Exploration of Heat Shock Protein Inhibitor Geldanamycin against *Aspergillus terreus*

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

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in

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by

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Under the supervision of

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To

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SELF-DECLARATION

I, Neha, a student of M.Sc. Biotechnology, Jaypee University of Information Technology, Waknaghat, Solan, Himachal Pradesh do hereby declare that the project entitled **"Exploration of Heat Shock Protein Inhibitor Geldanamycin against *Aspergillus terreus*"** with and without inhibitor Geldanamycin, submitted towards partial fulfilment for the award of the degree of Master of Science in Biotechnology of Jaypee University of Information Technology. I do declare that a literature review and original research work has been written by me and carried out by me under the guidance and supervision of Prof. Jata Shankar, in accordance with the academic rules and ethics. This work has not been submitted partially or wholly to any other university or institute for the award of this or any other degree or diploma.

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CERTIFICATE

This is to certify that the work titled "**Exploration of Heat Shock Protein Inhibitor Geldanamycin against *Aspergillus terreus***" submitted by "Neha", roll number- 225111011 in partial fulfilment for the award of the degree of M.Sc. Biotechnology of Jaypee University of Information Technology, Waknaghat has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

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Thank you

Neha

Date-

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LIST OF ABBREVIATIONS

nccpf	National culture collection of Pathogenic Fungi
LAFH	Laminar Air Flow Hood
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
RPMI 1640	Roswell Park Memorial Institute
FBS	Fetal Bovine Serum
PBST	Phosphate Buffer Saline
Tween 20 PBS	Phosphate Buffer Saline
EDTA	Ethylene Diamine Tetra-acetic acid
TAE	Tris-acetate-EDTA
RNA	Ribonucleic acid
Etbr	Ethidium Bromide
GA	Geldanamycin
AmB	Amphotericin B
Hsp	Heat shock protein
µg	Micro gram
µl	Microlitre
cDNA	Complementary DNA
RT-PCR	Real time polymerase chain reaction
NTC	Non template control
DMSO	Dimethyl Sulfoxide
DCFDA	2'-7'-Dichlorodihydrofluorescein diacetate

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ABSTRACT

Aspergillus terreus is a rising pathogen among individuals with weakened immune systems. Due to intrinsic resistance to amphotericin B and acquiring resistance to different azoles, echinocandins, and other antifungal agents, it is important to explore alternative methods to combat its infection. On the other hand, the coexistence of cancer and *Aspergillus* infection presents a complex clinical situation. Thus, in this project we have explored a new drug molecule Geldanamycin that inhibit the growth of fungus as well as the growth of the cancer cells. Geldanamycin disrupts the activity of the Heat shock proteins which is important for the morphogenesis of the fungal cell, and plays important role in the Replication, Posttranscriptional process, Transcription, Translation, Posttranslational processes, and the activation of signalling pathways. We determined the effect of Geldanamycin drug in treated versus control *Aspergillus terries* culture. The expression of the Hsp90 at 24 hours observed to be decreased which may disrupt the function of chaperone such as Hsp90. Thus, the stress due to drug possibly lead to the inhibition of *A. terreus*.

Key words: - *Aspergillus terreus*, Geldanamycin, Hsp70, Hsp90, Resistance, Minimal Inhibitory Concentration, RT-PCR.

CHAPTER 1

INTRODUCTION

Fungal infections have been increasing recently due to increase in resistance towards certain drugs, antifungals and antimicrobials majorly in patients with weakened immune systems. Among the various fungal infections globally, *Aspergilli*-mediated infections have been particularly on the rise in humans, domestic animals, and birds. *Aspergilli* are spread in the environment through the air, soil, plant debris, food sources, and indoor environments, causing significant economic losses by damaging crops. The subgenera *fumigatus, flavus, terreus,* and *niger* of *Aspergillus* are the most common, highlighting their significant impact as opportunistic pathogens in humans [1, 2, 3]. In recent years, there has been a significant increase in infections caused by opportunistic fungi. This rise can be attributed to the growing antimicrobial and antifungal resistance observed in certain medications. Out of the 120,000 fungal species identified to date, the *Aspergillus* family is particularly notorious for causing infections. These give rise to both acute and chronic infections in humans, along with various allergic reactions including Invasive Aspergillosis, Allergic Bronchopulmonary Aspergillosis, Allergic Aspergillus sinusitis, Hypersensitivity pneumonia, and IgE-mediated asthma [3]. As a widely distributed sporophyte fungus in the air, *Aspergillus terreus* can lead to infections in individuals with a compromised immune system. It holds significance as one of the invasive pathogens. The prevalence of immunocompromised individuals has risen in recent years, primarily due to factors such as cancer chemotherapy, organ transplantation, and HIV/AIDS [4]. Consequently, opportunistic fungal pathogens have found it easier to infect these vulnerable hosts.

Furthermore, individuals who suffer from respiratory conditions such as asthma, alergies, and chronic obstructive respiratory disease are particularly susceptible to fungal manifestations due to the direct interaction between lung epithelium cells and *Aspergilli* [5]. In particular, *Aspergillus terreus* conidia have the ability to continue in antigen-presenting cells, leading to increased dispersal of conidia and reduced effectiveness of drug treatments. Moreover, in people with respiratory disorders (asthmatic, allergic, and chronic obstructive respiratory disease), the lung epithelium cells are in direct contact with *Aspergilli* which makes them vulnerable to fungal manifestations. *Aspergillus terreus* conidia continue in antigen-presenting cells that allow more dispersal of conidia and lower efficacy against the drugs [6].

According to the latest research, it has been discovered that *Aspergillus terreus* is the primary cause behind Invasive aspergillosis (IA), particularly in individuals with cancer [3]. Moreover, the inherent resistance of *Aspergillus terreus* to amphotericin B (AmB), the widely used antifungal medication for over four decades, poses a challenge in devising an effective treatment plan [7]. The mechanism of drug resistance in *Aspergillus terreus* remains unclear, although approximately 5-10% of isolates are resistant to azole [8].

There exist numerous known drug targets as well as newly recognized ones. Heat shock proteins, a family of proteins present in all types of alive cells, play a crucial role in ensuring proper protein folding. In fungal cells, they play a significant role in cell morphogenesis and are vital for fungal cell survival. In case of cancerous cells, the amount of heat shock proteins also increases. Consequently, inhibiting these proteins through different drugs and methods could potentially be a promising approach in eliminating fungal infections and the inhibition of growth of cancerous cells [9].

In summary, there remains much that is yet to be understood about fungal cells and their role in causing infections. The rise in drug resistance necessitates the exploration of new drug components or alternatives that are more effective, as well as new drug combinations.

As part of this research project, we conducted a transcriptional analysis of Hsp70 and Hsp90 proteins in *Aspergillus terreus* at time intervals of 24 hours both with and without Geldanamycin. This specific time point was chosen to coincide with the germination time of *Aspergillus terreus* at 16 hours, and to examine Hsp gene expression during periods of minimum and maximum drug activity at 24 hours.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Members of genus *Aspergilli*

Aspergillus is a fungus. Fungus are of eukaryotic organisms that contain microbe such as yeasts and molds, as well as the mushrooms and lichens, classified under the kingdom Fungi. While some fungi are microscopic, others are visible to the naked eye. These organisms are widely distributed in nature, thriving in various environments including land, soil, and air [10]. They play a vital role in the ecological by breaking down dead organic matter and returning nutrients to the soil [11].

Classification of Fungus

Fungi can be classified as: -

1) **Based on types of fungus**

   Molds: Molds are multicellular filamentous fungi that grow in thread-like structures called hyphae. They are common in moist environments and can reproduce quickly, forming fuzzy colonies on food, plants, and other organic matter. Some molds produce mycotoxins, which can be harmful if inhaled or ingested.

   Yeasts: Yeasts are single-celled fungi that reproduce asexually by budding. They are found in a variety of habitats, including soil, plants, and animals. Some yeasts are beneficial, such as those used in baking and brewing, while others can cause infections [11].

   Lichens: Lichens are not technically fungi, but rather a symbiotic association between a fungus and an algae or cyanobacteria. The fungus provides protection and structure, while the algae or cyanobacteria photosynthesizes and produces food for both partners. Lichens are found in a variety of habitats, including on rocks, trees, and soil.

   Mushrooms: Mushrooms are the fruiting bodies of certain fungi. They are the above-ground reproductive part of a much larger underground network of hyphae called mycelium. Mushrooms come in all shapes and sizes, and some are edible while others are poisonous [12].

2) **Based on the mode of nutrition:**

   Saprophytic: Saprophytes: Saprophytic fung obtain nutrients from the dead and decaying organic matter. They play an vital role in decomposition and nutrient cycling in ecosystems.

   Examples- Aspergillus, Penicillium and Rhizopus.

Parasites: Parasitic fungi live on or in other living organisms and obtain nutrients from their host. They can cause disease in plants, animals, and even other fungi.

Examples- Puccinia and Taphrina.

Symbiotic: Some fungi form Mutualistic Relationships with other organisms, such as algae and plants. In these partnerships, both organisms benefit from each other.

Example- Mycorrhizal fungi live in association with the roots of plants and help them to absorb water and nutrients from the soil. Lichens are a symbiotic association between a fungus and an alga or cyanobacteria. The fungus provides protection and structure for the alga or cyanobacteria, which produce food through photosynthesis [13].

3) Based on spore formation:

Asexual Spores: Many fungi reproduce asexually through spores produced by specialized structures called sporangia. These spores can be airborne or carried by water to germinate and form new fungal colonies [12].

Sexual Spores: Sexual reproduction is due to the fusion of two compatible mating types. This can result in the formation of specialized sexual spores, which may be more resistant to harsh conditions than asexual spores. There are three main types of sexual spores:

- Ascospores**:** Ascospores are produced inside a sac-like structure called an ascus. Fungi in the phylum Ascomycota reproduce sexually using ascospores. asexual spores are known as conidiospores.

  Example- Saccharomyces
- Basidiospores: Basidiospores are borne on a club-shaped structure called a basidium. Fungi in the phylum Basidiomycota reproduce sexually using basidiospores and asexual reproduction is done by conidia formation, bud formation or through fragmentation.

  Example- Agaricus
- Zygospores: Zygospores are thick-walled spores produced by the fusion of hyphae from two compatible individuals. They are characteristic of zygomycetes fungi. Their Sexual spores are known as zygospores and Asexual spores are known as sporangiospores. Their hyphae do not contain septa.

  Example- Mucor
- Deuteromycetes: Grout of this fungi are also known as imperfect fungi because they do not follow the regular reproduction cycle like other members of fungi kingdom. Asexual reproduction one by conidia and they do not reproduce sexually [14].

Due to the rise in anti-microbial resistance in microorganisms, it is quite a task to treat such infections. In the recent last few years, *Aspergillus terreus* known to be leading cause of Invasive aspergillosis in patients with weak immunity, especially in cancer patients [15]. Therefore, there is a requirement of more therapeutic options and drugs to beat such infection.

2.2 Taxonomic classification of *Aspergillus terreus*

Kingdom: Fungi

Phylum: Ascomycota

Order: Eurotiales

Family: Trichocomaceae

Genus: *Aspergillus*

Species: *terreus*

2.2.1 Morphology and Distribution of *Aspergillus terreus*

Aspergillus terreus, also known as *Aspergillus terrestris*, is a fungus (mold) found worldwide in soil, contains 30-35 Mbp and roughly 10,000 protein-coding genes. It is commonly found in warm and humid regions, as well as in tropical and sub-tropical areas worldwide. Moreover, it has the ability to survive in extreme environmental conditions, including high salinity, alkalinity, and temperature [16]. Generally, conditions required to the fungus is the level of moisture present in the material. It has been documented that the fungus can thrive at a minimum water activity (Aw) of 0.78. The ability to tolerate low Aw conditions may contribute to the widespread presence of this species, as it can grow in various environments [17].

Initially, its colonies grow rapidly and have a white cotton-like appearance, which gradually turns brownish-grey as they age with a wrinkled texture. The hyphae of *Aspergillus terreus* have septa and typically measure 2-4 μm in diameter. For asexual reproduction, it produces unbranched conidiophores that contain small, round conidia. These conidiophores are smooth-walled and approximately 200-500 μm in length. The conidia themselves are spherical in shape, smooth-walled, and around 2-4 μm in diameter. They are produced in large quantities and dispersed in the air, making *Aspergillus terreus* an airborne contaminant and easily inhaled by humans [18].

2.2.2 Life cycle of *Aspergillus terreus*

The life cycle of *Aspergillus terreus* typically, filamentous fungi consist of several stages these are:

1. **Spore germination**:
   Spore dispersal: - *Aspergillus terreus* reproduces through asexual reproduction, where spores(conidia) are produced in large numbers on specialized structures called conidiophores.
   Germination: - Under favourable conditions, the spores undergo germination, giving rise to hyphae – elongated, branching, thread-like structures
2. **Vegetative growth**:
   Formation of Mycelium: The spores that have germinated undergo a process of developing into a complex network of hyphae, referred to as mycelium. This mycelium acts as the fungal organism's vegetative structure.
   Assimilation of Nutrients: Through the process of branching and elongation, the mycelium continues to expand, enabling the fungus to effectively absorb essential nutrients from its surrounding environment [19].
3. **Asexual reproduction**:
   Conidiophore formation: - *Aspergillus terreus* reproduces asexually by forming spores known as conidia. These spores are formed at the tips of specialized hyphae called conidiophores.
   Conidiation: - Conidia are formed at the tips of conidiophores through a process known as conidiation. When the conidia are mature, they are released into the environment and can germinate to start a new life cycle [20].
4. **Sexual reproduction**: In certain conditions, *Aspergillus terreus* can also reproduce sexually. Occurs when two hyphae of different mating types fuse and forms a structure what is known as zygospore. The zygospore is a thick-walled structure that can survive adverse environmental conditions and when conditions become favorable, the zygospore will germinate and give rise to a new mycelium [21].

Almost all filamentous fungi have the same life cycle and comprise of same above steps.

2.2.3 Ecological and Environmental Roles: -

Plant Growth Promotion and Biocontrol: *Aspergillus terreus* exhibits biocontrol properties and plant growth-promoting effects, making it highly valuable in agricultural and ecological settings. It also produces some secondary metabolites that have anti-microbial properties and protect plants from disease-causing pathogens [22].

