Isolation and Characterization of Xanthine Oxidase Inhibitor from Endophytic Fungi of *Aegle marmelos*

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In
Chemistry

Submitted by

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June - 2018

CANDIDATE's DECLARATION

I hereby declare that the thesis entitled **"Isolation and Characterization of Xanthine Oxidase Inhibitor from Endophytic Fungi of *Aegle marmelos*"** is an authentic record of my work carried out as partial requirement for the award of the degree of Master of Science in Chemistry at School of Chemistry and Biochemistry, Thapar Institute of Engineering and Technology, Patiala, India under the supervision of Dr. Manmohan Chhibber and Dr. Sanjai Saxena during December 2017 to June 2018. 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.

Pallavi

Date: June, 2018

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

30/6/2018

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I owe my gratitude to all those people who have made this research work possible and because of whom my project experience has been one that will cherish forever.

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I also express my sincere thanks to all the lab members and my friends for their whole-hearted support and cooperation.

I also take this opportunity to thank my family for their constant support and blessings.

Pallavi Garg

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Abbreviations

XO	Xanthine Oxidase
NBT	Nitroblue Tetrazolium
PCR	Polymerase Chain Reaction
DNA	Deoxyribonucleic Acid
NSAID	Non-Steroidal Anti Inflammatory Drugs
ULT	Urate Lowering Therapy
ROS	Reactive Oxygen Species
ATP	Adenosine triphosphate
GTP	Guanosine triphosphate
NADH	Nicotinamide adenine dinucleotide
XDH	Xanthine Dehydrogenase
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
TEAC	Trolox Equivalent Antioxidant Capacity
TPC	Total Phenolic Content
TFC	Total Flavonoid Content
MHA	Mueller Hinton Agar
SDA	Soubard's Dextrose Agar
SNA	Synthetic Nutrient Agar
MEA	Malt Extract Agar
PLA	Pine Leaf Agar
WA	Water Agar
TAE	Tris Acetate EDTA
FTIR	Fourier Transform Infrared
ATR	Attenuated Total Reflection
NMR	Nuclear Magnetic Resonance

Abstract

Hyperuricemia is initiated by the overproduction or underexcretion of uric acid in the body ultimately leading to gout. The key enzyme responsible for this pre-disposing factor is xanthine oxidase (XO) which oxidises xanthine to uric acid. The work presented here aims to isolate the xanthine oxidase inhibitors of the pre-screened endophytic fungi isolated from *Aegle marmelos*. The fungus extract after evaluation of XO inhibition activity was subjected to coloumn chromatography for isolation of purified compounds. All the fractions obtained were subjected to qualitative NBT test for maximum inhibition towards XO. Fraction 1 which is having the least polarity showed best results that was then subjected to antioxidant and antimicrobial assay. The fraction was subjected to different phytochemical tests for the presence of various secondary metabolites. Genomic DNA of the fungus was isolated and amplified using PCR. The pure fraction was then subjected to different spectroscopic techniques for characterizing the compound.

Keywords: Hyperuricemia, Xanthine, Xanthine oxidase, Allopurinol, *Aegle marmelos.*

INTRODUCTION

Gout is one of the most prevalent inflammatory arthritis which is activated by crystallization of monosodium urate ions inside the joints and is usually linked with hyperuricemia [1]. Since the pk_a value of uric acid is 5.8 thus it is a weak acid, so it particularly exists as urate ion at physiological pH (Figure 1). This increase in concentration of urate in physiological fluids is responsible for escalation in crystal formation ultimately leading to super-saturation. Various studies conducted on human population demonstrate a positive relationship between how serum urate levels enhance the future possibility of gout [2].

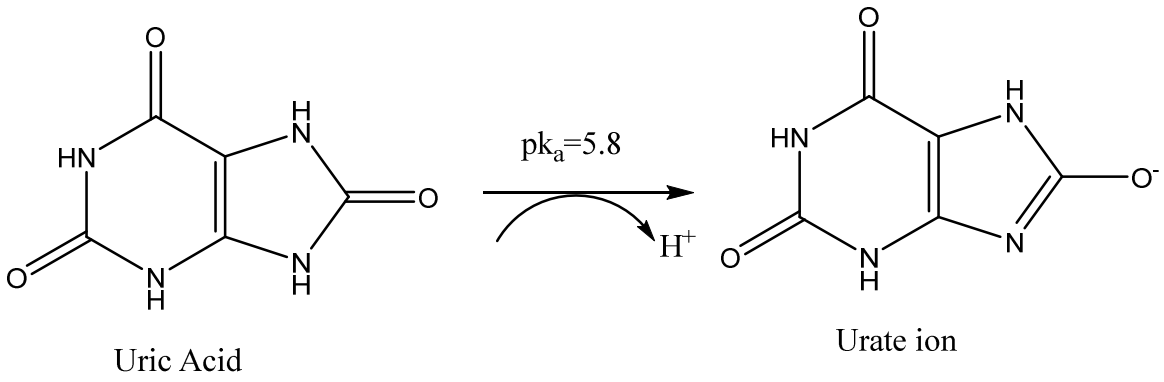


Figure 1 : Acidity of uric acid

The enzyme responsible for the conversion of urate to allantoin which is quite soluble and is the end product of purine metabolism i.e., uricase is absent in humans. When the serum urate level reaches 6.8 mg dl^{-1}, the solubility of urate at the physiological pH and temperature is limited. This leads to a biochemically abnormality called hyperuricemia and it can be attributed to either overproduction of uric acid or impairment in handling of renal uric acid causing under-excretion [3,4]. It is known that hyperuricemia is the primary cause of gout. However some factors such as lifestyle, body mass index, high blood pressure, age and cholesterol level contribute significantly towards the prevalence of this metabolic disorder [5,6].Annual frequency of gout is 2.68 for every 1000 people and it is observed that men are 2 to 6 times more prone of this abnormality than women [7]. Reduction in monosodium urate ion can be accomplished either by change in lifestyle, drug therapy or both. Despite the fact that medication drug therapy is the most influential strategy to bring down urate in serum, modification in lifestyle is quite often prescribed for people with hyperuricemia with or without gout [8]. Treatment of gout mainly falls into two classes:

1. Symptomatic relief amid an acute scene of gout which can be achieved by using therapeutic agents like colchicine, corticosteroids and non-steroidal anti-inflammatory drugs (NSAIDs).

2. Urate lowering therapy (ULT) for prevention against long term gout attacks. However the intake of ULT should be regular for attaining effective results. Urate lowering drugs are further categorized into three types:

. Xanthine Oxidase/dehydrogenase inhibitors including allopurinol, febuxostat and topiroxostat.

. Uricosuric agents i.e., the drugs that enhance the uric acid excretion like probenecid, sulfinpyrazone and benzbromarone.

. Urate degrading enzyme such as rasburicase and pegloticase [8,9].

Among all these therapeutic practices, inhibition of xanthine oxidase is the most reliable strategy as it involves the central enzyme responsible for uric acid production [10]. Oxidation of Purines in eukaryotes is catalysed by Xanthine oxidase which catabolizes hydroxylation of hypoxanthine to xanthine and further xanthine into uric acid [11]. When xanthine is oxidised, O_2 accepts electron resulting in formation of hydrogen peroxide along with superoxide radicals (Figure 2). These reactive oxygen species (ROS) lead to oxidative stress which is associated with numerous pathological processes including inflammation, cancer,atherosclerosis and aging [12].

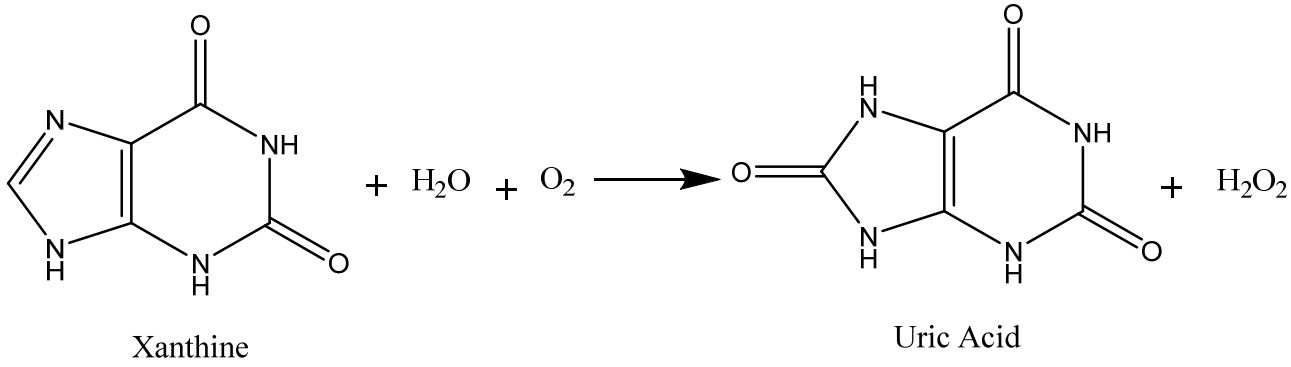


Figure 2 : Oxidation of Xanthine to Uric Acid

So by inhibiting xanthine oxidase enzyme the production of uric acid as well as other ROS can be cut down to a great extent. Xanthine oxidase inhibitors can be categorized into two

types i.e., purine analogous and others. Amongst all allopurinol (purine analogue) and febuxostat (others) are used frequently [13]. It is well known that purine analogues have more side effects than non-purine XO inhibitors. Intake of allopurinol can cause some adverse effects like skin rashes, gastrointestinal distress and high fever. Febuxostat is also associated with many side effects [14]. Therefore, exploration of non-purine based XO inhibitors with lesser side effects is need of an hour.

In addition to natural sources such as plants and animals, endophytic fungi also serve as a vital source of present day drugs. Endophytic fungi resides inside the plant and show symbiotic relationship with the host plant by attaining nutrition and shelter from the host and in turn producing secondary metabolites which are helpful in the growth and survival of host plant [15]. These secondary metabolites show great potential use in medicine, agriculture and industry [16].

This research aims at isolation and characterization of non-purine based xanthine oxidase inhibitors from endophytic fungus which is isolated from the leaves of *Aegle marmelos*, a well renowned medicinal plant.

REVIEW OF LITERATURE

The abundance of uric acid in the body causes hyperuricemia. Uric acid gets accumulated as urate ions in the joints and kidneys which is responsible for the prevalence of gout. Uric acid is the end product of purine catabolism and the enzyme which catalyses the uric acid production is known as xanthine oxidase. Thus by inhibiting Xanthine oxidase, production of uric acid can be lowered, reducing risks of hyperuricemia and gout.

2.1 Purine metabolism

A variety of functions in the cell are performed by purines. They help in neurotransmission and are the monomeric precursors of DNA and RNA. Higher nucleotides such as ATP, GTP comprise of structural component of purines. Besides this, purines also play an eminent role in energy metabolism in the body. However there should be a perfect balance between the synthesis and degradation of purines for the growth and subsistence of the cells and certain enzymes are involved in maintaining this purine metabolism [17].

The final product of purine catabolism in humans as well as primates is uric acid. However, uric acid is further degraded to allantoin in lower vertebrates, ultimately leading to release of ammonia through a series of steps involving different enzymes [18].

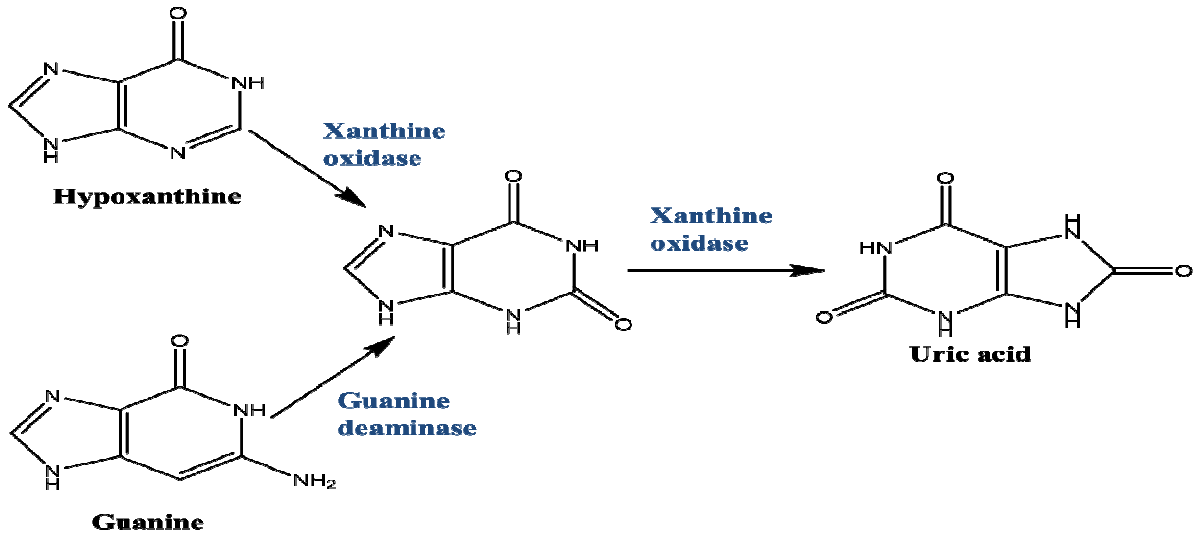


Figure 3 : Purine catabolism in humans.

