Bioactive potential of fungal endophytes isolated from medicinal plants

A thesis submitted in partial fulfillment of the requirements for the award of the degree of

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Submitted by

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CERTIFICATE

I hereby declare that the work presented in Ms. Manmeet Kaur's thesis "**Bioactive potential of Fungal endophytes isolated from Medicinal plants** " submitted in partial fulfilment of the requirement for the award of the degree of Master of Technology in Biotechnology, TIET, Patiala, is a record of student's own work carried out under my supervision and guidance. This report has not been submitted for consideration for any other degree or certificate at this or any other institution.

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DECLARATION

I hereby declare that the work presented in this thesis, "**Bioactive potential of Fungal endophytes isolated from Medicinal plants**," submitted under the supervision of **Dr M. Vasundhara** in partial fulfilment of the requirement for the award of the degree of Master of Technology in Biotechnology, Department of Biotechnology, TIET, Patiala, is my own original research work completed between August 2020 and July 2022. This report has not been submitted for any other degree or certificate at this or any other institute.

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ABSTRACT

A variety of therapeutic compounds may be found in medicinal plants, which are a natural and well-known source.

Endophytic fungi that colonise medicinal plants are a hot topic of investigation because they have the potential to create biologically active compounds with antibacterial, antioxidant, cytotoxic, and immunomodulatory activities.

Endophytic fungi were isolated in the current study using *Eucalyptus teretecornis*. Three different fungal endophytes from the leaf of *E. teretecornis* were isolated. T6 isolated from *T.baccata* L subsp. *wallichiana* (Zucc.) Pilger and AL2 isolated from *Terminalia arjuna* cultures were offered by the lab. These extracts underwent additional preliminary screening to determine their potential for bioactivity. All of these extracts were subjected to a variety of activities, including antibacterial, anti-oxidant, and anti-inflammatory to determine their bioactive potential.

The inhibition of *S. aureus* by AL2 at 500µg/ml was 43.8 percent, compared to a 25.7percent inhibition by T6. In terms of antioxidant power, it was shown that AL2 at 500µg/ml had 61.3 percent radical scavenging activity, compared to 44.8 percent for T6. Additionally, EL1showed its highest level of scavenging activity at 1000µg/ml, of 67.5 percent. When AL2 was subjected to the protein denaturation inhibition experiment it was revealed that AL2 crude had a remarkable inhibition percentage of 91.3 percent.

AL2 once again demonstrated its superiority as an extract to be employed in future studies on the bioactivities of screened extracts.

A preliminary TLC evaluation was performed, then fractionation was accomplished using column chromatography. Fourteen fractions of AL2 were produced after column chromatography; these were then put through a protein denaturation inhibition assay, where fractions 12, 13, and 14 were shown to be effective.

The LOX inhibition experiment was then performed on these fractions, and fraction 12 displayed the highest percentages of inhibition. This led to the decision to characterise it using UV, FTIR, NMR and HR-MS studies.

INTRODUCTION

On the surface of the world, nature has a slew of life forms. Microorganisms are everywhere in nature. Among these life forms endophytes are a group of microorganisms that are very appealing. In a single plant or microbe, endophytes are said to create a multitude of bioactive metabolites that have potential uses in the food, cosmetics, pharmaceutical, and agricultural sectors as well as being a great source of therapeutics for treating a wide variety of ailments (Godstime et al., 2014). Endophytes are named after the Greek word endon, which means "within the plant" (phyton = plant).

According to De Bary (1866), endophytes are organisms that permeate the tissue of a host plant without harming it. Endophytes are organisms that thrive inside without inflicting immediate harm to the plants, the interior tissues of plants, according to Bacon and White (2000).

They can be present in almost every plant on the planet. Endophytic fungi have a wide biological variety, with around three hundred thousand terrestrial host plant species found in temperate and tropical rainforests. These are microorganisms that dwell inside the host plant's single tissues. Fungi, bacteria, and actinomycetes are examples of endophytes.

Endophytes are biological repositories for new compounds, bringing up new options for medication development. Plant-associated bacteria, which populate the interior tissues of all plant species, are becoming more popular as bio-prospecting targets in the hunt for new chemical entities. Endophytic fungi create bioactive compounds that are unique from those generated by their host plants, and these molecules are essential for enhancing endophytic fungi and their hosts' adaptability, such as tolerance to biotic and abiotic stimuli. Furthermore, these substances can produce a range of recognized and unknown physiologically active secondary metabolites (Firáková et al., 2007), which humans can employ as valuable therapeutic resources (Rodriguez et al., 2009).

The discovery of the well-known anticancer drug paclitaxel boosted endophytic biology research, resulting in promising "drug candidates" with antibacterial, immunosuppressive, antioxidant, and anti-neurodegenerative properties. The pharmaceutical industry is currently using high-throughput techniques to screen plant secondary metabolites as prospective lead compounds, demonstrating the importance of natural products in clinical application.

According to research by Newman and Cragg, 141 of the 1562 drugs authorized by the FDA were botanical pharmaceuticals, 64,320 were natural product derivatives, and 61 were synthesized based on natural pharmacophore.

Endophytic fungi are polypletic organisms that live in close proximity to their host plant, most commonly in the leaves, stems, and roots (Bacon and White 2000). They have a wide spectrum of relationships with their hosts, ranging from mutualistic to aggressive.

After a lengthy period of coexistence, each endophytic fungus and its host plant developed a pleasant relationship. This relationship is beneficial to both organisms. The host plant provides endophyte fungi with food, shelter, and protection in exchange for which they give the host plant with a variety of advantages, including enhanced immunity, increased resistance to herbivores and diseases, and increased tolerance to abiotic and biotic stressors (Saikkonen et al., 1998).

These organisms can also produce secondary metabolites identical to those produced by their hosts (Tan and Zou, 2001). *Taxomyces andreanae*, an endophytic fungus from the Pacific yew (*Taxus brevifolia*) that can create taxol, a very powerful anticancer treatment derived from its host plant, is an example of fungus-produced new chemicals (Stierle et al., 1993, Wani et al., 1971). Endophytic fungi may have undergone genetic recombination through time, inheriting the ability to produce metabolites in the same manner as host plants do.

The ability to manufacture host metabolites is also thought to be owing to genetic recombination during evolution. There are also a significant number of endophytic fungi that are formed from a single plant tissue, necessitating endophytic fungi screening. Screening aids in the identification of fungus with the ability to create chemicals with medicinal potential (Stierle et al., 1993; Zhang et al., 2006).

Alkaloids, steroids, tannins, terpenoids, quinones, lignans, phenols, and lactones are some of the bioactive substances derived from endophytic fungi. Many valuable secondary metabolites have been extracted from fungal endophytes, with the potential to be used as antibacterial, herbicidal, antioxidant, insecticidal, cytotoxic, and anticancer chemicals, according to the literature (Vasundhara et al., 2016).

Azila et al. examined the anti-inflammatory effects of *Trametes lactinea* and suggested that the growth and activities rely on the culture conditions, such as medium composition, pH, inoculum volume, temperature of incubation, and incubation period. Terrein, brefeldin A,

and asperlin are examples of polyketide fungal metabolites that have been shown to have unique anticancer effects (Y.Y Azila 2014).

From the medicinal plant *E. crista-galli*, Weber et al. purified the new antibiotic component Phomol and isolated Phomopsis. This substance has mild cytotoxic, anti-inflammatory, and antibacterial properties (Weber 2004).

With drug-resistant bacteria and antimicrobial resistance (AMR) on the rise, it's more important than ever to find new antimicrobials to combat the problem. However, to compensate for the drying drug pipeline, the slow pace of development and indiscriminate use of current antibiotics has prompted the study of new antimicrobial properties. Due to the widespread use of antibiotics to treat common bacterial diseases, antibiotic resistance is rapidly growing, which is a sign that there are no more powerful medicines available. Recent corona pandemics have made this more blatant. Similar to this, increased antimicrobial resistance (AMR) is increasing the virulence and pathogenicity of infectious microorganisms. Endophytes have been shown to express several novel ,bioactive substances with noteworthy biological activity. Bioactive metabolites with distinctive skeletons have been found, namely in endophytic fungi, and they may aid in reducing the development of antibiotic resistance.

An innovative technique used in the production of bioactive chemicals employing recombinant microorganisms as hosts is co-culture engineering. This technique uses microbial resources and metabolic engineering to reconstruct the target compound's biosynthesis pathway. This innovative approach also enables overcoming the metabolic limitations of each strain by distributing the metabolic loads among many hosts. Additionally, it offers a fresh viewpoint on how to address problems such as the expression of different proteins, the efficient use of complicated substrates with diverse compositions, the quick reconstruction of novel biosynthetic pathways, and other hitherto unrecognized difficulties.

By merging cutting-edge methods like machine learning and artificial intelligence, co-culture is also expected to develop into a powerful instrument. It enables control over gene induction, restriction against the inhibition of intermediates, export of products, and balancing of co-culture system to sustain constantly autonomous performance on an industrial scale. As a result, the field of metabolic engineering as a whole may see extensive use of modular co-culture engineering.

OBJECTIVES

The objectives of the project are listed underneath:

1. To study the bioactive potential of endophytic fungi isolated from medicinal plants
2. Fractionation, purification and characterization of crude extract from endophytic fungi
3. Co-culture of isolated endophytic fungi for secondary metabolite production.

REVIEW OF LITERATURE

2.1 Endophytes

Different academics have defined the term endophyte in various ways in recent years.

De Barry (1866) was the first to coin the term "endophyte" to distinguish fungi that dwell within host tissues from epiphytes. Except for mycorrhizal and pathogenic fungi, Carroll (1986) defined these organisms as those that live in plant tissues without infecting the host plant asymptomatically.

Carroll's definition was then broadened by Petrini (1991) to encompass all organisms that permeate plant tissue without affecting the host at any stage of their life cycle. According to Wilson, endophytes are fungi and bacteria that live in plants for all or part of their life cycle and do not cause disease (1995).

2.2 The transmission and categorization of endophytic fungi

Endophytes can transmit in two different ways: horizontally and vertically. The majority of endophytes use the horizontal mode of transmission to reach their host plants via airborne spores. Other endophytes may use seeds as a vertical mechanism of transmission. Endophytes that never leave their host and are transferred vertically to the host are known as true endophytes (Hartley and Gange 2009). Based on variations in host plant, taxonomy, ecological features, and evolutionary relatedness, (Rodriguez 2009) categorized endophytic fungi into two categories (Clavicipitaceous endophytes and non clavicipitaceous endophytes) or four classes (class 1, 2,3, 4) (Figure 1).

Class 1 Clavicipitaceous endophytes:

- Endophytes of the Clavicipitaceous class infect grasses, sedges, and rushes.
- Endophytes, saprophytes, and pathogens infiltrate as endophytes, saprophytes, and pathogens, all of which are thought to create bioactive chemicals.

Class 2 Non-clavicipitaceous endophytes:

- Ascomycetes account for the majority, whereas basidiomycetes account for the minority.
- They live inside the host plant's stems, roots, and leaves.

Class 3 Non-clavicipitaceous endophytes:

- These endophytes enter plant stems and belong to the dikaryomycota kingdom.

Class 4 Non-clavicipitaceous endophytes:

- These endophytes are dark septate endophytes that solely live inside roots of plants.

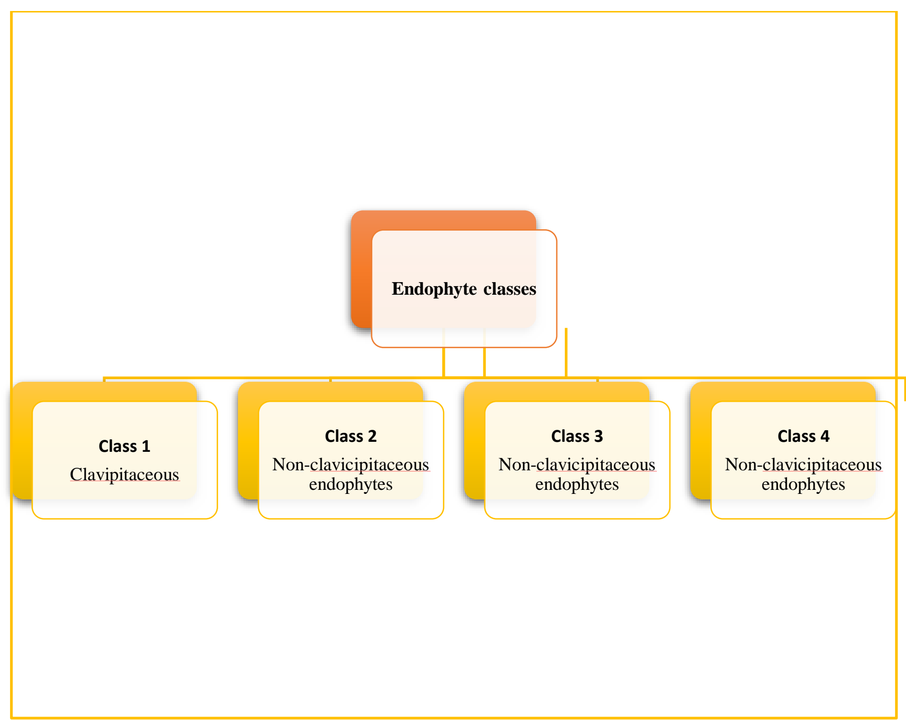

Figure 1: Classification of endophytic fungi

2.3 Endophytic fungus and their hosts' interactions

Endophytic fungus can be found asymptomatically in the stems, leaves, and roots of broad range of plants. Their hosts share a symbiotic relationship with them. They receive sustenance, shelter, and protection from the host plant in return for better tolerance to biotic and abiotic factors, as well as increased resistance to herbivores, diseases, and other risks. Endophytes and their hosts interact in a variety of ways, ranging from antagonistic to mutualistic, according to many studies (Saikonnen et al., 1998). These create a large range of metabolites that aid in the promotion of plant development. Some fungal endophytes, particularly those that spread horizontally, infect woody plants and have no beneficial effect on the host's growth or resistance.

To begin with, certain endophytic fungi may create a variety of plant hormones to aid in the development of their host plants (Waqas et al., 2012). *Azospirillum sp*., for example, might boost wheat (*Triticum aestivum L.*) growth in drought conditions (Dingle and McGee, 2003). Second, certain endophytic fungi create bioactive chemicals such alkaloids, diterpenes, flavonoids, and isoflavonoids to help their host plants withstand biotic and

abiotic challenges (Firáková et al., 2007; Rodriguez et al., 2009). Finally, some endophytic fungi may aid in the accumulation of secondary metabolites (such as vital therapeutic components or medications) generated by plants.

Various variables, such as the pattern of infection, environmental circumstances, mechanism of transmission, and genetic background, may have an impact on the endophyte's relationship with the host plant (Aly et al., 2011). The endophyte and its host plant have a complicated connection. Depending on a variety of conditions, the consequence of the connection might be symbiotic, parasitic, commensalism, or harmful. Mutualism is the result of a harmonious relationship between the host and endophytes in a given environment, whereas parasitism/pathogenesis/antagonism is the result of an imbalanced condition in which one benefits while the other suffers unfavorable consequences Endophytes that spread by seeds typically have a symbiotic relationship with their hosts, but those that spread vertically via spores have a hostile relationship with their hosts Another facet of this connection is the length of time that the fungus spends within the host.

Endophytic fungi live inside plants for the whole of their lives or for a specific length of time without ever creating disease signs (Hartley and Gange 2009). The disease triangle, according to Schulz and Boyle (2005), comprises of three elements that determine the outcome of the interaction between host and endophytes, including endophyte virulence, host defensive response, and environmental parameters.

A plant's endophyte might be a pathogen for other plants. They may adapt to a variety of life patterns when colonizing new hosts, ranging from mutualism to pathogenicity (Kogel et al., 2006). They behold a biotrophic relationship with the host. Depending on the availability of resources to endophytes and the metabolic condition of the host, this long-term relationship may evolve into mutualism or hostility (Lahrmann et al., 2013).

Under severe conditions, a fungus isolated as an endophyte has the potential to become a pathogen. It may serve as an endophyte in one host under certain conditions and then become a pathogen in another host or in a different environment (Garcia-Guzman and Heil 2013). Endophytes might be thought of as haphazard opportunists or latent pathogens that produce illness when conditions are stressful.

Discula quercina is a symptomless endophyte of Quercus cerris that becomes a weak pathogen when exposed to harsh environmental conditions (Moricca and Ragazzi 2008).

Below is a list of fungal endophytes offering defensive responses to host medicinal (Table 1).

Table 1: Endophytic fungi and their defensive response to host medicinal plants

S.No.	Host	Endophytic fungi	Functioning	References
i.	*Atracty lancea*	*Sclerotium sp*	Increase the host's leaf metabolic capacity and cell protection	Chen et al., 2008
ii.	*Cucumis sativus*	*Phoma glomerata*	Phytohormones	Waqas et al., 2012
iii.	*Pedicularis sp*	*Dark septate endophytic fungi*	Boost the efficiency with which they use nutrients.	Li and Guan, 2007
iv.	*Cirsium arvense*	*Chaetomium cochliodes*	Make some pathogen-toxic compounds.	Gange et al., 2012
v.	*Oryza sativa.*	*Sordariomycetes sp.*	Inhibition of electron transport from the quinone acceptor QA to QB.	Li and Zhang, 2015

2.4 Endophytes of fungi isolated from medicinal plants

Since ancient times, a broad varied plants have been investigated for the treatment of various maladies. Herbal treatments and their preparations are documented in ancient texts such as the Vedas and the Bible to be valuable in treating various diseases, and these are found to be helpful in healing sickness (Kaul et al., 2012). Within medicinal plants, a diverse microbiota produces innovative and diverse secondary metabolites.

Endophytic fungi have been seen to generate a wide range of phytochemicals, including, including paclitaxel (also known as taxol) (Stierle et al., 1993), podophyllotoxin (Eyberger

et al., 2006; Puri et al., 2006), deoxypodophyllotoxin (Kusari et al., 2009a), camptothecin, and structural analogues (Puri et al., 2005)

These are microorganisms that live inside the host tissue and use a specific metabolic route to survive. Because the metabolic pathways of the host and endophytes are similar, the likelihood of the creation of 13 secondary metabolites identical to those of the host plant increases. Under stressful situations, the rate and synthesis of these chemicals rises.

In response to the activated plant defence system against the invading pathogen, fungal endophytes produce a range of defensive components in the form of secondary metabolites. During this time, the ability to synthesis new secondary metabolites improves as well. Various environmental factors influence the host plant, which influences the endophytes' metabolic composition.

The endophyte and its host plant have a complicated connection. Depending on a variety of conditions, the consequence of the connection might be symbiotic, parasitic, commensalism, or harmful. Mutualism is the result of a harmonious interplay between the host and endophytes that is governed by environmental, physiological, and genetic factors, whereas parasitism/pathogenesis/antagonism is the result of an imbalanced condition in which one benefits while the other suffers unfavourable consequences (Kogel et al., 2006). Endophytes that transmit by seeds typically have a symbiotic relationship with their host, whereas those that transfer vertically via spores have an adversarial relationship with their host (Aly et al., 2011).

Researchers are attempting to isolate new chemicals from therapeutic plants that have yet to be found, as well as the endophytes that live on them. This might be beneficial to the pharmaceutical industry's disease-fighting efforts. Therefore, studying fungal endophytes is crucial. This group of plants might produce interesting substances in an environmentally favourable, inexhaustible, and low-cost manner, hence aiding biodiversity conservation. These also give researchers the opportunity to identify medicinal chemicals from indigenous species. Fermentation may be used to manufacture these chemicals on a big scale. Their capacity to produce bioactive compounds has sparked interest in bioprospecting therapeutic plants for them (Kusari et al., 2012).

Endophytes found in the Taxaceae family can produce taxol, which has anticancer effects. *Taxomyces andreanae*, an endophytic fungus isolated from the bark of *Taxus brevifolia* in

1993, was demonstrated to generate Taxol under in vitro axenic culture conditions (Stierle et al., 1993). Numerous researches have been carried on the wide spectrum of Taxol production from various endophytic fungus isolates in various batch cultures (Gangadevi and Muthumary, 2009). Paclitaxel (taxol) is a class of diterpenoids discovered by American scientists in the 1960s as a natural secondary metabolite in Pacific yew extract. It possesses potent anti-tumor properties, particularly in tumours with a high incidence. As a result, these important discoveries need additional investigation.

The most beneficial use is to take advantage of the benefits of endophytic fungi, which can boost the accumulation of secondary metabolites generated by plants. By introducing certain endophytic fungus to the plants, we can improve the production and accumulation of bioactive compounds in the host medicinal plants, resulting in higher grade crude pharmaceuticals. Given that the link between endophytic fungus and their host medicinal plants is well recognised, this application might open up a whole new world for the creation of very efficacious natural medicines.