Soil Microorganism: As a saprophytic fungus present in soils, *Aspergillus terreus* plays a important role in the breakdown of organic matter and cycling of nutrients within ecosystems. It is an important decomposer of cellulose, lignin and hemicellulose which are complex polymers that are found in the plant cell wall, by breaking those *Aspergillus terreus* releases variety of nutrients into the soil which are then used up by other organisms and plants itself.

Pathogenicity and Plant Pathogen: Although it has positive effects on soil-plant systems, *Aspergillus terreus* can also act as a plant pathogen, causing infections in important crops such as rice, wheat, potato, sugar cane, maize, and soybean.

Mycotoxin Production: *Aspergillus terreus* is recognized for its ability to generate mycotoxins, including tremorgenic mycotoxins, which induce tremors in mammals and can have harmful effects on both humans and animals [18].

2.2.4 Industrial applications: -

1. Itaconic acid production:

   Aspergillus terreus is utilized for the industrial production of Itaconic acid, a bioproduct in the green chemistry field, with a broad range of applications.

   Itaconic acid is a versatile platform chemical used in the production of:

   a. Plastic (e.g., biodegradable polymers)
   b. Resins
   c. Lubricants
   d. Dyes
   e. Cosmetics

2. Source of Lovastatin:

   Aspergillus terreus was historically the primary source of lovastatin, a drug used to lower cholesterol levels. Lovastatin acts by inhibiting an enzyme involved in cholesterol production in the liver.

Although chemically modified versions of lovastatin (like simvastatin) are now more commonly used, *Aspergillus terreus* played a significant role in the development of cholesterol-lowering medications [23].

3. Enzyme Production (Limited):

   Aspergillus terreus has shown some promise in producing specific enzymes with potential industrial applications. One notable example is xylanase, an enzyme capable of breaking down xylan, a key component of plant cell walls. Xylanase can be useful in various industries like:

   - ✓ Pulp and paper production
   - ✓ Biofuel production

   However, enzyme production from *Aspergillus terreus* is not as extensive as that of other industrial fungi.

4. Potential for Other Bioactive Compounds:

   Research indicates that *Aspergillus terreus* is capable of producing a range of bioactive compounds that could be utilized in the pharmaceutical sector. These compounds possess characteristics such as anti-inflammatory, antimicrobial, and anticancer properties. It is essential to conduct additional research to fully understand the capabilities of these compounds for drug development [24].

2.2.5 Germination in *Aspergillus terreus*

The first stage in the life cycle of a filamentous fungus is germination. Once its spores are dispersed in the environment, they start to germinate under favourable conditions. The germination process of *Aspergillus terreus* spores is greatly influenced by environmental factors such as temperature, moisture, and nutrient availability. Providing optimal conditions is crucial for the successful germination and subsequent growth of the fungus. The germination of *Aspergillus terreus* spores typically involves the following steps:

1. Activation: Once the spores land on a suitable surface, they become active and prepare to start the germination process.
2. Swelling: After activation, the spores undergo a swelling process, increasing in size as they absorb water from the surrounding environment.

3. Germ tube formation: Following the swelling, a germ tube emerges from the spore, serving as the initial extension of the fungal hypha. This germ tube plays a crucial role in establishing the fungal colony.
4. Hyphal growth: The germ tube further develops into a network of branching, thread-like structures known as hyphae. These hyphae form the mycelium, which is the vegetative part of the fungus responsible for absorbing nutrients and promoting growth.
5. Maturation: As the germinating spores continue to mature, they eventually develop into a fully formed mycelium, allowing the fungus to thrive and reproduce [25,26].

2.2.6 Diseases caused by *Aspergillus terreus*

As increase in number of immunosuppressant patients the *Aspergillus terreus* infections are also rising. There is high mortality of its infections due to persistence in immune cells, immune suppression and intrinsic resistance to amphotericin B. When conidia inhaled by a person, it resides in the immune cells of the host of long period of time and get transferred to other secondary organs via macrophages. At that time the immune system of the host gets compromised, the spores start to germinate and initiates their life-cycle.

1. **Invasive aspergillosis (IA)**

IA is a serious fungal infection caused by members of Aspergillus genus that typically affects people with compromised immune system. This infection can cause severe fungal invasion in vital organs such as lungs, brain and others, potentially leads to death. Symptoms of IA includes, cough, pain in chest, fever, shortness of breath, headache and neurological symptoms. Diagnosis of IA can be done by examining the symptoms, although symptoms are similar to other Aspergillus infection which makes the job challenging. Other than symptoms, radiographic findings and lab tests such as, blood tests like for checking the levels of galactomannan and beta-D-glucan which are the cell wall components of many fungal species, imaging studies like, computed tomography (CT) scans or magnetic resonance imaging (MRIs) and biopsy. Treatment of IA involves usage of various anti-fungal drugs such as, isavuconazole, voriconazole and amphotericin B, these are given intravenously [27]. A combination of these drugs is also used at times. Other than medications, supportive care is also necessary to prevent respiratory failure or sepsis that includes mechanical ventilation, supplemental oxygen, etc. Surgical intervention can also be done in some cases to remove the infected part of the tissue. Prevention of IA involves reducing the exposure to the fungus but avoiding contact with

contaminated food, soil or vegetation. Maintaining good hygiene practice in the surrounding is the key [28].

2. **Acute Bronchopulmonary Aspergillosis (ABPA)**

ABPA caused by the allergic reaction of Aspergillus fungus. It typically affects the people with asthma and cystic fibrosis but some cases also seen in individuals with no lung disease. Inhaled spores trigger the immune system which leads to excessive production of antibodies especially IgE takes place that leads to inflammation and symptoms like coughing, wheezing and chest tightness [29]. Over the time if infection left untreated, severe damage to lungs can occur which can also lead to chronic aspergillus which is a life-threatening condition. Diagnosis involves checking for symptoms, blood tests for elevated levels of IgE and eosinophils and CT scans and MRIs. Treatment of ABPA involves combination of corticosteroids for reducing inflammation and other anti-fungal agents to depress the growth of the fungus, surgery is also done in some cases to remove the damage part of the lung, oxygen therapy is also provided to the patients with extreme conditions to help them breathe easily [30].

3. **Aspergilloma**

Aspergilloma or mycetoma or fungal ball also affects lungs primarily as other infections caused by Aspergillus. It grows like a ball mass within lung and consist of complex network of fungus, mucus and other debris. Symptoms are more likely the same as all other infections caused by Aspergillus, such as coughing, chest pain etc. Diagnosis done usually by symptoms examination and imaging such as chest X-ray or CT scans, which shows the presence of fungal ball in lungs, sputum culturing and biopsy of lung tissue. Treatment involves anti-fungal medication and surgical intervention. This basically depends on the size and location of aspergilloma and over health of the patient. Relapse of aspergilloma is seen in many cases [31]

4. **Chronic Pulmonary Aspergillosis**

Chronic Pulmonary Aspergillosis (CPA) is a persistent fungal infection that mainly impacts the lungs. Individuals with underlying lung conditions like tuberculosis or chronic obstructive pulmonary disease (COPD) are commonly affected by CPA, resulting in gradual and incapacitating respiratory symptoms [32]. The development of CPA begins with the inhalation of *Aspergillus* conidia (spores), which then sprout in the lungs, leading to the formation of a fungal mass (aspergilloma) or invasive aspergillosis, depending on the immune status of the host. Symptoms of CPA caused by *Aspergillus terreus* are usually vague but may include a

chronic cough, hemoptysis (coughing up blood), weight loss, fatigue, and difficulty breathing. If left untreated, the disease can cause significant lung damage and respiratory failure over time. Diagnosis of CPA requires a combination of clinical, radiological, and microbiological assessments. Treating CPA caused by *Aspergillus terreus* can be difficult due to its natural resistance to specific antifungal medications, particularly amphotericin B [33].

2.2.7 Concomitant illness; Cancer and *Aspergillus* infection

The coexistence of cancer and *Aspergillus* infection presents a complex clinical situation, characterized by the interaction between cancer-induced immunosuppression and opportunistic fungal infection. Cancer patients often have a weakened immune system, either as a direct result of the cancer itself or due to the immunosuppressive effects of cancer treatments like chemotherapy. This weakened immune system creates an environment that is favorable for opportunistic infections [34].

The relationship between cancer and *Aspergillus* infection is multifaceted. Firstly, cancer and its treatment can suppress the immune system, particularly affecting natural killer cells that play a crucial role in identifying and eliminating cancer cells and pathogens. Secondly, the presence of a malignancy can causes chronic inflammation and tissue damage, which can facilitate fungal colonization and infection. Additionally, the inflammatory environment characterized by elevated levels of cytokines like interleukin 6 (IL-6) and tumor necrosis factor can further impair immune responses [35]. The coexistence of cavitary lung lesions, a common manifestation of pulmonary aspergillosis, with lung carcinoma can create challenges in diagnosis. Distinguishing between fungal infection and cancerous growths is crucial but difficult, often requiring careful evaluation of radiographic features and histopathological examination.

The management of aspergillosis in cancer patients requires the administration of antifungal medications. Nevertheless, it is essential to take into account the possible interactions with cancer therapies when selecting the treatment. For example, voriconazole, which is a primary choice for treating invasive aspergillosis, has shown effectiveness in cancer patients; however, close monitoring is vital due to potential drug interactions and adverse effects. Having a comprehensive understanding of the immune system suppression and inflammation in cancer patients is critical for devising preventive and therapeutic approaches for fungal infections in this susceptible group [36].

Aspergillus terreus is a subject that has not been extensively researched, yet it is crucial to conduct studies on it due to the high fatality rate associated with its infections. The emergence of antimicrobial resistance towards AmB, Echinocandins and drug toxicity in certain patients further emphasizes the necessity of developing new drugs and identifying novel compounds with antifungal properties. On the other hand, the coexistence of cancer and *Aspergillus* infection presents a complex clinical situation, characterized by the interaction between cancer-induced immunosuppression and opportunistic fungal infection. Distinguishing between fungal infection and cancerous growths is crucial but difficult. However, the task of discovering potential new antifungal or drug candidates is complicated by the structural similarities between fungal and human cells.

2.3 Antifungal drugs

2.3.1 Introduction

Fungal infections are a significant concern in the field of infectious diseases, posing a major threat to public health and impacting millions of people worldwide. These infections can range from mild skin conditions to severe diseases that can severely damage vital organs. Antifungals are a class of drugs used to treat and prevent fungal infections [37]. Among the various antifungal agents available, Amphotericin B, azoles and Echinocandins has emerged as a crucial component in the treatment of serious fungal infections. This potent antifungal compound, discovered in the 1950s, has proven its effectiveness over time and has become an essential tool in combating fungal pathogens. Its unique mechanism of action, wide range of activity, and the challenges associated with its administration and side effects necessitate a comprehensive evaluation to fully understand its clinical significance [38].

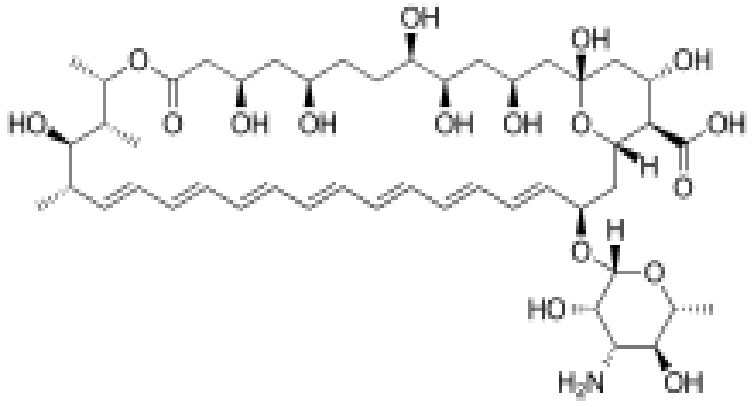


Figure 2.3.1 Structure of Amphotericin B

2.3.2 Mechanism of action

Amphotericin B, azoles, and echinocandins are the main classes of antifungal drugs used in clinical settings. Each of these classes targets specific cellular components that are essential for the survival of fungi.

Amphotericin B works by disrupting the cell membranes of fungi through its binding to ergosterol, a component somewhat similar to cholesterol in human cells. This disruption leads to the leakage of cell contents and ultimately, the death of the fungus [39].

Azoles, on the other hand, inhibit the biosynthesis of ergosterol, which is crucial for maintaining the integrity of the fungal cell membrane. They achieve this by blocking the

enzyme 14α-demethylase. Majorly, triazoles fluconazole and itraconazole were formulated, exhibiting a wider range of antifungal efficacy and enhanced safety characteristics [40].

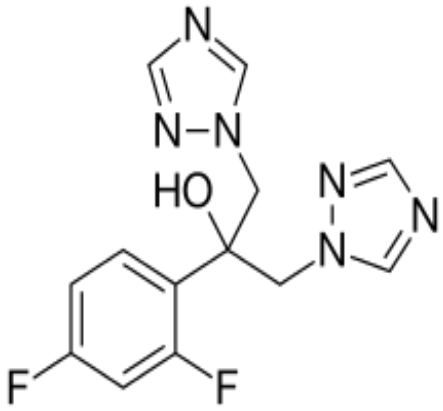


Figure 2.3.2.1 structure of Fluconazole

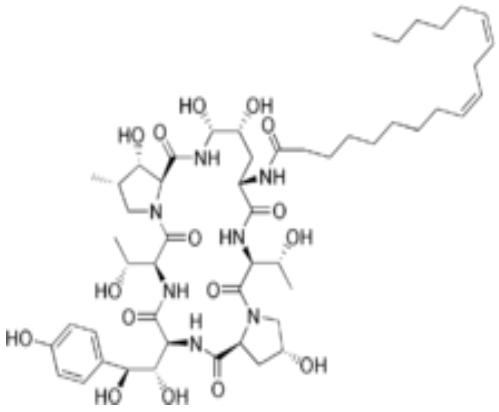


Figure 2.3.2.2 Structure of Echinocandin B

Echinocandins, the third class of antifungals, target the fungal cell wall. They do so by inhibiting the enzyme β-1,3-d-glucan synthase, which is crucial for the formation and maintenance of the cell wall. Echinocandins exhibit exceptional effectiveness against yeasts by targeting the cell wall, a structure that is absent in human cells. This targeted action reduces toxicity and minimizes side effects [41].