In humans, degradation of purines initiates with the transformation of two nucleotides, adenosine monophosphates (AMP) and Guanine monophosphate (GMP) into nucleosides

called inosine and guanosine respectively in the presence of nucleotidase. Inosine thus formed is converted to hypoxanthine whereas guanosine gets transformed to guanine by purine nucleoside phosphorylase (PNP). Both guanine as well as hypoxanthine is then converted to xanthine. Whereas former undergoes deamination in the presence of guanine deaminase to form xanthine, latter is oxidised to xanthine by xanthine oxidase. Xanthine is then oxidised to uric acid, the end product of purine metabolism by xanthine oxidase (Figure 3) [17, 18].

2.2 Xanthine Oxidase

A normal healthy cell consists of XO primarily in the form of Xanthine Dehydrogenase (XDH) which can be converted to XO either by oxidation of sulfhydryl group, limited proteolysis or both. XO oxidises hypoxanthine and xanthine to uric acid producing superoxide radicals and hydrogen peroxide, whereas the basic role of XDH is to reduce NAD^+ to NADH (Nicotinamide adenine dinucleotide, a coenzyme present in all living cells)at the flavin nucleotide (FAD) reaction site [19,20]. This formation of XO from XDH is of great concern as it is associated with diseases which can be categorized as tissue damage caused due to oxygen radicals like postichemic reperfusion injury [21]. Xanthine oxidase is mainly found in the gut and the liver along with heart, lungs, brain, plasma and kidneys. For studying various properties such as structure and activity of XO it is mainly obtained from the bovine milk serum.

2.2.1 Crystal structure of XO

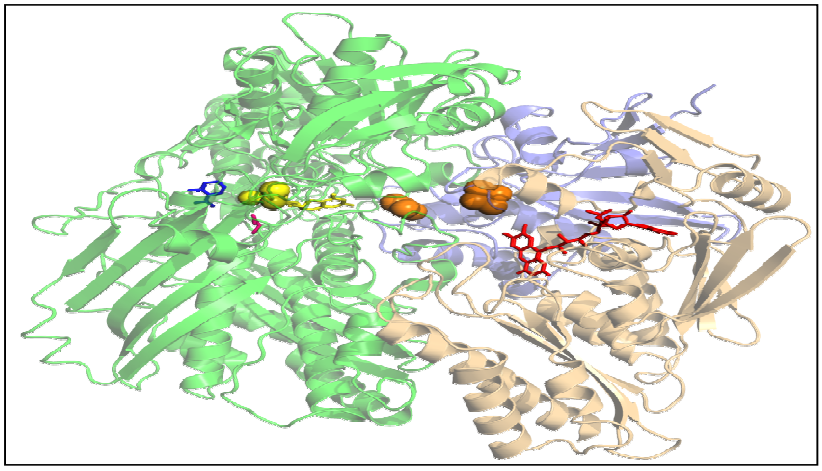


Figure 4 : Monomeric structure of xanthine oxidase crystal obtained from bovine milk.

Active XO exists as a homodimer having molecular mass of 290 kDa, where each monomer acts independently for catalysis. In mammalian xanthine oxidoreductases, each subunit is consisting of a 85 kDa C-terminal molybdopterin-binding centre, 20 kDa N-terminal comprising of two spectroscopically iron sulphur centres, and a 40 kDa central FAD [20]. Figure 4 represents the crystallographic structure of XO where red, orange, yellow and blue colour portrays bounded FAD, FeS-cluster, molybdopterin cofactor with molybdenum and salicylate respectively.

2.2.2 Functional Mechanism of XO

Besides the hydroxylation of hypoxanthine and xanthine, XOR is responsible for catalytic hydroxylation of a variety of aldehydes and N-heterocyclic substrates as well [22]. Conversion of xanthine to uric acid involves the reductive half reaction, where oxygen is transferred from the molybdenum centre i.e., xanthine is oxidatively hydroxylated with the reduction of molybdenum centre (Mo^{6+} to Mo^{4+}) of the enzyme. Reformation of Mo centre takes place with the addition of water. The oxygen atom added to the substrate by the enzyme originates from the water and not from O_2. The oxidative half reaction incorporates the removal of reducing equivalents from the enzyme through FAD (Figure 5). O_2 accepts the electrons from the oxidase form of the XOR (XO) whereas NAD^+ serves as an electron acceptor from the dehydrogenase form of the XOR (XDH) [22, 23].

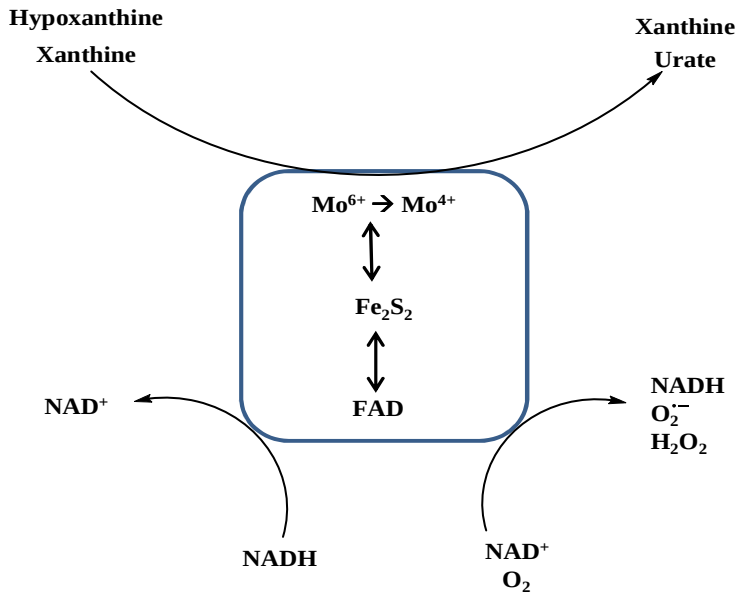


Figure 5 : Mechanistic approach of conversion of hypoxanthine to xanthine.

As shown in Figure 5, reactive oxygen species are generated during the purine metabolism and these ROS are responsible for the oxidative stress leading to various physiological and

pathophysiological responses indicating the adverse effects of increased levels of xanthine oxidase enzyme responsible for various inflammatory and cardiovascular diseases [12, 16].

2.3 Inflammatory arthritis

At physiological pH, uric acid exists as urate in the body (Figure 1). With the increase in urate concentration in blood, formation of crystals of urate takes place in the body leading to various inflammatory diseases. The increase in concentration of uric acid concentration in blood is responsible for an abnormality termed hyperuricemia which is considered as one of the main reasons for the prevalence of gout. 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 [17]. Formation of monosodium urate crystals followed by their deposition in the joints and kidneys takes place if level of uric acid increases from 6.8 mg/dL [1, 3]. It is highly recommended that individuals suffering from gout should inculcate low fat dietary products and vegetables into their diet and should avoid food items such as sweet breads, alcohol and organ meats [24].

In humans, uric acid and urate gets accumulated in the joints or connective tissues as calculi ultimately leading arthritis and rheumatic pain whereas deposition in kidneys may even lead to kidney failure [7]. Various epidemiologic studies suggest that hyperuricemia alone may lead to renal failure [9].

Pervasiveness of gout is not similar in the world. Frequency of people suffering from this arthritis abnormality is found to be more in Pacific countries. Also it is found that there are higher incidents of gout in the developed countries as compared to that of developing countries. Gout is more dominant in men as compared to women. Usually men after the age of 40 years and women after their menopausal stage are more prone this abnormality [25].

2.4 Therapeutic treatment of Gout

2.4.1 Management of acute gouty arthritis

Acute gout is an ailment which persists for short span of time and its treatment provides relief from the pain and also fastens the recovery process. Therapeutic agents which are used for the treatment of acute gout can be classified as:

(a) Nonsteroidal anti-inflammatory drugs (NSAIDs) like ibuprofen and steroids such as corticosteroid.

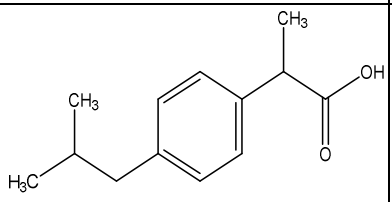
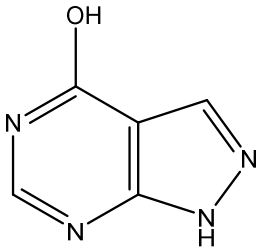
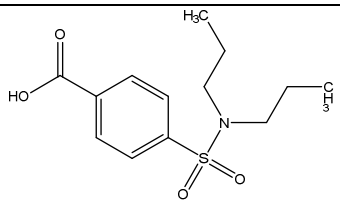
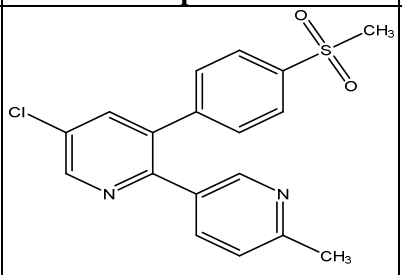
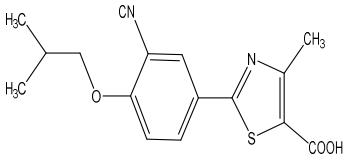
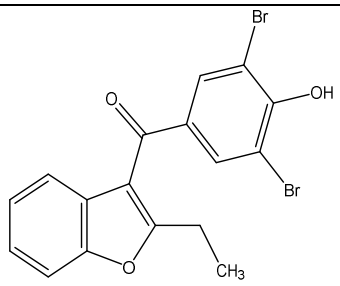
(b) Anti-inflammatory drugs including colchicine and glucocorticoid.

NSAIDs are preferred for patients not having any comorbid illnesses. However patients with congestive heart failure, renal damage and peptic ulcer disease cannot be given these drugs and are thus given anti-inflammatory drugs. Moreover, high intake of NSAIDs for acute gout can prompt gastric toxicity. Neither of these drugs is found to be quite effective and safe for the treatment of gout. Thus for the management of acute gout, new therapies are being evolved and it is discovered that anakinra which is an IL-1 receptor antagonist, showed fast and complete pain relief without causing any side-effects in patients [26].

2.4.2 Treatment of long-term hyperuricemia

Long term hyperuricemia can be cured by urate lowering therapy. Urate lowering drugs can be classified into two types as uricostatic drugs and uricosuric drugs. Uricostatic drugs including allopurinol and febuxostat reduce the production of uric acid by inhibiting the enzyme responsible for its production whereas uricosuric drugs such as sulphinpyrazone, benzbromarone and probeneceid increase the uric acid excretion (Table 1).

Table 1: Various classes of common anti-gout drugs.

Sr. No.	NSAIDs	Uricostatic drugs	Uricosuric drugs
1.	 **Ibuprofen**	 **Allopurinol**	 **Probeneceid**
2.	 **Etoricoxib**	 **Febuxostat**	 **Benzbromarone**

Allopurinol (purine based) with an efficacy of 300 mg/day and febuxostat (non-purine based) having efficacy at 80-120 mg/day are most widely used urate lowering drugs. The reason for

this low dosage of febuxostat may be attributed to the selectivity of drug towards xanthine oxidase in the purine metabolism [27].

2.5. Need for novel XOI

Although allopurinol (1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one), which is an analogue of hypoxanthine is a safe drug, however around 2% of patients exhibit hypersensitivity towards it. Common drug effects include headache, gastrointestinal distress, nausea, liver function abnormalities and deteriorating 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 hypersensitive to allopurinol. Uricosuric drugs can also be given to such patients for example probeneceid and the urate inhibitor. Febuxostat is usually well tolerated, but some patients (≈1 %) may suffer from side effects like nausea, headache and diarrhoea.

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 non-purine based xanthine oxidase inhibitors [27].

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

2.6. Plants as a source of XOI

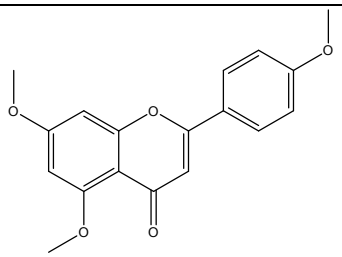
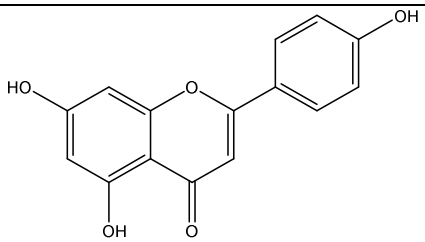
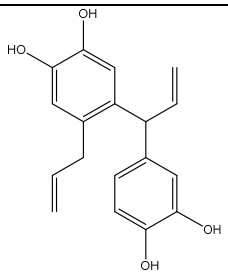
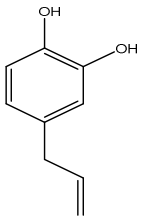
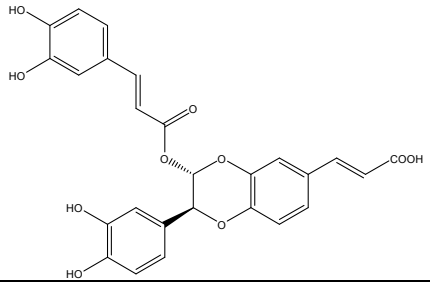
The use of plant materials is gaining renewed interest for treatment of different diseases. Natural products isolated from medicinal plants propose an opportunity for the advancement of new therapeutic agents. Table 2 provides an insight to Xanthine oxidase inhibitors that have been isolated from different plant species.

