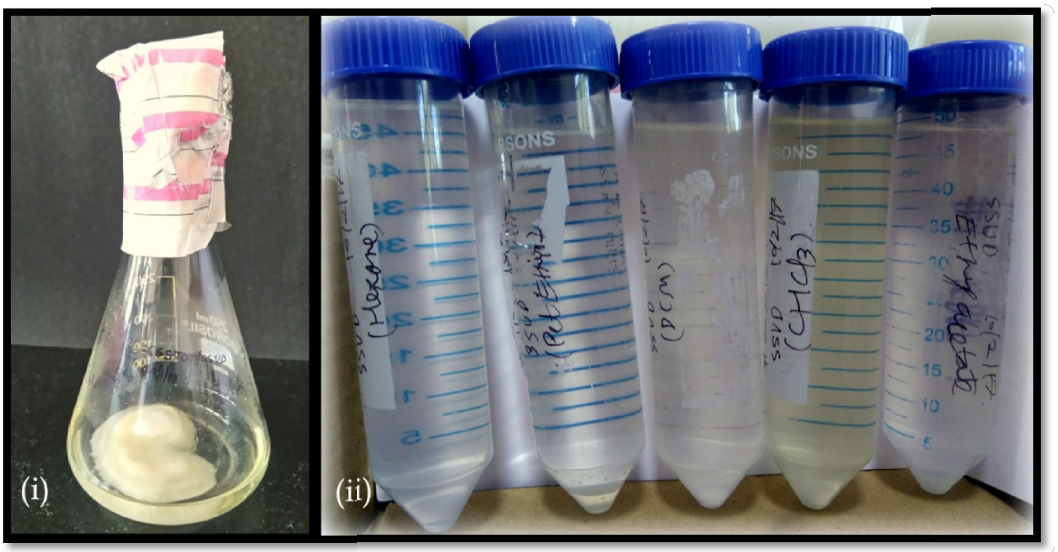


Figure 5.2: (i) Growth of fungus (#1CSSTOT) after 8-10 days (ii) Solvent extraction of cell free filtrate with various solvents

Isolation of bioactive fractions

The light yellow coloured cell free filtrate was subjected to liquid-liquid extraction (three times) using different solvents (Figure 5.2 (ii)).The organic layer containing secondary metabolites was made free from traces of moisture by using anhydrous sodium sulphate and evaporating to get crude mixture. Table 5.1 shows that maximum amount of secondary metabolites was obtained from chloroform fraction (25 mg) using 50 ml of filtrate.

Table 5.1: weight of secondary metabolites obtained after solvent extraction

S. No.	Solvent	Yield of bioactive residue (mg)
1.	Hexane	7
2.	Pet ether	9
3.	Dichloromethane	12
4.	Chloroform	25
5.	Ethyl acetate	19

The dried organic mass after evaporation of the solvent was reconstituted in DMSO/Methanol.

Screening of XOI from endophytic fungi

All the reconstituted extracts were screened for XO inhibition using Xanthine-NBT plate assay with allopurinol as standard. The assay is based on appearance of blue colour due to reduction of NBT into formazan. However, in case XO is inhibited by the secondary metabolites of the fungus there will be reduction in the blue colour (Figure 5.3).

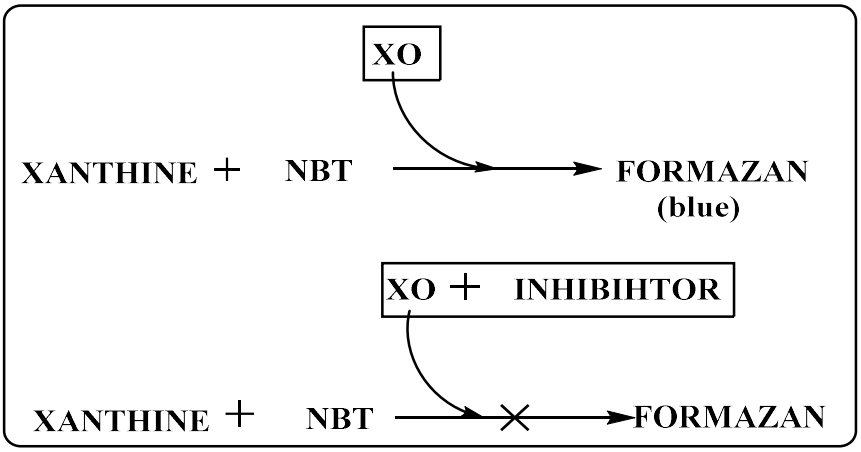


Figure 5.3: Formazan formation in the presence of XO

The chloroform extract of secondary metabolites isolated from the fungus(#1CSSTOT) displayed the best potential XO inhibition and were taken up further for isolation of pure compounds from the crude extract (data not given).