2.3.3 Efficacy of antifungals against different fungal species

The effectiveness of these antifungal medications differs among various types of fungi. These antifungal classes possess unique mechanisms of action and ranges of effectiveness, which enhance their effectiveness in treating fungal infections.

AmB demonstrates efficacy against a wide range of fungi, encompassing Candida spp., Cryptococcus spp., and diverse molds like Aspergillus spp., Fusarium spp., and agents of mucormycosis. Its extensive spectrum and enduring post-antifungal impact establish it as a fundamental component in the management of serious fungal infections [39].

Azoles demonstrate significant efficacy against Candida spp. and Aspergillus spp. Nevertheless, the presence of resistance mechanisms, such as mutations in the erg11 gene, overexpression of efflux pumps, and changes in sterol composition, can reduce their effectiveness. Despite these obstacles, azoles continue to be a crucial group of antifungals for treating fungal infections, even those that are unresponsive to other antifungal categories [40].

Echinocandins are highly effective against most Candida species, including strains resistant to fluconazole. Although resistance to echinocandins can develop through mutations in the FKS1 gene, it remains relatively rare compared to resistance mechanisms against other antifungal classes. Their favourable safety profile and efficacy against Candida spp. make echinocandins preferred agents for treating invasive candidiasis [41].

These mechanistic differences play a crucial role in selecting the most suitable antifungal therapy based on the specific fungal infection and the patient's condition.

2.3.4 Resistance against antifungals in India and worldwide

Antimicrobial resistance (AMR) poses a significant threat to global health, especially in low- and middle-income countries like India, where it resulted in approximately 297,000 deaths in 2019 and in global 4.95 million people died and suffered from drug-resistant infections in 2019. The issue is exacerbated by the widespread use of fungicides in agriculture, as many antifungal drugs approved for human medicine are also used in agriculture, thereby compromising the effectiveness of these drugs [42].

The important antifungal classes such as Amphotericin B (AmB), Azoles, and Echinocandins, there resistance are significant, especially for high-risk populations.

From a global perspective, the emergence of multidrug-resistant Candida has brought attention to the issue of antifungal drug resistance. This particular strain exhibits resistance to multiple antifungal classes, including azoles and echinocandins. The concern lies in its potential for rapid spread and the limited treatment options available. One medical centre noted a 14% resistance rate among bloodstream isolates, which poses a challenge as echinocandins are often the first line of therapy for invasive candidiasis. Azole resistance, especially in *Aspergillus* species, has also been observed globally. This has an impact on the management of invasive aspergillosis, considering the widespread use of azoles for prophylaxis and treatment [43].

In India, there has been a significant increase in antifungal resistance, specifically in cases of dermatophytosis caused by *Trichophyton indotineae*. This particular strain, has shown resistance to terbinafine. The resistance is attributed to mutations in the squalene epoxidase (SQLE) gene [42].

2.3.5 Side effects and precautions

Amphotericin B formulations, such as AMB-d and L-AMB, have been linked to significant side effects such as hypotension, chills, headache, and renal insufficiency, with L-AMB demonstrating a slightly lower occurrence of renal function abnormalities [44]. Azoles are generally well-tolerated but have the potential to cause hepatotoxicity and, depending on the specific medication, may result in severe liver damage or skin reactions. Echinocandins may lead to hepatotoxicity and other less common side effects like hypotension and peripheral edema.

It is crucial to strictly adhere to the prescribed dosages and treatment duration when using antifungals to ensure safety. Patients must be informed about the significance of following their treatment plan to avoid resistance development and minimize side effects. Healthcare professionals need to take into account individual patient factors, like existing health conditions and possible drug interactions, when recommending antifungal treatments. Regular monitoring for adverse effects and making adjustments to therapy based on patient response and side effect profiles are essential [45].

Due to their toxicity and developed resistance towards drugs an alternative therapy needs to be investigated to beat this infection. There are various drug targets that are known and various new that are recognized. Additionally, some studies suggest that heat shock protein 90 could also serve as a viable target for combating these infections. Heat shock proteins are the proteins family that are present in all types of living cells and plays role in proper protein folding.

2.4 Heat shock protein

Heat shock proteins were first identified by Italian scientist Ferruccio Ritossa in the early 1960s in *Drosophila melanogaster* as a consequence of increased synthesis after the heat shock in house fly [46]. These proteins become a significant area of research because they are ubiquitous, present in all living organisms, from bacteria to human cells. They act as chaperones, helping in the folding and refolding of client proteins to maintain their functional conformation [47]. Additionally, they play a role in protein trafficking, immune system regulation, and protects cells from damage caused by stress. Hsp are also associated with repairing damaged proteins and facilitating the transport of proteins within the cytosol, endoplasmic reticulum, and mitochondria [48]. Their expression levels increase not only in response to heat shock but also in reaction to other environmental stressors such as Inflammation, Hypoxia, Infection, and Nutrient Deprivation [49].

Heat-shock proteins are categorized according to the molecular mass of their monomer, which can range from 10 to over 100 kDa, as well as their structure and function. The main families of heat-shock proteins include Hsp100, 90, 70, 60, and small Hsp (sHsp) [50,48]. Our specific interest lies in Hsp90. Hsp90 is a subgroup of heat-shock proteins with a molecular mass of 90 kDa. Its expression level is typically around 1-2% of total proteins in normal circumstances, but this expression can increase significantly, by 2-10-fold, in cancer cells or under stressful conditions [51].

Fungi generally need warm and humid conditions for expansion. A drop in temperature makes fungal latent because spores are cold-resistant, while an increase in temperature leads to destruction of fungi. Therefore, temperature begins stress responses in fungi, which can be either heat-shock or cold-shock affecting the life cycle and cellular processes [11]. The Hsps are involved in the morphogenesis of fungi and play a major role in the replication, transcription, translation and the activation of signalling pathways [52].

In *Aspergillus terreus* Hsp90 and Hsp70 is well studied and involved in folding, morphogenesis, activation of variety of other proteins and biosynthesis of secondary metabolites. Understanding role of Hsps in *Aspergillus terreus* or any other *Aspergillus* species is necessary for the development of novel strategies to control the fungal infection and production of valuable secondary metabolites on the other hand understanding the role of Hsps in cancer cells.

2.4.1 Hsp90 and Hsp70

Hsp90 and Hsp70 play crucial roles in cellular processes, such as protein folding, maturation, and trafficking. The HSP90 family encompasses four essential members: Hsp90AA1 and Hsp90AB1 found in the cytosol, GRP94/Hspp90B1 located in the Endoplasmic Reticulum, and TRAP1 situated in mitochondria. Similarly, the Hsp70 family consists of 13 members, including cytosolic proteins Hsp70/HspA1A, HSC70/HspA8, mitochondrial mortalin/GRP75, and ER member HSPA5/GRP78, also referred to as BiP [53].

2.4.2 Structure of Hsp90

Heat shock protein 90 (Hsp90) is a widely distributed molecular chaperone present in every eukaryotic cell. It plays a crucial role in various cellular processes, including protein folding, stabilization, and activation. Hsp90 client proteins fulfil essential roles in Signalling Cascades, including Cell Cycle, Cell Proliferation, Differentiation, and Apoptosis. These proteins are indirectly participated in several cellular processes and found in distinct cellular compartments. In addition to Hsp90's function as a molecular chaperone, it serves an essential role in overall cellular homeostasis [54].

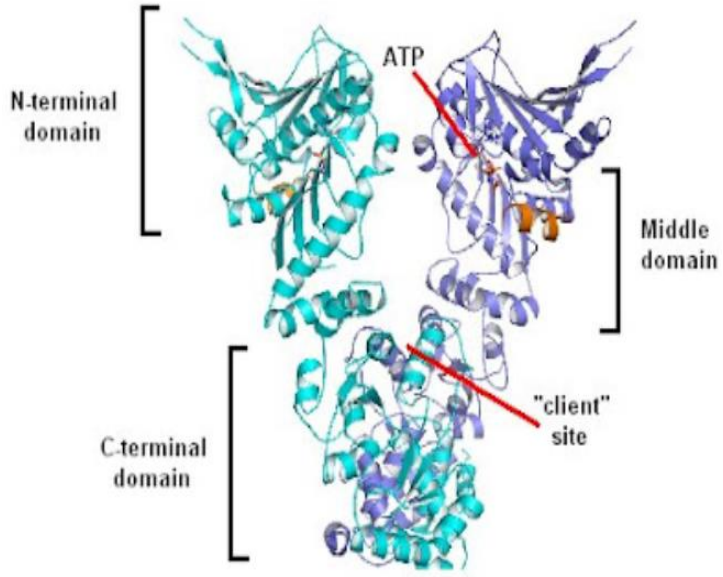


Figure 2.4.2: - Structure of Hsp90

Heat shock proteins: a therapeutic target worth to consider

The mammalian Hsp90 family of proteins is conserved and exists in cells in the form of a homodimer, each monomer includes three domains, there are:

1. **N-terminal nucleotide-binding domain (NTD)**: This domain contains a conserved ATP binding pocket, thus called the nucleotide binding site. ATP binding and hydrolysis are essential for HSP90's chaperone activity, driving conformational changes in the protein.
2. **Middle domain (MD)**: This domain is responsible for substrate binding and is composed of a beta-sheet sandwich structure. It contains several hydrophobic pockets that interact with specific client proteins. The NTD and MD are connected to each other through a charged linker region.
3. **C-terminal domain (CTD)**: This domain is involved in dimerization and co-chaperone binding. It is composed of a helical structure with a flexible linker connecting it to the MD. CTD adenine nucleotide-binding pocket activates only when the N-terminal site is occupied [55,60].

2.4.3 Chaperone Mechanisms and Cellular Influence

Hsp70 usually initiates the protein folding process, while Hsp90 intervenes at a later stage to guarantee correct protein maturation. The functions of these chaperones are controlled by post-translational modifications like acetylation, phosphorylation, and ubiquitination, which adjust the functional cycles of these proteins. Working together, Hsp70 and Hsp90 not only assist in protein restructuring but also have a significant impact on maintaining proteome stability. Natural compound showed a potential in inhibiting the various cell component and including stress proteins paving way to a choice of drugs [56].

2.4.4 Interaction and Regulation

The molecular chaperones work together in a synchronized way, often needing the help of co-chaperones such as Hsp40 and different nucleotide exchange factors that control the substrate binding and release process. This process is crucial for the chaperone function of Hsp70 proteins, driven by ATP binding and hydrolysis. In the case of Hsp90, the stability of client proteins is preserved through a series of ATP binding, resulting in structural changes and the activation of client proteins [57].

2.4.5 Role of Hsp90 and Hsp70 in Fungal Stress Tolerance

The critical functions of Hsp90 and Hsp70: Both proteins play vital roles in regulating stress responses within fungal cells. Hsp90 acts as a safeguard against cellular and molecular damage caused by heat stress by controlling the levels of reactive oxygen species (ROS). Hsp70 aids in enhancing temperature resistance in yeast by accumulating trehalose [58].

Expression patterns during stressful conditions: These proteins exhibit distinct expression patterns in response to various stress conditions. For example, Hsp60, Hsp90, Hsp104, Hsp30, and Hsp10 are activated during heat stress, while Hsp12 is essential for adapting to cold stress. Additionally, Hsp30, Hsp70, and Hsp90 are responsive to changes in pH, and small Hsps, along with Hsp60, play a role in managing osmotic stress [59].

2.4.6 Hsp90 and Hsp70 in Fungal Cell Morphogenesis

Hsp90 and Hsp70 are crucial in morphological changes, specifically in morphogenesis and dimorphism, which are essential for the fungal lifecycle. HSP90, in particular, has been recognized as a promising target for antifungal treatments because of its involvement in these mechanisms.

Hsp90 as an Antifungal Target: - Hsp90 has emerged as a promising target for antifungal treatments, with compounds like Geldanamycin and its derivatives showing antifungal activity [51]. These compounds have displayed synergistic effects with caspofungin against pathogens such as Aspergillus fumigatus and Candida albicans.

Hsp70's Role in Enhancing Antifungal Efficacy: - On the other hand, Hsp70 plays a crucial role in enhancing the efficacy of antifungal drugs by modulating the effects of caspofungin through its interaction with Hsp90-Hop/Sti1 co-chaperones in Aspergillus fumigatus, underscoring its potential in improving antifungal treatment outcomes [60].

Aspergillus terreus is an understudied organism that warrants further investigation. Dysfunction of Hsps can result in the accumulation of misfolded and damaged proteins, leading to cell death or the development of various diseases, including cancer in human or animal cells. Consequently, inhibiting these proteins can enhance the susceptibility of *Aspergillus* species to stress and drugs, thereby reducing pathogenesis. Numerous studies have indicated that inhibitors of Hsp70 and Hsp90 exhibit anti-fungal activity against *Aspergillus* species, although the underlying mechanism remains poorly understood. Additionally, research has explored the use of phytochemicals and natural compounds like curcumin, resveratrol and Geldanamycin,

which have shown the ability to inhibit Hsp activity. These compounds hold potential as anti-fungal agents for treating *Aspergillus* infections. However, in-depth research is necessary to fully comprehend the mechanism of action of Hsp inhibitors and optimize their application in infection treatment.

2.5 Geldanamycin

2.5.1 Introduction

Geldanamycin (GA) was isolated from the broth of *Streptomyces hygrocopicus* in 1970 as tyrosine kinase inhibitor [61]. Initially, it was identified as an antibiotic with antifungal properties. Now a days, it is known as inhibitor of hsp90. GA binds to Hsp90 and disrupts its normal function [1]. GA also become a compound of interest in cancer research due to its unique mechanism of action against cancer cells, as in case of cancer cells the expression of Hsp90 also increases 2-10-fold and GA effectively inhibits the function Hsp90 protein [62]. GA clinical advancement has been hindered by its poor physicochemical characteristics and undesirable side effects, including elevated levels of hepatotoxicity and ocular toxicity [63].