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: Some XOI isolated from plants

S. No.	Structure	Name	Source	Reference
1.		4,5,7-Trimethoxyflavone	Perillafrutescens	[28]
2.		Apigenin	Perillafrutescens	[28]
3.		Neotaiwanensol B	Piper nudibaccatum	[29]
4.		Hydroxychavicol	Piper nudibaccatum	[29]
5.		Epihyprhombin B	Hyptis rhomboids	[30]

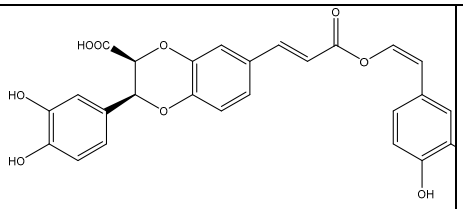
6.		Hyprhombin C	Hyptis rhomboids	[30]

Table 3: Some XOI isolated from bacteria

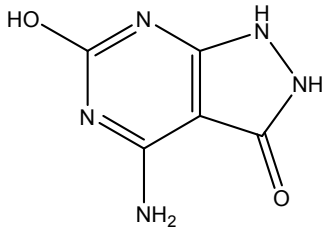
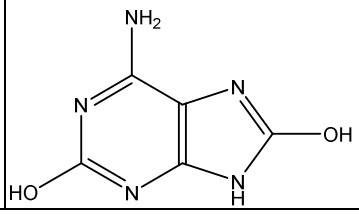
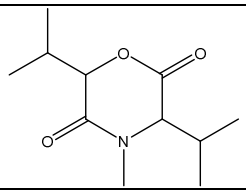
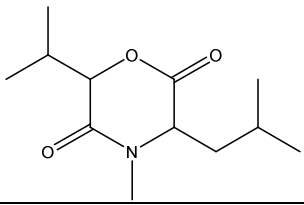
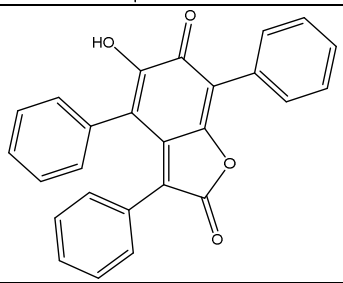
S. No.	Structure	Name	Source	Reference
1.		Hydroxyakalone	Agrobacterium aurantiacum	[31]
2.		2,8-dihydroxyadenine.	Alcaligenes aquamarines	[32]

Table 4: Some XOI isolated from Fungi

S. No.	Structure	Name	Source	Reference
1.		3,6-di(propan-2-yl)-4-methyl-morpholine-2,5-dione	Fusarium sporotrichioides	[33]
2.		3-(2-methylpropyl)-6-(propan-2-yl)-4-methyl-morpholine-2,5-dione	Fusarium sporotrichioides	[33]
3.		5-Hydroxy-3,4,7-triphenyl-2,6-benzofurandione	Peniophora sanguine	[34]

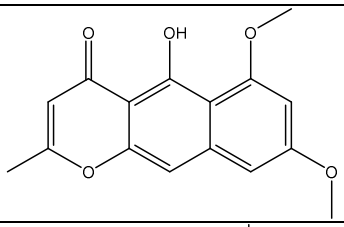
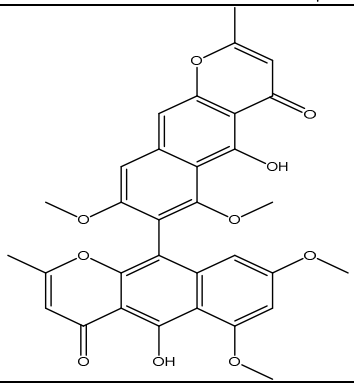
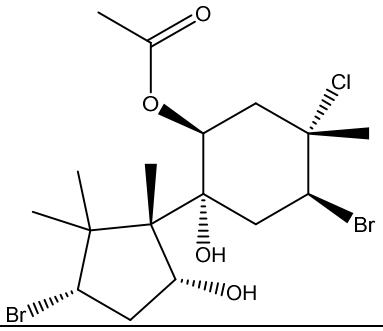
4.		Rubrofusarin B	Aspergillusniger	[35]
5.		Aurasperone A	Aspergillusniger	[35]

Table 5: XOI isolated from algae

S. No.	Structure	Name	Source	Reference
1		Algoane	Laurencianatalensis	[36]

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 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 invansion of pathogens [37]. 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 [15]. Such bioactive metabolites find wide-

ranging application as agrochemicals, antibiotics, immunosuppressants, antiparasitics, antioxidants, and anticancer agents [16].

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 ($C_{32}H_{30}O_{14}$) [38, 39, 40]. Pestacin ($C_{15}H_{14}O_4$) and "isopestacin", 1,3-dihydro isobenzofurans, isolated from the endophytic fungus *Pestalotiopsismicrospora*displayed potential antioxidant activity [41, 42].

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 [43].

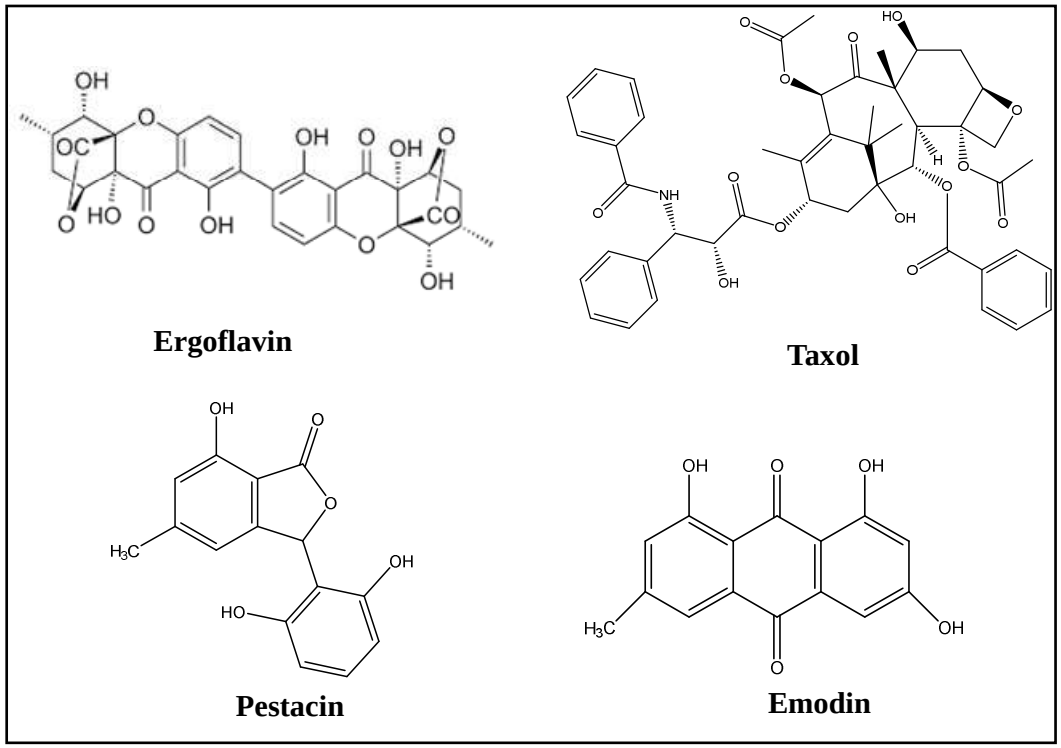


Figure 6 : Few compounds isolated from Endophytic Fungi

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

AIM OF THE STUDY

1. Isolation of endophytic fungi from *Aegle marmelos.*

2. Isolation and Purification of Bioactive fractions from endophytic fungi.

3. Screening of Xanthine Oxidase inhibitors from endophytic fungi.

4. Identification of endophytic fungi.

5. Characterization of Xanthine Oxidase Inhibitors.

MATERIALS AND METHODS

General: All the apparatus, media and discard as and when required were autoclaved at 121°C, 15 psi for 15 minutes. All types of culture handling (isolation, inoculation and production) was done in Thermadyne Bio Safety Class II Cabinet (Laminar Flow) after cleaning it with spirit followed by exposure to UV light for 15-20 minutes.

4.1. Isolation of Xanthine oxidase Inhibitors

4.1.1. Preparation of Potato Dextrose Agar Plates:PDA media was prepared in distilled water by dispensing it in Erlenmeyer flask covered with cotton plug followed by autoclaving. After cooling, the media was dispensed onto 90 mm petri-plates (20-25 ml) in the laminar flow and allowed to solidify at room temperature [44].

4.1.2. Sub-culturing of Endophytic Fungi: Culture from the glycerol stock was inoculated aseptically using sterilized loop onto the fresh PDA plates and incubated at 26 ± 2°C to promote the augmentation of the endophytes for 5-6 days till the growth was observed.

4.1.3. Production of the Culture Filtrate: Production of endophytic culture was carried out by inoculating 5mm mycelial disk, of 7-10 days old culture, into the pre-sterilized potato dextrose agar broth (PDA) in the Erlenmeyer flask that was incubated in the shaker at 26 ± 2 °C, 120 rpm for 8-10 days. The spent broth was separated from the mycelia mass by filtration through Whatman Filter Paper No. 4. The filtrate was centrifuged at 12,000 rpm for 10 minutes for obtaining the cell free filtrate and stored at -20°C till further use [45].

4.1.4. Isolation of Bioactive Fractions: The bioactive fraction [46] of the cell free filtrate was extracted, using liquid-liquid extraction, with different solvents ranging from non-polar (hexane) to polar (ethyl acetate) in the ratio of 1:2 for filtrate to solvent. The organic layer was pooled together for each solvent and the residual moisture removed using anhydrous sodium sulphate. Evaporation of the organic layer using rotator evaporator gave crude bioactive fraction that was weighed and reconstituted in DMSO. The fractions were stored at -20°C till further use.

4.1.5. Qualitative Screening of Xanthine Oxidase Inhibitors: Xanthine oxidase inhibition assay was done using xanthine nitroblue tetrazolium (NBT) agar plates prepared with agar (0.8% w/v), NBT (0.11 mg/ml) and xanthine (1.5 mg/ml) and allowed to solidify in

dark.After solidification to each of the 5mm wells were suspended with 5µl tris HCl solution (10 mM) followed by addition of 10 µl of xanthine oxidase (0.04U) and 20 µl of culture filtrate. In two of the wells, DMSO and allopurinol were replaced with culture filtrate to be used as solvent and positive controls respectively. The plates were kept in dark for incubation at 37 ^{0}C for 24 hours. After the incubation period, the plates were observed for the reduction of blue coloured halo formation attributed to the presence of xanthine oxidase inhibitory activity in the isolated culture filtrate [47].

4.2. Purification of Xanthine Oxidase Inhibitors

4**.2.1. TLC analysis:** Thin layer chromatography of the reconstituted chloroform extract was done with different solvent systems to analyse the number of compounds present in the extract. Solvent system which gave more resolved spots was optimized. Isolated culture filtrate was dissolved in chloroform and spotted on TLC with the help of a capillary tube. The spots were allowed to move in the optimized solvent system (2% ethyl acetate in hexane), till the solvent reaches ¾ th height of the TLC plate. The TLC plate after air drying, so as to evaporate the solvent system, was observed in the UV light (254 nm).

4.2.2. Column Chromatography: Based on TLC results different compounds present in the culture filtrate were separated by the means of column chromatography. A glass column of length 90 cm and diameter 2cm was packed with silica gel suspension of hexane without any air gap in the bed. This silica gel stationary phase was loaded with culture filtrate adsorbed over silica gel and eluted with solvent mobile phase having gradient (starting from hexane to 2% ethyl acetate in hexane). The eluted solvent mixed with different fractions of the pure compounds was collected in test tubes. All the test tubes were analysed for the purity and among the pure fractions, the compounds appearing at same height were pooled together. Evaporation of the solvent using rotatory evaporator gave pure product that was stored for the characterization and various assays.

4.3. Screening of Xanthine Oxidase Inhibitors

4.3.1. Quantitative Screening by NBT assay: Done as reported in 4.1.5 [48].

4.3.2. Phytochemical Screening: The fraction with highest inhibition activity towards XO i.e., fraction 1(1 mg/ml in methanol) was screened for the presence of various secondary metabolites. Various tests were performed to serve the purpose.

i) Liebermann Burchardt Test: The test is used to detect terpenoids and the steroids. In a 1 ml of the extract of the purified fraction is added 2 ml of chloroform and 2 ml of aceticanhydride

followed by slow addition of 2 ml of conc. sulphuric acid along the walls of the test tube. A change of colour ranging from pink to red indicates the presence of terpenoids while same reagents if give dark green colour indicate presence of steroids[49].

ii)Wagner Test: This test determines the presence of alkaloids. It is performed by adding first 1 ml of the purified compound followed by addition of 2ml HCl and and heating on the hot water bath. Addition of few drops of Wagner reagent gives creamy white precipitates that indicate presence of alkaloids [50].

iii) Ferric Chloride Test:The test confirms the presence of phenolic compounds. To a 1 ml extract of the purified compound, when 2 ml of 2% ferric chloride solution is added the colour change to blue-green or red confirms the presence of phenols [51].

iv) Zinc Hydrochloride Test:This test is used for the determination of flavonoids. To a 0.5 ml of the purified compound taken in a test tube, addition of 5-10 drops of diluted HCl followed by addition of small piece of zinc should give reddish pink or brown coloured precipitates upon boiling [52].

v) Ninhydrin Test:The test confirms the presence of amines on the TLC plate itself. The purified compound as well as the standard amine, when spotted on the TLC plate followed by dipping in the Ninhydrin solution should give appearance of purple colour after heating.