Although there is still a substantial demand from the pharmaceutical industry for synthetic pharmaceuticals owing to cost and time constraints, endophytic fungi's bioactive chemicals show promise as prospective instruments that might be helpful in relation to safety and human health problems. As they encourage the development of synthetic techniques, natural products have been a rich source of therapeutic medicines because it is now possible to create analogues of original lead compounds that have better pharmacological qualities. Because of their high degree of biodiversity and unique chemical compounds with intriguing biological functions, endophytic fungi are a valuable source of these materials (D. Prabhavaty, 2014).

In biotechnology, the study and creation of secondary metabolites from endophytic fungus has become a fascinating area. Pharmaceutically significant chemicals are being found and isolated in various forms. As potential makers of new and physiologically active chemicals, fungal endophytes have attracted the attention of several scientists. Many beneficial bioactive substances with antibacterial, anticancer, antioxidant, antimalaria, cytotoxic antiviral, and antituberculosis properties have been effectively isolated from the endophytic microflora during the past two decades (N.C Valli, 2013).

Below are fungal endophytes that were bio prospected from various medicinal plants and their activities (Table 2)

Table 2: The findings of bioprospecting of fungal endophytes from diverse medicinal plants

S.No.	Endophytic Fungi	Host Plant	Bioactivity of secondary metabolite	References
i.	*Alternaria sp*	*Phellodendron amurense*	Antibiotic	Duan, 2009
ii.	*Fusarium solani*	*Apodytes dimidiata*	Antitumor	Shweta et al., 2010
iii.	*Blastomyces sp., Botrytis sp*	*Phlegmariurus cryptomerianus*	Anticholinesterase	Ju et al., 2009
iv.	*Pestalotiopsis guepinii*	*Wollemia nobilis*	Antitumor	Strobel et al., 1997
v.	*Ozonium sp*	*Taxus chinensis var. mairei*	Antitumor	Zhou et al., 2007
vi.	*A. oryzae*	*Ginkgo biloba*	Anti-inflammatory	Qiu et al., 2010
vii.	*Rhizopus oryzae*	*Iris germanica*	Anti-inflammatory	Zhang L. et al., 1999
viii.	*Monilia sp*	*Dysosma veitchii*	Antitumor	Yang et al., 2003
ix.	*Sordariomycete sp.*	*Eucommia ulmoides*	Antimicrobial and antitumor	Chen et al., 2010
x.	*Alternaria sp.*	*Ginkgo biloba*	Antitumor	Kim and Ford, 1999
xi.	*Colletotrichum gloeosporioides*	*Piper nigrum*	Antibacterial Antidepressant Anti-inflammatory Cancer-fighting properties	Chithra et al., 2014

As a result, studying fungal endophytes is important. This group of plants might produce interesting substances in an environmentally favourable, inexhaustible, and low-cost manner, therefore aiding biodiversity conservation. This also gives researchers the opportunity to identify beneficial chemicals from indigenous plants. Fermentation may be used to create these chemicals on a big scale. Their capacity to produce bioactive metabolites has sparked interest in bioprospecting for these compounds in medicinal plants.

The use of extracts from Eucalyptus species leaves treating different diseases, particularly respiratory disorders, has a long folkloric tradition, particularly among Australian Aborigines (Galan et al., 2020). The genus *Eucalyptus* was initially described in 1788 by botanist named Charles, based on a specimen of *Eucalyptus obliqua* from Adventure Bay.

Eucalyptus oil is a popular essential oil (EO) with a wide range of medicinal properties, including analgesic, antibacterial, CNS stimulant antimicrobial, anti-oxidant, antiviral, sedative, pulmonary decongestant, and antispasmodic properties (Figure 2).

With a proportion of 39.00, β-pinene is the most abundant component in *Eucalyptus tereticornis*. (Ogunwande et al., 2003)

From prehistoric times to the present, Eucalyptus species have proved their remarkable health advantages. EEO's major component, 1,8-cineole, is responsible for its therapeutic efficacy and biological activities include those with anti-cancer, anti-bacterial, antiseptic, anti-oxidant, and anti-inflammatory properties.

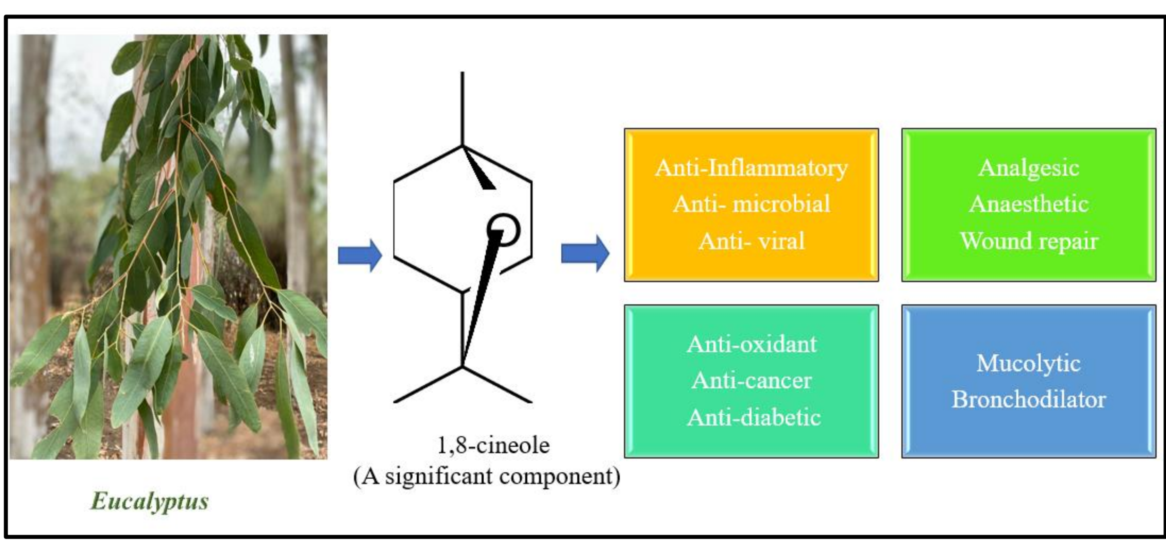

Figure 2: Bioprospection of *Eucalyptus*

The various health benefits of the Eucalyptus genus might be a game-changer in the future of healthcare, addressing issues like multi-drug resistance, pain management, and the treatment of COVID-19, cancer, diabetes, Alzheimer's, hypertension, and other significant disorders. The present state-of-the-art of the Eucalyptus genus in the realm of health sciences has been rigorously reviewed and completely summarized in this study, which includes insights into their molecular processes. The medicinal efficacy of the Eucalyptus genus is also confirmed by the preclinical and clinical investigations included in this study. In addition, this evaluation took into account the many Eucalyptus-based drug delivery methods and patents established to improve the oil's targeting, stability, and solubility (Table 3).

Table 3 Recent advancements in formulations based on *Eucalyptus*

S.No.	System of transport	Form of administration	Indication	References
i	Liposomes	Gel	Antifungal	Moghimipour et al., 2012
ii	Microemulsion	Intranasal spray Topical	Migraine Antimicrobial	Tiwari et al, 2007 Nirmala et al ,2018
iii	Nano emulsion	Topical Transdermal	Antimicrobial Wound healing Analgesic	Sugumar et al.,2014 Aziz et al, 2019
iv	Lipid nano capsules Nano emulsion	Topical	Antifungal	Hussein et al, 2020

2.5 Exploration of endophytic fungi has several advantages

1. Resistance in infectious microbes has resulted in the rise of a spate of new diseases, as well as a reduction in the number of treatments available to treat them. Endophytic fungi generate bioactive substances with a variety of biological and pharmacological characteristics that might facilitate the creation of several substances with therapeutic potential for a number of disorders.

2. Microbial natural products may be produced on a large scale by altering the factors required for fermentation, such as the content of the nutrition medium, pH, and temperature. This might be ideal for large-scale production.
3. Using endophytic fungus to increase the quality of medicinal plants is thus a potential and long-term strategy.

2.6. Secondary metabolite synthesis by endophytic fungi

Endophytes from fungi are thought to have a wealth of structurally and physiologically active secondary metabolites. These aid in enhancing nutrient intake, inhibiting the growth of plant pathogens, promoting plant growth, and reducing disease severity. The therapeutic characteristics of plants can sometimes be found in the endophytes that live on the host plant, and the endophytes generate the same sort of physiologically active secondary metabolites as the host plant.

Endophytes from medicinal plants are valuable resources for the identification of natural products and bioactive substances with biotechnological potential in agriculture, industry, medicine, and other fields. Endophytes have been extracted from the roots, stem, leaves, fruits, flowers, bark, and even the meristem of medicinal plants. Medicinal plants, which have been used as a medicine replacement for ages, are a valuable source for bioprospecting endophytes.

Plants adapt their biological systems in competitive and unfavorable ecological situations by developing a variety of defense responses, the most visible of which is the synthesis of protective secondary metabolites. More than 70,000 genes are predicted to be present in plant genomes ,15–25% of which are involved in encoding secondary metabolism enzymes Somerville and Somerville, 1999). As a result, defensive secondary metabolites vary widely within and between groups.

Fifty one percent of bioactive compounds extracted from endophytic fungi were novel, as per literature review whereas thirty eight percent of bioactive compounds identified from soil fungi were. As a consequence, endophytic fungi give the researcher an opportunity to delve further into this domain because the vast of them are still unexplored (Strobel, 2003).

Exploring an array of heretofore unrecognized endophytic fungal species aids in the emergence of new bioactive compounds with a wide range of chemical characteristics,

which may be used in a variety of domains including the environment, agriculture, and pharmaceuticals.

Endophytic fungi also assist in the establishment of host defense mechanisms through secondary metabolites, which boost the host's ability to adapt to a variety of biogeographical settings. (Violle et al., 2014). The variable arrangement of coding genes in the secondary metabolite synthesis pathway is one cause for the heterogeneity of an endophytic fungi species' secondary metabolite. Because endophytic fungi coding genes for metabolite synthesis pathways are frequently grouped (Andersen et al., 2013), divergent rearrangements of the clusters within or across endophytic fungi species may also contribute to metabolite variety.

Endogenous biology is currently gaining popularity owing to the wide range of applications that chemicals released by Endophytic –host symbiotic interactions have in sustainable agriculture and medical. Endophytism's underlying processes, as well as its biological and ecological roles, are of interest to scientists. Its significance is established by the many findings in the area of endophytic biological research.

2.7 Endophytic fungi exploration meets substantial challenges

Despite the fact that endophytic fungi research has gotten a lot of attention, there are still a lot of issues that need to be solved in the future decades, including:

1. Researchers can discover and identify novel endophytic fungi and their associated mutualistic or antagonistic signaling systems during symbiosis by selecting suitable host plants and their healthy organs or tissues.
2. Due to the aseptic or non-culturable features of some fungal strains, artificial culture may be challenging. To mitigate this problem, new bioengineering techniques must be established or current isolation methods must be altered. (Zhang P. et al., 2019).
3. It's also difficult to maintain the "competency" of effectively separated endophytic fungi in culture while monitoring there in vivo stimulation under quasi-natural conditions.
4. It is necessary to address the degradability of secondary metabolites retrieved from target endophytic fungi. In vitro or in axenic culture, compounds extracted from a

symbiotic continuum may be exceedingly unstable. As a result, obtaining these new chemicals in an artificial medium is difficult.

2.8. Host plant

To isolate endophytic fungi, the medicinal plant *Eucalyptus teretecornis* was adopted from the TIET campus.

Eucalyptus species have proved their remarkable health advantages since long. Major component termed as 1,8-cineole, is responsible for its therapeutic efficacy and biological benefits, including antibacterial, antiseptic, antioxidant, anti-inflammatory, anticancer, and a variety of other biological functions. By modulating the NF-B and MAPK signaling pathways, 1,8-cineole possesses anti-inflammatory and antioxidant properties.

The term *Eucalyptus* comes from the prefix "Eu," which implies "true," and "calyptus," which implies "to cover," and refers to the flower's bottom bud, which is made up of united calyx and corolla components that seal the flower until it blooms. Eucalyptus oil is a popular essential oil (EO) with a variety of therapeutic characteristics, notably analgesic, antimicrobial, anti-oxidant, antibacterial, antiviral, sedative, CNS stimulant, pulmonary decongestant, and antispasmodic (Table 4).

Figure 3*: Eucalyptus tereticornis*

With a proportion of 39.00, β-pinene is the most abundant component in *Eucalyptus tereticornis* (Ogunwande et al., 2003. *Eucalyptus* comes in a varied range of sizes and shapes, from little shrubs to large trees. *Eucalyptus* is made up of a variety of compounds. These compounds might have a variety of consequences on the human body. In addition, some study reveals that eucalyptus has antibacterial and antifungal properties. Many people use *eucalyptus* to treat asthma, bronchitis, flu (influenza), and other ailments, but there is no strong scientific evidence to back up these claims. Colds, influenza, rhinitis, sinusitis, and other respiratory illnesses have all been treated using *eucalyptus* leaves, which include antibacterial, antiviral, and antifungal properties. Endophytic fungi create secondary metabolites that are comparable to those produced by their hosts.

The outstanding wood characteristics of *Eucalyptus* spp. make it a species of choice for the manufacturing of paper pulp, and it is favored for bioenergy plantings (Labate et al., 2008). Endophyte associations with *Eucalyptus* species have been documented for *E. globulus* (Bettucci and Saravay, 1993), *E. urophylla* x *E. grandis* (Paz et al., 2012), *E. grandis x E.* u*rophylla* (Miguel et al., 2016).

Table 4: Bioprospection of fungal endophytes from *Eucalyptus* plant

S.No.	Species	Bioactivity	References
1.	*E. tereticornis* *E. camaldulensis* *E. Citriodora* *E. camaldulensis*	Antimicrobial activity	Chaudhari (1989)
2.	*E. camaldulensis,* *E. citriodora* *E. dalrympleana* *E. globulus*	Antifungal activity	Liapi (2007).
3.	*E. camaldulensis* *E. staigeriana*	Antioxidant	Sumitra (2005).

Its essential oils are used both commercially and medicinally. They are anesthetic, anodyne, antiperiodic, antiphlogistic, antiseptic, astringent, disinfectant, expectorant, febrifuge, fumigant, hemostat, inhalant, insect repellent, sedative yet stimulating, suppurative, tonic, and vermifuge, and they can be used as folk medicine. Numerous studies on the yield and chemical makeup of *eucalyptus* oils, as well as the development of extraction and analytical procedures, have been conducted due to the wide range of traditional usage and possible commercial possibilities of these oils.

In the future of healthcare, the abundance of health benefits provided by the *Eucalyptus* genus could represent a ground-breaking innovation, primarily addressing problems with multi-drug resistance, pain management, and the treatment of a number of serious illnesses like COVID-19, cancer, diabetes, Alzheimer's, hypertension, etc. The present state-of-the-art of the *Eucalyptus* genus in the realm of health sciences has been rigorously examined and succinctly explained in this study with insights into their molecular processes. The medicinal efficacy of the genus *Eucalyptus* is also confirmed by the preclinical and clinical trials presented in the current study. Additionally, the many *Eucalyptus*-containing drug delivery methods and patents created to improve the targeting, stability, and solubility of the oil and its active components have been taken into account in this analysis. Future advancements and uses for *eucalyptus*-containing formulations, including micro- and nano-formulations, for treating a variety of illnesses, are certain to be remarkable.

MATERIAL AND METHODS

3 Endophytic fungi from medicinal plant

A. Isolation of endophytic fungi from *Eucalyptus teretecornis.*
B. T6 isolated from *T.baccata* L subsp.*wallichiana* (Zucc.) Pilger and AL2 isolated from *Terminalia arjuna.* These two fungal isolates were provided from the lab.

3.1 Sample gathering

The samples were taken from the TIET Patiala campus. The stem and leaves of *Eucalyptus* from TIFAC -CORE were collected. The samples were placed in sterile jars and taken to the TIFAC-CORE for the further studies.

Additionally, cultures of T6 and AL2 fungal endophytic isolates were provided by the TIFAC - CORE lab to investigate its bioactive potential.

3.2 Surface disinfection of samples

To eliminate dust and debris from the surface of the *Eucalyptus* plant parts, these were rinsed with distilled water after being washed with running tap water. The samples were initially soaked in 70% ethanol for a minute before being dipped in 1% sodium hypochlorite aqueous solution for three minutes before being rewashed in 70% ethanol for ten seconds. The last wash was done using autoclaved water, followed by drying under laminar air flow (Strobel and Daisy 2003).

3.3 Isolation of the endophytic fungi from the plant samples

After drying, the samples were cut along their edges, and the top layer was removed with a sterile blade. The samples were then split horizontally into two halves and placed on plates made of water agar. Then, potato dextrose agar (PDA) plates were used to further transfer endophytic fungi that had emerged from the water agar plates. The plates were then kept at 27 ± 2°C. The endophytic fungus's development was then monitored every two to three days.

3.4 Fungal endophytes purification

Purification of fungal endophytes from a mixed population was carried out in order to get pure strains. On fresh potato dextrose agar plates, a mixed population of fungal endophytes was sub cultured using the hyphal tip method (Strobel et al. 1996a, b). The plates were incubated for 7 days at 27± 2°C. The master cultures were kept at four degrees °C for preservation.

3.5 Endophyte coding for fungi

The isolated and purified fungi from various plants parts of *Eucalyptus* were given names. The labelling scheme was as follows: For instance, endophytes isolated and purified from the leaves of the plant *Eucalyptus* were designated with the letters EL (E= Eucalyptus and L=leaves), followed by the digits EL 1, 2,...etc.

3.6 Endophytic fungi subculture

Endophytic fungi sub-culturing was carried out on a regular basis using sterile blades to cut out 1-2 cm discs of mycelia from the master plate and positioned in the centre of a new PDA plate. These were then kept for 7 days incubation at 27±2°C, in case of EL1. In case of T6 and AL2 discs were placed in the midst of new PDA plates, Czapek - Dox plates, and SDA plates and were kept at 27 ±2°C for 7 days.

Diverse media plates were created to observe how the fungus adapts and thrives in different environments.

3.7 Secondary metabolite production by fermentation

From the edges of mycelium that was actively growing, four to eight mycelial discs (5.0 mm in diameter) were aseptically introduced to a 500 ml Erlenmeyer flask holding 250 ml PDB. Under stationary circumstances, the cultivated flasks were incubated for 21 days at 27±2°C. The culture was filtered with sterile muslin cloth after a twenty-one-day incubation period to eliminate the mycelia from the broth (Prabavathy et al., 2011). The mycelia of each isolated fungus were dried until it achieved a uniform weight, and the dry weight was recorded. Whereas in case of T6 and AL2 the fresh mycelia from plates were transferred respectively to a 500 ml Erlenmeyer flask containing 250 ml PDB after being sub - cultured on PDA. A similar experiment was carried out with a 250ml Czapek dox broth. The cultivated flasks were incubated for 21 days at 27 ± 2°C in stationary conditions. After a 21-day incubation period, the culture was filtered with sterile muslin cloth to remove the mycelia from the broth. The dry weight of each isolated fungi's mycelia was recorded after it was dried to a uniform weight.

3.8 Solvent extraction to obtain metabolites

The fungal metabolites were extracted using ethyl acetate as a solvent using solvent extraction technique for EL1, AL2 and T6. In a separating funnel, an equal volume of ethyl acetate and filtrate were combined and agitated for fifteen minutes. It was then allowed to stand for a while until the two phases (organic and inorganic) had completely separated. In a separate flask, the organic phase was collected. The obtained organic phases were mixed in a flask and dried using a rota-evaporator (Ika® RV 10 digital, Germany) at 32°C water bath temperature and 4°C chiller temperature.

Before being weighed, the leftover crude extract was completely dried in a vial at room temperature. The biological activity of these crude extracts was next evaluated.

3.9 Bioactive screening of the fungal extracts

3.9.1 Antioxidant assay of crude extracts

DPPH assay: DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) is a commonly used antioxidant assay that uses electron transfer to create a violet solution in methanol. At normal temperature, free radicals are stable and are diminished when an antioxidant molecule is present. It transforms the solution from violet to colorless by scavenging free radicals.

Materials required: DPPH, ascorbic acid, water, 96 well microtiter plate, methanol, pipette, tips, extracts, aluminum foil and ELISA plate reader.

Procedure:

Following template was designed for this assay:

1. **Methanol (M)** = 200 µl
2. **Methanol + DPPH** = Methanol (50µl) + DPPH (150 µl).
3. **Ascorbic acid standard** = Methanol (20 µl) + DPPH (150 µl) + Ascorbic acid (30 µl)
4. **Ascorbic acid control** = Methanol (20 µl) + DPPH (150 µl) + Autoclaved water (30 µl)

Table 5: Template design - DPPH assay

Concentration	Total reaction volume 200 µl	Fungal crude extract (µl)	Methanol (µl)	DPPH (µl)
62.5 µg/ml		2.5 µl	47.5	150
125 µg/ml		5 µl	45	150
250 µg/ml		10 µl	40	150
500 µg/ml		20 µl	30	150

i. In a 96 well microtiter plate, endophytic fungal extracts dissolved in methanol were combined with 150µl of DPPH (100µM).
ii. The negative control was water, while the positive control was ascorbic acid (100µg/ml).
iii. After enclosing the plate in aluminum foil, it was placed in the dark for 45 minutes.
iv. Shift in color intensity (from deep violet to pale yellow) was recorded at 517 nm using an ELISA reader.