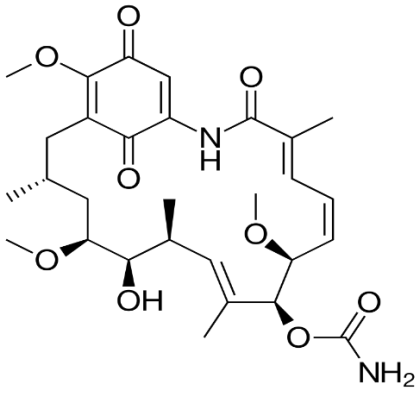


Figure 2.5.1: - Structure of Geldanamycin

2.5.2 Antifungal properties of GA

Initially, GA was first identified as an antibiotic with antifungal activity. GA as a benzoquinone ansamycins, it consists of a quinone ring with alternatively double bonds and carbonyl groups, participated in redox reactions, reactive quinone produces superoxide radicals causing cell death and a hydrophobic ansa bridge of aliphatic chain connects to aromatic core, which plays a role in its antifungal properties [64].

2.5.3 Anticancer properties of GA

The anticancer activity of GA is due to its ability to block the Hsp90 molecular chaperone function. Hsp90 is expressed at high levels in different types of human cancers and play a important role in controlling the stability and activity of oncogene proteins, known as “client proteins”. GA interacts to Hsp90 N-terminal ATP binding site [65] leading to the dissociation of mature Hsp90 complex and an increase of their turnover rate and breakdown of Hsp90

proteins via ubiquitin-dependent proteosomes through E3 ligase. Therefore, GA has been utilized as a dominant negative regulator of Hsp90 activities [66].

2.5.4 Mechanism of Action of Geldanamycin

1. **Binding to HSP90**: Geldanamycin binds to the N-terminal ATPase domain of Hsp90. This site is crucial for Hsp90 act as a molecular chaperone. This binding site overlaps with the natural ATP binding pocket, effectively preventing ATP hydrolysis.
2. **Inhibition of ATPase activity**: ATP hydrolysis is essential for the chaperone activity of Hsp90. By inhibiting ATPase activity, geldanamycin prevents Hsp90 from completing its chaperone cycle, leaving its client proteins in an unstable and unfolded state.

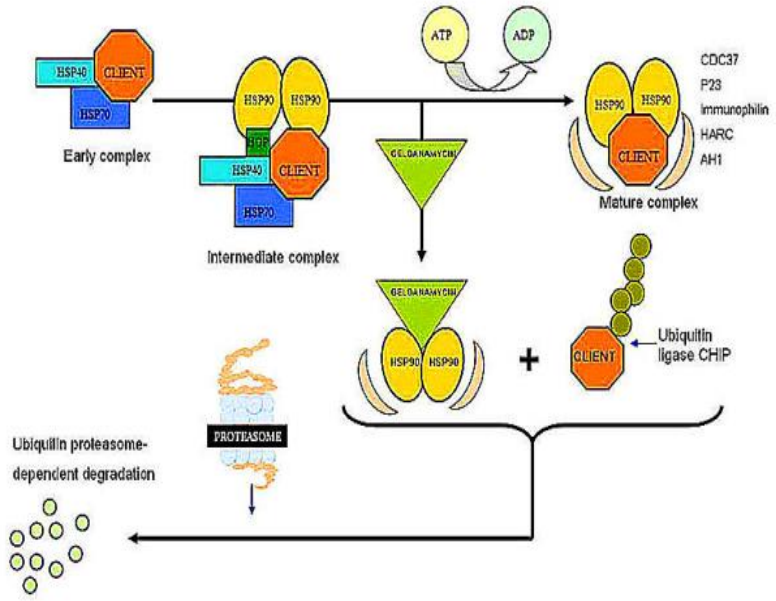


Figure 3: - Mechanism of action of GA

Geldanamycin and its derivatives as Hsp90 inhibitors

3. **Disruption of protein folding and stability**: Unstable client proteins are unable to fold properly and are thus targeted for degradation by the proteasome. This degradation leads to a reduction of oncogenic proteins and ultimately cell death.
4. **Inhibition of signalling pathways**: Many oncogenic proteins are involved in crucial signalling pathways that promote cell growth and survival. By disrupting the function

of these proteins, geldanamycin can effectively inhibit these signalling pathways and suppress the growth of fungal cell and tumor growth [67].

2.5.5 Efficacy of Geldanamycin as an Hsp90 Inhibitor in Cancer Treatment

The effectiveness of Geldanamycin as an Hsp90 inhibitor in treating cancer is backed by a growing body of preclinical and clinical data. Research indicates that Geldanamycin can hinder the growth of various types of cancer, including breast, lung, prostate, and melanoma. Its capacity to target multiple oncogenic pathways simultaneously is a notable advantage, potentially addressing the limitations of treatments that focus on a single molecular pathway. Preclinical studies have shown that Geldanamycin prompts the breakdown of several oncogenic proteins, resulting in the suppression of cancer cell proliferation, initiation of apoptosis, and inhibition of angiogenesis and metastasis. These results underscore the compound's ability to address different aspects of cancer progression, presenting a comprehensive approach to cancer treatment [68].

2.5.6 Side Effects and Limitations of Geldanamycin

Despite the impressive antifungal and anticancer properties that Geldanamycin possesses, its clinical application has faced obstacles due to various side effects and limitations. One major concern is its hepatotoxicity, which has been observed in animal models and early clinical trials. This toxicity restricts the maximum tolerable dose of Geldanamycin, potentially diminishing its effectiveness in cancer treatment. Furthermore, the poor solubility and stability of Geldanamycin in aqueous solutions present significant challenges in formulating and delivering the drug. These issues complicate the administration of the medication and may impact its bioavailability and therapeutic effectiveness. Moreover, the inhibition of Hsp90 by Geldanamycin affects a broad range of client proteins, which can result in unintended off-target effects. While the specificity of Geldanamycin for Hsp90 is advantageous, the essential role of Hsp90 in normal cellular functions means that inhibiting it can also disrupt non-cancerous cells, leading to adverse effects [69].

In this study, we examined whether GA-induced disruption of the HSP90 heterocomplex, could affect the expression of cellular proteins, and ultimately lead to the death of fungal cells or cancer cells.

2.6 Objectives

The current work on "**Exploration of Heat shock protein Geldanamycin against *Aspergillus terreus***" with and without inhibitor was performed with following objectives:

1. To calculate the MIC50 value of Geldanamycin on *Aspergillus terreus.*
2. To assess the transcripts of Hsp70 and Hsp90 genes at 24 hours during the treatment of *Aspergillus terreus* with and without inhibitor.

CHAPTER 3

MATERIAL AND METHODS

3.1 Materials

3.1.1 Chemicals

S.no	Chemical	Manufacturer
1	Potato Dextrose Agar (PDA)	HIMEDIA
2	Potato Dextrose Broth (PDB)	HIMEDIA
3	RPMI 1640 medium	HIMEDIA
4	FBS	HIMEDIA
5	Sodium chloride	Vetec
6	Potassium chloride	HIMEDIA
7	Sodium phosphate dibasic	HIMEDIA
8	Potassium phosphate monobasic	HIMEDIA
9	Tween 20	Merck
10	Lactophenol cotton blue	HIMEDIA
11	Trizol Reagent	Takara
12	Chloroform	HIMEDIA
13	Isopropyl alcohol	Loba Chemie
14	Ethanol	HIMEDIA
15	Nuclease free water	Affymetrix
16	Agarose (1.2%)	HIMEDIA
17	Tris	HIMEDIA
18	Glacial acetic acid	Merck
19	EDTA (0.5M)	Sigma-Aldrich
20	EtBr	HIMEDIA
21	6X DNA loading dye	Thermo Scientific
22	DNA ladder	Thermo Scientific
23	Geldanamycin	Cayman
24	DMSO (dimethyl sulphoxide)	Qualigens
25	Water purification system	Elix
26	DCFDA (2, 7-dichlorofluorescein diacetate).	LOBA CHEMIE

3.1.2 Instrumentation

S.no	Instrument	Manufacturer
1	Laminar air flow	Microsil India
2	Incubator with shaker	Labnet
3	Weighing balance	Denver
4	Centrifuge	HITACHI, Eppendorf
5	Autoclave	SANYO
6	Agarose gel electrophoresis setup	Bio-RAD
7	Gel doc. System	Bio-RAD
8	Freezer (4℃, -20℃, -80℃)	Celfrost/Vestfrost solutions
9	Spectrophotometer	Thermo Fisher scientific
10	Bright field Microscope	Olympus
11	Fluorescence Microscope	Olympus
11	Haemocytometer	Rohem India
12	PCR	Applied Biosystem
13	Rt-PCR	Bio-RAD
14	Ice machine	Manitowac
15	Vortex	REMI

3.1.3 Equipment

S.no	Equipment	Manufacturer
1	Micropipettes	Eppendorf/Thermo Scientific
2	Microtips	Tarson
3	Conical flasks	JSGW
4	Microcentrifuge tubes (1.5ml and 2ml)	Eppendorf
5	Falcon tubes (50ml, 15ml)	Tarson
6	Petri plates	Genaxy
7	Test tubes	JSGW
7	Reagent bottles (1 litre and 100 ml)	JSGW
8	Mortar and pestle	JSGW
9	Spatula	JSGW
10	Ice bucket	Cello
11	Surgical blade	JSGW
12	Forceps	JSGW

3.1.4 Primers

Primers	Sequence
40s Ribosomal Protein	F- 5' CATTGGCCGTGAGATCGAG3' R- 5' CCCTTGTCATCGGTGGTAGA3'
HSP 70	F- 5'GACCACGGAAATCGAGCAGA3' R- 5'CATGGTGGGGTCGGAAATGA3'
HSP 90	F- 5'CTCGCCAAGAGCCTCAAGAA3' R- 5'GCTCCTTGATGATGGGGGAC3'

3.1.5 Biological material

Aspergillus terreus nccpf strain 860035

3.2 Methodology

3.2.1 Revival of *Aspergillus terreus* culture

Revival of the *Aspergillus terreus* nccpf (national culture collection of pathogenic fungus) strain 860035 was done by placing a small piece of fungus in the centre of the petri plates containing PDA media from master plate with the help of autoclaved scalpel and surgical blade inside LAFH. After inoculation the plates were sealed using parafilm and placed in incubator at 37℃ (this optimised temperature suggested by nccpf catalogue). Full growth can be seen within 4-5 days.

3.2.2 Harvesting of spores

The fresh fungal culture plates were treated with 10ml of PBST (0.05% Tween 20) and incubated for 2-3 minutes. Subsequently, the surface was slightly rubbed with the help of tip of a 1ml pipette, and the resulting suspension was collected and transferred to 15 ml falcon tubes. Centrifugated at 5000rpm for 10 minutes which separates the cell pellet from the PBST, discarded the supernatant. Added 10ml of PBST again to the cells and centrifugated, discarded the supernatant. The cell pellet was then resuspended in PBS.

3.2.3 Cell count

Took 100µl of cell suspension from the harvested spores added to a microcentrifuge tube along with 100µl of lactophenol cotton blue dye making the total volume of cell suspension 200 µl which have 2 dilution factors, then thoroughly mixed using a pipette and the mixture allowed to set for few minutes. Cell counting was done using haemocytometer, which is a chamber device designed for counting cells, in which 10µl of cell suspension was loaded and viewed under microscope.

3.2.4 Minimal Inhibitory Concentrations (MICs)

It is defined as the lowest concentration of the drug that inhibits the growth of the fungal cells (*Aspergillus terreus*). MIC of *Aspergillus terreus* is detected by MTT assay [70].

The MTT assay is a colorimetric assay commonly used to assess cell metabolic activity, which can be an indirect indicator of cell viability and proliferation.

Principle: The assay relies on the ability of NAD(P)H-dependent cellular oxidoreductases, enzymes present within living cells, to reduce a yellow tetrazolium dye called MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to a purple formazan product.

Only metabolically active fungal cells with functioning mitochondria and these enzymes can convert MTT. Dead cells or those with compromised metabolism lack the necessary machinery and cannot participate in the reaction.

The amount of formazan produced is proportional to the number of viable metabolically active cells. As the amount of drug is increased there is more no. of dead cells and the amount of formazan production is less.

Drug - Geldanamycin

Media - RPMI + FBS

Spores - *Aspergills terreus*

Procedure for MIC of Geldanamycin

1. Prepared the different concentrations the drug Geldanamycin 1,2,4,8,12 and 16µl/ml in DMSO (dimethyl sulfoxide) from the stock solution of 1mg/ml.
2. Added components in the 96 well plate with the inoculation of $1*10^4$ spores [71] making the final volume 200 µl by adding the RPMI media, along with the control which contain only media and spores.
3. Incubated at 37ºC for 24 hours.
4. Added 10µl of MTT in each well.
5. Incubated at 37ºC for 3 hours.
6. Measured absorbance at 570nm.

Then mycelial growth inhibition calculates was analysed using the following formula.

$$\text{Inhibition of mycelial growth (\%)} = \frac{\text{Mycelial growth (control)} - \text{mycelial growth (treatment)}}{\text{Mycelial growth (control)}} \times 100$$

3.2.5 Antifungal susceptibility testing

3.2.5.1 Disc diffusion assay

The disc diffusion assay, also known as the Kirby-Bauer disk diffusion susceptibility test, is popular technique used in microbiology to assess the susceptibility of bacteria to various antimicrobial agents. To determine the susceptibility or resistance of bacteria or fungus to different antibiotics or drugs. PDA plates was formed. Streaked the 10µl spores of *Aspergillus terreus* onto the surface of the Potato dextrose agar plate with the help of spreader to create a confluent lawn. 5mm disc containing the 11µg/ml of GA was inoculated in the middle of the surface of the agar plate with the help of sterile forceps. Incubated the plate at 37ºC. After incubation, examine the plate for inhibition.

2.2.5.2 Agar Dilution Assay

The agar dilution method is another standard technique used in microbiology to determine the Minimum inhibitory concentration (MIC) of an antimicrobial agent (antibiotic, antifungal etc.) against a specific microorganism. In which the drug solution was added in the media after autoclaving the media. Poured the media containing drug into the plate, spread 10µg/ml of spores into the plate with the help of spreader. Incubated at 37ºC and examine the plate for inhibition.

2.2.5.3 The food poisoning technique

It is a simple and effective method to assess the antifungal activity of various drugs against fungal pathogens. In which the drug solution was added in the media. With the help of sterile cork borer, agar plugs of fresh and actively growing fungal mycelium from the original fungal culture was obtained and placed one plug in the centre of the PDA plate containing drug. Incubate all plates at 37ºC. After incubation examine the plate for inhibition.