4.3.3. Antioxidant assay

In order to calculate the antioxidant activity of the extract, various tests were conducted for scavenging of different active radicals responsible for the oxidative stress in the body.

i) DPPH scavenging assay: 2,2-Diphenyl-1-picrylhydrazyl scavenging assay was used to calculate the antioxidant activity of the extract [53]. Dilutions of the purified compound as well as quercetin (standard) were prepared from 10-50 ppm range. Each dilution of the purified compound and the standard (50 µl each) were mixed with 100 µl of freshly prepared DPPH. As a negative control 50 µl of methanol was mixed with DPPH. Absorbance at 517 nm using ELISA reader was noted after 30 minutes at room temperature. Percentage free radical scavenging at different concentrations was calculated using formula

$$\%FRS = \frac{Absorbance(Control) - Absorbance(Sample)}{Absorbance\ (Control)} \text{ X } 100$$

IC_{50} value can be calculated from the graph plotted between % FRS and different concentrations of the purified compound.

ii) TEAC assay:Trolox equivalent antioxidant capacity assay is also used to measure the antioxidant activity of the compounds by determining their ability to scavenge 2, 2'-azino-

bis-3-ethylbenzothiazoline 6-sulphonic acid (ABTS) radical [54]. For this, radicals are generated by mixing 7mM ABTS in 0.1 M phosphate buffer saline (PBS) solution of pH-7.4 with 2.45 mM potassium persulphate in equal volume followed by incubation at room temperature in dark for 16 hour. The radical solution is diluted in PBS to reach the absorbance of 0.9-1.0 at 734 nm. Different dilutions of extract along with piceatannol from 50-250µg/ml were prepared. 10 µl of each dilution was mixed with 1 ml of ABTS solution and was incubated for 6 minutes. Mixture of ABTS and methanol was taken as negative control and the absorbance was noted at 734 nm. Percentage free radical scavenging and IC_{50} values were calculated in the same manner as calculated in DPPH assay.

ii) Total Phenolic Content (TPC) assay : For determining the total phenolic content, Folin-Ciocalteu (FC) reagent was used [55]. 150 µl of pure compound (1 mg/ml) was mixed with 2.25 ml of deionized water and 150µl of FC reagent followed by incubation for 30 minutes at room temperature. A 300 µl of sodium carbonate (5% w/v) solution was then added into the reaction mixture and kept for 1 hour at room temperature. The absorbance noted at 760 nm with quercetin as a standard gave concentration of the phenols in the sample by plotting a linear regression between the concentrations of the standard and their absorbance. The results are expressed as µg of quercetin equivalent per mg of the compound.

iii) Total Flavonoid Content (TFC) assay:Total flavonoid content was determined as described by LuximonRamma et al., 2002 [56]. In a 200 µl solution of the compound having 1mg/ml concentration was added 60 µl of sodium nitrate (5% w/v) and 800 µl of deionised water. This reaction mixture was left undisturbed for 5 minutes at room temperature and then added with 60 µl of aluminium chloride (10% w/v) and 400 µl of 1 N sodium hydroxide. Ttotal volume of the solution made up to 2 ml with the deionized water to measure the absorbance at 510 nm. Quercetin was used as a standard. Total flavonoid content was calculated from the linear regression, plotted between the concentrations and absorbance, both for the standard and the sample. The results were expressed as µg of quercetin per mg of extract.

4.3.4. Antimicrobial assay

The *invitro* antimicrobial activity was measured using agar well diffusion method. The endophytic fungus was screened for its antimicrobial activity against following four different microbial cultures. 1. Gram-positive bacteria MTCC 441 (*Bacillus subtilis*), 2. Gram-negative bacteria MTCC 647 (*Pseudomonas aeruginosa*) and two fungus including ATCC 227 (*Candida albican*) and MTCC 3011 (*Candida albican*). Test organisms were taken from pre-

existing depository maintained by Dr. Sanjai Saxena, Professor, Thapar Institute of Engineering and Technology, Patiala. The cultures were firstly revived by inoculating them in Mueller Hinton Broth (MHB; Hi-Media, India) one day before performing the assay. Organisms were diluted in 0.9% w/v NaCl solution and visually adjusted with 0.5 McFarland solution to achieve 106 CFU/ml followed by spreading on Mueller Hinton Agar (MHA, Hi-Media, India) for anti-bacterial assay and Soubard's Dextrose Agar (SDA) for anti-fungal assay using cotton swab. The plates were incubated with 30 µl of the test compounds in the 5mm wells for 16-24 hours at 37°C. Uninoculated PDB was used as the negative control. The zone of inhibition against the test organisms was noted in millimetre and all the tests were performed in triplicates. The zone of inhibition corresponds to the antimicrobial activity and is expressed as mean ± SD [57].

4.4. Identification of Endophytic Fungi

4.4.1. Morphotaxonomy: The fungus was examined under microscope (Nikon Eclipse E100) for its identification based on its morphological appearances. Growth of the fungal culture was optimized by growing on different media (PDA, SNA, PLA, MEA and WA) plates at 26 ± 2 ^{0}C for four weeks. Microscopic characterization was done with a small drop of water was put on glass slide and mycelia mass placed over it. The mass was teased with the fine needle tip followed by staining with lactophenol cotton blue dye. Before mounthing by DPX [58], it was covered with a cover slip (18 x 10 mm) to prevent trapping of any air bubbles. Morphological observations like colour, appearance were noted at microscope magnifications of 10X, 40X and 100X.

4.4.2. Molecular Identification

i) DNA isolation:The fungal genomic DNA isolation was done using Genomic DNA purification kit (Promega, USA). Mycelial mass (70 mg) of 5-7 days old culture was crushed in fine powder using liquid nitrogen in a sterile mortar and pestle. It was crushed again using nuclei lysis buffer (700 µl) and was then transferred to a micro centrifuge tube and vortexed followed by heating at 65°C in the water bath. After 15 minutes, the tubes were centrifuged at 12,000 rpm for 5 min to remove the cell debris followed by addition of 5 µl of RNase and keeping at 37°C for 15 minutes. Protein precipitation solution (200 µl) was added and the tubes centrifuged again at 12,000 rpm for 3 minutes to remove protein contamination. The aqueous phase containing DNA was then transferred to another micro centrifuge tube containing isopropanol to centrifuge at 13,000 rpm for 3 min. The DNA pellet thus formed was rinsed with 70% ethanol and finally centrifuged at 13,000 rpm for 1 min. The pellet thus

obtained was air dried and dissolved in 50 µl of tris- EDTA buffer (pH 8) and estimated qualitatively by agarose gel electrophoresis.

ii) Agarose Gel Electrophoresis: Agarose gel (0.8%) comprising of ethidium bromide (0.5µg/ml) was prepared in 1X TAE (Tris Acetate EDTA) buffer and casted in the electrophoresis apparatus. The electrophoretic tank was then poured simultaneously with running buffer (1X TAE). The wells on the gel were loaded with the solution of DNA sample and 6X loading dye and allowed to run for 1 hour at the voltage of 50 V. The DNA fragments thus obtained were visualized under trans UV illuminator and the imaging done in Bio-Rad gel documentation system.

Table 6: Reagents used in PCR Amplification

S.No.	Reagent	Stock Concentration	Quantity (for 25 µl)	Final Concentration
1.	Sterile double distilled water	-	14.5µl	-
2.	Taq buffer	10□	2.5 µl	1□
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 µM
6.	Taq DNA Polymerase	3 U/µl	1.0 µl	2.5 U
7.	DNA Template	25 ng/µl	1.0 µl	25 ng

iii) PCR Amplification: PCR amplification was done using universal primer pair ITS 1(5'TCC GTA GGT GAA CCT GCG G 3') and ITS 4 (5' TCC TCC GCT TAT TGA TAT TGA TAT GC 3') [59]. Amplification of isolated DNA using 25 µl of reaction mixture (table-6) comprising of extracted fungal DNA, primers (ITS 1 and ITS 4), dNTP, Taq DNA Polymerase in 10X Taq buffer. Thermal cycling conditions during the whole process are described in table-7. Analysis of the PCR products was done with gel electrophoresis in agarose gel (1.5%) and gel imaging was done as described earlier.

Table 7: Temperature Range for PCR reaction

Step No.	Name	Temperature	Time
1	Initial denaturation	96 °C	5 min
2	Denaturation	95 °C	45 sec
3	Annealing	60 °C	45 sec
4	Extension	72 °C	45 sec
5	Step 2 to 4 repeated 39 times	-	-
6	Final extension	72 °C	5 min
7	Store	4 °C	-

4.5. Characterization

^{1}H NMR was performed for the sample dissolved in $CDCl_3$ by JEOL nuclear magnetic resonance spectrometer at the frequency of 400 MHz.

RESULTS AND DISCUSSIONS

Endophytic fungi has been a matter of interest in medicinal and green chemistry as they show symbiotic relationships with the host plant because they encompass all the characteristic properties of the host species. Thus, endophytic fungi produce same secondary metabolites as that of host offering a great source for development of various therapeutic agents. Present work aims at the exploration and characterization of the secondary metabolites of a fungus named #6AMLWLS isolated from the leaves of a well-recognized medicinal plant *Aegle Marmelos*. The isolated secondary were evaluated for their inhibition towards xanthine oxidase, an enzyme responsible for production of uric acid in the body. Fungus samples was collected from Wayanad Wildlife Sanctuary, Kerala, India.

5.1. Isolation of Secondary Metabolites

The fungus was sub cultured on PDA, SNA, MEA and PLA plates as shown in **Figure 7 (i)**. from the slant of fungus #6AMLWLS present in stock. Also fresh slants were prepared for preserving the fungal culture. Sub cultured mycelia of 7-8 days were inoculated in PDB under sterile conditions for another 8-10 days to produce secondary metabolites into the media as shown in **Figure 7 (ii)**. After complete growth mycelia mass was separated from the media to obtain a mixture of secondary metabolites produced that was stored at -20°C after ultracentrifugation. The obtained mixture was treated with different solvents, starting from least polar to most polar, to carry out solvent- solvent extraction of the organic secondary metabolites'.

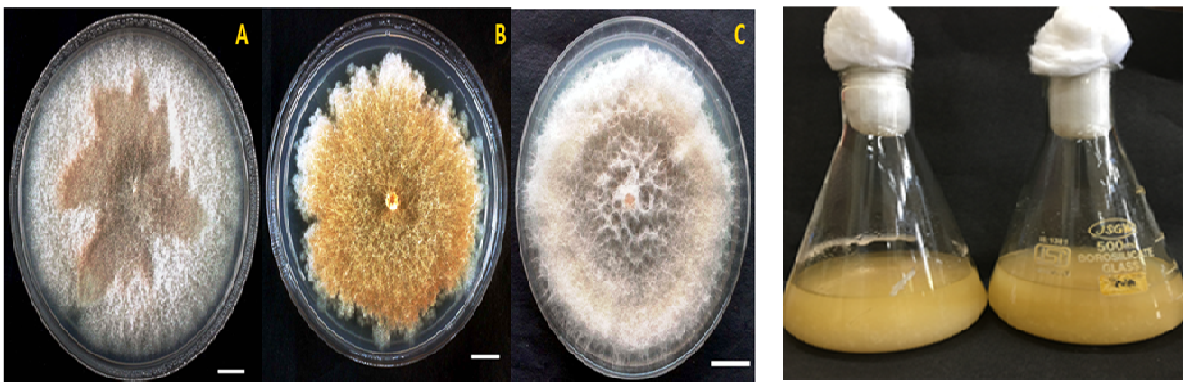


Figure 7: (i) Pure culture of #6AMLWLS grown on (A) PDA, (B) SNA, (C) MEA media plates. **(ii)** Fungal growth in PDB media.

The solvents used were hexane, chloroform, ethylacetate and dichloromethane. The crude product obtained was reconstituted in both methanol and dimethyl sulphoxide (DMSO) for further characterization and evaluation.

All the reconstituted extracts of different solvents were qualitative screened for the xanthine oxidase inhibition by nitroblue tetrazolium plate (NBT) assay. **Figure -8** below shows the molecular principle of this assay where formazan formation due to reaction of NBT with superoxide radical gives blue colour. Thus, the extracts that have xanthine oxidase inhibition activity prevent the formation of super oxide radical thus leading to less formation of formazan and blue colour. This assay works on the principle that more is the decrease in formation of blue colour, more potent is the inhibitor [60, 61]. **Figure -2** above describes the principal of xanthine oxidase inhibition.