The formula used to compute free radical scavenging activity was

Free radical scavenging activity = (A Control - A Sample / A Control) x100.

The Shen, *et al*. 2010, methodology was used to conduct this experiment.

The entire test was performed in triplicate.

3.9.2 Antibacterial activity (Broth microdilution assay)

Materials required: Extracts, Dimethyl sulfoxide (DMSO), MHB, PDB, ampicillin, 96 well plate, pipette, tips, ELISA reader.

Procedure: One of the simplest techniques for determining an antimicrobial's susceptibility is the microdilution of broth. Using a 96-well microtitration plate (microdilution), two-fold dilutions of the antimicrobial agent were prepared and poured into the liquid growth medium in lower proportions. Then, after diluting a standardised microbial suspension and adjusting it to 0.5 McFarland scale, each well was injected with a microbial inoculum made in the same medium. Depending on the test microorganism, the 96-well microtitration plate were incubated under the proper conditions after being well mixed.

Following template was again prepared for carrying out this assay:

1. **MHB** = 100µl
2. **Cell culture** = 50µl MHB + 50µl cell culture (cc)
3. **Sample control** = 50µl cc + 10µl DMSO + 40µl MHB
4. **Ampicillin** (Test antibiotic) = 50µl cc + 5µl water + 45µl MHB
5. **Sample** = 50µl cc + x µl extract + y µl MHB (x, y = varied concentration)

i. Well-grown bacterial colonies from the nutrient agar plate were streaked onto the Mueller-Hinton agar plates and incubated at 37°C overnight.

ii. A colony was selected from the MHA plates and transferred to the MHB plates using a sterile loop. To get the O.D. of 0.5 McFarland, this was then shaken at 37°C for 1-2 hours.

iii. Ampicillin stock (50µg/ml) was made in sterile distilled water.

iv. The experiment was carried out on a 96-well plate, with total volume of 100 µl of cell suspension, media and different fungal crude extracts dissolved in DMSO (dimethyl sulfoxide) at varying concentration (62.5µg/ml,125µg/ml,250µg/ml and 500µg/ml) were added into the wells.

v. MHB served as a blank, MHB with bacterial cells suspended was used as a positive control, and MHB with cells suspended and ampicillin was used as the test antibiotic control.

vi. The ELISA plate reader was set to 600 nm to record the absorbance first at 0 hours (initial reading), following which the plate was incubated with bacterial test cultures at 37°C for 24 hours, and the absorbance was once again measured at the same wavelength 24 hours later (final reading).

The formula used to compute percentage inhibition was

Percentage Inhibition = (A Control - A Sample / A Control) x100.

The Sroka and Cisowski ,2005, methodology was used to conduct this experiment.

The entire test was performed in triplicate.

3.9.3 Protein denaturation assay

Protein denaturation causes protein molecules to lose their biological capabilities. Inflammatory diseases such as rheumatoid arthritis, diabetes, and cancer have all been linked to protein denaturation. As a result, a substance's capacity to inhibit protein denaturation may also aid in the prevention of inflammatory illnesses.

Protein denaturation assay was done according to the method described by Gambhire *et al.*, 2009 with some modifications as described in Gunathilake *et al.*,2018.

Materials required: 2% BSA (bovine serum albumin), 10% DMSO (Dimethyl sulfoxide), test tubes, test tube stand, water bath, pipettes, distilled water, PBS (pH= 6.5), diclofenac sodium

To prepare 500 ml PBS (phosphate buffered saline), (1X, pH=6.5)

i. In an appropriate container, added 300 ml of distilled water.
ii. To this added 0.1 g of potassium chloride and 4 g of sodium chloride.
iii. Subsequently, 0.72 g of dibasic sodium phosphate and 0.1225 g monobasic potassium phosphate were added (pH =6.5).
iv. Distilled water was added up until the level reached 500ml.

Procedure:

i. 450µl of BSA as well as fungal crude extracts dissolved in dimethyl sulfoxide were added to test tubes.
ii. These were then incubated for 30 minutes at 37 degrees Celsius, then for 5 minutes at 70 degrees Celsius.
iii. After that it was allowed to cool down and 2.5ml PBS was added to all the samples.
iv. Using a UV/VIS spectrometer (Shimadzu UV-1800) after cooling, the turbidity was measured at 660 nm.
v. Diclofenac sodium (100µg/ml) served as positive control.

The formula used to compute protein denaturation inhibition was

Percentage inhibition = (A Control - A Sample / A Control) x100.

The entire test was performed in triplicate.

3.9.4 Lipoxygenase inhibition assay:

The enzymatic test is defined as an ongoing assessment of lipoxygenase activity during a period of incubation determined by introducing the extract (start of the reaction) and the FOX (ferrous oxidation xylenol orange) reagent for colorimetric measurement of hydroperoxides accumulated in the medium (end of the reaction)

Materials required**:** 96 well titer plate, fungal crude extracts, sodium linoleate, enzyme – 5 LOX (Lipoxygenase enzyme) FOX (ferrous oxidation xylenol orange) reagent, Tris - hydrochloride buffer, ice box, tips, pipettes

Tris- hydrochloride buffer preparation (pH=7.4,50mM):

Tris (molecular weight = 121.14g/mol)

Required components: tris base, distilled water and hydrochloric acid

i. 60.57g trisodium phosphate and 7.5 mL water in appropriate container.
ii. After a thorough stirring, hydrochloric acid was added to bring the pH up to 7.4.
iii. The buffer was then autoclaved.

Procedure:

i. In a final volume of 300µl, the lipoxygenase enzyme was pre-incubated with extracts dissolved in dimethyl sulfoxide, for 5 minutes at 37°C, followed by linoleic acid (substrate) for 20 minutes in the dark, with 50 mM tris-hydrochloride present.
ii. Sulphuric acid (30mM), xylenol orange (100µM), iron (II) sulphate (100µM), methanol/water (9:1) were used as the FOX reagent to halt the reaction, which was then allowed to develop in the dark for 20 minutes before being detected at 540 nm.
iii. A reagent blank was run in each experiment.
iv. Diclofenac sodium (50 µg/ml) served as positive control.

The percentage of inhibition of hydroperoxide generation was calculated from changes in absorbance values at 560 nm after 30 minutes at 25°C to assess the lipoxygenase inhibitory activity.

The formula used to compute percentage inhibition was

$$\text{Percentage inhibition} = \frac{(\text{A Control - A background / A Sample - A background})}{\text{A Control - A background}} \times 100$$

The Gay et al., 2012 technique was modified to conduct this assay.

The entire test was performed in triplicate.

3.10 Identification through morphological studies

Lactophenol cotton blue dye was used to examine the endophytic fungi under the microscope. The slides were first cleaned in 70% ethanol, then 2-3 drops of lactophenol cotton blue dye were dropped onto the slide with a dropper. The needle was used to pick mycelia off the plate. The slide was properly covered with a sterile cover slip. A microscope was used to examine the slide.

3.11 Separation of various fractions

3.11.1 Thin layer chromatography (TLC)

Materials Required: Glass plates, sample applicator, capillary tube, glass chamber, and UV torch

TLC is a quick and easy way to figure out how many components are present in a crude extract. TLC plates (Silica Gel 60 F254 – Merck) were used to identify ethyl acetate extracts of the fungus spotted over 2cm from the base. In an optimum mobile phase, the spotted TLC plates were run. The components were split into Rf ranges by the mobile phase. Under UV light of wavelength 254, the developed chromatograms were observed.

There were several steps involved:

- Choosing a stationary phase
- Choosing a mobile phase
- Using a sample application
- Developing chamber type and size
- Developing chamber mode
- Visualization and detection

All solvents of A.R. grade were used throughout the analysis.

Mobile phase optimization

The typical technique for optimizing the mobile phase in TLC is to alter the solvent strength according to the polarity index or to replace a pure solvent with another, so that the Rf values are in the 0.15–0.85 range. In extracts containing more than one component to be separated, separation may not be possible using a mixture of two solvents, and a combination of a greater number of solvents may be employed instead.

The mobile phase employed hereby was dichloromethane : methanol (90:10)

3.11.2 Separation of active fractions by silica gel column chromatography

Requirements: Glass column, stationary phase-silica gel G (60-120 mesh), Mobile phase – hexane, ethyl acetate and methanol, cotton, test tubes, test tube stand

Preparation of sample: The crude ethyl acetate extract was evaporated to dryness, weighed, and crushed. The distinct fractions were separated employing 150 mg of sample.

Column Packing: On stationary phase, the extract chosen (AL2 ethyl acetate crude extract) for fractionation was adsorbed. Wet packing was used to charge the stationary phase (silica gel G, 60–120 mesh) into the glass column, which measures 60 cm by 25 mm. To eliminate impurities, a selected column was washed with solvent. To block the passage of stationary phase, a cotton pad was firmly affixed at the bottom. While pouring eluting solvents from the top, a second layer of cotton pad was placed above the mixture to prevent messing up the stationary phase.

Procedure:

i. The slurry was poured gently on top of column to avoid the production of air bubbles.
ii. After that, the column was washed with hexane and prepared by passing a sufficient volume of the initial solvent combination across it.
iii. The crude extract was first dissolved in a suitable solvent before being put onto the column.
iv. Hexane and ethyl acetate gradients were utilized.
v. The column was rinsed with pure methanol once more.

Next, a gradient of hexanes, ethyl acetate and methanol were used to elute the column (Table 6).

The column's eluted solvents were collected in test tubes. On a TLC plate, each fraction was concentrated and marked. TLC was generated with the appropriate mobile phase and observed in an UV transilluminator or under long UV (365nm). Similar fractions were grouped together based on the TLC profile. A rotary evaporator was used to evaporate the final fractions.

Table 6: Gradient used for column chromatography:

Solvent	Gradient
Hexane	100%
Hexane: Ethyl acetate	95: 5
Hexane: Ethyl acetate	90: 10
Hexane: Ethyl acetate	80: 20
Hexane: Ethyl acetate	70:30
Hexane: Ethyl acetate	60: 40
Hexane: Ethyl acetate	50: 50
Hexane: Ethyl acetate	40: 60
Hexane: Ethyl acetate	30: 70
Hexane: Ethyl acetate	20: 80
Hexane: Ethyl acetate	10: 90
Ethyl acetate	100%
Methanol in Ethyl acetate (5% ethyl acetate)	
Pure methanol (100%)	

3.12 Screening of bioactive fractions

Final bioactivities (antimicrobial, antioxidant (DPPH), and anti-inflammatory activities- Bovine Serum assay and Lipoxygenase inhibition Assay) were assessed on the fractions as per methodology described in section 3.9.

Three fractions: F12,13 and F14 were chosen, out of which with fraction 12 proved to be the most efficacious. Henceforth was put forward for characterization studies.

3.13 Characterization of the fractions exhibiting maximum activity

3.13.1 Compound spectroscopic study in UV-Visible light

Analytical approaches based on the measurement of light absorbed by substances in the wavelength range of 190 to 900 nm are used in ultraviolet and visible spectrophotometer techniques. The UV section of the spectrum is between 190 and 380 nm, whereas the visible area is between 380 and 900 nm. Electronic transitions between molecules aid in molecule absorption in the UV - visible range. A wide range of equipment may be used to

measure the spectrum's light absorption. In general, the UV spectrum is used to validate the identification of substances. It's also utilized in HPLC as a detector.

Hereby the purified fraction AL2-F12 was diluted in methanol. Using a UV-Visible spectrophotometer (Shimadzu UV-2600, Japan), the UV absorbance of these extracts was measured between 200 and 500 nm.

3.13.2 Fourier-transform infrared spectroscopy (FTIR): Analytical method that uses extractive Fourier transform infrared spectrometry to measure the concentration of fluorinated compounds.

Procedure: F12-AL2 was sent to CEEMS lab for FTIR analysis. FTIR was recorded using a Perkin Elmer RX (Spectrum RX FTIR Spectrometer, USA) instrument. The IR spectra's range was 250 to 4000 cm^{-1}.

3.13.3 Nuclear magnetic resonance spectroscopy (NMR): NMR spectroscopy is an essential tool that uses a magnetic field and radio frequency pulses to characterize the resonant frequency of an atomic nucleus in relation to its chemical or environmental surroundings (for example, the most prevalent stable isotopes 1H, and 13C). On a 400 MHz JEOL, NMR for fraction F12-AL2 was performed.

3.13.4 HR- MS: Utilizing this technique it separates each component of a mixture separately before ionizing and separating the ions according to their mass/charge ratio. After being separated, the ions are sent to a photo or electron multiplier tube detector, where each ion is identified and measured. As molecular level analysis is used in this method, its main benefits are sensitivity, specificity, and accuracy. The analyte's structural features can also be understood.

Fraction 12 from crude AL2 was considered for HRMS analysis and was sent to SAIF, lab, Chandigarh. F12 was dissolved in methanol and used as such for analysis in HR-MS testing. Utilizing the UPLC Acquity H class series technology, chromatographic separation was completed. At a flow rate of 0.3 ml/min, 0.6µl of sample were injected into the column. With the supply pressure of N2 kept at 6-7 bar (90-100 psi) and that of argon at 5-6 bar, separation was conducted using 0.1 percent formic acid in water solvent A and 0.1 percent formic acid in Acetonitrile (ACN) solvent B, respectively, at a ratio of 20/80. The resulting m/z values were further examined to determine the compound.

4.Co-culture of AL2 and T6

Many bioactive metabolites are produced by medicinal plants. The co-cultivation approach is based on the idea that competition for restricted resources usually results in the activation of silent genes that do not ordinarily contribute to the creation of essential bioactive chemicals. The cultured broths of AL2 and T6 will be analyzed for secondary metabolite potential. The impact of co-culturing on the production of secondary metabolite will be investigated.

4.1 Sample gathering - Cultures of T6 and AL2 which were introduced in lab were further taken up to conduct co-culture studies.

4.2 Endophytic fungi sub-culture: Subculturing of AL2 and T6 on fresh PDA and Czapek dox agar was carried out as explained in previous section (section – 3.1.6).

4.3 Secondary metabolite production by fermentation: PDB and Czapek dox broths were made in use. Same procedure was followed as discussed in earlier section (section 3.1.7).

4.4 Solvent extraction to obtain metabolites: The fungal metabolites were extracted using dichloromethane as a solvent in a solvent extraction technique (3.1.8)

4.5 Purification of crude extracts

4.5.1 Thin layer chromatography (TLC)

The extracts underwent TLC in a manner similar to that which was previously mentioned (3.4.1).

4.6 Characterization of the highest-activity fractions

4.7 Compound spectroscopic study in UV-Visible light

RESULTS AND DISCUSSION

Endophytes are an endosymbiotic group of microorganisms that colonize in plants and microbes that can be readily isolated from any microbial or plant growth medium. They act as reservoirs of novel bioactive secondary metabolites. While plant sources are being extensively explored for new chemical entities for therapeutic purposes, endophytic fungi constitutes an important source for drug discovery.

In the present study *Eucalyptus teretecornis* was selected for the isolation of endophytic fungi. The above was chosen for research since there hasn't been much work reported on endophytic fungal isolation or characterization of bioactive compounds produced from these plants.

Additionally, AL2 isolated from *Terminalia arjuna* and T6 isolated from *T.baccata L sub sp.wallichiana* (Zucc.) Pilger cultures received from TIFAC-CORE to determine their bioactive potential, and the influence of co-culturing on the production of secondary metabolites was studied.

5.1 Sample gathering

The samples were taken from the TIET, Patiala campus. The stem and leaves of *Eucalyptus* from TIFAC -CORE were collected (Figure 3,4). The materials were placed in sterile jars being sent to the TIFAC-CORE laboratory for further analysis. T6 and AL2 cultures from the lab were also introduced and employed to conduct preliminary screening (Figure 5).

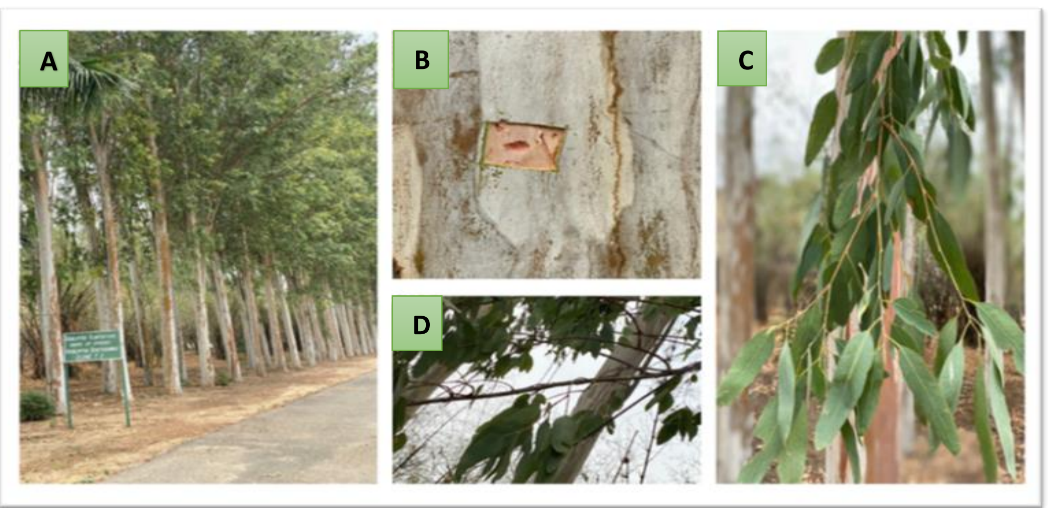

Figure 4: Sample collection from varied parts of *Eucalyptus tereticornis* A) *Eucalyptus* tree B) *Eucalyptus* bark C) *Eucalyptu*s leaves, D) *Eucalyptus* stem

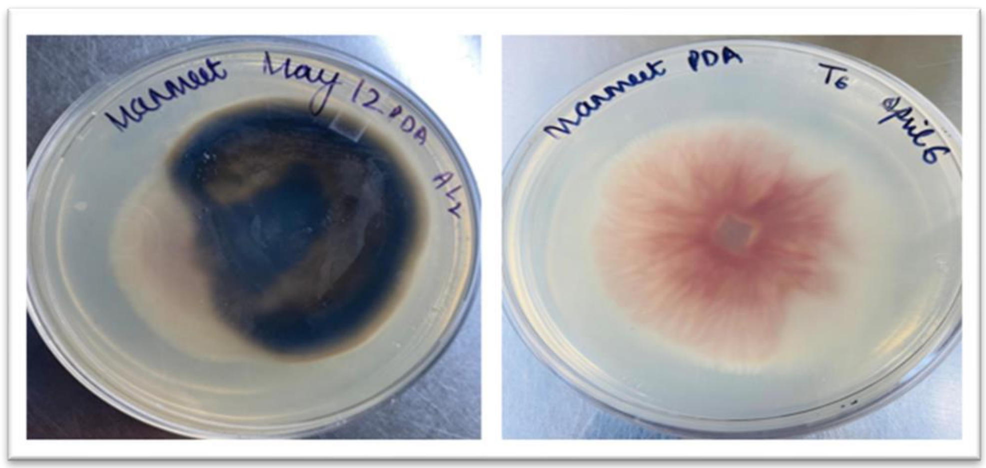

Figure 5: AL2 and T6 fungal isolates on PDA

Table 7: Sample collection

S.No	Source (Host plant)	Endophytic fungal extract
1	*Eucalyptus teretecornis*	EL
2	*Terminalia arjuna*	AL2
3	*T.baccata*	T6

5.2 Isolation of endophytic fungi

The samples were edge-trimmed and the outer layer was scraped with a sterile blade after drying. After then, the samples were split horizontally into two halves. These were placed on PDA plates for 7 days at 27 ± 2 °C. (Figure 6). The endophytic fungi growth was examined every two to three days.