3.2.6 Cellular ROS estimation

The effect of GA on intracellular ROS levels in *Aspergillus terreus* was evaluated using ROS assay. Intracellular ROS activity refers to the production of reactive oxygen species (ROS) within a cell. ROS are molecules that contain oxygen and are highly reactive due to the presence of unpaired electrons. The assay involved the use of the florescent dye DCFDA (2,7-dichlorofluorescein diacetate). DCFDA is a common dye used to estimate Reactive Oxygen Species (ROS) levels within cells. A cell suspension of *Aspergillus terreus* $1*10^6$ cells/ml was inoculated in both control and GA treated (MIC50 11µg/ml) samples. The samples were then incubated at 37ºC for 24 hours in RPMI + 10% FBS medium with constant shaking. After incubation, the cells were collected by centrifugation, suspended in PBS, and washed twice in PBS. They were treated with DCFDA (5µg/ml) and incubated at 37ºC for 45 minutes with constant shaking. The fluorescence intensity of the cell suspensions (100µl) was observed under a fluorescence microscope using filters with excitation at 485 nm and emission at 520 nm, under similar conditions. The fluorescence intensities were analysed using software.

3.2.7 Growth of culture in broth

3.2.7.1 For control cultures

After the four days of growth of *Aspergillus terreus* on plates, spores were harvested and counted. Inoculated $1*10^6$ spores in 45ml RPMI-1640 medium + 5ml FBS which making total 50ml media under the LAFH. After inoculation flask covered with cotton plug and aluminium foil and placed in incubator shaker at 37℃ at 100rpm shaking for 24 hours.

3.2.7.2 For treated cultures

For treated culture $1*10^6$ spores of *Aspergillus terreus* in combination with 550µl of drug Geldanamycin (MIC value= 11µg/ml of Geldanamycin) added to 50ml media containing 45ml RPMI-1640 media and 5ml FBS. Once the media is prepared covered the flask with cotton plug and aluminium foil and placed in an incubator shaker at 37℃ at 100rpm shaking for 24 hours.

3.2.8 RNA extraction

RNA extraction was done by using TRIZOL-CHOLOROFORM method at time point of 24hour for both treated and control samples.

RNA extraction process is as follows: -

1. After the growth of 24 hour the cell suspension in the media was transferred to the 50ml tarsons and centrifuged for 6000 rpm for 10 minutes.
2. Discarded the supernatant which was just the media by avoiding the cell loss.
3. 1ml of Trizol reagent is added to the cell pellet, mixed and transferred to the chilled mortar and pestle.
4. Liquid nitrogen is added to the cells to homogenized cells until forms powder
5. 2-3ml of Trizol reagent is added to the mortar, mixed using pestle.
6. The homogenised sample was incubated at room temperature for 5 min, it will convert in liquid form used.
7. 0.2 ml of chloroform was added per 1 ml of Trizol reagent.
8. The tubes were shaken strongly by hands for 15 seconds and then incubated for 2-3 min at room temperature.
9. The samples were centrifuged at 12000 ×g for 15 min at 2- 8℃.
10. After centrifugation mixture separates into lower red organic phase, interphase and upper colourless aqueous phase, RNA resides in the upper aqueous phase.
11. Aqueous phase is carefully transferred into fresh tube.
12. 0.5 ml of isopropyl alcohol was added to the freshly transferred sample per 1 ml of Trizol reagent. Then sample was incubated for 45 min at -20℃.
13. Sample was centrifuged at 12000 rpm at 2-8℃ for 10 min.
14. The RNA gets precipitated and forms a gel like pellet at the bottom.
15. The supernatant was removed and pellet was washed with 75% ethanol. 1 ml of 75% ethanol was added per 1ml of Trizol reagent.
16. Sample were mixed using vortex and centrifuged at 7500 ×g at 2-8℃ for 5 min.
17. Remove the supernatant and left the pellet air dry.
18. Stored in 25 µl of nuclease-free water.

3.2.9 Agarose gel electrophoresis

Once the RNA is extracted gel electrophoresis was run to check its quality and yield. To achieve this 450 ml of 1X TAE buffer working solution was prepared in distilled water from 50X TAE buffer stock. For 0.6% agarose, 0.6 grams of agarose was weighed and dissolved in 50 ml of 1X TAE buffer, heated till become transparent, followed by the addition of 1µl of EtBr when the flask of gel is bearable to hold. Gel was carefully poured in the casting tray with a comb in and allowed to solidify for 15 minutes. Once the gel is solidified, it was put in the buffer chamber and the remaining 400ml of the buffer poured in, carefully remove the comb. Loading of samples involved placing 6X DNA ladder in the first well and RNA samples in the remaining wells. The Setup was run initially at 50V for 10 minutes when the samples was run out from the wells then at 100V for 45 min followed by visualisation of the RNA bands in gel doc. System.

Different RNA was pulled which are as follows-

¬ 24-hour control

¬ 24 hours treated with Geldanamycin

Sample preparation

DNA ladder preparation- 3µl ladder

RNA sample preparation- 2µl loading dye and 5µl RNA sample

450 ml of 1X TAE buffer preparation from stock

For preparation of 450ml of 1X working from 50X stock of TAE buffer, 9ml of the stock was added to 441ml of distil water.

Table 1: - 1 litre of 50X TAE buffer preparation (stock)

Components	**Quantity**
Tris	242g
Glacial Acetic acid	57.1ml
0.5 M EDTA	100ml
Distilled water	Up to 1L

3.2.10 RNA Quantification using Spectrophotometer

RNA quantification, also known as nucleic acid quantification. It determines the concentration (amount) of RNA present in a sample.

Following the visualization of the bands, a spectrophotometer was utilized to quantify the RNA. The spectrophotometer analysis yields the mean concentrations of nucleic acids (DNA or RNA) found in a mixture, while also determining their purity. This is possible because nucleic acids exhibit a distinct absorption of UV light, with DNA and RNA specifically absorbing light at 260nm. The analysis relies on the unique pattern of UV light absorption by nucleic acids at 260 nm while absorbance at 280nm is used to estimate the amount of Protein in the sample [72].

Beer-Lambert law establishes a linear relationship between absorbance and concentration the, therefore can be used to relate the amount of light absorbed to the concentration of the molecule.

Before analysis the RNA sample was diluted as, in a cleaned quartz cuvette, 5µl of RNA sample added to 995µl of distilled water and the optical density was determined at 260nm and 280 nm using a spectrophotometer.

Once the OD was known, 260/280 ratio was calculated. Ratio

- approximately or near to 1.8 implies the purity for DNA
- near to 2.0 implies purity for RNA
- lower than or equal to 1.6 that may idicates presence of protein contamination.

The formula to calculate the amount of RNA is as follows-

40 µg/ml * Abs at 260nm * Dilution Factor

3.2.11 cDNA synthesis

cDNA, also known as copy DNA or complementary DNA is synthetic DNA molecule that is transcribed from mRNA. Unlike normal DNA, cDNA does not contain the non-coding regions. To generate cDNA, an enzyme called reverse transcriptase is used. cDNA was synthesised at 24-hour temporal points of treated as well as control samples using Thermo scientific cDNA synthesis kit. Based on the amount of RNA obtained, cDNA is synthesised. The reaction mixture was prepared in PCR tubes.

Table 2: - Reaction mixture concentration of different components

Final volume of the reaction mixture was kept 20µl.

Components	Volume (in µl)
5x cDNA Synthesis buffer	4
dNTPs	2
Oligo dt	1
Random Hexamers	1
RT enhancer	1
Enzyme mix	1
Template RNA	1-5
Nuclease free water	Up to 20

Volume of template RNA used depends on the amount of RNA (in ng) present per microliter and based on that volume of template RNA was added to the reaction mixture as cDNA is synthesised using 1 microlitre of RNA.

Table 3: Cyclic program for cDNA synthesis

Steps	Temperature	Time	No. of cycles
cDNA synthesis	42ºC	30	1
Inactivation	95 ºC	2	1

3.2.12 Real-Time PCR (RT PCR)

RT PCR is commonly used to measure the fold of gene expression. In this study, we analysed the expression of HSP70 and HSP 90 genes of *A. terreus* at time points of 24hr control and treated samples. To ensure accuracy, a 40s ribosomal protein was used as a positive control, as it is a housekeeping gene and is expressed all the time in the cell.

Autoclaved distilled water was used as non-template control or NTC. The reaction mixture was prepared in PCR vials with a total volume of 20µl. For each sample, 40 cycles ran which took approximately 1 hour 32 minutes. The reaction mixture was prepared in total of 15 PCR vials containing, 3 NTCs, 4 positive controls, and 8 samples.

Table 4: Reaction mixture concentration of different components

For 20µl of total volume.

Components	Volume (in µl)
Syber Green	10
cDNA template	1
Primers	1 each forward and reverse
Nuclease free water	7

Primers working solution was prepared from the stock primer by adding 10µl of stock primer to 90µl of the nuclease-free water in an autoclaves micro centrifuged tube.

Table 5: Cyclic program for qRT PCR

Steps	Temperature	Duration
Initial denaturation	95 ℃	3 minutes
40 cycles of		
Denaturation	95 ℃	10 seconds
Annealing	The annealing temperature for every primer is different	45 seconds
Extension	72 ℃	30 seconds

Table 6: Annealing temperature of the selected primers

Primers	Annealing temperature (in ℃)
HSP 70	65.1
HSP 90	65.1
40s Ribosomal Protein	64

CHAPTER 4

Results

4.1 *Aspergillus terreus* culture revival

The culture of *Aspergillus terreus* was revived from the master culture by plating onto the PDA plates. Incubation was done at 37℃ for 4-5days.

Figure 4.1: - *Aspergillus terreus* culture on PDA on day 5th

4.2 Harvesting of Spores

The fresh fungal culture plates were treated with 10ml of PBST (0.05% Tween 20) and incubated for 2-3 minutes, slightly rubbed using the tip of 1ml pipette and the suspension was transferred to the 15 ml falcon tubes. Centrifugation at 5000rpm for 10 minutes was done which left us with cell pellet and PBST in supernatant. Cell pellet was suspended in PBS.

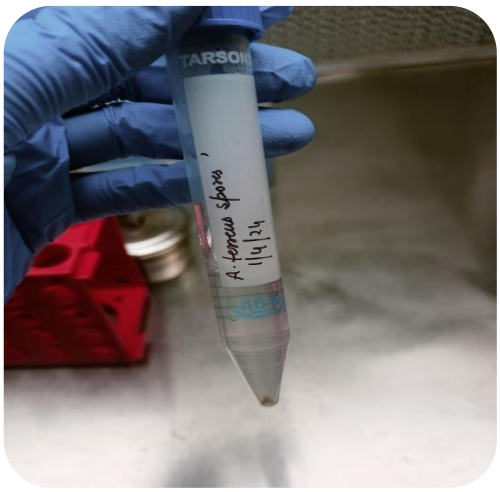


Figure 4.2: - Harvesting of spores

4.3 Cell counting

After the growth, fungal spores were counted using haemocytometer device.

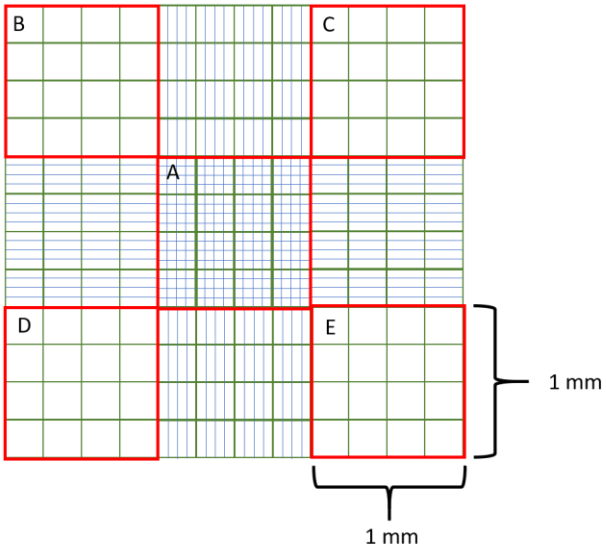


Figure 4.3.1: - Haemocytometer

https://bitesizebio.com/13687/cell-counting-with-a-hemocytometer-easy-as-1-2-3/

Calculations for Cell Counting:

Cells at the top and right edge were considered and cells at bottom and left edge were ignored.

Four boxes at the corners were considered for counting and middle one was ignored.

Total volume of cell suspension- 200µl (100 µl cell suspension + 100µl lactophenol cotton blue dye).

Loaded 10 µl on haemocytometer

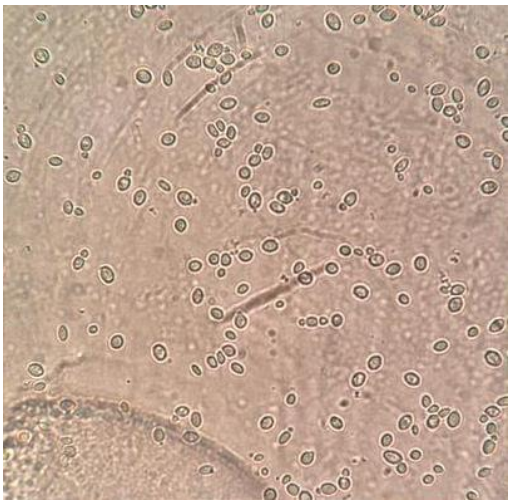


Figure 4.3.2 Spores under 40x

Counting of cells by formula

Concentration of cells/ml = Average no. of cells per square* Dilution factor* 10^4

Concentration of cells/ml = 278/4 * 2 * 10^4

= 139 * 10^4 cells

= 1.39 * 10^6 cells/ml

Once the concentration of cells known, *Aspergillus terreus* was grown in 50 ml of media containing 45 ml RPMI 1640 and 5 ml of FBS at 24 hr time point in normal conditions and in presence of geldanamycin.