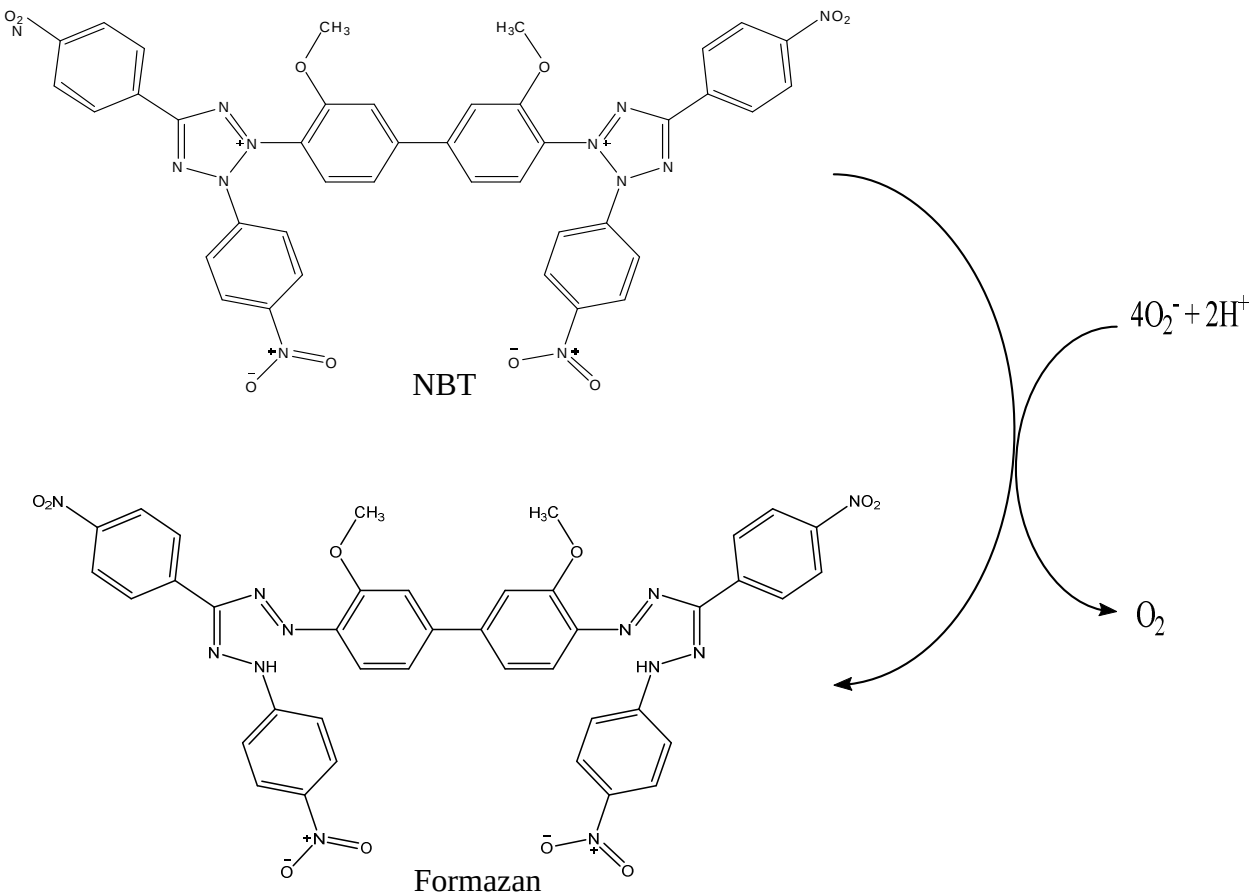


Figure 8: Nitroblue Tetrazolium (NBT) gives the formation of blue coloured formazan compound upon reaction with superoxide radical produced due to conversion of Xanthine to Uric acid.

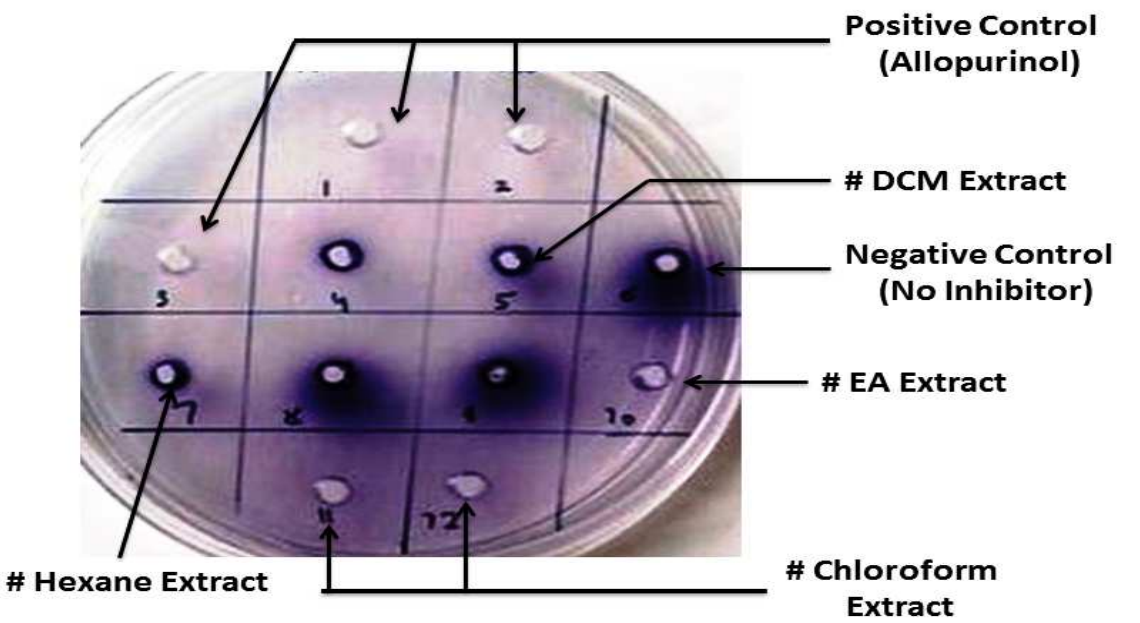


Figure 9: Xanthine-NBT agar plate displaying the results of XOI assay of different solvent extracts. Chloroform extracts (well no 11 and 12) shows best XOI activity

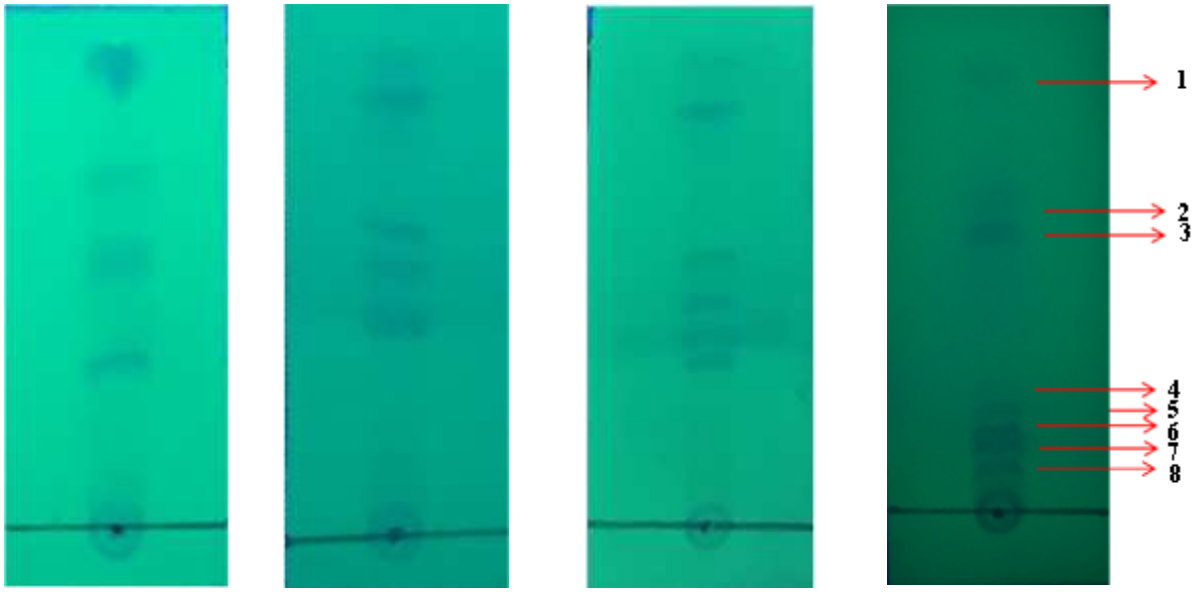
Figure-9 above shows that of all the four extracts (hexane, dichloromethane (DCM), chloroform and ethyl acetate (EA)), chloroform shows best activity for XOI and was taken up further for the isolation of pure compounds.

Purification of the Secondary Metabolites

Above results formed the basis for the purification of secondary metabolites from chloroform extract. Thus, thin layer chromatography (TLC) analysis was performed in different solvent systems to prior to column chromatography. **Figure –10** below shows that the best separation was obtained in case of 2 % ethyl acetate in hexane. The TLC shows that extract has 8 compounds with fairly good separation. Table -8 also shows the retention factor (Rf Vale) for each of the separated spots.

Table 8: R_f values of the spots as optimized in case of 2 % ethyl acetate in hexane by TLC

Sr. No.	Compound	R_f value
1	Spot 1	1
2	Spot 2	0.72
3	Spot 3	0.64
4	Spot 4	0.29
5	Spot 5	0.24
6	Spot 6	0.18
7	Spot 7	0.13



(a) Toulene **(b)** 7 % EA in Hexane **(c)** 5% EA in Hexane **(d)** 2 % EA in Hexane

Figure 10: Chloroform extract as optimised in different solvent systems comprising of ethyl acetate and hexane. Best separation was achieved in case of 2% ethyl acetate (EA) in hexane.

Column chromatography using silica as a stationary phase and pure hexane - ethyl acetate as the solvent system gave a total of six fractions. Starting with pure hexane, a gradient of mobile phase was used up to 2 % ethyl acetate in hexane. After column a total of 6 fractions were obtained comprising of 5 pure fractions and one a mixture of 3 compounds having spots 4, 5 and 6 (Fraction 4). Each of these spots was then subjected to quantitative test for inhibition of xanthine oxidase enzyme.

Xanthine Oxidase Inhibition Activity

All the six fractions were evaluated for XOI by quantitatively NBT assay by measuring the absorbance in the visible region at 575 nm. Decrease in absorbance was noted for the fractions that inhibited XO because of non-formation of superoxide radicals which are responsible for conversion of NBT to insoluble formazon. Allopurinol was used as a positive control while DMSO as a negative control because it was also used as a solvent to dissolve the fraction. **Table-9** gives comparative XOI activity with respect to negative control, DMSO. It can be seen that fraction 1, that eluted with the non polar hexane. Fraction-5 that eluted later from the column was expected to be polar, also gave impressive XOI activity of 40.49 %. Other biological assays like antioxidant activity and anti microbial activity were however carried out further with fraction-1 only.

Table 9:Quantitative Xanthine Oxidase Inhibition of the fractions separated using column for quantitative NBT assay at 575 nm.

Sr. No.	Compound	% Inhibition
1	Allopurinol	79.41 ± 0.59
2	Fraction 1	50.28 ± 0.12
3	Fraction 2	11.28 ± 0.78
4	Fraction 3	5.16 ± 0.52
5	Fraction 4*	18.34 ± 0.41
6	Fraction 5	40.49 ± 0.33
7	Fraction 6	2.62 ± 1.25

* Fraction 4 is mixture of spots 4,5,6

5.2. Antioxidant Activity

Antioxidant activity was carried out by two methods. DPPH and TEAC assay. The basis of DPPH assay lies on the fact that any compound that can donate hydrogen as a radical can act as an antioxidant.The method is helpful in determining the radical scavenging activity of any compound.Following results**(Figure-11)** show antioxidant activity carried out with DPPH and TEAC method using Quercetin and Piceatannol as standards. The antioxidant activity of the fraction was impressive with results having been expressed as equivalent of the respective standards. It is well known that naturally occurring compounds like phenols and flavonoids possess antioxidant activity. Therefore, phytochemical tests to determine phenols and flavonoids were carried out.

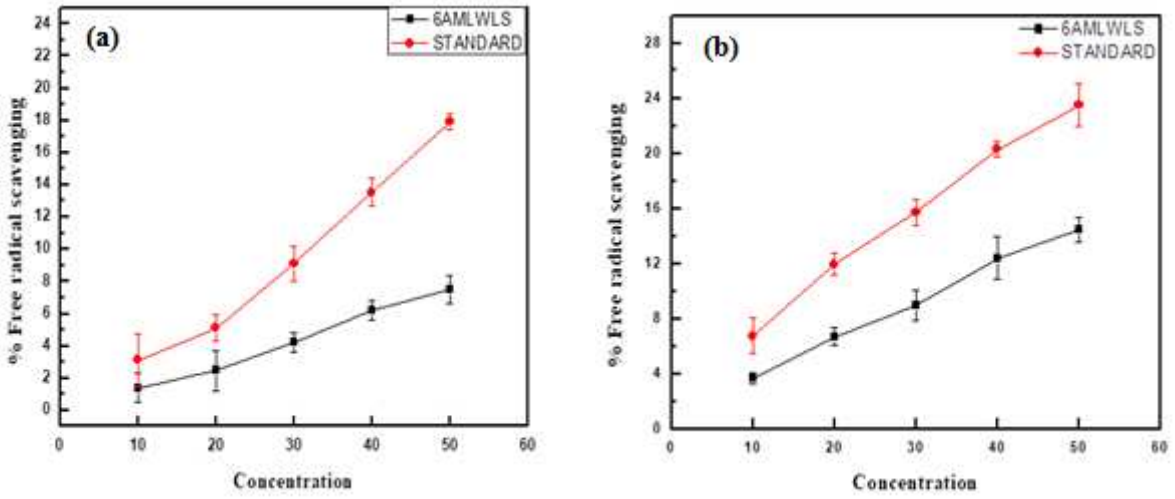


Figure 11: Free radical scavenging in **(a)** DPPH and **(b)** TEAC assay in comparison to their respective standards.