(ii) Purification of Crude Extract:

TLC Analysis and Column Chromatography

Chloroform fraction was further worked on to isolate pure compounds with XO inhibitory activity. A combination of different solvent systems was used to optimize the best separation. The results of solvent system showing best separations have been compiled in table 5.2.

From Figure 5.4 it can be seen that toluene (100 %) gave best separation of five spots and R_f values of all the spots has been mentioned in Table 5.3.

Table 5.2: TLC separation using various solvent systems

S. No.	Solvent system used	Ratio used	No. of spots
1.	Hexane	100%	6
2.	Hexane: Ethyl acetate	80:20	2 merged
3.	Hexane: Ethyl acetate	50:50	2 merged
4.	Toluene	100%	5

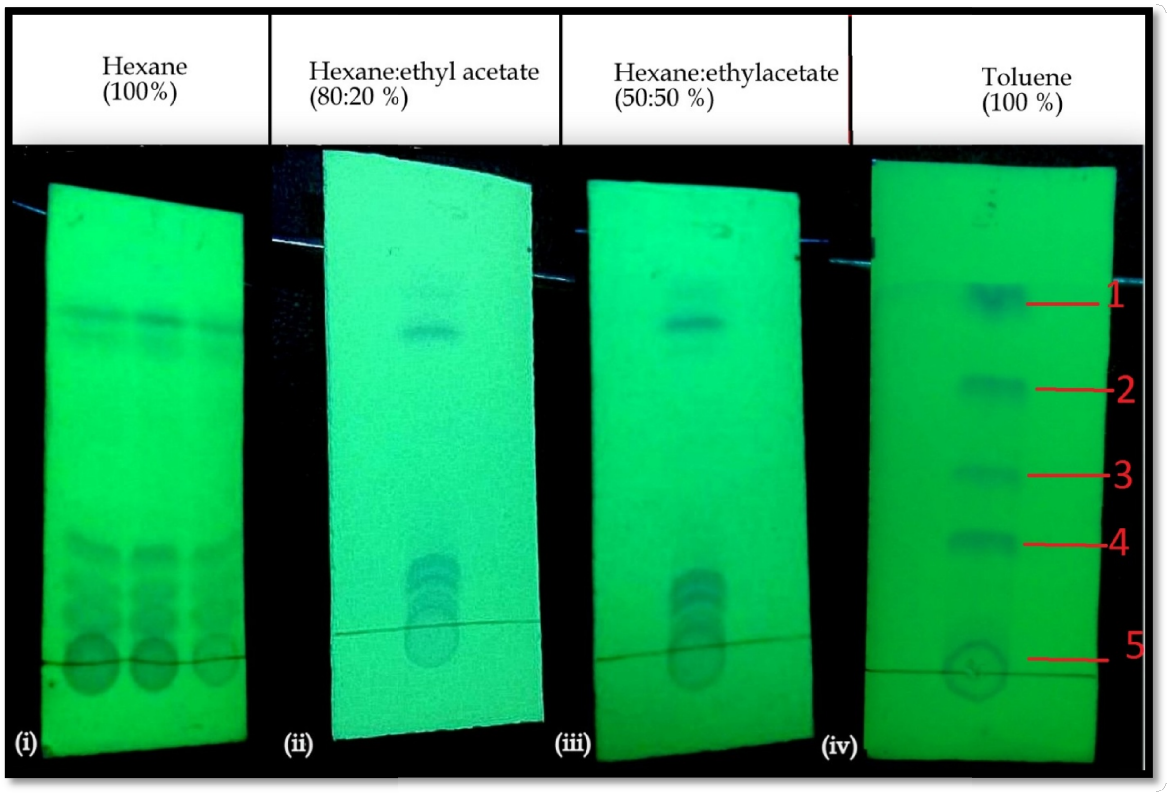


Figure 5.4: TLC run indifferent solvent systems

Retention factor was calculated for every spot using the formula:

$$\text{Retention factor} = \frac{\text{distance travelled by the solute from base line}}{\text{distance travelled by the solvent front from the base line}}$$