4.4 Minimal Inhibitory Concentrations (MIC)

MIC is calculated by MTT assay.

$$\text{Minimal Inhibition Concentration} = \frac{C_t - OD * 100}{C_t}$$

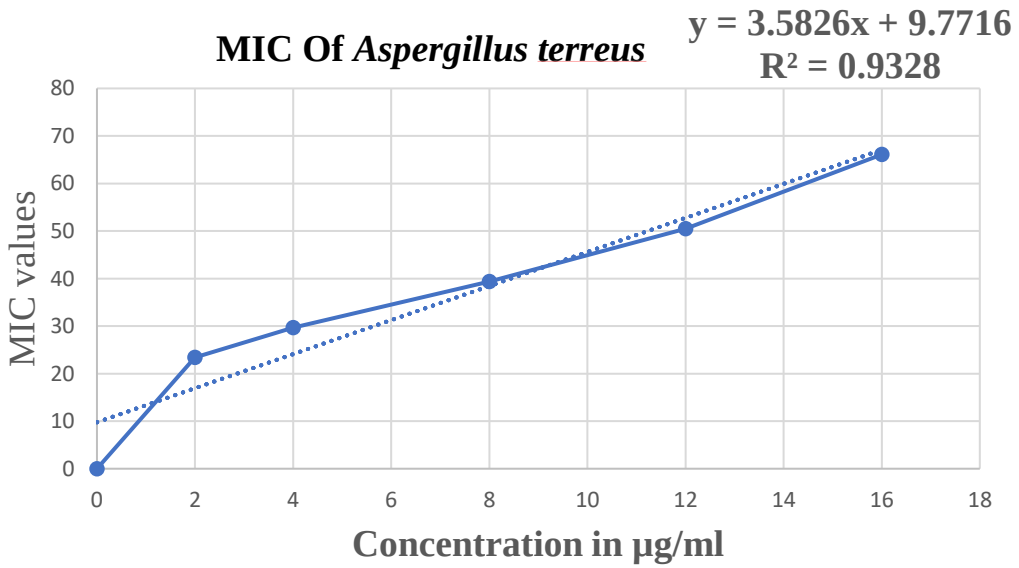


Figure 4.4 Graph showing the MIC value of Geldanamycin

MIC_{50} by MTT assay

$$y = 3.5826x + 9.7716$$

MIC_{50}

$$50 = 3.5826x + 9.7716$$

$$x = 11.2$$

MIC_{50} is the drug concentration that inhibits the growth of 50% of the fungal cells. MIC_{50} of *Aspergillus terreus* nccpf 860035 with drug Geldanamycin is 11.2.

4.5 Antifungal susceptibility test

4.5.1 Disc diffusion assay

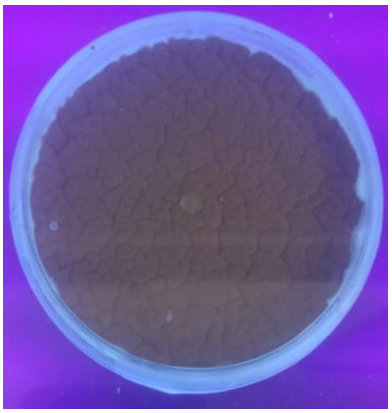
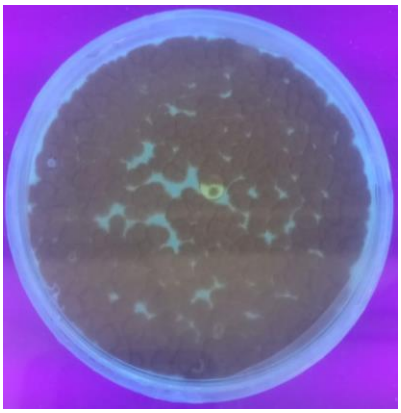
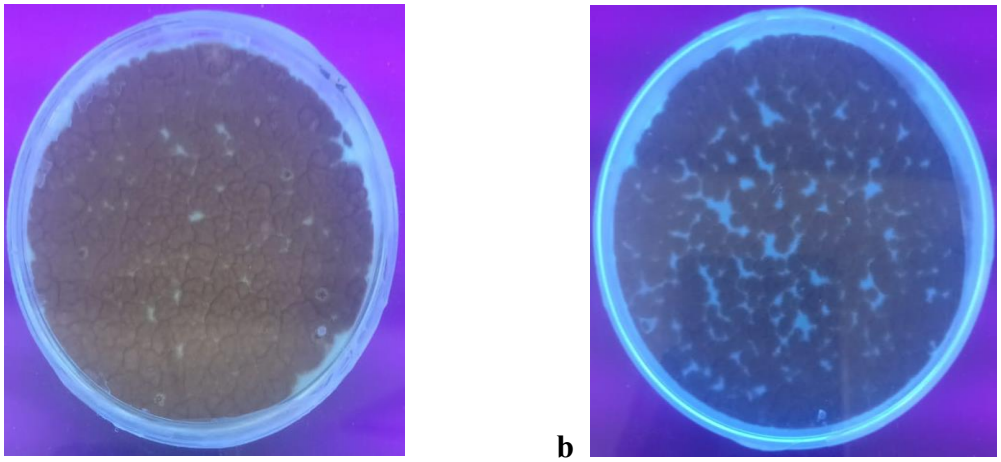
a  **b** 

Figure 4.5.1: - (a) Plate containing Sterile strip only (b) Sterile strip with Drug GA

The growth of *Aspergillus terreus* showing in the plates after 72 hours, the first plate containing the sterile strip as a positive control shows the full growth of the fungus, the second plate contains the drug GA loaded on the sterile strip showing the inhibition of fungal growth.

4.5.2 Agar dilution method

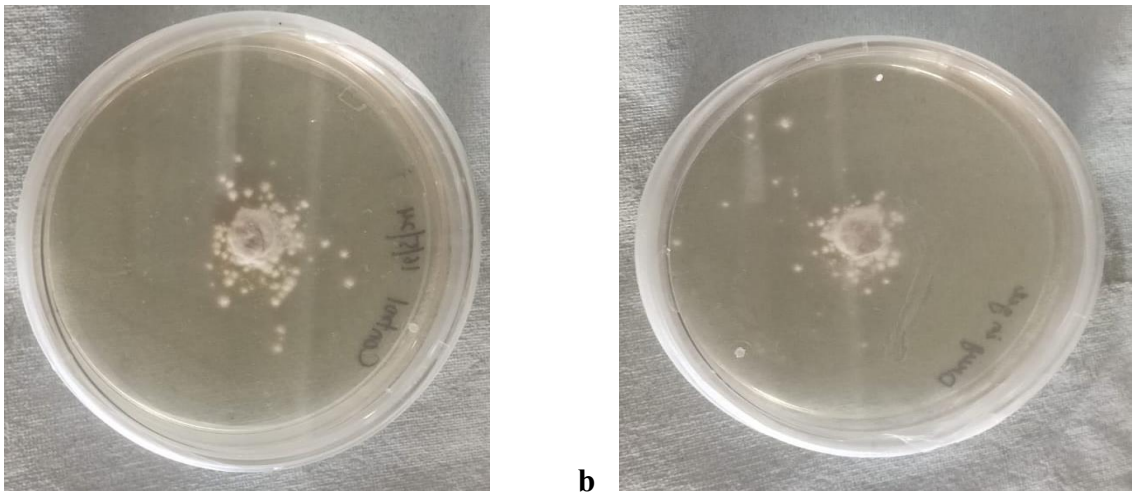


a **b**

Figure 4.5.2: - (a) PDA plate containing Media without drug (b) Media with Drug GA

The growth of *Aspergillus terreus* showing in the plates after 72 hours, the first plate containing the media only act as a positive control shows the full growth of the fungus, the second plate contains the drug GA in the media showing the inhibition of fungal growth.

4.5.3 The food poisoning technique



a **b**

Figure 4.5.3: - (a) PDA plate containing Media without drug (b) Media with Drug GA

The growth of *Aspergillus terreus* showing in the plates after 24 hours, the first plate containing the media only act as a positive control shows the more growth of the fungus as comparison to the plate containing the drug in the media.

4.6 Cellular ROS estimation

4.6.1 Control Culture

Bright field green fluorescence

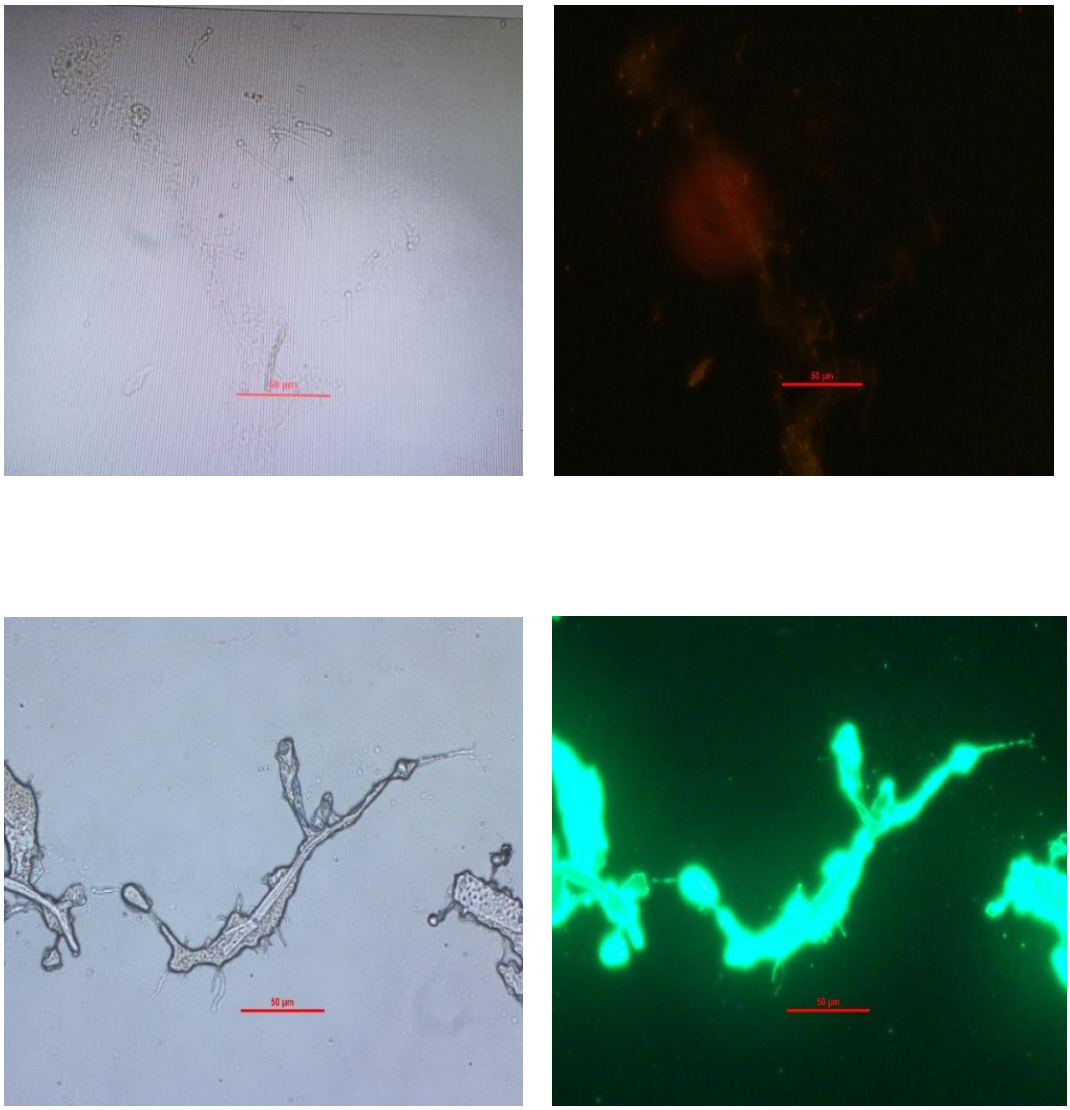


Figure 4.6.1.1: - ROS activity of *Aspergillus terreus* without drug

4.6.2 treated culture

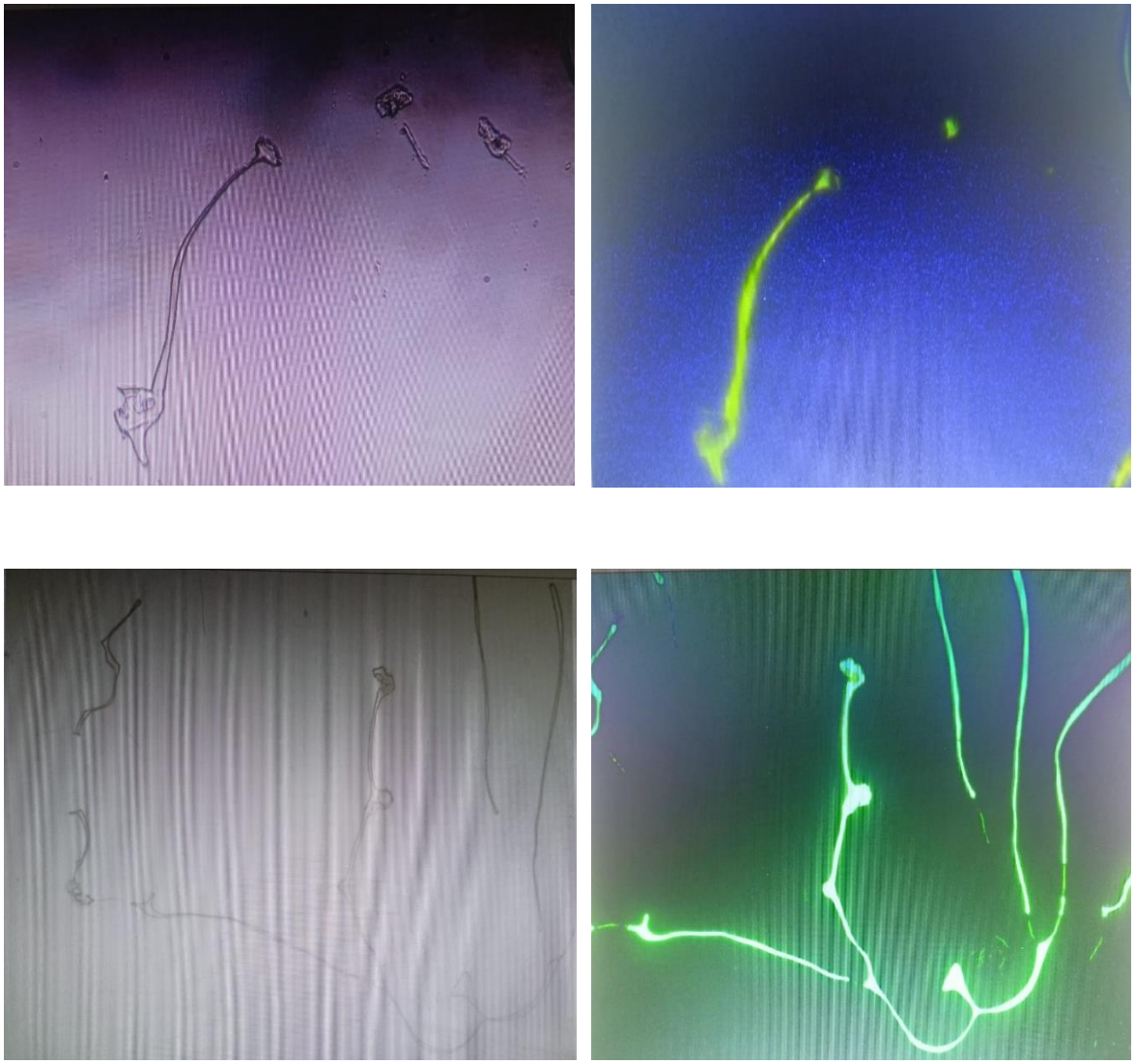


Figure 4.6.2.1: - ROS activity of *Aspergillus terreus* with GA-drug

As observed under fluorescent microscope, the intensity level in both GA-treated and untreated *Aspergillus terreus* were not significantly different. Primarily, it indicated that the ROS activity in both sample are similar suggesting the inhibition mechanism of Geldanamycin adapt different approach.