S. No.	Activity	Method Used	Value	Std. Used
1.	Anti oxidant activity	DPPH	IC_{50}= 299.81 ± 29.24µg/ml.	Quercetin
2.	Anti oxidant activity	TEAC	IC_{50} = 178.72 ± 14.90µg/ml.	Piceatannol.

However, the total phenolic and total flavonoid content were found to be 16.55 ± 1.96 and 20 ± 2.82 µg quercetin equivalents/ mg of the sample. The low value of TPC and TFC suggests that although compound has antioxidant activity but it is neither a phenoilc or flavanoid molecule.

5.3. In-vitro Antimicrobial Assay

Antimicrobial drugs are in demand due to resistance developed by various organisms against prevalent drugs. Thus, evaluating any compound for its antimicrobial activity becomes important. The isolated pure compound of fraction 1 was evaluated for both antibacterial and antifungal activities against four microbial cultures two of which were bacteria while other two were fungus *Bacillus Subtilis*(MTCC 441), *Pseudomonas aeruginosa* (MTCC 647)and *Candida albican* (ATCC 227, MTCC 3011). The activity was carried out by calculating the zone of inhibition. **Table -10** shows an impressive zone of inhibition for antibacterial activity as compared to antifungal activity as compared to control.

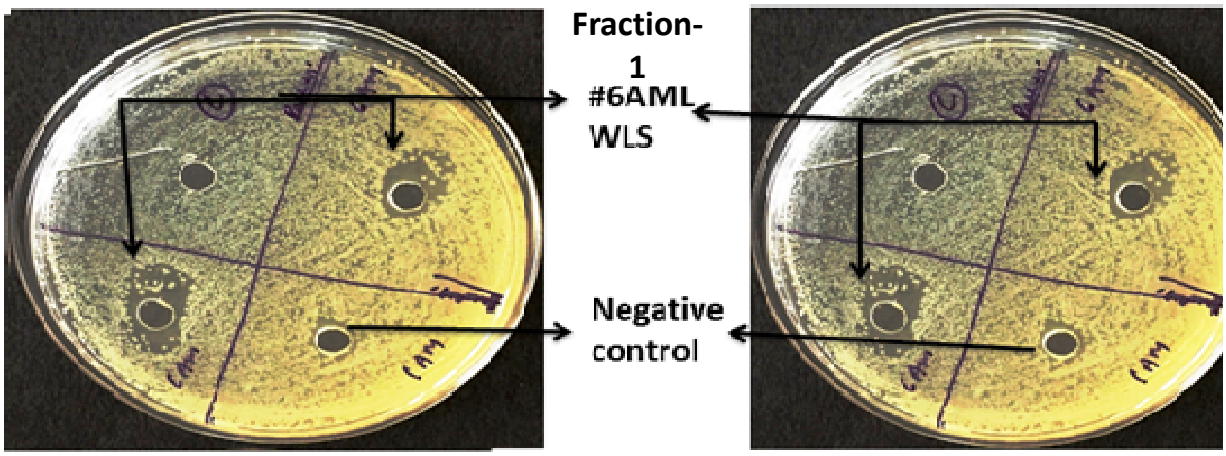


Figure 12: **Antimicrobial activity of #6AMLWLS towards MTCC 441 showing zone of inhibition.**

Table 10: Zone of inhibition (in mm) against the microbial cultures

Sample	Antibacterial assay		Antifungal assay	
	MTCC 441	**MTCC 647**	**ATCC 227**	**MTCC 3011**
#6AMLWLS	10.22 ± 0.87	12.67 ± 0.74	9.66 ± 1.15	11.33 ± 0.57
Positive Control	17.00 ± 1.00	16.33 ± 0.58	15.33 ± 0.57	16.66 ± 0.57
Negative Control	5 ± 0	5 ± 0	5 ± 0	5 ± 0

5.4. Identification of Endophytic Fungi

Morphotaxonomy of #6AMLWLS : The endophytic fungus #6 AMLWLS produced white, fast growing (90 ± 0) floccose aerial mycelium over PDA and MEA. Initially white in color and later becomes pale brown with smooth margin over PDA and MEA. Over Pine Leaf Agar (PLA) and Water Agar (WA) the margins were flat. On SNA medium it was white in color with wooly appearance. On PLA medium it was brown in color. On PDA and MEA hyphae were thick, septate and branched. Conidiophores were present in the aerial mycelium. Conidia developed over aerialconidiophores are generally fusi form to falcate in shape, usually 3-7 septate and both macro and micro conidia were present. On WA and SNA medium, colonies were moderate to fast growing, white in color, hyphae were septate and thick, conidia were fusi-form to slightly curved with foot shell, mostly 5-6 septate and both macro and micro conidia were present **(Figure-13)**. Based on these morphological features the fungus was identified as *Fusarium* sp.

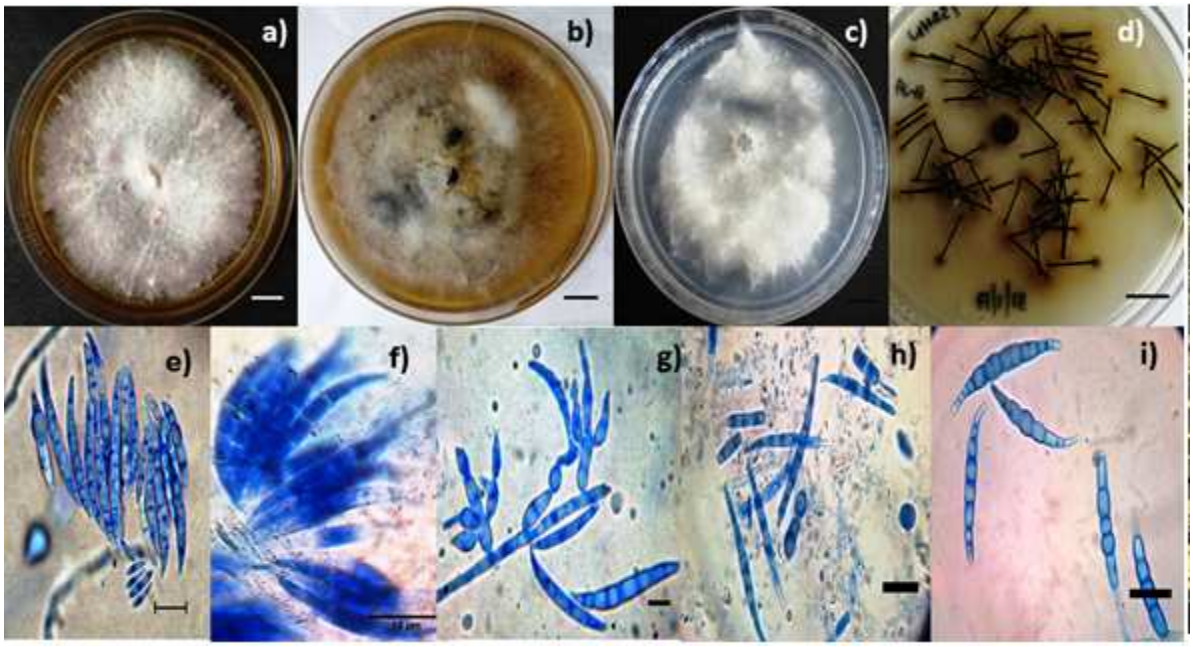


Figure 13:Morphological and microscopic feature of #6AMLWLS; colony morphology on **a)** PDA medium; **b)** MEA medium; **c)** SNA medium; **d)** PLA medium (Bar : 10mm); **f)** and **i)** macro conidia over PDA medium; **e)** micro and macro conidia on SNA medium; **g)** and **h)** macro conidia with chlamydospores (Bar : 10µm).

Molecular Identification of #6AMLWLS

The genomic DNA was isolated and the concentration of the isolated DNA used for amplification was found to be 35ng/µl isolated from endophytic fungi. Absorbance of the sample was taken at range 260/280 nm, which defines the purity and quantity of the sample.

For qualitative estimation, DNA was run at 0.7% agarose gel electrophoresis as shown in **Figure 14**.

Molecular identification of the endophytic fungi was done by using primers specific PCR (a rapid and accurate DNA amplification process in order to get large amount of DNA fragments of specific sequence). PCR amplicons were resolved at 1.5% agarose by using agarose gel electrophoresis and compared with 500 bp ladder. The size of the PCR amplicon was found to be approx. 550 bp for ITS specific primers **(Figure 14)**.Sample was amplified in bulk, purified and was sent for sequencing at Eurofins Genomics India Pvt. Ltd., Bangalore.

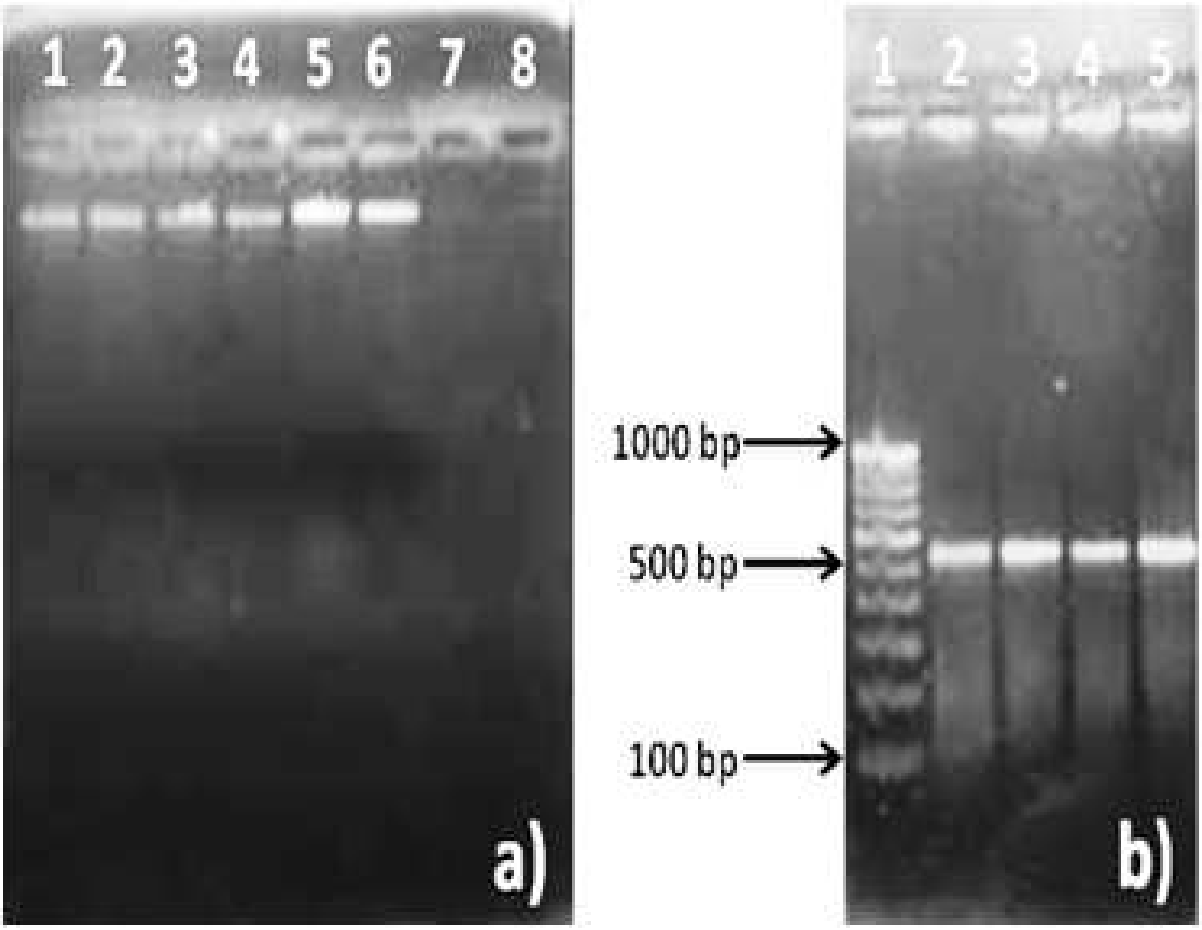


Figure 14: Agarose gel electrophoresis of **(a)** genomic DNA isolated from #6AMLWLS and **(b)** PCR product of isolated genomic DNA where lane 1 is ladder of 1000 bp and lane 2-5 are PCR products.

Sequence Assembly and alignment

The final sequence of PCR amplicon of #6AMLWLS was sent to genbank under the accession number KC960885 and its sequence similarity was searched so as to establish the

positional homology of final sequence using BLAST. It was found that BLAST analysis found 100% sequence similarity with *Fusarium equiseti* strain Fe3 (Table 11).