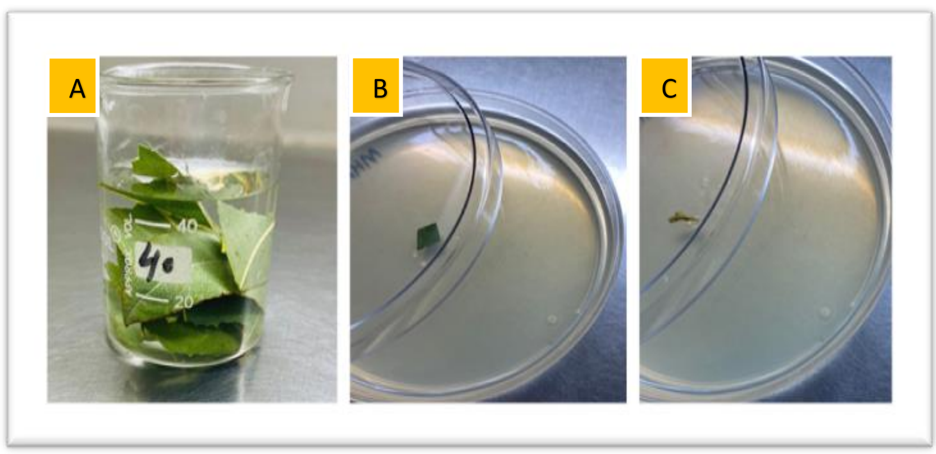

Figure 6: Tissue surface sterilization A) Ethanol washing of tissues, B) Culturing of sterilized tissues on PDA plates

In total, three different endophytic fungal isolates were obtained from *Eucalyptus* leaf (Figure 7).

Purification of fungal endophytes from a mixed population was carried out in order to get pure strains (Figure 7,8). The inoculum was extracted off the master plate and deposited onto the potato dextrose agar medium using a sterile blade. The plates were kept for 7–14 days at 27 ±2°C. The master samples were kept at 4 °C.

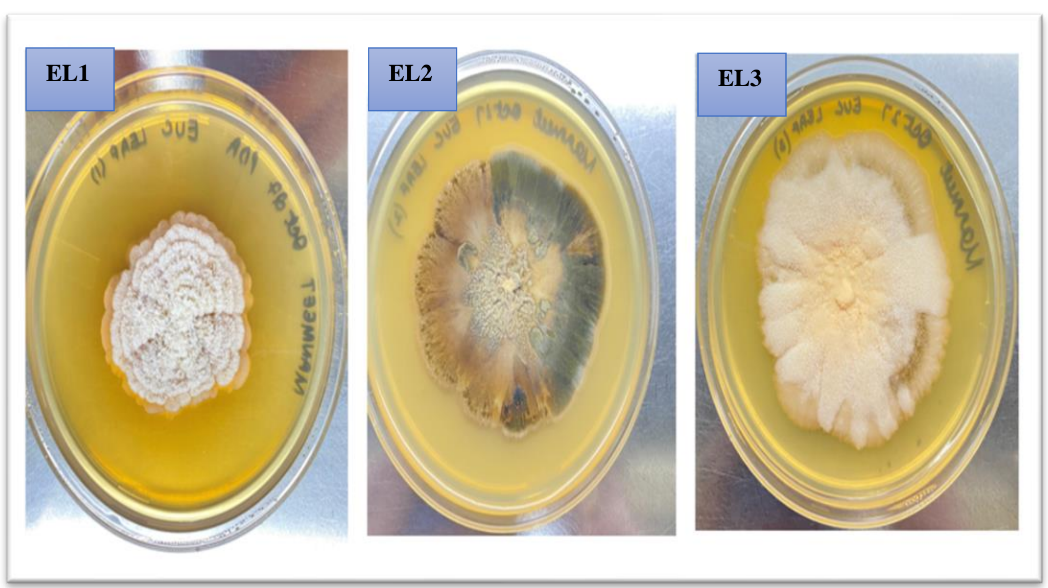

Figure 7: Different fungal isolates obtained from *E. tertecornis*

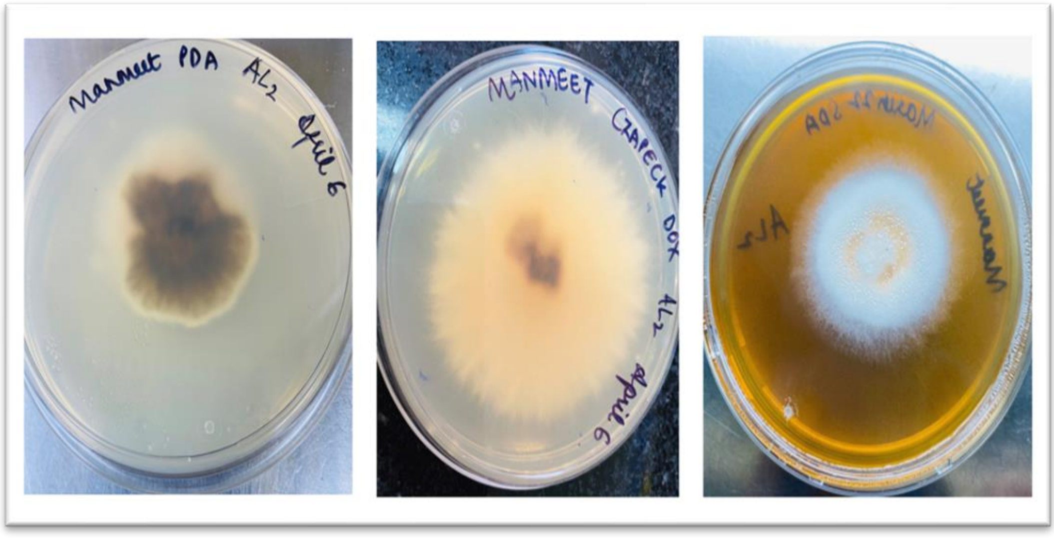

Figure 8: AL2 fungal isolates on PDA, Czapek agar, and Sabouraud agar plates

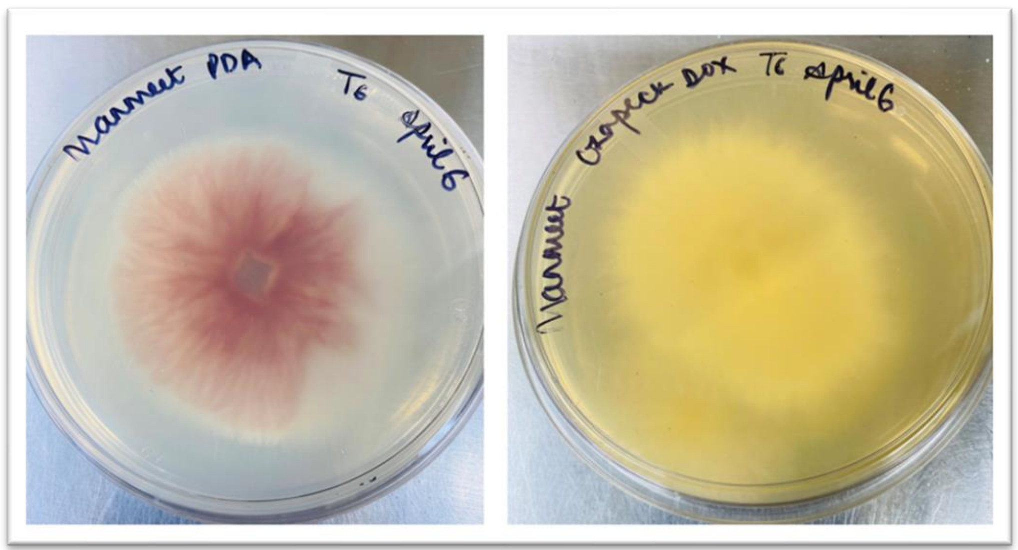

Figure 9: T6 fungal isolates on PDA and Czapek agar plates.

5.3 Endophytic fungi subculture

By periodically removing 1-2 cm discs of mycelia from each fungus master plate (T6 and AL2) using sterilized blades, the endophytic fungi were sub-cultured. These discs were incubated for seven days at 27 ± 2°C, placed between a fresh PDA plate and a Czapek-Dox plate (Figure 9,10). The growth of the fungus was noted every two to three days.

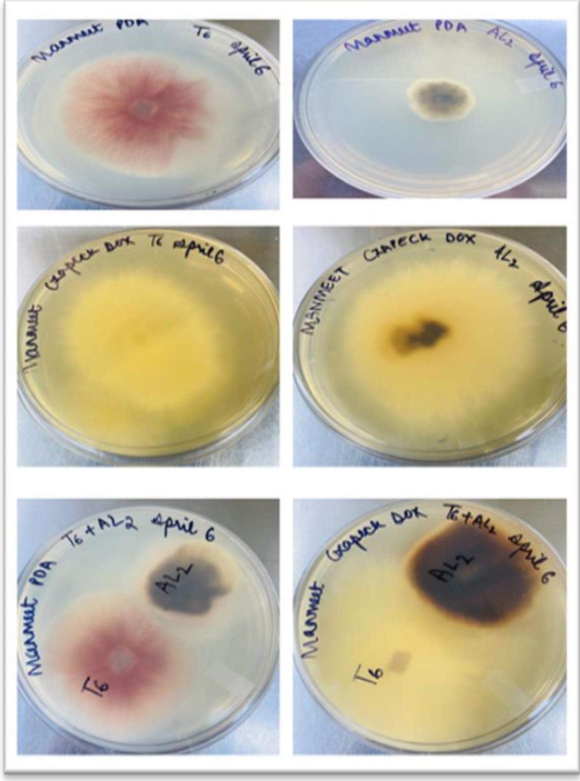

Figure 10: T6 and AL2 fungal isolates grown on PDA and Czapek's agar

5.4 Fermentation to produce secondary metabolites and broth extraction to obtain the crude extract

Endophytic fungus obtained from EL1, T6 and AL2 were introduced into the PDB for fermentation These were incubated for 21 days at 27± 2°C under stationary conditions. After 21 days of incubation, the broth was separated from the mycelia (Figure 11,12) and the organic phase containing metabolites was extracted using ethyl acetate. To acquire the fungal crude extract, this was dried further (Figure 13,14) .

Figure 11 : Broths with fungal inoculum after fermentation after 21 days

5.5 Dry weight of mycelia : Fresh mycelia discs were transferred from PDA plates to a 500 mL Erlenmeyer flask containing 250 mL PDB. Under stationary circumstances, the cultured flasks were incubated for 21 days at 27+2°C. The mycelia from the broth were removed from the culture after twenty one days of incubation using sterilised muslin cloth. The mycelia-containing sterile muslin fabric was dried in an oven until it reached a consistent weight. For all of the isolated fungus, the dry weight of mycelia was assessed (Table 8).

Table 8: Isolated fungi's dried mycelial weight

S.no	Extracts	Dried mycelia weight (mg)
•	AL2(PDB)	250
•	AL2 (Czapeck)	210
•	T6 PDB	220
•	T6 (Czapeck)	210
•	Eucalyptus L1	150

* The mycelial weight per 100ml is the average of all the batches.

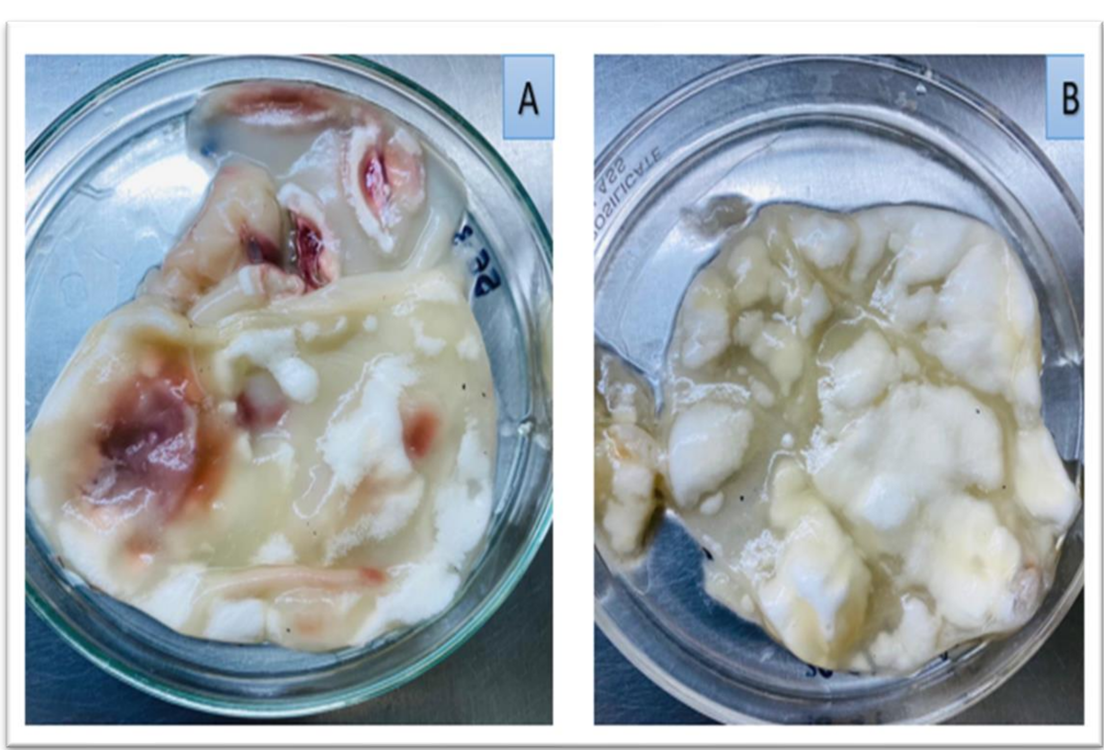

Figure 12 : Mycelia of A) T6, B) AL2

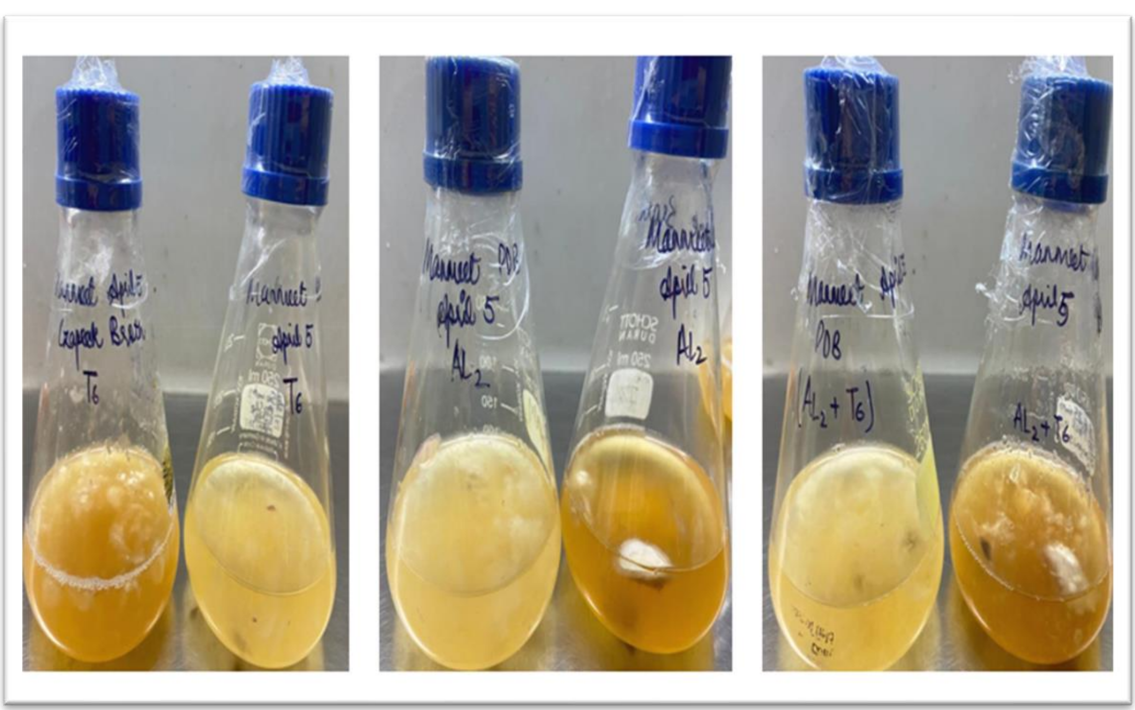

Figure 13 : Broths with fungal inoculum after fermentation

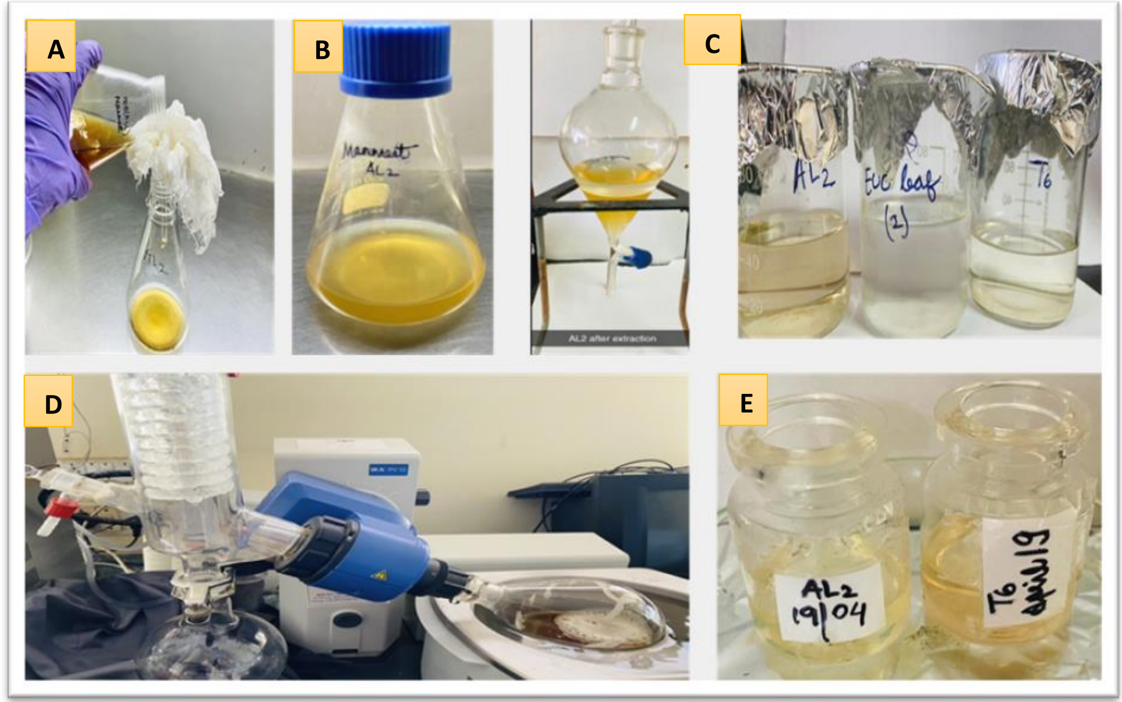

Figure 14 : A) Broth filtering B) Broth collection after filtration C) Broth extraction with an organic solvent in a separating funnel D) Rotaevaporator evaporation of organic solvent E) crude extract collection

5.6 Screening of crude fungal extracts for bioactivities

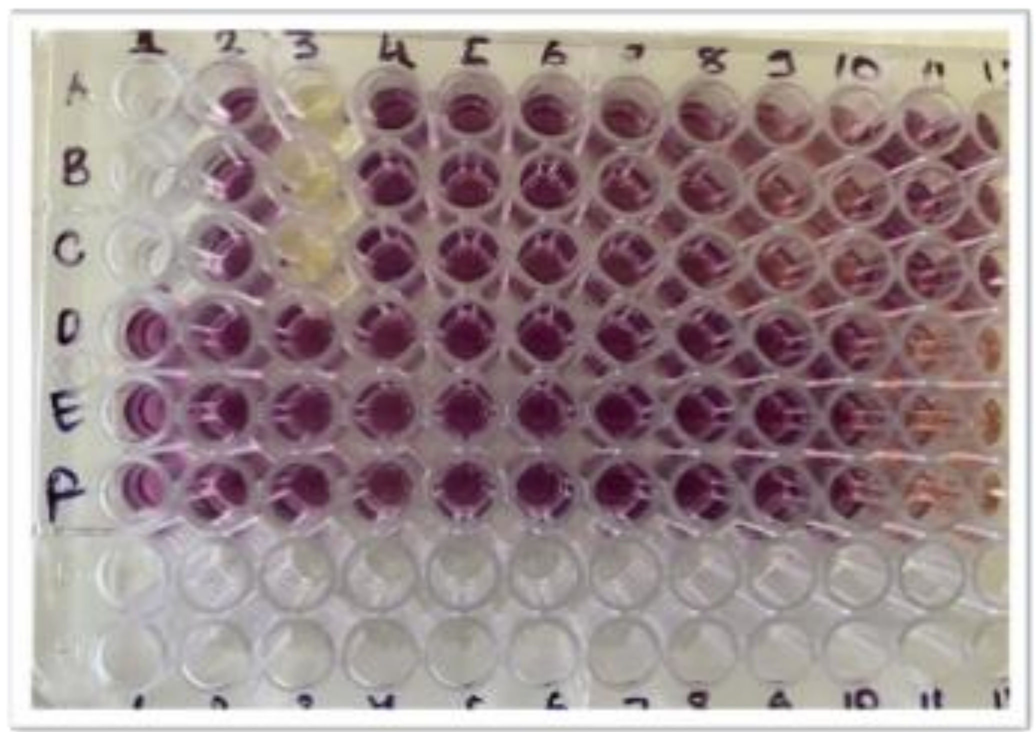

Figure 15: DPPH assay on EL1 extract

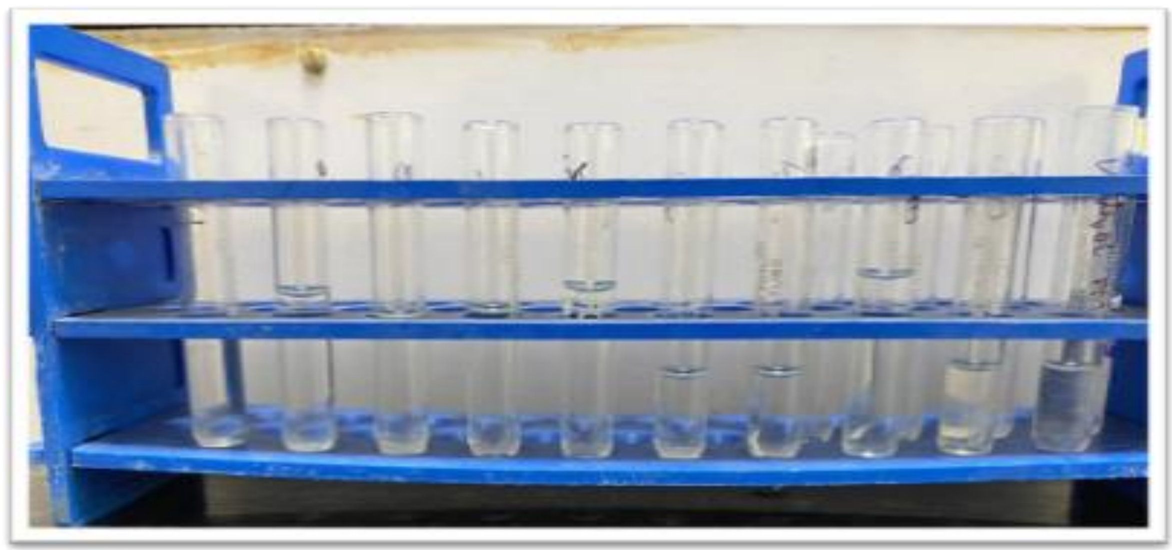

Figure 16 BSA activity on crude AL2 extract

5.6.1 Free radical scavenging activity (DPPH): The ability of crude fungal isolates of EL1, T6, and AL2 to scavenge free radicals was examined at different concentrations. Ascorbic acid was employed as a positive control, while methanol was used as a negative control. Anti-oxidant activity of T6, AL2 and EL1 fungal isolates at 62.5µg/ml, 125µg/ml, 250µg/ml and 500µg/ml is depicted beneath (Figure17, 18 and 19).