4.7 RNA extraction

RNA was extracted from normal and treated fungal broth culture at time point of 24 hours.

Figure 4.7: Upper colourless phase that contains RNA, interphase and lower organic phase

4.8 RNA bands visualisation on 1.2% agarose gel

Gel electrophoresis is the most crucial technique for fractionation of RNA. As RNA is polyanion polymer thus it will migrate from negative terminal (black wired) towards the positive terminal (red wired) on the application of electric field.

0.6% of the agarose was prepared for RNA visualisation and electrophorese at 100V for 45 minutes. Later the gel was visualised on a UV transilluminator. Two bands of rRNA were seen while visualising on transilluminator, the upper one is 28S and lower 18S. The 28S rRNA bands are seem to be more intense than 18S rRNA which shows the 2:1 ratio (28S:18S) and tells that the RNA is intact.

24-hour control

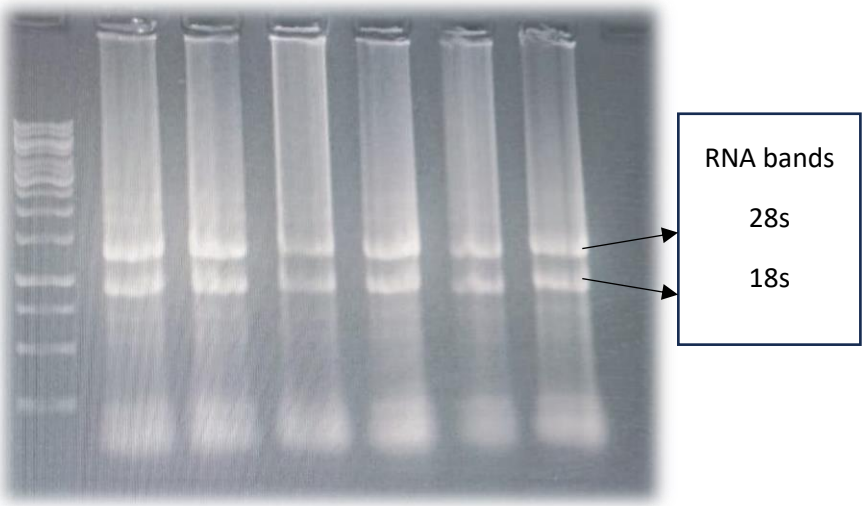


Figure 4.8.1: - 24-hour control RNA

24 hours treated with Geldanamycin

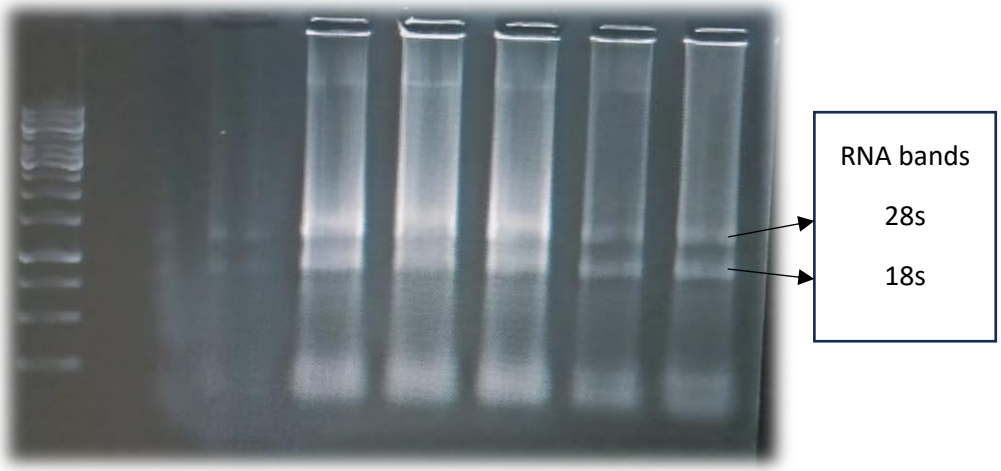


Figure 4.8.2 Treated RNA with GA

4.9 RNA quantification using spectrophotometer

The formula to calculate the amount of RNA mg/ml is as follows -

40 µg/ml * Abs at 260nm * Dilution Factor

4.10 RT PCR result analysis

Real time polymerase chain reaction analysis was carried on using cDNA which provides us with the amplification plot and cycle threshold (Ct) values. The reaction mixture was prepared in 15 PCR vials, containing 3 NTC, 4 positive control (40S ribosomal protein) and 8 sample vials containing samples of different time points of normal conditions as well as treated conditions. Below are the amplification plots and Ct values for time point and condition.

4.10.1 24-hour control Hsp70

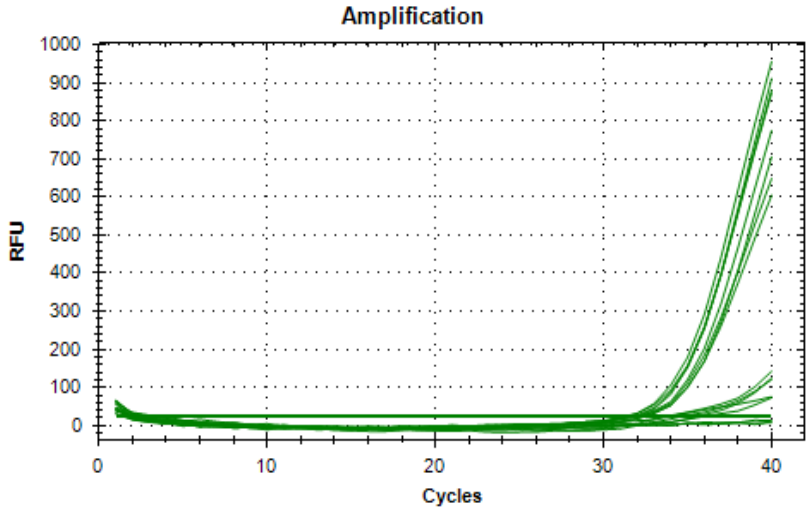


Figure 4.10.1.1: - Amplification plot for 24-hour control Hsp70 gene

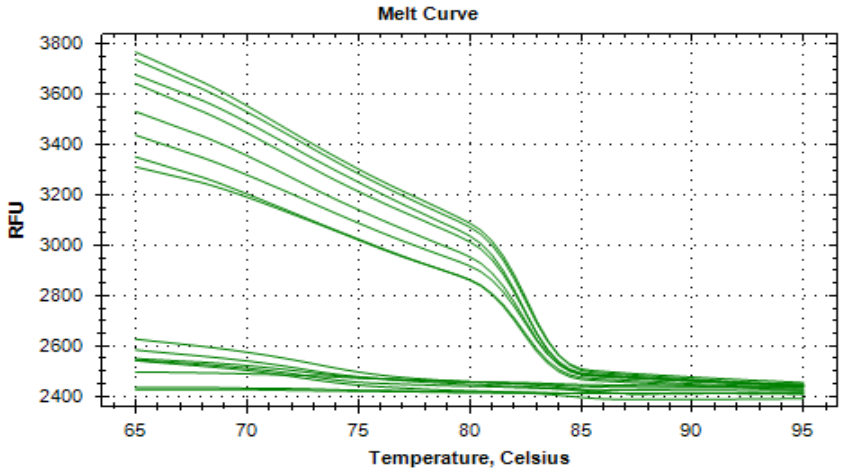


Figure 4.10.1.2: - Melt curve plot for 24-hour control Hsp70 gene

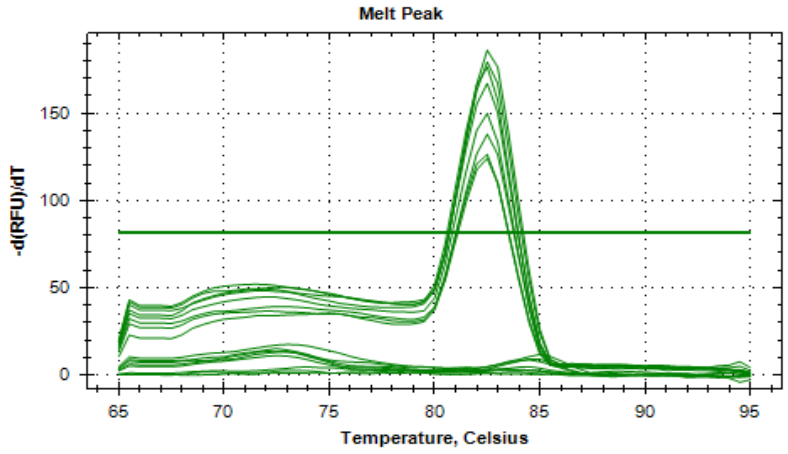
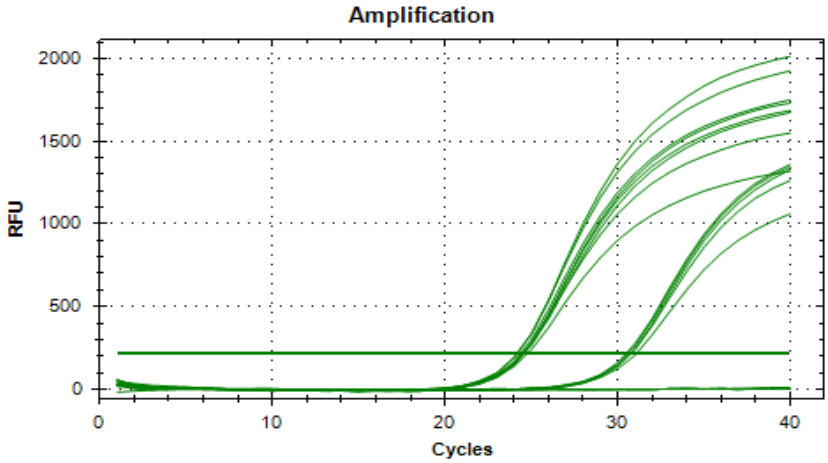
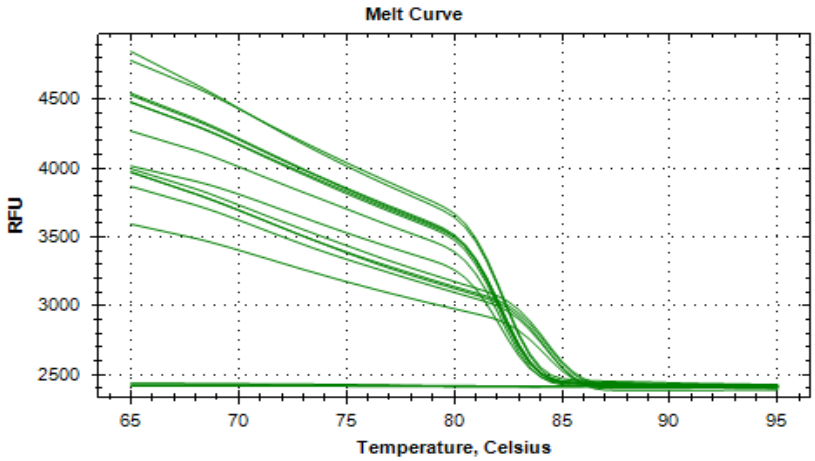


Figure 4.10.1.3: - Melt peak plot for 24-hour control Hsp70 gene

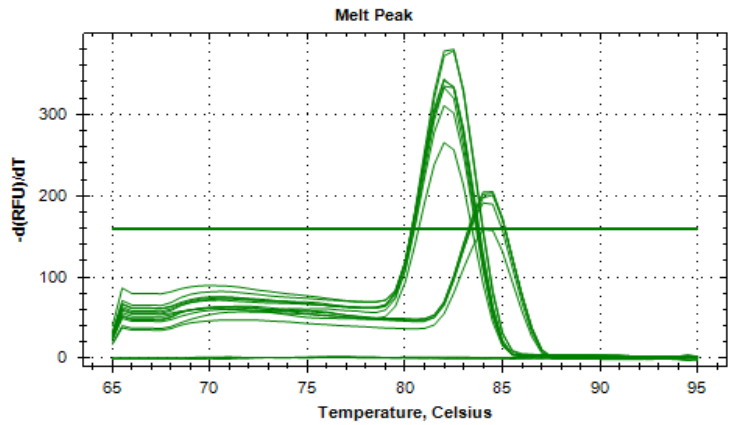
4.10.2: - 24 hour Treated Hsp70 with Geldanamycin