Table 11: BLAST analysis of final sequence of #6AMLWLS

Accession no.	Description	% similarity	Query coverage	e-value
	#6AMLWLS ITS1-5.8-ITS4 gene			
HQ718414	*Fusarium equiseti* strain	96	100%	0.0
KF913195	*Fusarium* sp.	96	99	0.0
KJ412504	*Fusarium equiseti* isolate JG55	96	99	0.0
FJ459976	*Fusarium equiseti* isolate T34	96	99	0.0
GQ352488	*Fusarium* SP. 141GP/S	96	99	0.0
KC513507	*Fusarium equiseti* strain MTAM	96	100	0.0
KJ412509	*Fusarium equiseti* isolate JG65	96	99	0.0
AB425996	*Fusarium equiseti* genes	96	99	0.0
KJ412508	*Fusarium equiseti* isolate JG64	96	99	0.0
HQ718416	*Fusarium equiseti* strain Fe3	100	96	0.0

The final sequence of #6AMLWLS is as follows:

>6AMLWLSITS1 & ITS4

GGNANNAGGCGGAGGATTCGGAGGTGAGCTGCGGAGGNATCATTAATAGGAGG
AGGCCGCCGGTGGCCTTCAGCGGGACGGCCCGCCCGAGGACCCCTAAACTCTGT
TTTTAGTGGAACTTCTGAGTAAAACAAACAAATAAATCAAAACTTTCAACAACG
GATCTCTTGGTTCTGTGATCGATGAAGAACGCAGCAGAATGCGATAAGTAATGTG
AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGT
ATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCAGCTTGGT
GTTGGGACTCGCGGTAACCCGCGTTCCCCAAATCGATTGGCGGTCACGTCGAGCT
TCCATAGCGTAGTAATCATACACCTCGTTACTGGTAATCGTCGCGGCCACGCCGT
AAAACCCCAACTTCTGAATGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACT
TAAGCATATCAATAAGCGGAGGAACGC

NAGGCAATCCCTCCGCTTATTGAGTGCTCCNCAGGGGTCCTCCGGAAGCCTCCCC
CCATTACTAGGCTCCCCCCCTGGGCCTACTACGGACTGCCCCCTCGATGTGTCCG
CCNATCGATTTGGGGAACGCGGGTTACCGCGAGTCCCAACACCAAGCTGAGCTT
GAGGGTCGAAATGACGCTCGAACAGGCATGCCCGCCAGAATACTGGCGGGCGCA
ATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCG
CATTTGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTT
TGATTTATTTGTTTGTTTTACTCAGAAGTTCCACTAAAAACAGAGTTTAGGGGTCC

TCGGGCGGGCCGTCCCGTTTTACGGGGCGCGGGCTGATCCGCCGAGGCAACGTA
TAGGTATGTTCACAGGGGTTTGGGAGTTGTAAACTCGGTAATGATCCCTCCGCAG
GTTCACNTACNGAAG

5.5. Chemical Characterization

Chemical characterization of the pure fraction-1 was carried out by phytochemical tests that included alkaloids, terpenoids, steroids, phenols, flavonoids and amines. Among theses, the test for the presence of terpenoids was confirmed due to appearance of light pink colour in the Liebermann Burchardt reagent.

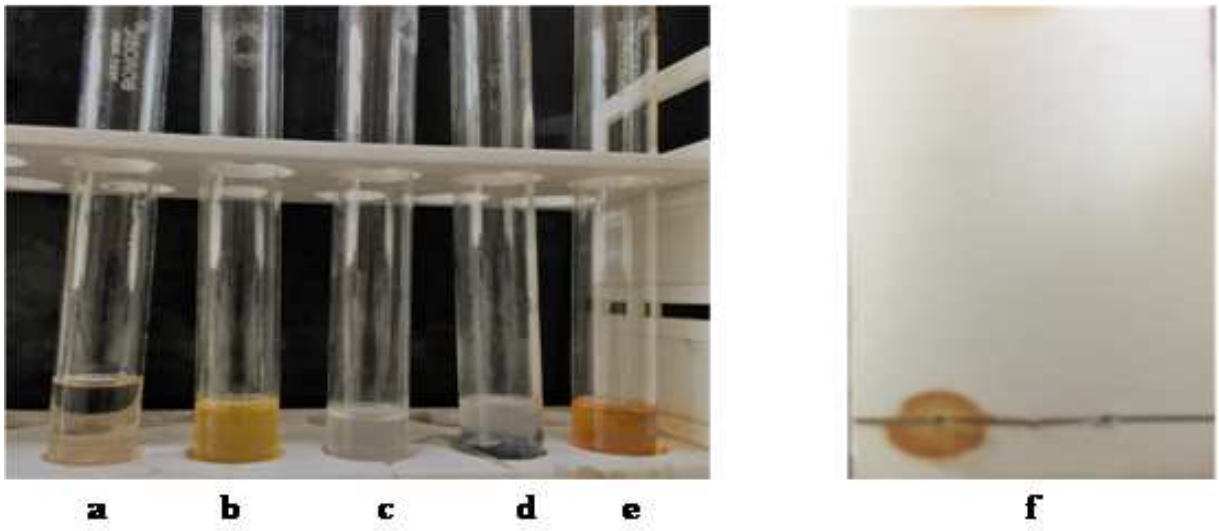


Figure 15: Test results for (a) Presence of terpenoids and absence of steroids, and absence of **(b)** Phenols, **(c)**&**(d)** Flavonoids **(e)** Alkaloids and **(f)** Amines (coloured spot for standard amine and no colour on second spot).

Spectroscopic characterization of the compound was done using ^{1}H NMR technique. Absence of peak in the aromatic region was in line with the low phenolic and flavanoid content of the pure compound. Appearance of two signals, a multiplet at 5.8 ppm for one proton and a double doublet at 5.0 ppm for two protons indicated the presence of a terminal double bond. Besides this 56 protons in the aliphatic region that included three protons corresponding to a triplet at 0.9 ppm and others at 1.3 ppm indicated the presence of a terpene group.

A look at the literature revealed that pythol is a terpenoid obtained from *Aegle Marmelos* plant, from which the endophytic fungus has been isolated has strikingly similar NMR spectra as that of pure fraction-1[62]. Thus, **figure -16** gives a hint of the isolated compound.

However further spectroscopic studies are required to completely elucidate the complete structure of the isolated pure fraction.

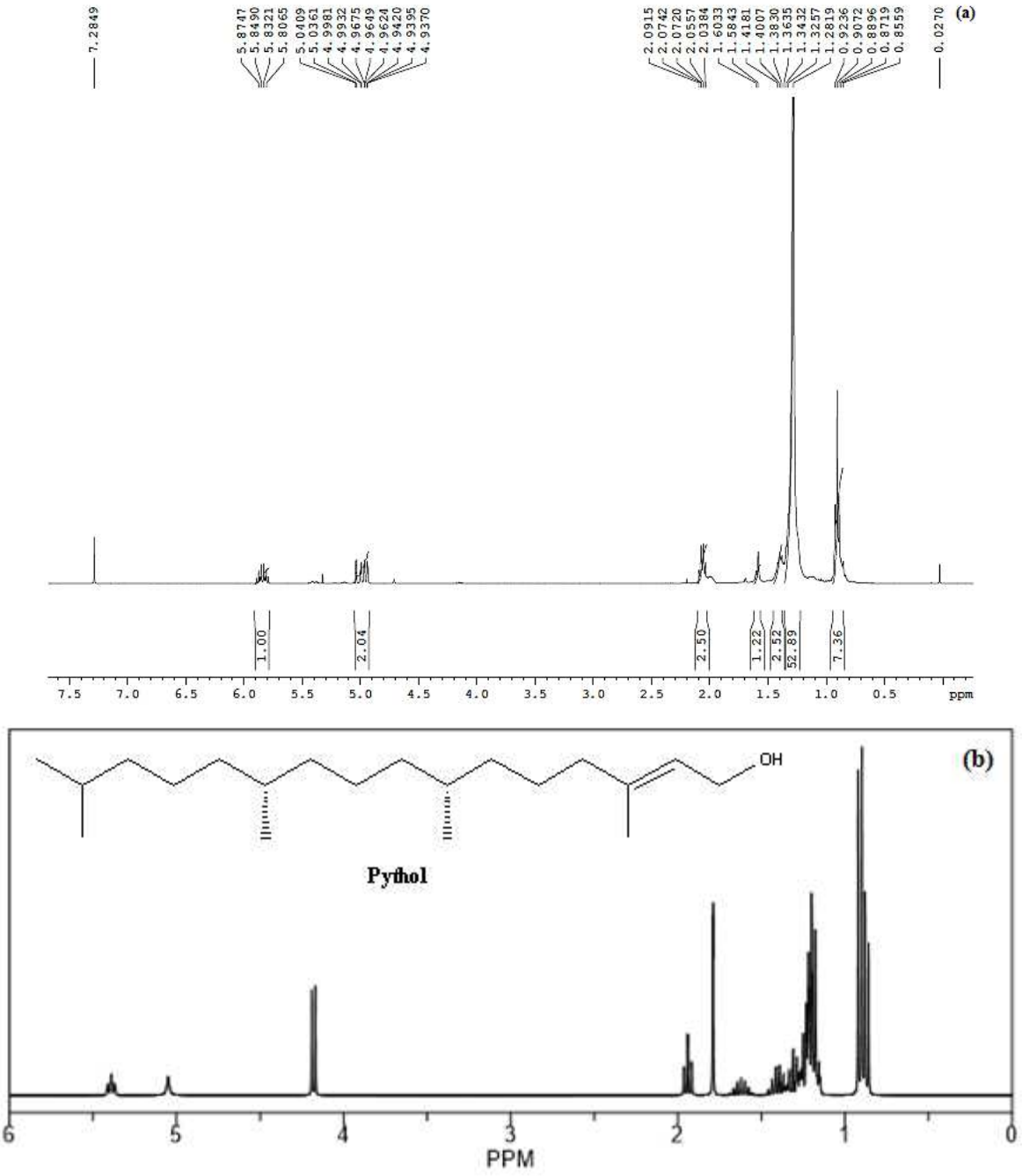


Figure 16: (a) Comparing the ^{1}H NMR of isolated fraction-1 with that of (b) predicted structure of pythol shows striking similarity.

Conclusion

The work describes isolation of a pure compound from fungus named #6AMLWLS that grows on *Aegle Marmelos* and its xanthine oxidase inhibition activity besides antioxidative and antimicrobial activity. Besides complete morphological and genetic characterization of fungus an attempt has been made to elucidate its structure.

REFERENCES

1. Bardin, T. and Richette, P.; Definition of hyperuricemia and gouty conditions. *Current Opinion in Rheumatology*, **(2014)**, 26, 186-191.
2. Choi, H. K., Mount, D. B. and Reginato, A. M.; Pathogenesis of gout. *Annuals of Internal Medicine*, **(2005)**, 143, 499-516.
3. Neogi, T.; Gout. *New England Journal of Medicine*, **(2011)**, 364, 443-452.
4. Lioté, F.; Hyperuricemia and gout. *Current Rheumatology Reports*, **(2003)**, 5, 227-234.
5. Campion, E. W., Glynn, R. J. and Delabry, L. O.; Asymptomatic hyperuricemia. Risks and consequences in the Normative Aging Study. *The American Journal of Medicine*, **(1987)**, 82, 421-426.
6. Gaffo, A. L., Edwards, N. L. and Saag, K. G.; Gout. Hyperuricemia and cardiovascular disease: how strong is the evidence for a causal link? *Arthritis Research & Therapy*, **(2009)**, 11, 240.
7. Ragab, G., Elshahaly, M. and Bardin, T.; Gout: An old disease in new perspective–A review. *Journal of Advanced Research*, **(2017)**, 8, 495-511.
8. Kamatani, N.; Treatment of gout and hyperuricemia, an update. *Gout and Hyperuricemia*, **(2014)**, 1, 1-6 (DOI: 10.3966/231191872014030001001).
9. Robinson, P. C. and Horsburgh, S.; Gout: joints and beyond, epidemiology, clinical features, treatment and co-morbidities. *Maturitas*, **(2014)**, 78, 245-251.
10. Kapoor, N. and Saxena, S.; Xanthine oxidase inhibitory and antioxidant potential of Indian Muscodor species. *3 Biotech*, **(2016)**, *6*\, 248.
11. Bajaj, K., Burudkar, S., Shah, P., Keche, A., Ghosh, U., Tannu, P. and Ahire, Y.; Lead optimization of isocytosine-derived xanthine oxidase inhibitors. *Bioorganic and Medicinal Chemistry Letters*, **(2013)**, *23*, 834-838.