Table 5.3: R_f value of various spots on the TLC run in toluene

S. No.	Compound	R_f value
1.	Spot 1	1
2.	Spot 2	0.66
3.	Spot 3	0.5
4.	Spot 4	0.35
5.	Spot 5	0

Column chromatography of the chloroform fraction was performed using SiO_2 as stationary phase and toluene as mobile phase after absorbing the reconstituted chloroform extract in dichloromethane (DCM). Fractions were identified using TLC and pooled together to get five components corresponding to five spots. Each of these fractions was subjected to other biological assays besides the quantitative estimation of XO inhibition.

Qualitative Assay of Xanthine Oxidase Inhibition

In the qualitative screening of five fractions of the chloroform extract, using xanthine-NBT plate assay, allopurinol depicted reduction in halo size by 38.35% indicating XO inhibition. It was observed out of the five fractions fraction 4 and fraction 2 showed the best activity of inhibition of 32.06 % and 26.75 % respectively. These two fractions were further tested for functional group identification. Further, the inhibitory potential of the fractions were established with spectrophotometric NBT assay.

Table 5.4: Xanthine- NBT agar plate assay showing XO inhibition

S. No.	Compound	Diameter of halo (mm)	% Inhibition
1.	Allopurinol	11.5 ± 0.57	38.3 ± 0.03
2.	Fraction 1	15.3 ± 0.57	17.8 ± 0.02
3.	Fraction 2	13.6 ± 0.57	26.7 ± 0.02
4.	Fraction 3	14 ± 0.57	24.9 ± 0.03
5.	Fraction 4	12.6 ± 0.57	32.0 ± 0.04
6.	Fraction 5	14.5 ± 0.57	22.2 ± 0.03
7.	Control	18.4 ± 0.57	0

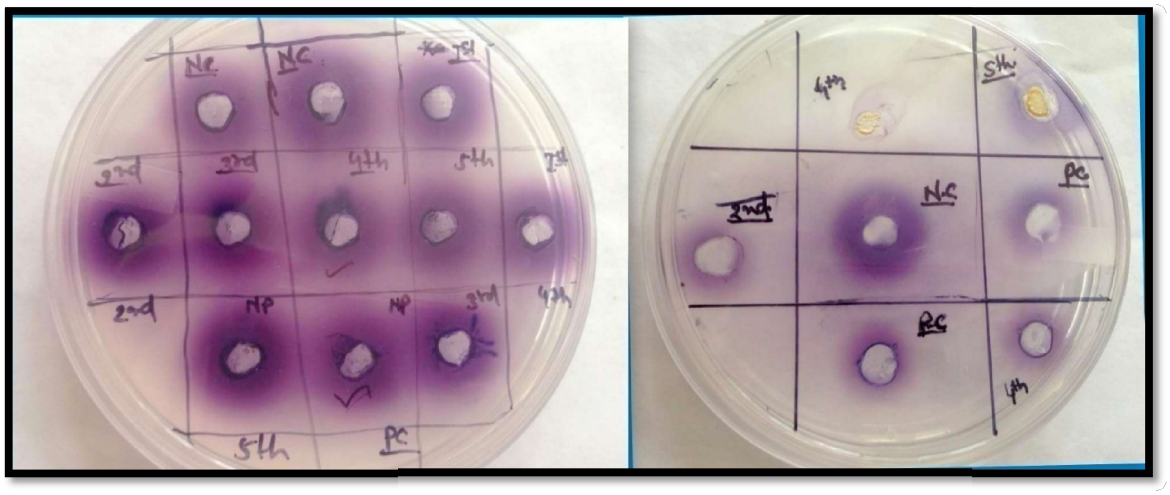


Figure 5.5: Xanthine-NBT agar plate assay of different fractions of chloroform.