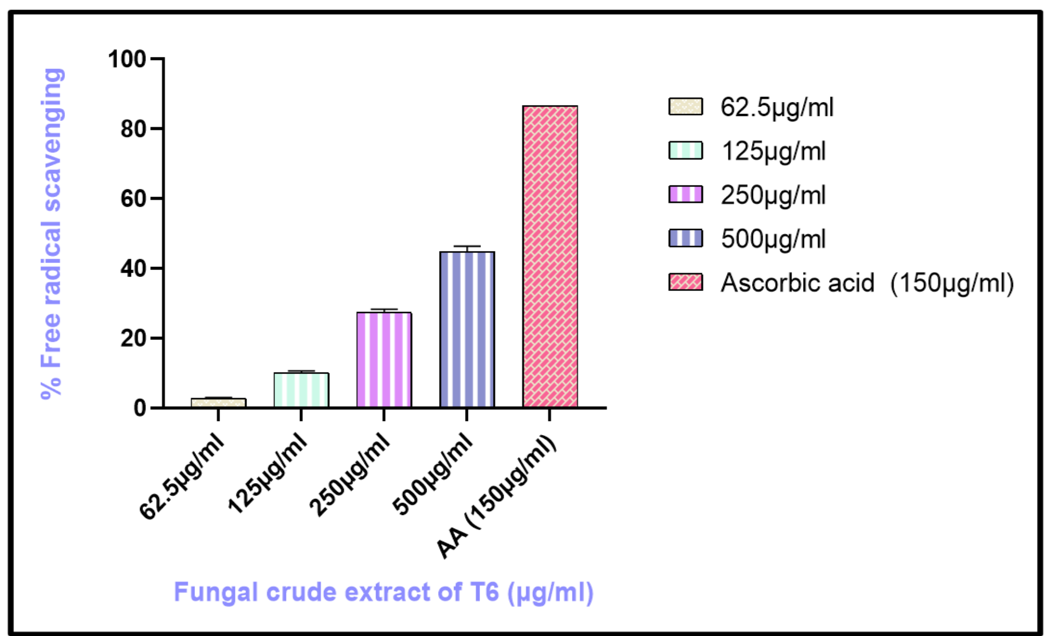

Figure 17: Anti-oxidant activity of T6 extract

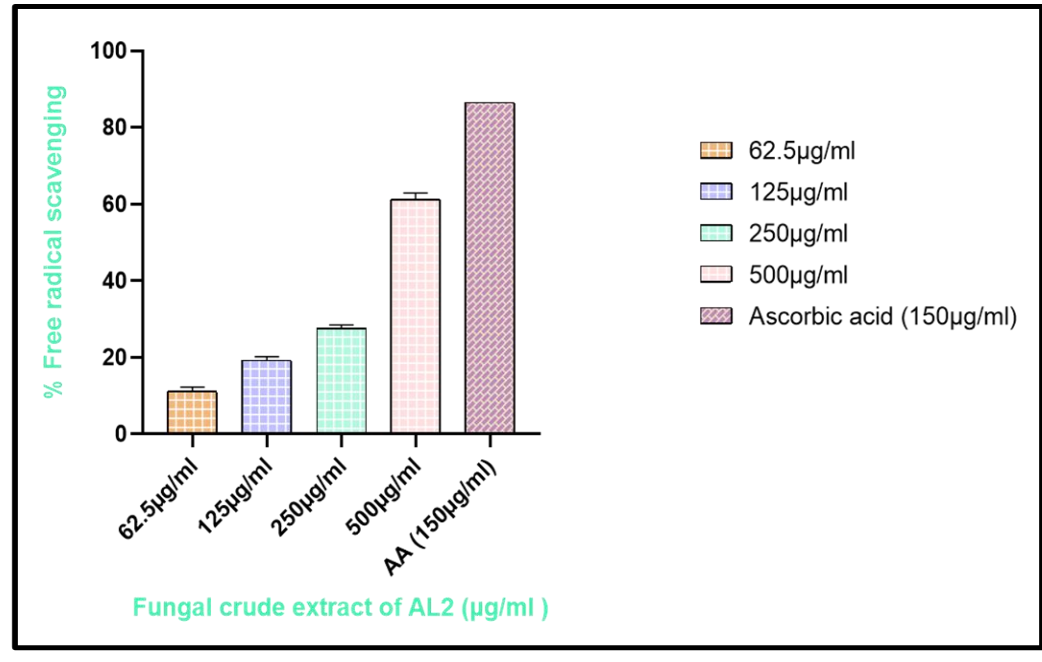

Figure 18: Anti-oxidant activity of AL2 extract

Ascorbic acid (100µg/ml) has a radical scavenging activity of 86.51 percent whilst methanol employed as negative control.

T6 free radical scavenging percentages were 2.9 percent, 10.1 percent, 27.4 percent and 44.8 percent at concentrations of 62.5µg/ml, 125µg/ml, 250µg/ml, and 500µg/ml, respectively.

In case of AL2 crude fungal extract again, several concentrations were used:62.5µg/ml, 125µg/ml, 250µg/ml, and 500µg/ml and it was discovered that the levels of radical scavenging activity were respectively 11.2 percent, 19.3percent, 27.6 percent and 61.3 percent.

From the foregoing, it can be inferred that AL2 crude extract has a notable antioxidant activity.

Geethanjali Dhayanithy *et al*., 2019 studied endophytic fungi isolated from *P. oxalicum* and reported that 21% of the isolated exhibited DPPH radical scavenging activity.

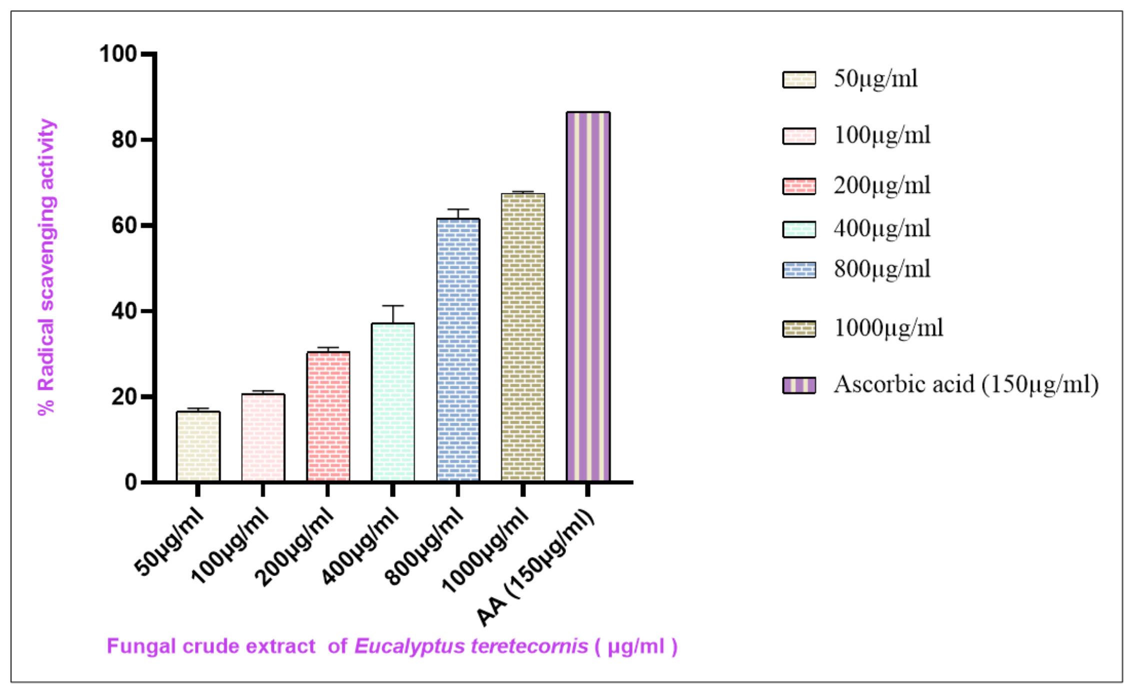

Figure19: Anti-oxidant activity of EL1

Different concentrations (50µg/ml, 100µg/ml, 200µg/ml, 400µg/ml, 800µg/ml, and 1000µg/ml) were used for the EL1fungal isolate in the graph (Figure 19) above, and the radical scavenging activity observed was 16.7 %, 20.8 %, 30.5 %, 37.2 %, 61.7 %, and 67.5%.

Ascorbic acid (150µg/ml) which was used as a positive control exhibited 86.56% radical scavenging activity and methanol was employed as negative control.

As a result, the minimal radical scavenging activity was seen at 50µg/ml of 16.7%, whereas the maximal radical scavenging activity of 67.5 % was reported at 1000µg/ml. As a consequence, we may infer that EL1 has a potent antioxidant activity.

Elena González-Burgos et al., 2018 study depicted that endophytic fungi obtained from *eucalyptus* at 125 µg/mL demonstrated strong scavenging action of 67.27 ± 0.62%, as opposed to crude extract 57.17 percent.

5.6.2 Antimicrobial activity of crude fungal extracts:

The ability of crude fungal isolates of T6, and AL2 to inhibit microorganisms at varied concentrations were was examined at different concentrations. Anti-bacterial activity of T6 and AL2 extracts against *S.aureus* at ,62.5µg/ml 125µg/ml, 250µg/ml and 500µg/ml are depicted beneath (Figure 20,21).

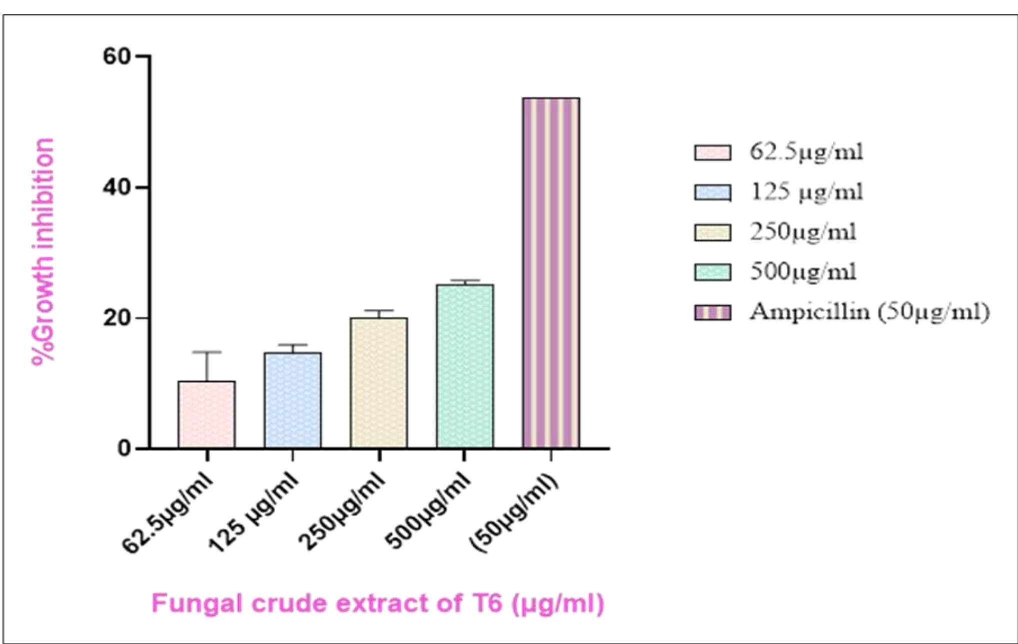

Figure 20: Anti-bacterial activity of T6 fungal extract against *S.aureus*

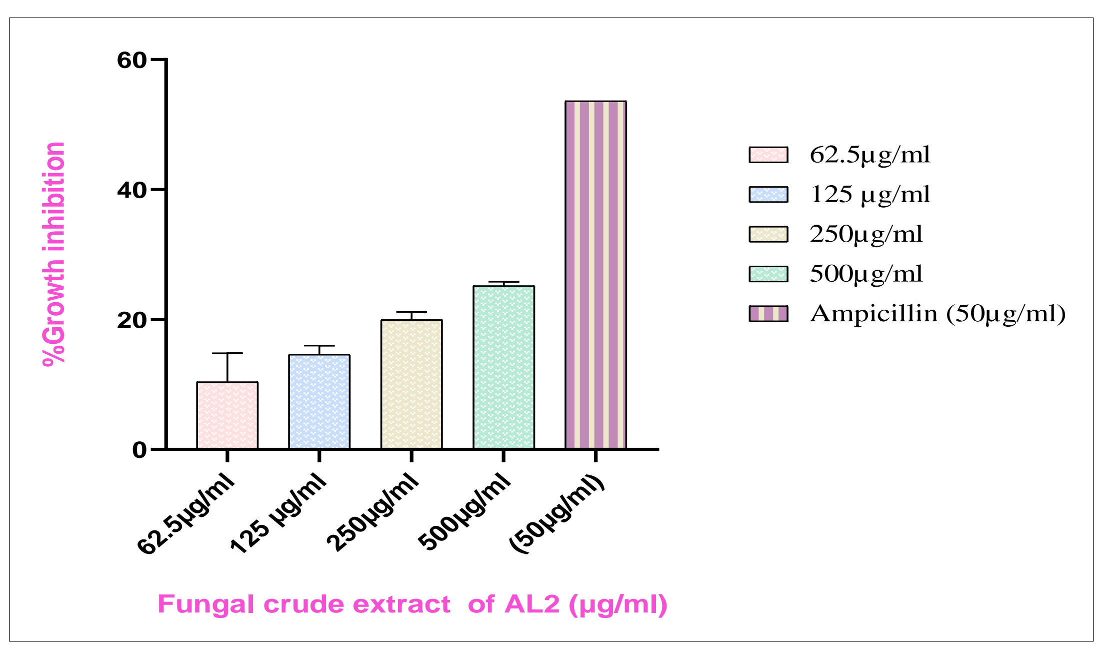

Figure 21: Anti-bacterial activity of AL2 fungal extract against *S.aureus*

Ampicillin (50µg/ml) inhibited 53.64% of cells, whereas fungal extracts of T6 and AL2 when employed at 62.5µg/ml, 125µg/ml ,250µg/ml and 500µg/ml showed varied inhibition percentages.

T6 inhibition percentages were 10.7%, 14.2%, 19.5%, and 25.7% for 62.5µg/ml, 125µg/ml, 250µg/ml, and 500µg/ml, respectively. From this, it can be inferred that the lowest level of inhibition was seen at 62.5 µg/ml, whereas T6fungal extract exhibited stronger antibacterial activity at 500 µg/ml.

For AL2 fungal extract the percentages of growth inhibition revealed were 9.1%, 16.8%, 25.2%, and 43.1%, respectively at concentrations of 62.5µg/ml, 125 µg/ml ,250µg/ml and 500µg/ml. Therefore AL2 extract demonstrated greater antibacterial activity at 500 µg/ml and the lowest amount of inhibition was seen at 62.5µg/ml.

The aforementioned information revealed that AL2 crude extract is a noteworthy antibacterial activity bearer.

Nine out of 35 species of endophytic fungus (accounting for 25.71 percent) displayed substantial inhibitory action against *E. coli*, according to Wen Du et al., 2020. For example, *S. aureus, P. aeruginosa,* and *E. faecalis* were significantly inhibited by four, six, and six different fungal species, respectively.

5.6.3 Protein denaturation inhibition assay (BSA Assay)

AL2 crude was employed for BSA Assay at 125 µg/ml and 500 µg/ml concentrations to test its anti-inflammation potential (Figure 22).

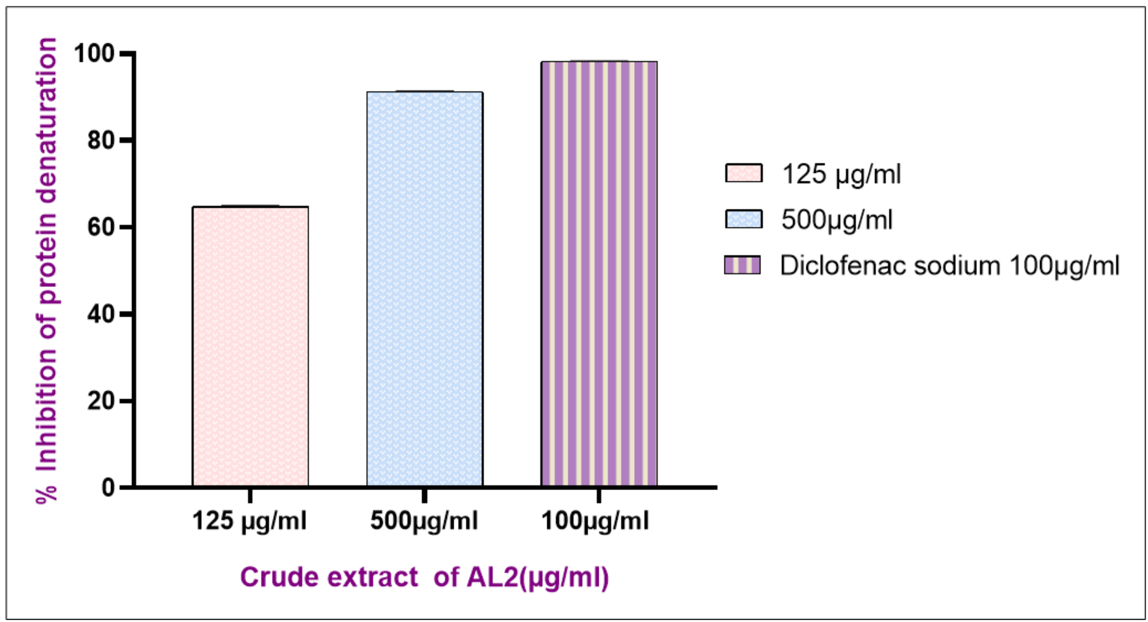

Figure 22: Anti-inflammatory activity of AL2 crude

Diclofenac sodium (100µg/ml) was used as control and showed inhibition of 98.2 %. It was further interpretated that at 125 µg/ml, 64.76 % inhibition was witnessed and as far as 500 µg/ml concentration is concerned, a percentage inhibition of 91.28 % was seen.

In an in vitro BSA experiment, *eucalyptus* extract at a concentration of 1000µg/ml demonstrated a percentage inhibition of 97.28 percent (M.Farmaha *et al.*, 2021)

5.7 Morphology based Identification

The slides of fungi were prepared with lactophenol dye and then were observed under the microscope. Under the microscope, AL2 featured long chain, branching conidiophores. The fungus on the PDA plate had a dark brownish appearance and growth that seemed velvet alike. Whereas in case of czapek's agar, AL2 seemed to have a white smooth growth. (Figure 23,24).