12. Mehta, S. K. and Nayeem, N.; Natural Xanthine Oxidase Inhibitors for Management of Gout: A Review. *Research and Reviews: Journal of Medical and Health Sciences*, **(2014)**, 3, 4-13.
13. Iwanaga, T., Kobayashi, D., Hirayama, M., Maeda, T. and Tamai, I.; Involvement of uric acid transporter in increased renal clearance of the xanthine oxidase inhibitor oxypurinol induced by a uricosuric agent, benzbromarone. *Drug Metabolism and Disposition*, **(2005)**, 33, 1791-1795.
14. Guan, Q., Cheng, Z., Ma, X., Wang, L., Feng, D., Cui, Y. and Zhang, W.; Synthesis and bio evaluation of 2-phenyl-4-methyl-1, 3-selenazole-5-carboxylic acids as potent xanthine oxidase inhibitors. *European Journal of Medicinal Chemistry*, **(2014)**, 85, 508-516.
15. Tan, R. X. and Zou, W. X.; Endophytes: a rich source of functional metabolites. *Natural Product Reports*, **(2001)**, 18, 448-459.
16. Pimentel, M. R., Molina, G., Dionísio, A. P., Maróstica Junior, M. R. and Pastore, G. M.; The use of endophytes to obtain bioactive compounds and their application in biotransformation process. *Biotechnology Research International*, **(2011)**. http://dx.doi.org/10.4061/2011/576286
17. Maiuolo, J., Oppedisano, F., Gratteri, S., Muscoli, C. and Mollace, V.; Regulation of uric acid metabolism and excretion. *International Journal of Cardiology*, **(2016)**, 213, 8-14.
18. Moriwaki, Y., Yamamoto, T. and Higashino, K.; Enzymes involved in purine metabolism-a review of histochemical localization and functional implications. *Histology and Histopathology*, **(1999)**, 14, 1321-1340.
19. Granger, D. N.; Role of xanthine oxidase and granulocytes in ischemia-reperfusion injury. *American Journal of Physiology-Heart and Circulatory Physiology*, **(1988)**, 255, 1269-1275.
20. Enroth, C., Eger, B. T., Okamoto, K., Nishino, T., Nishino, T. and Pai, E. F.; Crystal structures of bovine milk xanthine dehydrogenase and xanthine oxidase: structure-based mechanism of conversion. *Proceedings of the National Academy of Sciences*, **(2000)**, 97, 10723-10728.
21. McCord, J. M.; Oxygen-derived free radicals in postischemic tissue injury. *New England Journal of Medicine*, **(1985)**, 312, 159-163.
22. Harrison, R.; Structure and function of xanthine oxidoreductase: where are we now? *Free Radical Biology and Medicine*, **(2002)**, 33, 774-797.

23. Cao, H., Pauff, J. M., and Hille, R.; X-ray crystal structure of a xanthine oxidase complex with the flavonoid inhibitor quercetin. *Journal of Natural Products*, **(2014)**, 77, 1693-1699.
24. Ekpenyong, C. E. and Daniel, N.; Roles of diets and dietary factors in the pathogenesis, management and prevention of abnormal serum uric acid levels. *PharmaNutrition*, **(2015)**, 3, 29-45.
25. Kuo, C. F., Grainge, M. J., Zhang, W. and Doherty, M.; Global epidemiology of gout: prevalence, incidence and risk factors. *Nature Reviews Rheumatology*, **(2015)**, 11, 649-662.
26. Gliozzi, M., Malara, N., Muscoli, S. and Mollace, V.; The treatment of hyperuricemia. *International Journal of Cardiology*, **(2016)**, 213, 23-27.
27. Pacher, P., Nivorozhkin, A. and Szabó, C.; Therapeutic effects of xanthine oxidase inhibitors: renaissance half a century after the discovery of allopurinol. *Pharmacological Reviews*, **(2006)**, 58, 87-114.
28. Wang, Z., Kwon, S. H., Hwang, S. H., Kang, Y. H., Lee, J. Y. and Lim, S. S.; Competitive binding experiments can reduce the false positive results of affinity-based ultrafiltration-HPLC: A case study for identification of potent xanthine oxidase inhibitors from *Perilla frutescens* extract. *Journal of Chromatography B*, **(2017)**, 1048, 30-37.
29. Liu, H. X., He, M. T., Tan, H. B., Gu, W., Yang, S. X., Wang, Y. H. and Long, C. L.; Xanthine oxidase inhibitors isolated from Piper nudibaccatum. *Phytochemistry Letters*, **(2015)**, 12, 133-137.
30. Tsai, S. F. and Lee, S. S.; Neolignans as xanthine oxidase inhibitors from Hyptis rhomboides. *Phytochemistry*, **(2014)**, 101, 121-127.
31. Izumida, H., Adachi, K., Mihara, A., Yasuzawa, T. and Sano, H.; Hydroxyakalone, a novel xanthine oxidase inhibitor produced by a marine bacterium, Agrobacterium aurantiacum. *The Journal of Antibiotics*, **(1997)**, 50, 916-918.
32. Sunahara, N., Nogi, K. and Yokogawa, K.; Production of xanthine oxidase inhibitor, 2, 8-dihydroxyadenine, by Alcaligenes aquamarinus. *Agricultural and Biological Chemistry*, **(1977)**, 41, 1103-1109.
33. Smelcerovic, A., Rangelov, M., Smelcerovic, Z., Veljkovic, A., Cherneva, E., Yancheva, D. and Kocic, G.; Two 6-(propan-2-yl)-4-methyl-morpholine-2, 5-diones as new non-purine xanthine oxidase inhibitors and anti-inflammatory agents. *Food and Chemical Toxicology*, **(2013)**, 55, 493-497.

34. Härtl, A., Stelzner, A., Ritzau, M., Heinze, S. and Gräfe, U.; 5-Hydroxy-3,4,7-triphenyl-2,6-benzofurandione, a new xanthine oxidase inhibitor from *Peniophora sanguinea*. *The Journal of Antibiotics*, **(1998)**, 5, 528-530.
35. Song, Y. C., Li, H., Ye, Y. H., Shan, C. Y., Yang, Y. M. and Tan, R. X.; Endophytic naphthopyrone metabolites are co-inhibitors of xanthine oxidase, SW1116 cell and some microbial growths. *FEMS Microbiology Letters*, **(2004)**, 241, 67-72.
36. Rengasamy, K. R., Slavětínská, L. P., Kulkarni, M. G., Stirk, W. A. and Van Staden, J.; Cuparane sesquiterpenes from *Laurencia natalensis Kylin* as inhibitors of alpha-glucosidase, dipeptidyl peptidase IV and xanthine oxidase. *Algal Research*, **(2017)**, 25, 178-183.
37. Firáková, S., Šturdíková, M. and Múčková, M.; Bioactive secondary metabolites produced by microorganisms associated with plants. *Biologia*, **(2007)**, 62, 251-257.
38. Wani, M. C., Taylor, H. L., Wall, M. E., Coggon, P. and McPhail, A. T.; Plant antitumor agents. VI. Isolation and structure of taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia*. *Journal of the American Chemical Society*, **(1971)**, 93, 2325-2327.
39. Wall, M. E., Wani, M. C., Cook, C. E., Palmer, K. H., McPhail, A. A. and Sim, G. A.; Plant antitumor agents. The isolation and structure of camptothecin, a novel alkaloidal leukemia and tumor inhibitor from camptotheca acuminata1. *Journal of the American Chemical Society*, **(1966)**, *88*, 3888-3890.
40. Deshmukh, S. K., Mishra, P., Almeida, A.K., Verekar, S., Sahoo, M. R., Periyasamy, G., Khanna, A.,Goswami, H., Balakrishan, A. and Vishwakarma, R.; Anti-inflammatory and anticancer activity of ergoflavin isolated from an endophytic fungus. *Chemistry and Biodiversity*, **(2009)**, 6, 784-789.
41. Harper, J. K., Arif, A. M., Ford, E. J., Strobel, G. A., Porco, J. A., Tomer, D. P. and Grant, D. M.; Pestacin: a 1, 3-dihydro isobenzofuran from *Pestalotiopsis microspora* possessing antioxidant and antimycotic activities. *Tetrahedron*, **(2003)**, 59, 2471-2476.
42. Strobel, G., Ford, E., Worapong, J., Harper, J. K., Arif, A. M., Grant, D. M. and Chau, R. M. W.; Isopestacin, an isobenzofuranone from Pestalotiopsismicrospora, possessing antifungal and antioxidant activities. *Phytochemistry*, **(2002)**, *60*, 179-183.
43. Liu, X., Dong, M., Chen, X., Jiang, M., Lv, X. and Zhou, J.; Antimicrobial activity of an endophytic Xylaria sp. YX-28 and identification of its antimicrobial compound 7-

amino-4-methylcoumarin. *Applied Microbiology and Biotechnology*, **(2008)**, 78, 241-247.

44. Agarwal, G. P. and Hasija, S. K.; Microorganisms in the laboratory - A laboratory guide of mycology, microbiology and plant pathology. *Print House (India) Lucknow*, **(1986)**.
45. Rodrigues, K. F., Hesse, M. and Werner, C.; Antimicrobial activities of secondary metabolites produced by endophytic fungi from *Spondiasmombin*. *Journal of Basic Microbiology: An International Journal on Biochemistry, Physiology, Genetics, Morphology, and Ecology of Microorganisms*, **(2000)**, 40, 261-267.
46. Choudhary, M. I., Musharraf, S. G., Mukhmoor, T., Shaheen, F., Ali, S. and Rahman, A. U.; Isolation of bioactive compounds from Aspergillus terreus. *Zeitschrift für Naturforschung B*, **(2004)**, 59, 324-328.
47. Kapoor, N. and Saxena, S.; Potential xanthine oxidase inhibitory activity of endophytic *Lasiodiplodia pseudotheobromae*. *Applied Biochemistry and Biotechnology*, **(2014)**, 173, 1360-1374.
48. Agarwal, A. and Banerjee, U. C.; Screening of xanthine oxidase producing microorganisms using nitroblue tetrazolium based colorimetric assay method. *The Open Biotechnology Journal*, **(2009)**, 3, 46-49.
49. Kumar, G. S., Jayaveera, K. N., Kumar, C. K., Sanjay, U. P., Swamy, B. M. and Kumar, D. V.; Antimicrobial effects of Indian medicinal plants against acne-inducing bacteria. *Tropical Journal of Pharmaceutical Research*, **(2007)**, *6*, 717-723.
50. Parekh, J., Karathia, N. and Chanda, S.; Evaluation of antibacterial activity and phytochemical analysis of Bauhinia variegata L. bark. *African Journal of Biomedical Research*, **(2006)**, 9, 53-56.
51. Devi, P. S., Satyanarayana, B. and Naidu, M. T.; Phytochemical Screening for Secondary Metabolites in *Boswellia serrata* (Roxb.) and *Wrightia tinctoria* (Roxb.) R. Br. *Notulae Scientia Biologicae*, **(2014)**, 6, 474-477.
52. Samatha, T., Srinivas, P., Shyamsundarachary, R., Rajinikanth, M. and Rama Swamy, N.; Phytochemical analysis of seeds, stem bark and root of an endangered medicinal forest tree *Oroxylum indicum* (L) Kurz. *Int. J. Pharm. Bio. Sci*, **(2012)**, 3, 1063-1075.
53. Kitts, D. D., Wijewickreme, A. N. and Hu, C.; Antioxidant properties of a North American ginseng extract. *Molecular and Cellular Biochemistry*, **(2000)**, 203, 1-10.

54. Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M. and Rice-Evans, C.; Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine*, **(1999)**, 26, 1231-1237.
55. Sriplang, K., Adisakwattana, S., Rungsipipat, A. and Yibchok-Anun, S.; Effects of *Orthosiphon stamineus* aqueous extract on plasma glucose concentration and lipid profile in normal and streptozotocin-induced diabetic rats. *Journal of Ethnopharmacology*, **(2007)**, 109, 510-514.
56. Luximon-Ramma, A., Bahorun, T., Soobrattee, M. A. and Aruoma, O. I.; Antioxidant activities of phenolic, proanthocyanidin, and flavonoid components in extracts of Cassia fistula. *Journal of Agricultural and Food Chemistry*, **(2002)**, 50, 5042-5047.
57. Bariş, Ö.,Güllüce, M., ŞAHİN, F., Özer, H., Kiliç, H., Özkan, H. and Özbek, T.; Biological activities of the essential oil and methanol extract of Achillea biebersteinii Afan.(Asteraceae). *Turkish Journal of Biology*, **(2006)**, 30, 65-73.
58. Barnett, H. L. and Hunter, B. B.; Descriptions and illustrations of genera. *Illustrated genera of imperfect fungi, 4th edn. American Phytopathological Society, St. Paul, MN*, **(1998)**, 68-69.
59. White, T. J., Bruns, T., Lee, S. J. W. T. and Taylor, J. L.; Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: a guide to methods and applications*, **(1990)**, 18, 315-322.
60. Naraginti, S., Li, Y., Wu, Y., Zhang, C. and Upreti, A. R.; Mechanistic study of visible light driven photocatalytic degradation of EDC 17α-ethinylestradiol and azo dye Acid Black-52: phytotoxicity assessment of intermediates. *RSC Advances*, **(2016)**, 6, 87246-87257.
61. Park, S. H., Kim, J., Tak, Y. K., & Song, J. M.; On chip superoxide dismutase assay for high-throughput screening of radioprotective activity of herbal plants. *Nano/Molecular Medicine and Engineering,* **(2010)** *IEEE 4th International Conference,* 218-222.
62. Mujeeb, F., Bajpai, P. and Pathak, N.; Phytochemical evaluation, antimicrobial activity, and determination of bioactive components from leaves of *Aegle marmelos*. *BioMed Research International*, **(2014)**, 2014, 1-11.