Phytochemical Screening

All the fractions of the chloroform extract were subjected to phytochemical screening for various secondary metabolites in order to conclude the structural framework of the isolated and purified compound. The test for phytochemicals like phenol, steroids, alkaloids, phenols, tannin and terpenoids showed no positive result (Figure5.6).A deeper study of phytochemical is required.

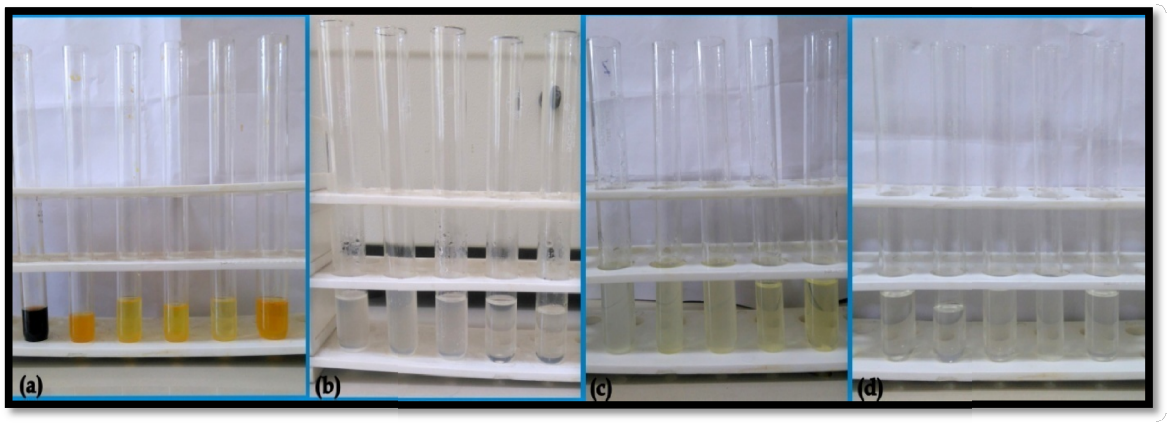


Figure 5.6 Phytochemical test results for (a) Phenols, (b) Flavonoids, (c) Alkaloids, (d) Libermann-Burchardt test.

Spot test for Amine and Carbohydrates

(i) Amine: Ninhydrin when reacts with primary or secondary amines gives a purple colour compound known as Ruhemann's purple. Figure 5.7(i) depicts that none of the two fractions (2&4) show positive result for amines.

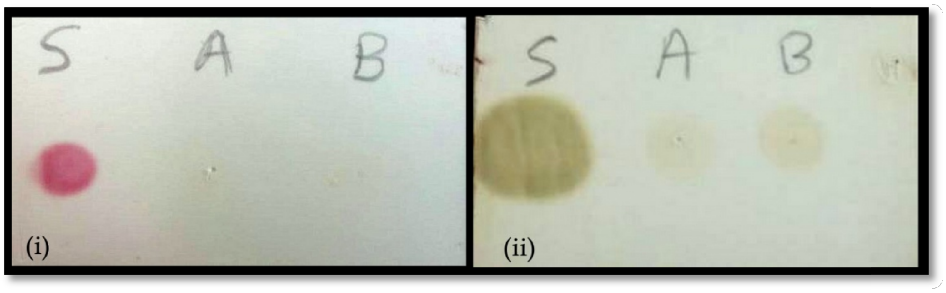


Figure 5.7: Spot test for (i) amines and (ii) carbohydrates where, A and B are fraction 2 and fraction 4 respectively

(ii) Carbohydrate: Sucrose was used as a standard for detection of carbohydrates. Sulphuric acid acts as a dehydrating agent and eventually causing charring of sugar which explains the black colour so formed. Both the fractions gave negative result for carbohydrates. This black color also appears as a result of loss of water of crystallization which can possible explain the lightly charred spot B as shown in Figure 5.7(ii).