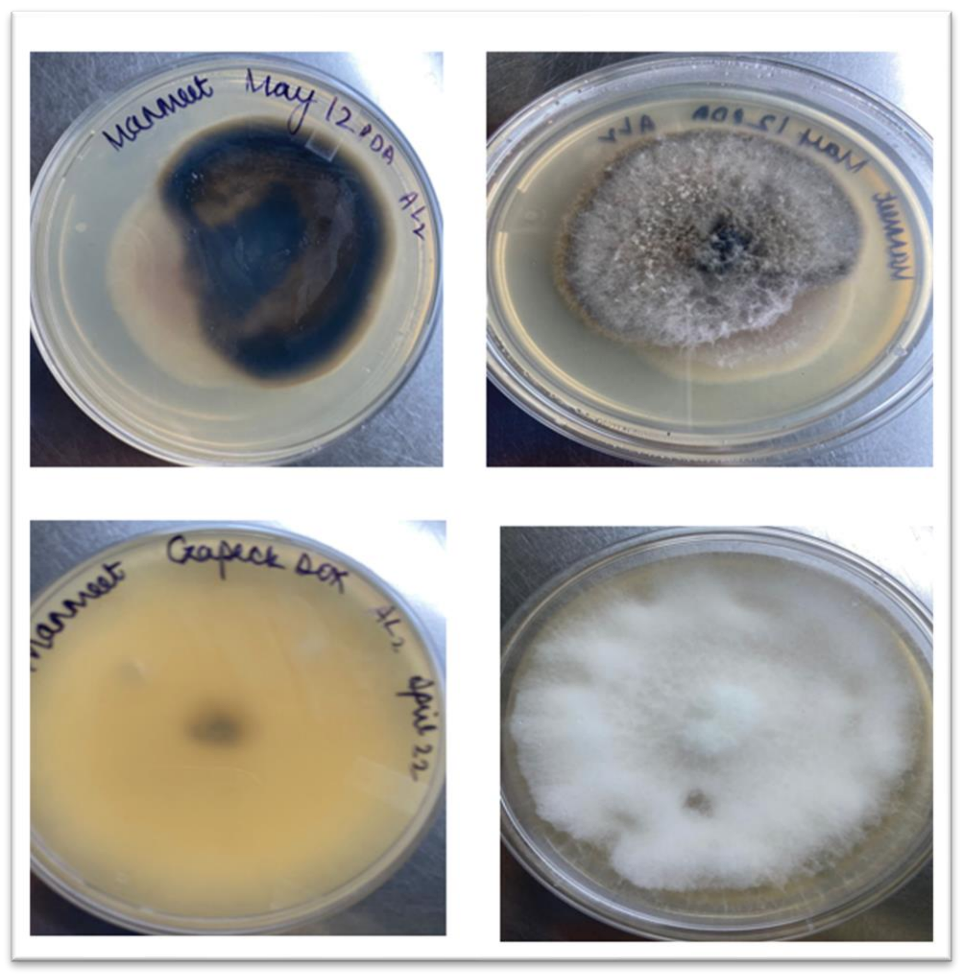

Figure 23: AL2 on PDA and Czapek's agar plate (front view and back side of plate)

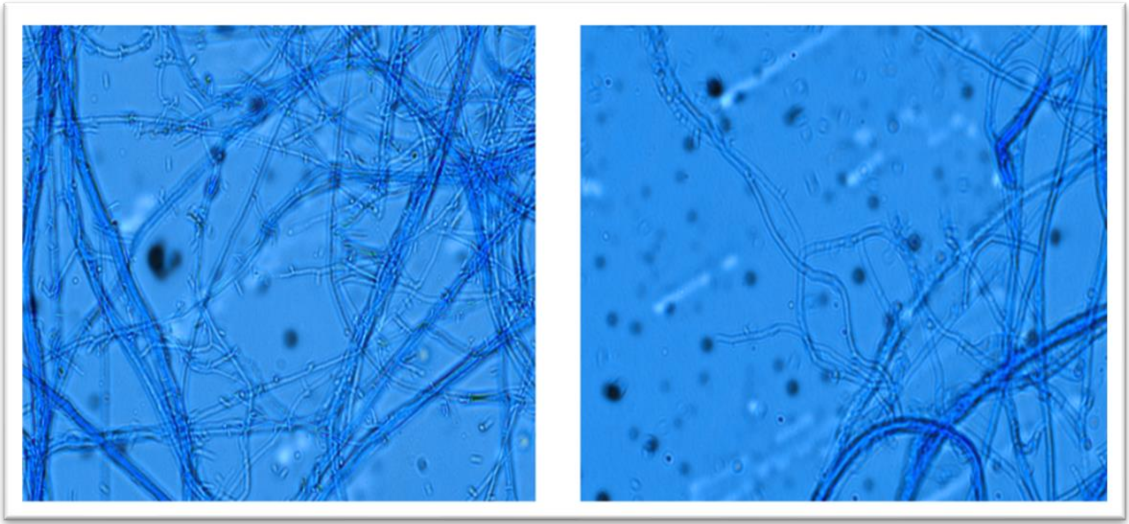

Figure 24:AL2 microscopic view (A) AL2 hyphae under a 40X microscope, (B) AL2 conidiophores under a 40X microscope

Figure 25: T6 on PDA and Czapek's agar plate (front view and back side of plate)

T6 isolated from *T.baccata* L sub sp.*wallichiana* . Endophyte isolated from this was *fusarium tricintum.* This was identified in the lab earlier (Figure 25,26).

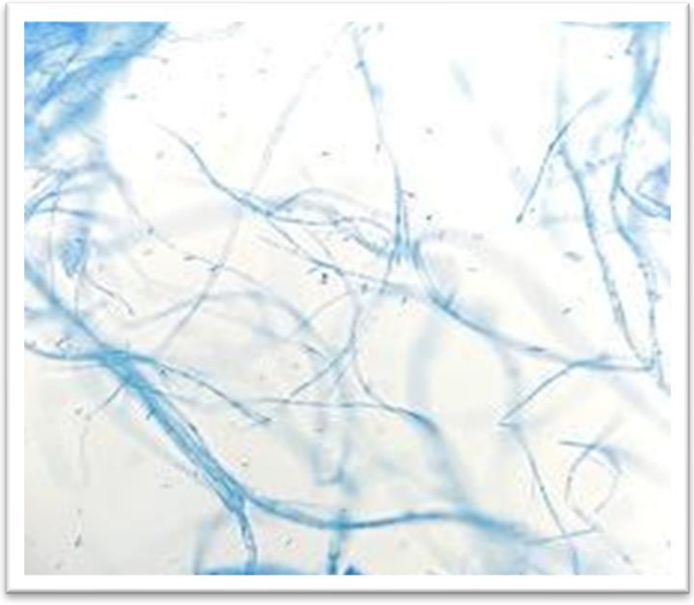

Figure 26: Microscopic view of T6 under 40X

5.8 Crude extract fractionation

5.8.1. Thin layer chromatography

Thin Layer Chromatography was used to analyze the AL2 methanolic extract. Depending on the polarity of the solvents, a variety of solvent systems were employed for the separation of fractions from the crude extract. These solvent systems were first used in a nuclear setting, subsequently in a binary setting (Figure 27,28,29,30 and 31).

Solvent system employed – Dichloromethane: Methanol (9:1)

Similar to this, silica Gel 60 F254-coated TLC plates were used to separate methanolic crude extract of T6 (Merck India, Mumbai). According to Strobel et al., the TLC plate was created using dichloromethane and methanol (9:1) v/v) (1996a).

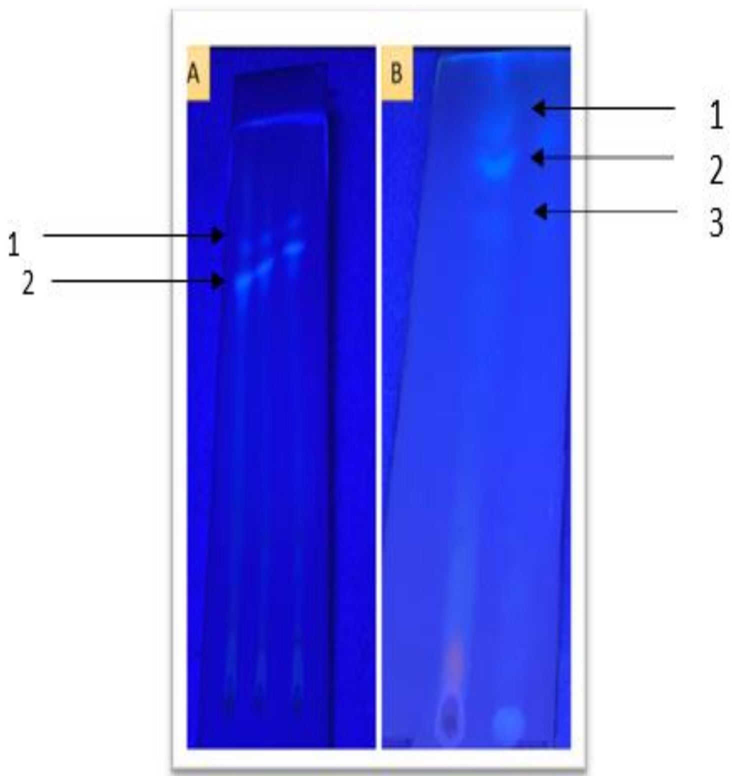

Figure 27: a) Chromatogram of AL2 b) Chromatogram of T6

Figure 28: Chromatogram for varied fractions obtained from AL2

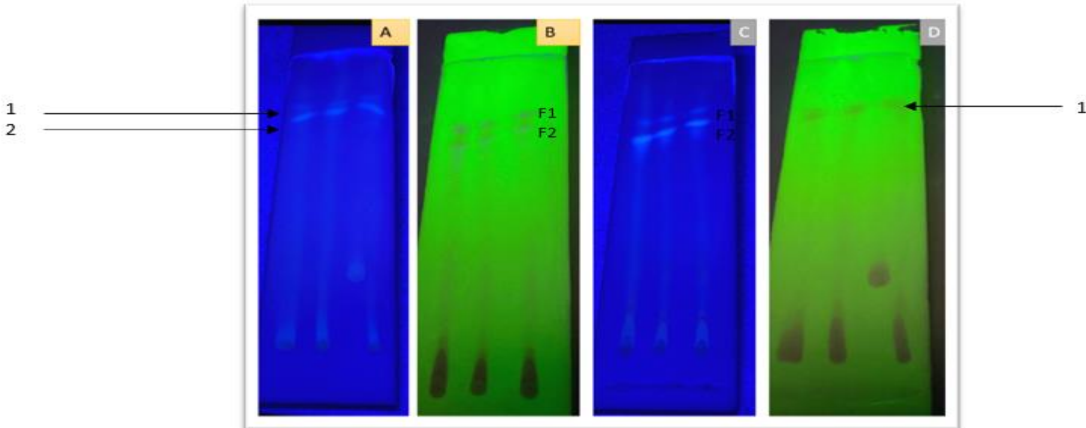

Figure 29: Chromatogram of T6 (PDB) A) under long UV B) under short UV C) Chromatogram of T6(Czapek agar) C) under long UV D) under short UV

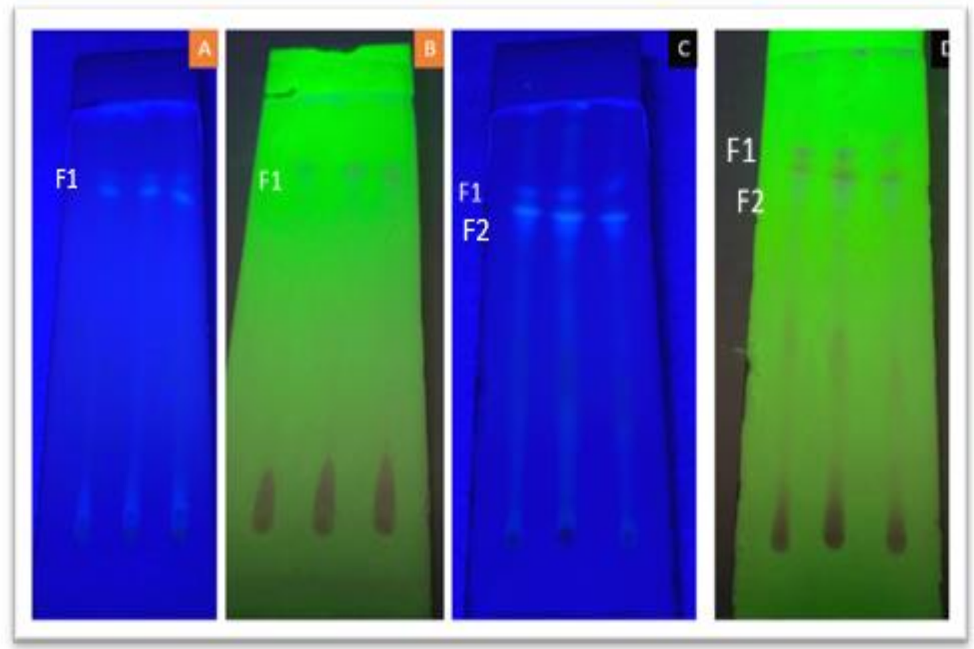

Figure 30: A) Chromatogram of AL2 + T6 (PDB) B) Chromatogram of AL2 + T6 (PDB) C) Chromatogram of AL2 + T6 (Czapek's agar D) Chromatogram of AL2 + T6 (Czapek's)

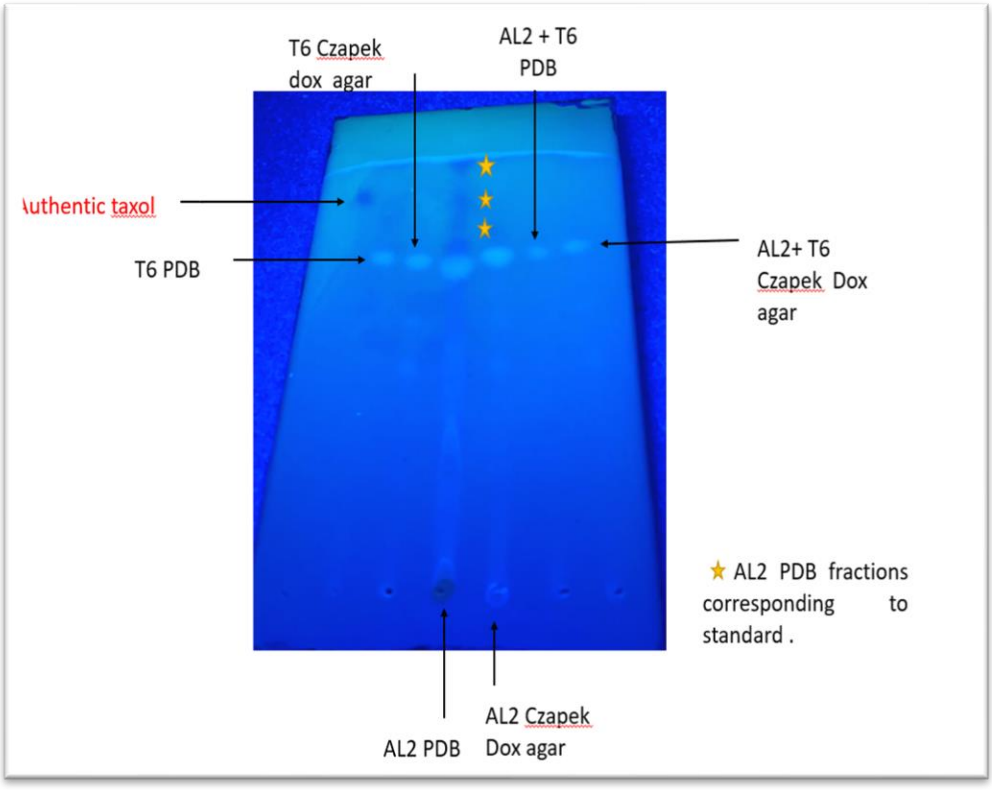

Figure 31: TLC Chromatogram for co-culture extracts

5.8.2. Column chromatography

The extraction adsorption, charging, and column saturation were all part of the column preparation. The crude extract was first dissolved in a suitable solvent before being added to the column. After that, the column was eluted with a gradient of hexanes and ethyl acetate, methanol in ethyl acetate, and methanol in ethyl acetate, followed by methanol in ethyl acetate (Figure 32).

After silica gel column chromatography, 14 fractions of endophytic crude extract AL2 were recovered. Coloured chemicals were contained in the AL2 mixture, making column monitoring simple. The coloured bands were pushed down the column according to the polarity of the solvent and collected in test tubes as they approached the end. Some organic compounds, on the other hand, are colourless. The reaction was monitored in this example by placing a spot on a TLC plate, and the chromatogram was created by placing the plate in an iodine chamber to determine the number of spots in each of the collected test tubes. The solvent was evaporated using a rota evaporator after the test tubes with the same spot were merged.

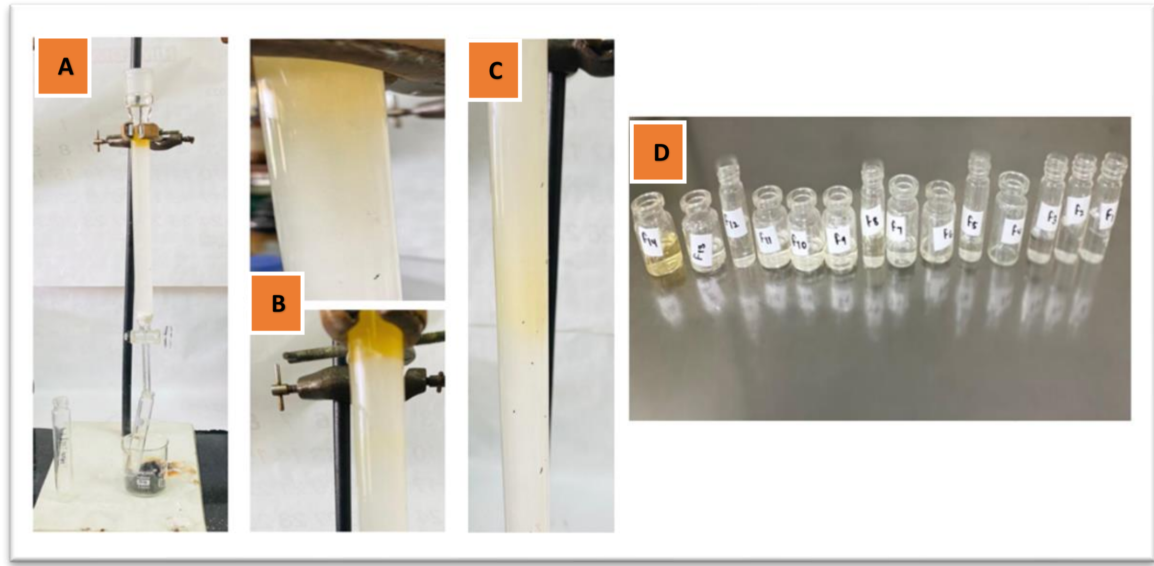

Figure 32: Fractionation of crude extract A) Loading the extract into the column B) Separating the crude with 100 percent hexane C) Increasing the polarity of the crude to separate it into a number of fractions D) Following rota-evaporation, fraction collection

5.9 Protein denaturation assay and Lipoxygenase Inhibition Assay on fractions obtained after column chromatography

5.9.1 Protein denaturation inhibition assay (BSA Assay):

Fractions obtained following column chromatography for AL2 were employed for BSA Assay at 25 µg/ml and 50 µg/ml concentrations to test their anti-inflammation potential (Figure 33,34).

Fractions of Al2 which were fourteen in number were then further tested for anti-inflammation assay. Diclofenac sodium (100µg/ml) was used as control and showed inhibition of 98.35%. Fractions of AL2 of concentration 25µg/ml and 50µg/ml were tested for anti-inflammatory potential. Out of these fourteen fractions, one's with 50µg/ml showed better potential at inhibition when compared to fractions at 25 µg/ml. When it came to fractions with 25 µg/ml - fraction12 showed remarkable inhibition percentage of 95.10% whilst at 50µg/ml again fraction 12 showed percentage inhibition of protein denaturation of 96 .23%.