4.10.2.1: - Amplification plot for 24 hours treated Hsp70 gene

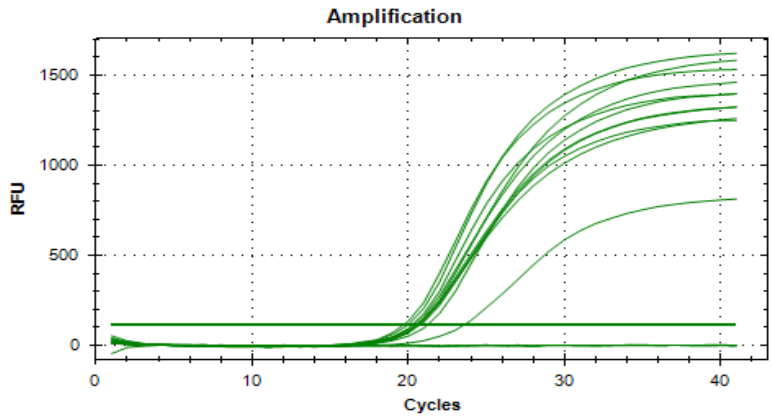


4.10.2.2: - Melt curve plot for 24 hours treated Hsp70 gene

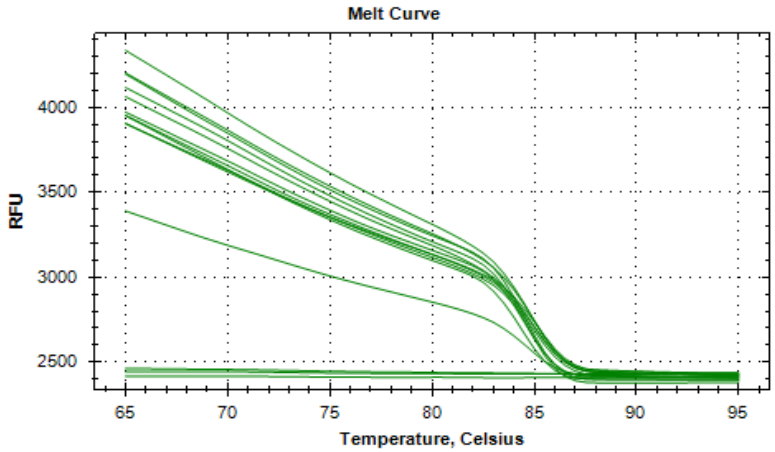


4.10.2.3: - Melt peak plot for 24 hours treated Hsp70 gene

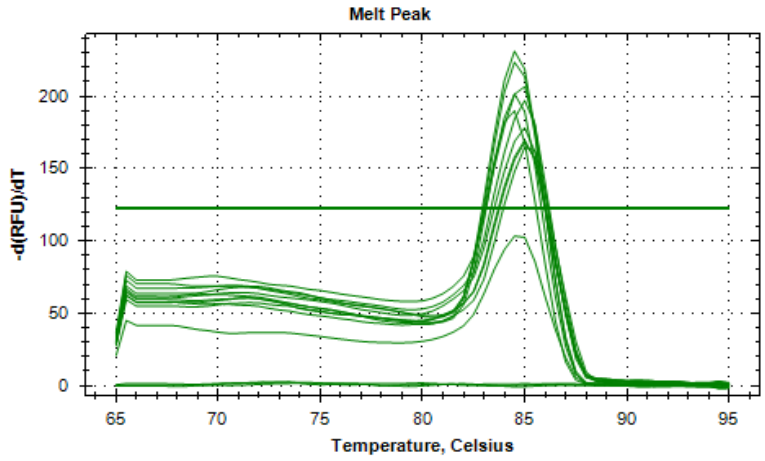
4.10.3: - 24-hour control Hsp90



4.10.3.1: - Amplification plot for 24-hour control Hsp90 gene

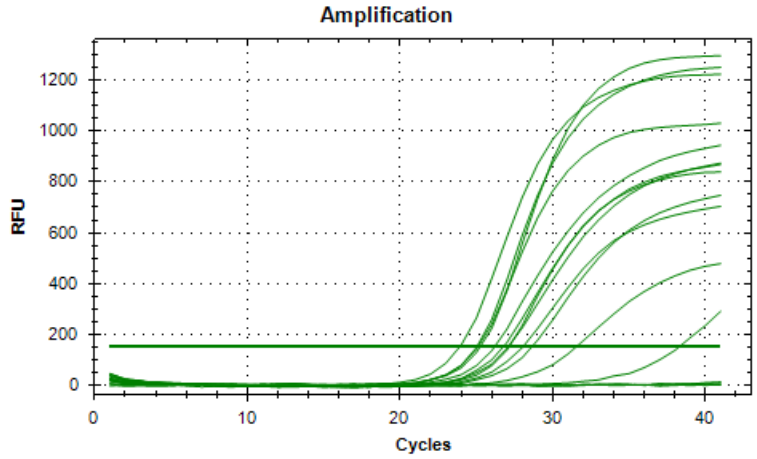


4.10.3.2: - Melt curve plot for 24-hour control Hsp90 gene

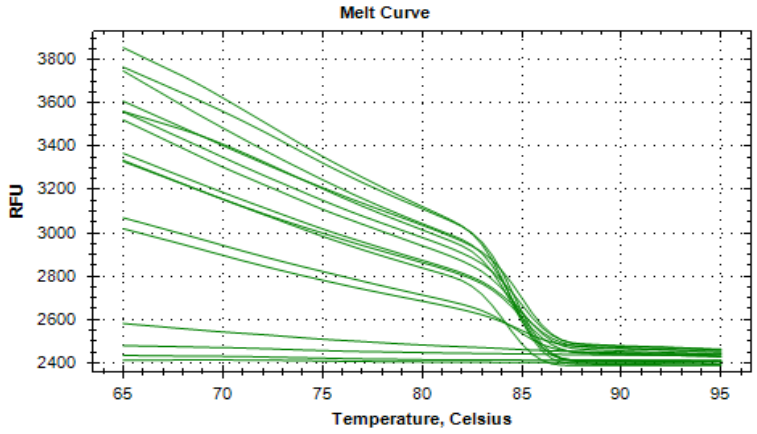


4.10.3.3: - Melt peak plot for 24-hour control Hsp90 gene

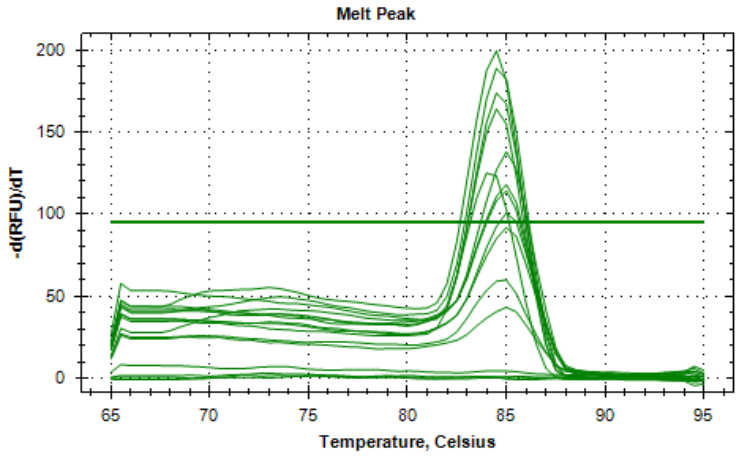
4.10.4: - 24 hour treated Hsp90 with Geldanamycin



4.10.4.1: - Amplification plot for 24 hours treated Hsp90 gene



4.10.4.2: - Melt curve plot for 24 hours treated Hsp90 gene



4.8.4.1: - Melt peak plot for 24 hours treated Hsp90 gene

4.11 C_t value calculation using double delta C_t method

To obtain the expression profile, expression fold and standard deviation of Hsp70 and Hsp90 genes in *A. terreus* under untreated and treated conditions, delta Ct value and double delta Ct values were calculation in Ms Excel using understated formulas:

Δ C_t value = Average C_t value of gene of interest - Average C_t value of housekeeping gene

ΔΔ C_t value = ΔC_t value of treated - ΔC_t value of control

Values of genes of interest that are HSP70 and HSP90 were normalised with 40S ribosomal protein (housekeeping gene).

Table 11: Double delta C_t values for untreated and treated samples –

Hsp70					
Samples	**Average C_t value of unknown**	**Average C_t value of housekeeping gene**	**ΔC_t value**	**$\Delta\Delta C_t$ value**	**$2^{-\Delta\Delta Ct}$**
Control	28.31	31.44	-3.13	-3.16	8.93
Treated	24.44	30.73	-6.29		
Hsp90					
Samples	**Average C_t value of unknown**	**Average C_t value of unknown**	**ΔC_t value**	**$\Delta\Delta C_t$ value**	**$2^{-\Delta\Delta Ct}$**
Control	21.02	20.31	0.71	1.35	0.39
Treated	27.36	25.30	2.06		

After the calculations of $\Delta\Delta C_t$ values for control and treated samples. It was seen that expression fold for Hsp70 was increases and the expression of Hsp90 was decreases. Expression fold for the gene Hsp70 was upfolded as comparison to the Hsp90. There is seen of downregulation of Hsp90 gene.

CHAPTER 5

CONCLUSION AND DISCUSSION

Aspergillus terreus causes serious illness in humans and the infection keeps on rising due to the increase in organ transplantation, cancer, and HIV-AIDS cases. Amphotericin B, a member of the polyene class of antifungals, has been utilized for nearly half a century in the treatment of fungal infections. However, *Aspergillus terreus* exhibits inherent resistance to this medication. Other concomitant cases of the fungus are also seen. There is a coexistence of *Aspergillus* infection with cancer which is characterized by the interaction between cancer-induced immunosuppression and opportunistic fungal infection. Cancer patients often have a weakened immune system, either as a direct result of the cancer itself or due to the immunosuppressive effects of cancer treatments like chemotherapy [73]. This weakened immune system creates an environment that is favourable for opportunistic infections.

Therefore, in this study, the expression of heat shock protein 70 and heat shock protein 90 of *Aspergillus terreus* was compared between normally grown cells and cells that are grown with Geldanamycin to determine the effect of Geldanamycin on fungal cells and cancer cells.

Performed the Minimal inhibitory concentration (MIC) rection of Geldanamycin on *Aspergillus terreus,* which shows the inhibition of the growth of fungus. Calculated the MIC50 in which the 50% growth of the fungus was inhibited. Data generated from RT PCR suggested that at 24 hours there is a decrease in Hsp90 gene in treated conditions may be due the drug action. Geldanamycin attaches to the N-terminal ATP-binding site of Hsp90, causing interference with its chaperone activity. Consequently, this disrupts the folding of client proteins and results in the degradation of these proteins by the proteasome. Research has not indicated a notable alteration in Hsp90 gene expression following treatment with Geldanamycin.

CHAPTER 6

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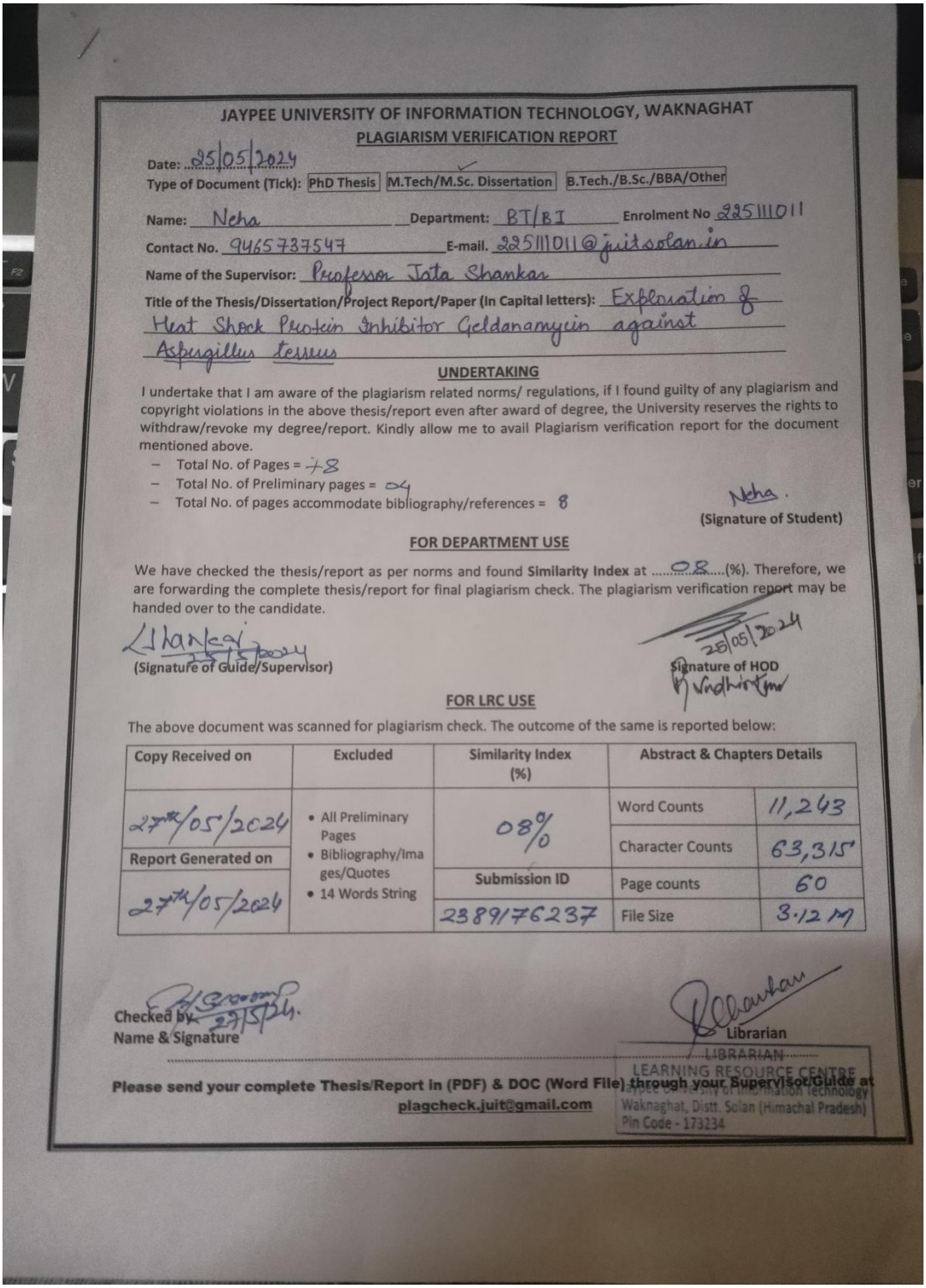

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