NBT assay for Quantitative estimation of XO inhibition

The assay was performed by measuring absorbance at 575 nm to estimate formazan formation in the presence of purified fraction. Decrease in the absorbance was observed for the fractions that inhibited XO due to non-formation of reactive oxygen species (ROS) that converts NBT into formazan. Control used in this case was allopurinol. Table 5.5 Shows that fraction-4 which was comparatively polar gave the maximum inhibition of 44.3 ± 0.1 %.

Table 5.5 Quantitative XO inhibition of various compounds using NBT assay

S. No.	Compound	% Inhibition
1.	Allopurinol	63.94 ± 0.01
2.	Fraction 1	24.65 ± 0.05
3.	Fraction 2	32.60 ± 0.01
4.	Fraction 3	15.61 ± 0.07
5.	Fraction 4	44.35 ± 0.09

6.	Fraction 5	3.34 ± 0.09

Antioxidant assay

This assay is based on decrease in the absorbance at 517nm for DPPH on addition of compound due to free radical scavenging activity. The assay was performed both for the fractions isolated with liquid-liquid extraction (Table 5.6) and the five purified chloroform spots (Table 5.7) with quercetin as standard.

DPPH scavenging activity (%) = $[A_{control} - A_{test}] / A_{control} \times 100$

Here again chloroform fraction showed best radical scavenging activity and the most polar spot (spot 5, figure 5.4) was the best for antioxidant activity**.**

Table 5.6: Antioxidant activity of different extracts of fungus #1CSSTOT

S. No.	Compound	% Free radical scavenging activity	Concentration (µg quercetin/ mg extract)
1.	Hexane	26.65 ± 0.94	10.78
2.	Pet ether	15.48 ± 0.70	3.41
3.	DCM	15.31 ± 0.74	3.30
4.	Chloroform	34.31 ± 0.89	15.83
5.	Ethyl acetate	10.67 ± 0.96	0.24

Table 5.7: Antioxidant activity of various fractions of the chloroform extract

S. No.	Compound	% Free radical scavenging activity	Concentration (µg quercetin/ mg extract)
1.	Fraction 1	44.33±0.33	22.45
2.	Fraction 2	37.11±3.07	17.68
3.	Fraction 3	33.70±0.74	15.43
4.	Fraction 4	33.58±1.07	15.36
5.	Fraction 5	47.79±0.24	24.73

Antimicrobial activity

Antibacterial activity of all the five fractions were performed against four stains namely, *Staphylococcus aureus* (MTCC 737, MTCC 96) *Bacillus subtilis* and *Escherichia coli.* Table 5.8 and figure 5.8 Shows the result against all the pathogens. The antimicrobial test aims at testing the sensitivity of the compound for pathogenic bacteria. This test helps us to determine the affect of the compound on microbial flora when administrated into the body.

In case of *E.coli*, Fraction 2, Fraction 4, fraction 5 showed appreciable antibacterial activity. Out of which fraction 4 depicts the best zone of inhibition. Whereas in *Bacillus subtilis,* all the fractions showed moderate activity. Fraction 1 can be considered to be the best among them.

In the antibacterial test against MTCC 96, fraction 4 depicts the best inhibition zone. All fractions showed moderate activity against MTCC 737, out of which fraction 5 showed the best activity.