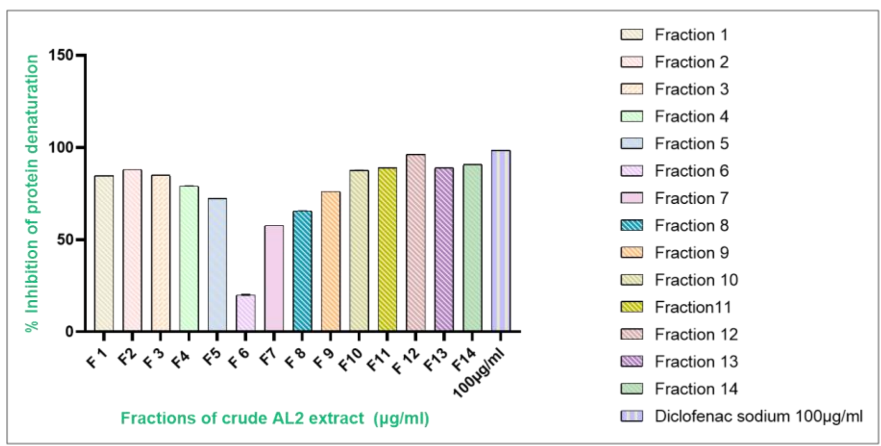

Figure 33: Fractions of AL2 crude at 25 µg/ml

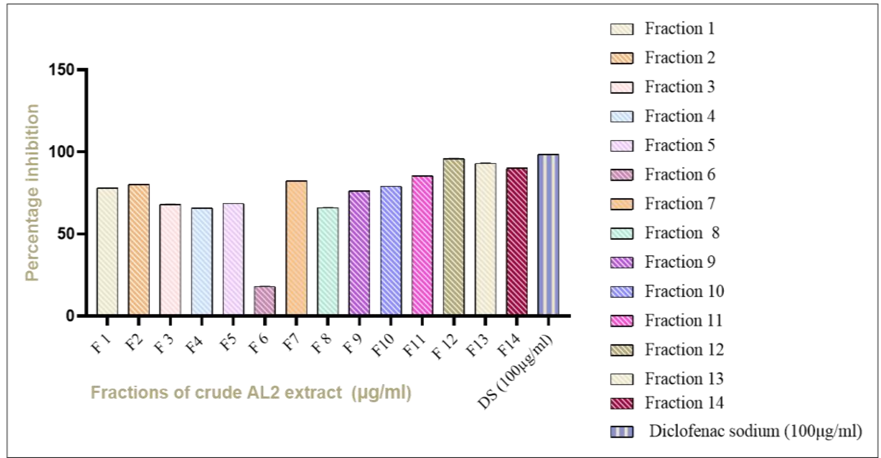

Figure 34: Fractions of AL2 crude at 50 µg/ml

5.9.2 Lipoxygenase Inhibition Assay: Further fractions 12,13, and 14 were chosen based on the results of an earlier anti-inflammation experiment. (Figure 35).

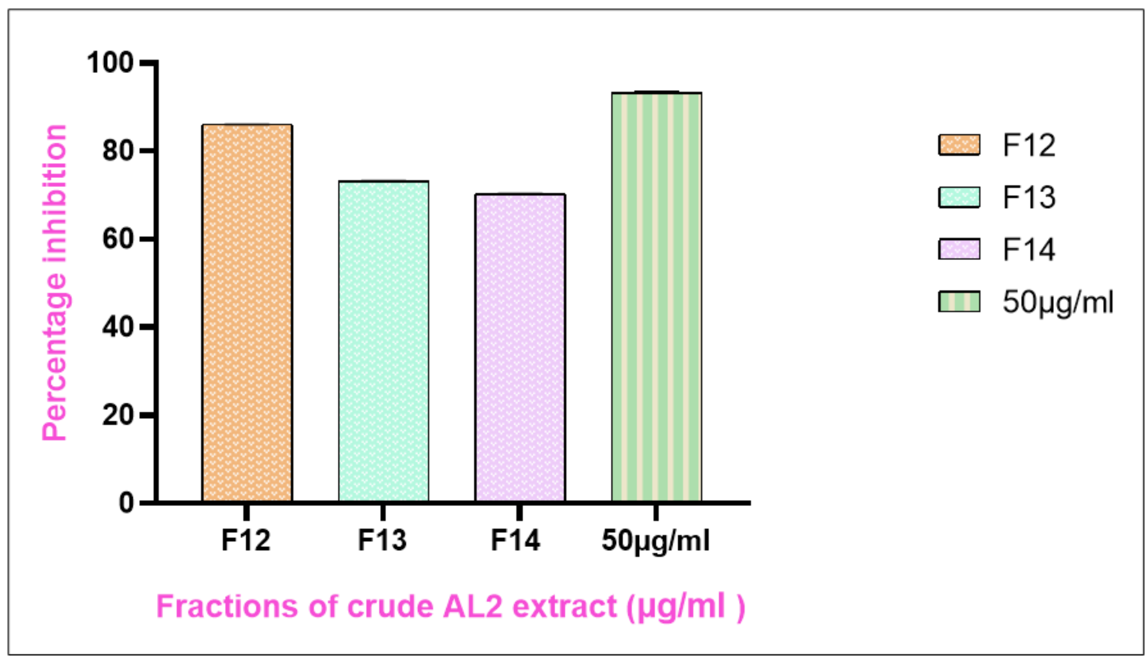

Figure 35: LOX assay on F12, F13 and F14

Fraction 12 inhibits 86.40 percent, fraction 13 inhibits 73.2 percent, and fraction 14 inhibits 70.30 percent, according to the graph above.

In the LOX experiment, fraction 12 from crude AL2 extract exhibited a significant inhibitory effect.

Based on the results of the aforesaid screening, AL2 remained a powerful extract with strong bioactive potential. When employed at 25µg/ml and 50µg/ml, respectively, fraction 12 in the protein denaturation experiment suppresses protein denaturation by 95.10 and 96.23 percent. Fraction 12 showed 86.4 percent inhibition of LOX at 500µg/ml during LOX inhibition.

A. fumigatus and *Fusarium sp*. had IC50 values for LOX inhibitory activity of 62µg/ml and 63µg/ml, respectively. Quercetin, the reference drug, displayed an IC50 of 1.45µg/ml (Prakash et al., 2013).

It was chosen, after much deliberation, to submit fraction -12 from crude extract AL2 for characterization testing.

5.10 Characterization of fraction:

5.10.1 Compound spectroscopic study in UV-Visible light: Using a UV-Visible spectrophotometer (Shimadzu UV-2600, Japan), the purified crude extracts from column chromatography were diluted in methanol and their UV absorption was determined between 200 to 500 nm (Figures 36, 37, 38, 39,40, 41,42 ,43 and 44).

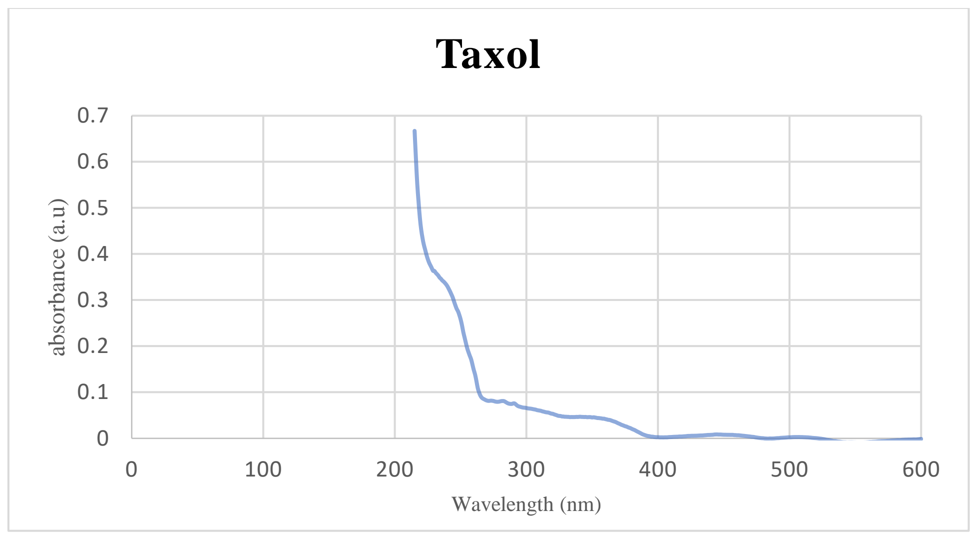

Figure 36: UV absorption spectra of authentic taxol

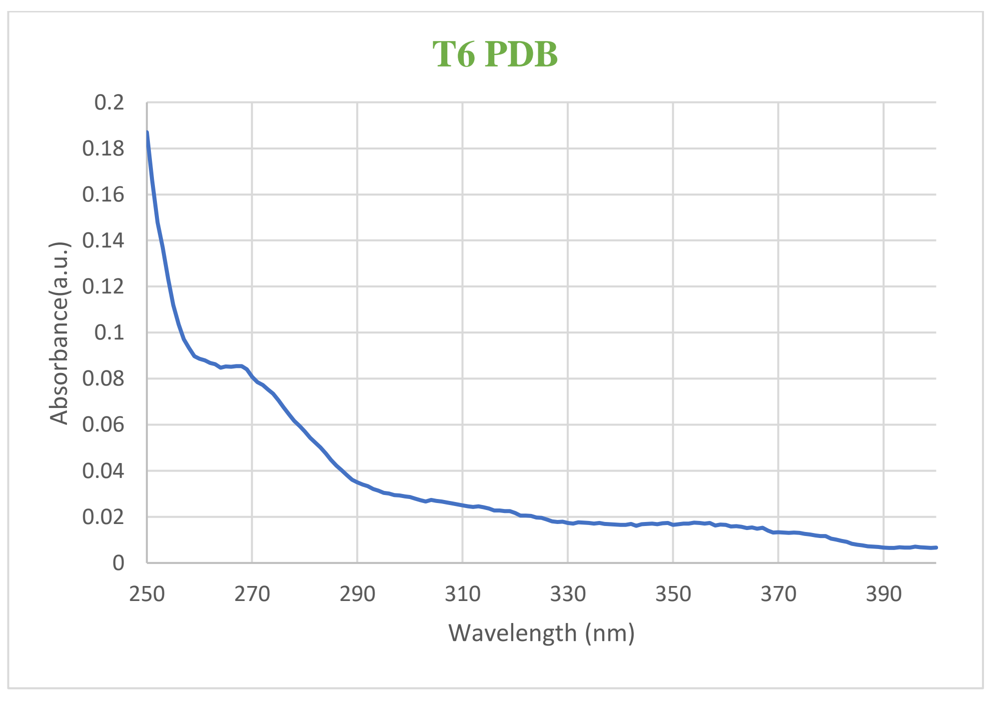

Figure 37: UV absorption spectra of T6 (PDB)

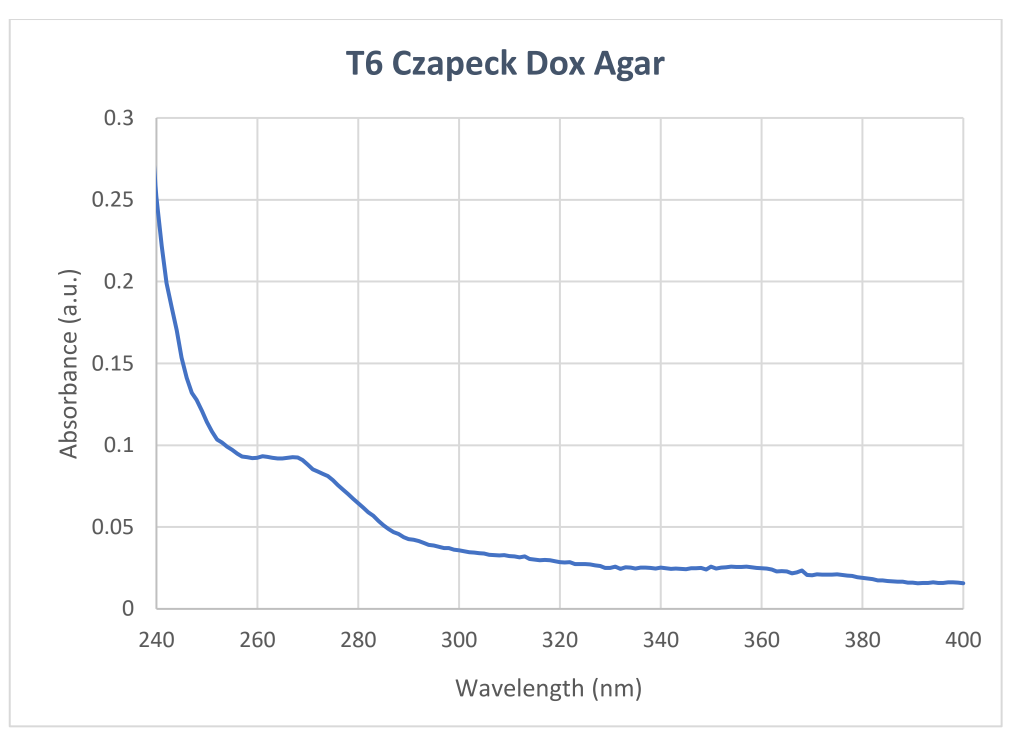

Figure 38: UV absorption spectra of T6 (Czapek Dox)

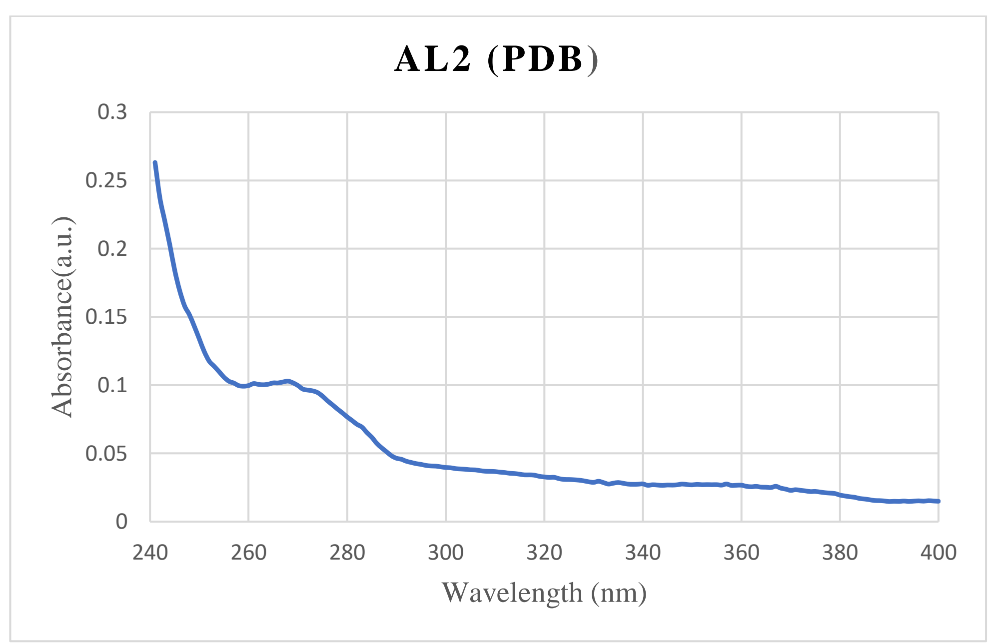

Figure 39: UV absorption spectra of AL2 (PDB)

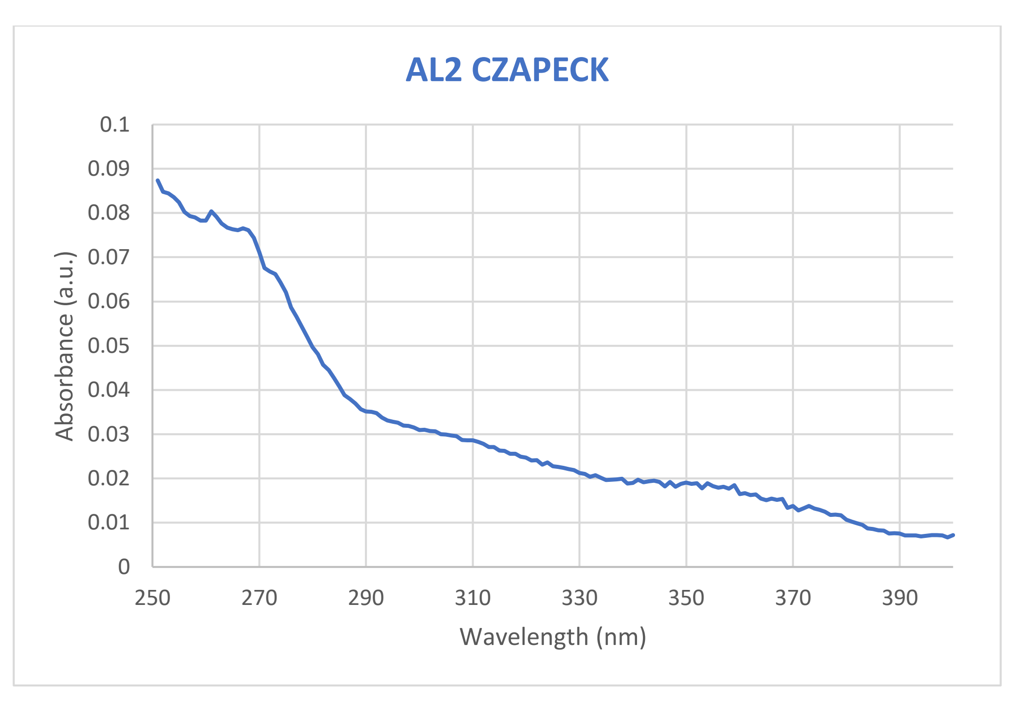

Figure 40: UV absorption spectra of AL2 (Czapek Dox)

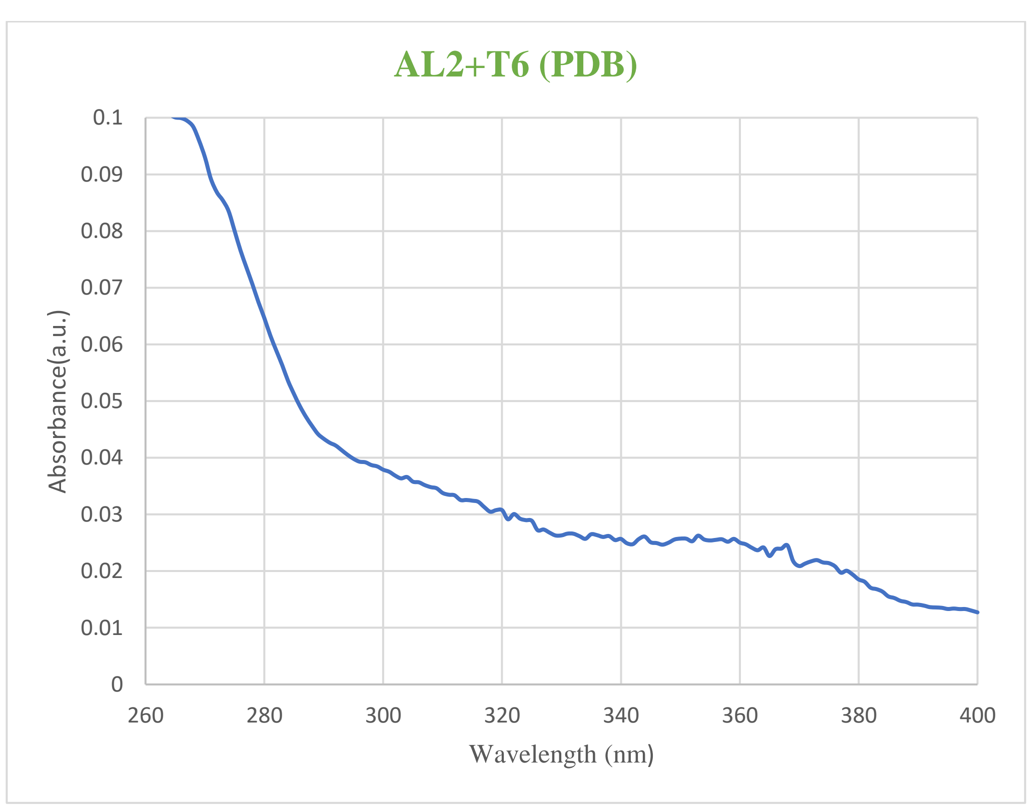

Figure 41: UV absorption spectra of AL2+ T6 (PDB)