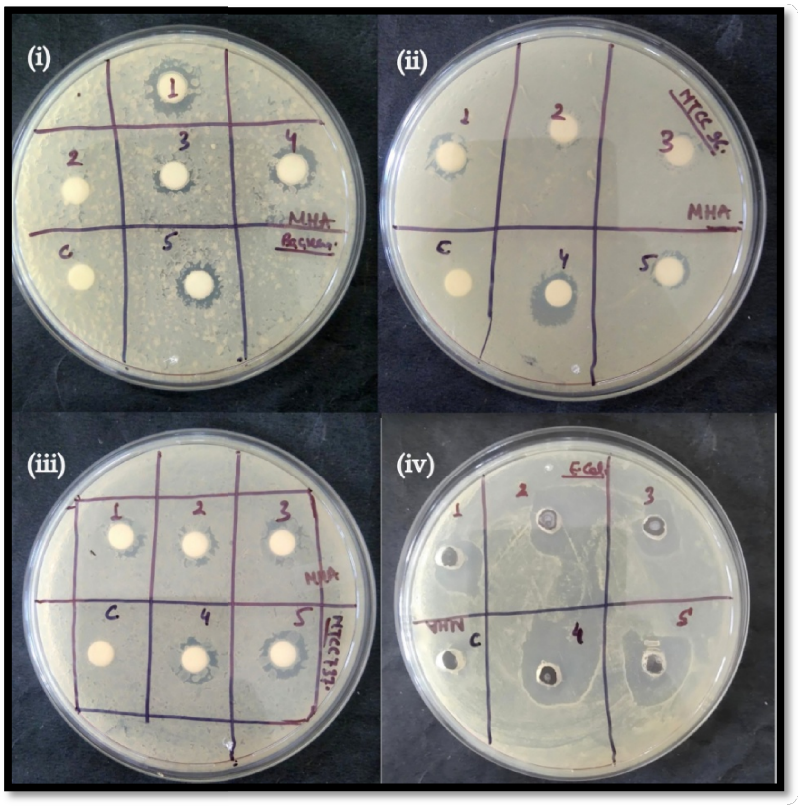


Figure 5.8: Antimicrobial activity of the five fractions against (i) *Bacillus subtilis*, (ii)MTCC 96, (iii) MTCC 737 and (iv) *E. coli*

Table 5.8: Inhibition zone diameter of various fractions of fungal extracts against different microbes.

S. No.	Name	Zone Diameter in case of *Bacillus subtilis* (mm)	Zone Diameter in case of *E. coli* (mm)	Zone Diameter in case of MTCC 737 (mm)	Zone Diameter in case of MTCC 96 (mm)
1.	Fraction 1	11.33±0.57	12±0	9±1	9.33±0.57
2.	Fraction 2	7±0	17.33±2.3	9.66±0.57	7.66±0.57
3.	Fraction 3	9.66±0.57	12.66±2.51	10.33±0.57	8±0
4.	Fraction 4	10.66±0.57	19±1.73	10.66±0.57	11.66±0.57
5.	Fraction 5	10±0	17±1	11.66±0.57	8.66±0.57
6.	Control	7	7	7	7

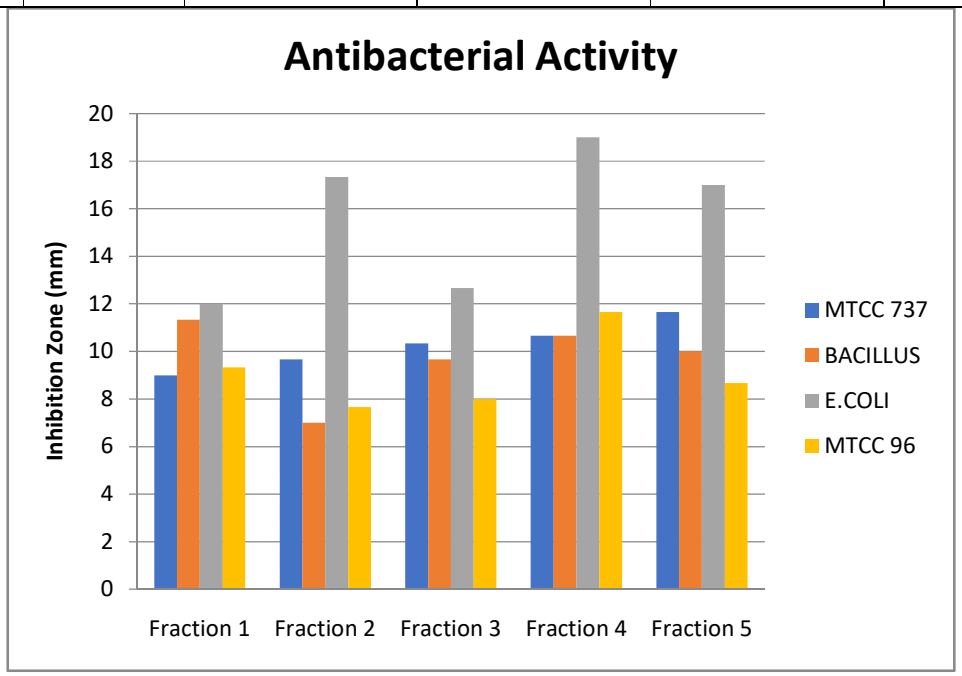


Figure 5.9: Antibacterial activity of various fractions against different microbes.