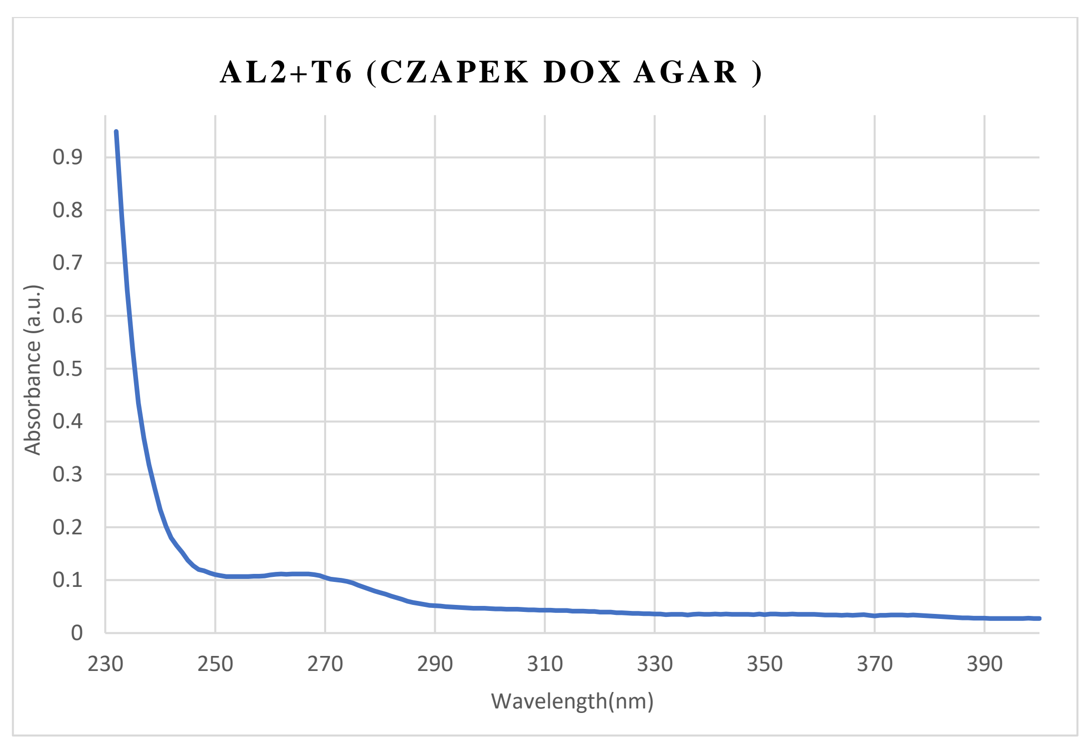

Figure 42: UV absorption spectra of AL2+ T6 (Czapek Dox)

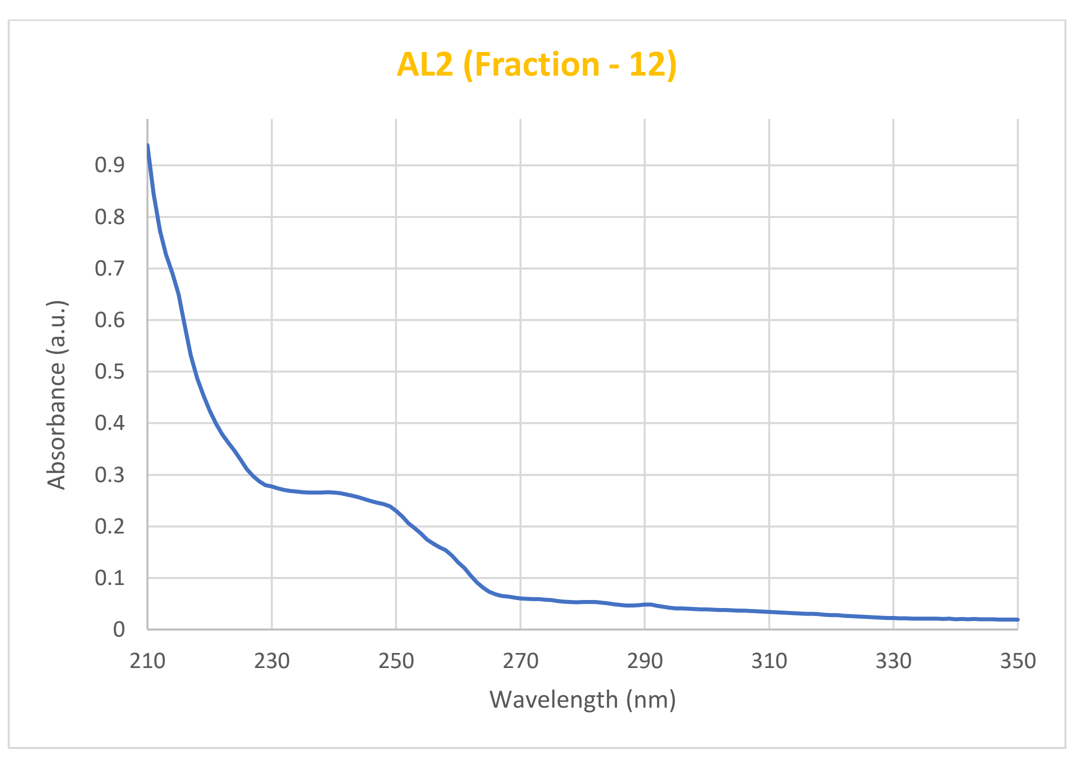

Figure 43: UV absorption spectra of F12

5.10.2 FTIR Analysis: The fraction 12 was sent for FTIR analysis. The acquired spectra were compared to the functional group's spectrum's wavelength. As per literature following findings (Figure 44) were revealed as listed in the table below (Table 9):

Table 9: Analysis of FTIR of F12

Sr. No	Frequency(cm^{-1})	Bond	Functional Group
i.	415	Mn-o Bond	Manganese bond
ii.	903.799	N-H wag Strong and broad	1º, 2º amine
iii.	952.78	C-H bend Strong	Alkenes
iv.	1012.77	C-O	Alcohols, carboxylic acid, esters, ether
v.	1313.70	C-O stretch	alcohols, carboxylic acids, esters, ethers
vi.	1416.68	C-C Stretch (in–ring)	Aromatics
vii.	1627.63	N-H bend	1˚ amines
viii.	3290	–C≡C–H: C–H stretch	Alkynes (terminal)

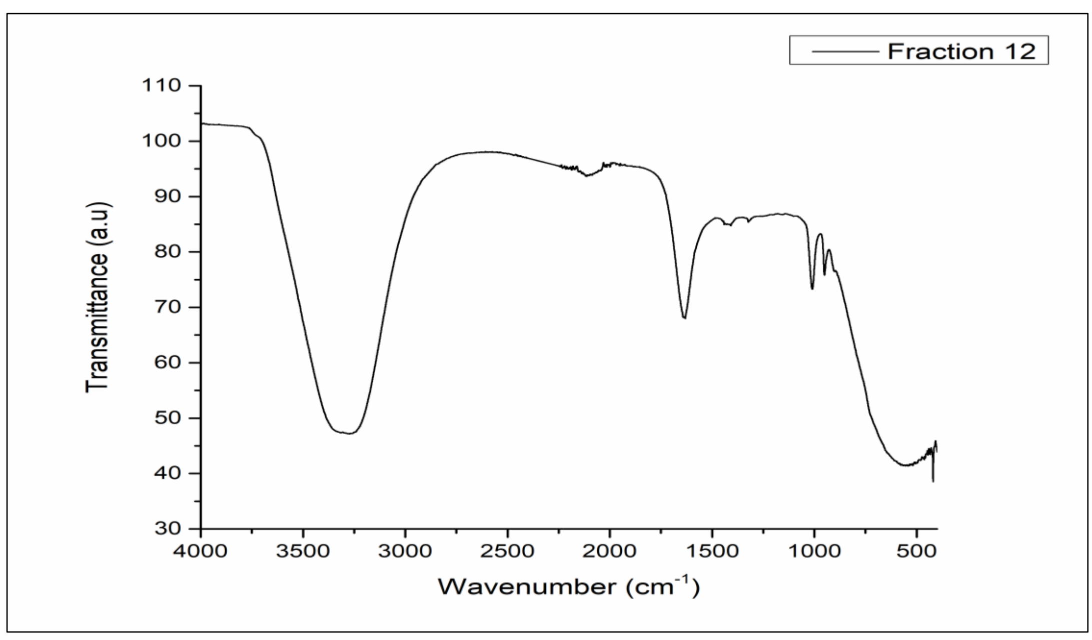

Figure 44: FTIR Spectrum of F12

5.10.3 Nuclear magnetic resonance: For NMR F12 was submitted to SAI labs. As per data obtained following graphs were generated. Additionally, the results were then compared to the literature and are listed in the table beneath (Table 10)

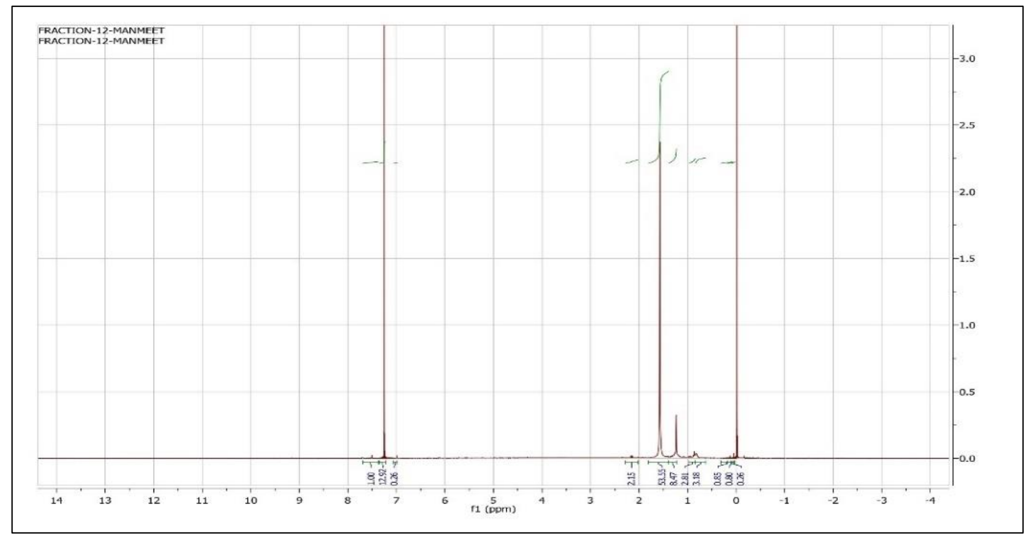

Figure 45: NMR Spectrum of F12

Table 10: Analysis of NMR data

S.No.	Obtained δ (ppm)	Type of C-H	Description of Proton
i.	1.00	ROH	Alcohol
ii.	12.92	$R-C(=O)-OH$	Carboxylic acid
iii.	0.26	ROH	Alcohol
iv.	2.15	$R-C(=O)-CH_3$	α to carbonyl (C is next to C=O)
v.	8.47	$R-C(=O)-NHR$	amide
vi.	2.81	$R-C(=O)-CH_3$	α to carbonyl (C is next to C=O)
vii.	3.18	$R-CH_2-X$	α to halogen (C is attached to Cl, Br, I)
viii.	0.85	$R-CH_3$	alkyl (methyl)
ix.	0.80	$R-CH_3$	alkyl (methyl)
x.	0.26	R - CH3	alkyl (methyl)

5.10.4 HR-MS data: Methanol was utilized to dissolve fraction 12 and use it as such for analysis. Utilizing the UPLC Acquity H class series technology, chromatographic separation was completed. After injecting the sample into the column, 0.1 percent formic acid in water (20:80 v/v) was used for elution, with a flow rate of 0.3 ml/min.

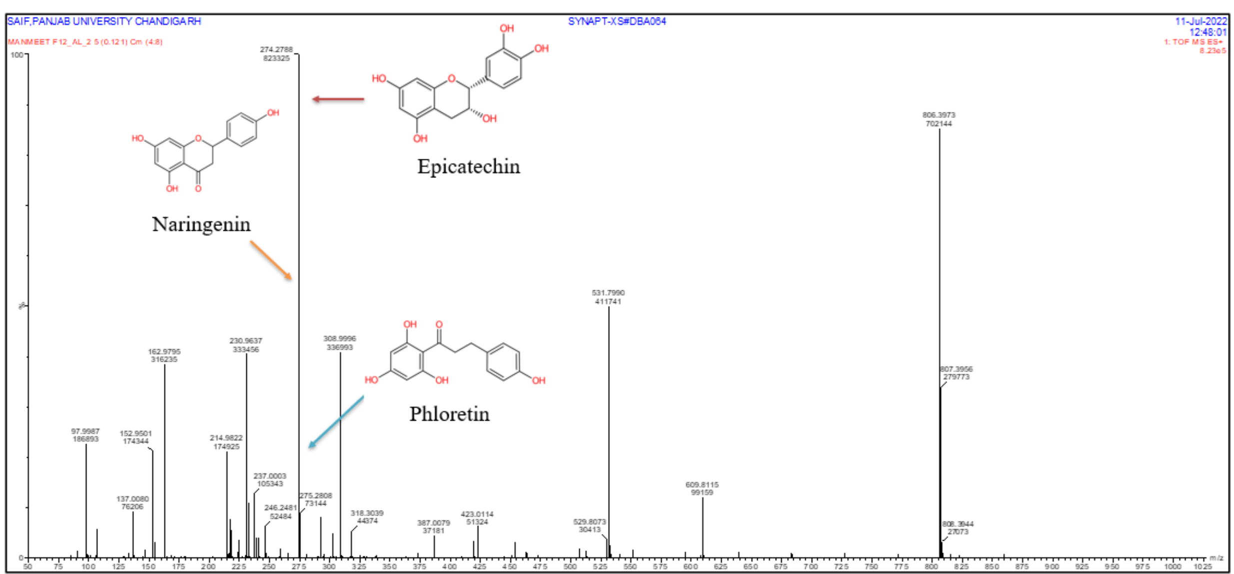

Figure 46: HR-MS results of F12

Putative compounds based on characterization studies of fraction 12 of AL2

Molecular ion m/z (measured value)	291.08
Ion type	[M+H]+
Molecular ion (reported value)	290.27
Molecular weight	290.2681
Structure	
Molecular formula	$C_{15}H_{14}O_6$
Putative compound	Epicatechin (Flavonoid)
Bioactivity	Anti-inflammatory
References	Three Pathways Assess Anti-Inflammatory Response of Epicatechin with Lipopolysaccharide-Mediated Macrophage RAW264.7 Cells (Deng-Jye Yang et al ., 2015)

Molecular ion m/z (measured value)	274.2788
Ion type	[M+H]+
Molecular ion (reported value)	274.08412
Molecular weight	272.25
Structure	
Molecular formula	$C_{15}H_{12}O_5$
Putative compound	Naringenin (Flavonoid)
Bioactivity	Anti-inflammatory
References	Naringenin, a flavanone with antiviral and anti-inflammatory effects: A promising treatment strategy against COVID-19 (Helda Tutunchi et al.,2020)

Molecular ion m/z (measured value)	274.2788
Ion type	[M+H]+
Molecular ion (reported value)	274.08412
Molecular weight	274.27
Structure	
Molecular formula	$C_{15}H_{14}O_5$
Putative compound	Phloretin (naringenin dihydrochalcone) Flavonoid
Bioactivity	Anti-inflammatory
References	Phloretin suppresses neuroinflammation by autophagy-mediated Nrf2 activation in macrophages (Tess Dierckx et al .,2021)

Table 11: Analysis of HR-MS data

S.No.	Obtained m/z	Reported m/z	Compound formula	Compound name	Bioactivity	Reference
i.	137.0080	138 $[M+H]^+$	$C_7H_9N_2O$	1-Methylnicotinamide	anti-inflammatory	Gebicki J *et al*., 2003
ii.	230.9637	232 $[M+H]^+$	$C_{13}H_{17}N_3O$	Aminopyrine	anti-inflammatory antipyretic agent analgesic	Shahzad *Murtaza et al*.,2019
iii.	237.0003	238.0857 $[M+H]^+$	$C_{15}H_{11}NO_2$	Viridicatin	strong antibiotic activity	Avnish Kumar *et al*.,2018
iv.	274.2788	274.08412 $[M+H]^+$	$C_{15}H_{14}O_5$	Phloretin	anti-allergic anti-carcinogenic anti-inflammatory anti-microbial anti-thrombotic antioxidant	Mariadoss *et al* .,2019
v.	275.2808	275.3535 $[M+H]^+$	$C_{15}H_{21}N_3O_2$	Physostigmine	anticholinergic	Olyn A. Andradee et *al*., 2022
vi.	308.996	318.03757 $[M+H]^+$	$C_{15}H_{10}O_8$	Myricetin	antioxidant anti-inflammatory	Kian C.ong *et al* .,1997
vii.	423.0114	423.06577 $[M+K]^+$	$C_{15}H_{21}NO_9S_2$	Gluconasturtiin	anti-inflammation antioxidant antimicrobial	Eun-Sun Hwang., *et al* 2019
viii.	529.8073	291.08683 $[M+H]^+$	$C_{15}H_{14}O_6$	Catechin	anticancer antidiabetic anticardiovascular anti-infectious	Mamrou Isemura *et al* .,2019

					hepatoprotective neuroprotective	
ix.	531.7990	291.08683 $[M+H]^+$	$C_{15}H_{14}O_6$	Epicatechin	antioxidant	Chem. Asian J *et al* ., 2013
x.	807.3956	806.50 $[M-H]^-$	$C_{44}H_{74}NO_{10}P$	Phosphatidylserine	anti-inflammatory	Darabi M.,*et al* 2020

CONCLUSIONS

Endophytic fungi were isolated in the current study using *Eucalyptus teretecornis*. Three different endophytes from the leaf of *E. teretecornis* were obtained. T6 isolated from *T.baccata* L subsp. *wallichiana* (Zucc.) Pilger and AL2 isolated from *Terminalia arjuna* were both cultures that were introduced in the lab. These extracts underwent additional preliminary screening to determine their bioactive potential. These extracts were subjected to a variety of activities, including antibacterial, anti-oxidant, and anti-inflammatory.

Since the aforementioned medicinal plant offers therapeutic potential, it may be investigated for endophytic fungi that have the ability to create bioactive chemicals. Sample collection was done from the Thapar campus. Additionally, cultures of T6 and AL2 were made available by the TIFAC - CORE lab for the purpose of testing their bioactive potential. For fermentation, the isolated endophytic fungi from *E. teretecornis*, T6, and AL2 were inoculated in PDB and czapek's broth.

When the crude extract from these isolates was subjected to a preliminary DPPH assay, AL2 crude fungal extract at 500μg/ml displayed levels of radical scavenging activity of 61.3 percent. At 1000μg/ml, EL1 fungal extract demonstrated a value of radical scavenging of 67.5 percent. As a result, we concluded that EL1 and AL2 extract both possessed strong antioxidant properties. The preliminary antibacterial activity of crude AL2 fungal extract was examined further using broth microdilution using *Staphylococcus aureus* as test organism. A 43.1percent inhibitory activity was detected. According to this, the lowest level of inhibition was seen at 62.5μg/ml, even though the AL2 fungal extract exhibited stronger antibacterial activity at 500μg/ml.

It was determined that AL2 extracts had additional protein denaturation inhibition action and that at 500μg/ml concentration, a percentage inhibition of 91.28 percent was detected.

According to the evidence above, AL2 crude extract was notable bioactive extract.

Based on morphological and molecular investigations, the lab-provided endophytic fungi (AL2) culture was identified as *Alternaria spp*. BLASTN was used to evaluate the acquired ITS sequence (569 bp), which revealed 100% query coverage and 100% similarity to *A. brassicicola.* The NCBI database has the sequence acquired in this investigation under the accession number MK158222. Phylogenetic study showed that AL2 was clustered with the species of *A. brassicicola* (M.Vasundhara *et al*., 2019).

Initial TLC analysis was performed for purification, and column chromatography was used for fractionation. TLC of co-culture extracts was performed in conjunction with an authentic taxol standard, and when this was done, bands corresponding to the standard could be seen when dichloromethane: methanol (9:1) was used. This offers a cue that taxol might be present in the co-culture extracts. But to determine taxol production, more quantitative research is needed.

The crude AL2 extract yielded fourteen fractions. Obtained fractions of AL2 were tested for anti-inflammatory potential at 25µg/ml and 50µg/ml concentrations. Out of these fourteen fractions, one's with 50µg/ml showed better potential at inhibition when compared to fractions at 25 µg/ml. When it came to fractions with 25 µg/ml - fraction12 showed remarkable inhibition percentage of 95.10% whilst at 50µg/ml again fraction 12 showed percentage inhibition of protein denaturation of 96 .23%. Further fractions 12,13, and 14 were chosen based on the results of an earlier anti-inflammation experiment. Fractions 12 and 13 inhibited 86.40 percent, 73.2 percent, and 70.30 percent, respectively. Fraction 12 of the crude AL2 extract showed a substantial inhibitory impact in the lipoxygenase inhibition assay.

In accordance with the findings of the aforementioned screening, F12 continued to be a potent extract with significant bioactive potential.

It was chosen, after much deliberation, to submit F12 for characterization testing which involved FTIR, NMR and HR-MS.

Therefore, it can be inferred from this research that AL2 crude extract is a noteworthy bioactive potential carrier. It showed notable antibacterial activity against *S. aureus* and free radical scavenging properties. Apart from this, when subjected to a protein denaturation inhibition assay and lipoxygenase inhibition assay, AL2 crude and fraction yielded from crude extract F12-AL2 showed an outstanding anti -inflammatory activity.

A variety of bioactive substances may be produced by the interactions between endophytic fungi, plant cells, metabolites, and other various environmental and biological variables (Pan et al. 2017). As a result, the endophytic fungi found in the plants under study may provide access to novel sources of chemicals useful for biotechnological applications or investigations of ecological interactions.

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