(iii) Molecular and Morphological Identification of Endophytic fungi

Morphotaxonomy

The endophytic fungus #1CSSTOT produced white, fast growing floccose to downy colonies over PDA and MEA medium, colony was white in colour from front and light brown colour from back (Figure5.10) over PLA, SNA and WA colony was white in colour from front and reverse side. Appearance was floccose to downy fungus does not produce any pigment or odour.

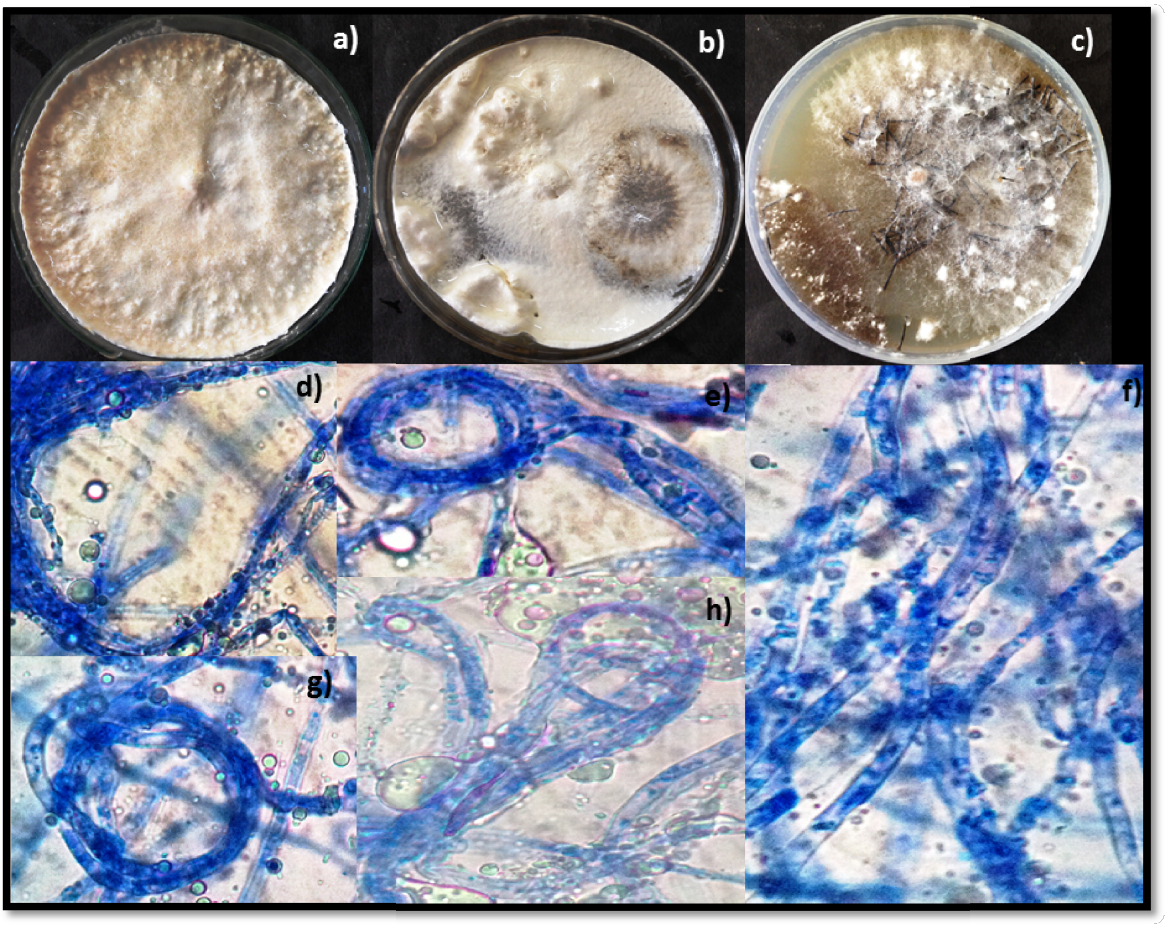


Figure 5.10: Morphological features of #1CSSTOT after twenty days of growth, (a-c): Culture characteristics on PDA, MEA and PLA, (f &h): Hyphae stained with lactophenol cotton blue on PLA, (d, e & g): Hyphae forming coils on SNA & WA

The fungus did not produce any reproductive structure like stromata, conidia, conidiogenus cells or ascospores after 2-4 weeks of incubation. Hence, on the basis of morphological and microscopic characteristics, we could not identify the potential endophytic fungi #1CSSTOT for identification and confirmation of fungus.

Molecular Identification of Endophytic Fungi

The concentration of the genomic DNA isolated from the fungus was found to be approximately 35 ng/µl .PCR is a process of rapid amplification of DNA by using specific primers so as to get large amount of DNA fragment of desired sequence length. ITS1– 5.8S – ITS 2 rDNA sequence was amplified using universal primer pair ITS 1 and ITS 4, synthesized by Integrated DNA Technologies (IDT).

Agarose gel (1.5%) was used to resolve PCR products. The size of the DNA was determined according to its mobility alongside of 5000bp ladder. The approximate size was determined to be between 550- 600 bp and was comparable to amplify ITS region (Figure 5.11). ITS was amplified in order to characterize the fungi at molecular level.

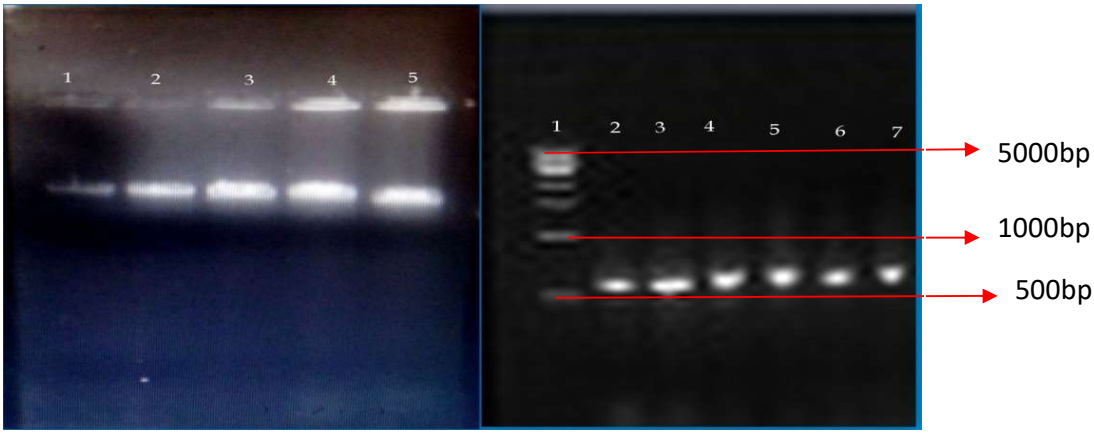


Figure 5.11: Agarose gel electrophoresis of (i) genomic DNA isolated from #1CSSTOT (Left) and (ii) PCR product of isolated genomic DNA where lane 1 is ladder of 5000 bp and lane 2-7 are PCR products (Right)

The amplified DNA was sent for sequencing of amplicon at Eurofins Genomics India Pvt. Ltd., Bangalore.

Chapter 6

CONCLUSION

6. Conclusion

The current study revealed that beside the natural sources like plants, bacteria and algae, endophytic fungi are capable of producing xanthine oxidase inhibitors. The fungus under study #1CSSTOT, isolated from *Camellia sinensis*, exhibited potential xanthine oxidase inhibition.

The chloroform extract of the fungus showed maximum activity and therefore was further taken up for purification using column chromatography. The five fractions obtained after the column chromatography were not only subjected to quantitative XOI assay but also antibacterial and antioxidant assays. Fraction 4 and Fraction 2 (from column chromatography) showed the best XO inhibition activity out of the five fractions.

Phytochemical screening and functional group detection was also performed to identify the structural framework of the pure compound responsible for the XO inhibition. Further work is required to confirm functional groups.

Future study aims at structure elucidation of the pure compounds which gave XO inhibitory activity, these compounds are a novel source for XO inhibitors and can help in the treatment of gout.

Chapter 7

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