Temporal transcriptional analysis of genes encoding heat shock protein family of *Aspergillus terreus* with and without Shikonin and Amphotericin B

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Submitted by:

ANANYA SHARMA

Enrolment number- 217825

Under the guidance of:

Prof. (Dr) JATA SHANKAR

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY,

WAKNAGHAT-173215, HIMACHAL PRADESH, INDIA

(MAY-2023)

SELF-DECLARATION

I, **Ananya Sharma**, student of M.Sc. Biotechnology, Jaypee University of Information Technology, Waknaghat, Solan, Himachal Pradesh do hereby declare that the project entitled "**Temporal transcriptional analysis of genes encoding heat shock protein family of *Aspergillus terreus* with and without Shikonin and Amphotericin B**" submitted towards partial fulfilment for the award of the degree of Master of Science in Biotechnology of Jaypee University of Information Technology is based on the results of the research work carried out by me and written by me under the guidance and supervision of Prof. Dr. Jata Shankar. This project or no part of this has been submitted elsewhere for awarding any degree or diploma.

____

Ananya Sharma

(217825)

DEPARTMENT OF BIOTECHNOLOGY AND BIOINFORMATICS

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY, WAKNAGHAT

CERTIFICATE

This is to certify that the work titled "**Temporal transcriptional analysis of genes encoding heat shock protein family of *Aspergillus terreus* with and without Shikonin and Amphotericin B**" submitted by Ananya Sharma (217825) is in partial fulfilment for the award of the degree of Master of Science in 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.

____

Prof. (Dr) Jata Shankar

Designation: Professor

Department of Biotechnology and Bioinformatics

Jaypee University of Information Technology,

Waknaghat, Distt. Solan,

Himachal Pradesh, 173215,

India

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LIST OF ABBREVIATIONS

Nccpf – National culture collection of Pathogenic Fungi

LAFH– Laminar Air Flow Hood

RPMI 1640 – Roswell Park Memorial Institute

FBS – Fetal Bovine Serum

PBST – Phosphate Buffer Saline Tween 20

PBS – Phosphate Buffer Saline

AmB – Amphotericin B

EDTA – Ethylene Diamine Tetra-acetic acid

TAE – Tris-acetate-EDTA

Ng – Nanograms

NTC – Non Template Control

RT PCR – Real time Polymerase Chain Reaction

Δ – Delta

ΔΔ – Double delta

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ABSTRACT

Aspergillus terreus is an emerging pathogen in patients with compromised immune system. Due to its intrinsic resistance towards amphotericin B and developing resistance towards various azoles and other antifungal agents an alternative to beat its infection needs to be investigated. Thus in this project we have explored a new combination of antifungal agents, shikonin + amphotericin B, as shikonin is known to increase the efficiency of other anti-fungal agents. We tried determining effect of this drug combination on heat shock protein 70 and 90 in treated versus control expression fold analysis. Expression of HSP90 seems to increase at 24 hours which might be due to the drug combination as it is putting the *Aspergillus terreus* cells cells under some stress condition which leads to increase in heat shock protein production.

CHAPTER 1: INTRODUCTION

In the recent decades infections due to opportunistic fungus has been raised to large extent. One of the major reason for that is antimicrobial/fungal resistance towards certain drugs. Among the 120,000 fungal species known till now *Aspergillus* family is associated with causing infection in humans, animals, birds, and plants. The infections can be range from acute to systemic as well as allergic in nature such as IgE-associated asthma, hypersensitivity pneumonitis, etc. In the *Aspergillus* family, *Aspergillus flavus, Aspergillus terreus, Aspergillus fumigatus* are the main cause for fetal infections in humans [1]. In recent times the number of immunocompromised individuals have been increased due to cancer chemotherapy, organ transplantation, HIV/AIDS and such conditions made opportunistic fungal pathogen to infect host easily [2]. Hence drug resistance in this harsh scenario is making it more complicated to produce new drugs. With the recent study it is know that *A. terreus* is the leading cause of invasive aspergillosis (IA) especially in cancer patients [3] and intrinsic resistance of *A. terreus* for amphotericin B (AmB) which is the most common anti-fungal agent and have been used for treating fungal infections over 40 years, complicates the treatment protocol [4]. As we know germination is the most crucial step in fungal biogenesis to invade host cells or tissues and initiates the infection [5]. Remolding mechanism in fungal cell wall is essential for conidial germination. As the germination begins the melanin and rodlet layer of the conidia vanishes and protiens and peptides that helps in establishing the infection exposes to facilitate the fungal infections [6]. Therefore, knowing the proteins that plays role in initiating the infection is important to understand the mechanism of invasion into host and also these proteins prove to be potent biomarkers for the diagnosis of infections. These proteins can be promising drug targets and vaccine candidates for fungal associated infections.

In humans, lungs are the primary organs to get infected with any air-borne pathogen and so in the case of Aspergillus. Inhaled conidia resided in lungs of the host finds the perfect time to germinate and establish its invasive infection. The alveolar and bronchial epithelial cells are the primary cells in lungs that encounter these inhaled conidia and fight to prevent the establishment of infection. These cells prevent infection by recognising pathogen associated molecular patterns (PAMPs) that are present on the fungal cells, using pathogen recognition receptors (PRRs) that are found on the epithelial cells [7]. On recognition these cells activates effector molecules such as cytokines and chemokines or antimicrobial peptides. In the individuals with weaken immune system, the phagocytic function of immune cells are not

properly function-able which allow fungus to cause infection. Therefore in conditions like these, lung epithelial cells plays the major role in eradication of the infectious cells [8]. People with respiratory disorders are more prone to fungal manifestations as their lung epithelial cells are in direct contact of *Aspergilli.* Despite the major role of lung epithelial cells there is very less information about their interaction with *A. terreus* cells [9]. Therefore it is crucial to know and understand the proteins that are expressed by epithelial cells to recognise *A. terreus* which allow us to understand various biochemical pathways involved in the mechanism.

There are various drug targets that are known and various new that are recognized. Heat shock proteins are the proteins family that are present in all types of living cells and plays role in proper protein folding. In fungal cells they plays major role during morphogenesis of cells and they are important for fungal cell survival. Therefore, inhibition of these using various drugs and other methods may prove to be promising in eradication of fungal infections [10].

In conclusion, there are still a lot that we are not aware about fungal cells and how they cause infection, increasing drug resistance is forcing us to find new drugs components or their alternative which are more reliable and new drug combinations. Therefore, in this research project we have done the transcriptional analysis of Hsp 70 and Hsp90 proteins in *A. terreus* at different time points such as at 16 hour, 24 hour and 48 hour with and without the Amphotericin B (AmB) + Shikonin (ShK) drug combination. These time points were taken, as 16 hour is the germination time of *A. terreus* and 24 and 48 hours were taken to study the Hsp gene expression at minimum and maximum drug activity.

CHAPTER 2: REVIEW OF LITERATURE

2.1 Members of genus *Aspergilli*

Fungi are the group of organisms that includes moulds, yeasts and mushrooms, they are eukaryotic organisms and comes under of the five kingdoms of classification- kingdom Fungi. Some of them are microscopic and some can be visible from naked eyes. These organisms are ubiquitous in nature and found in wide range of environments such as land, soil as well as in air [11]. They are crucial for environment as they breakdown dead organic matter and giving nutrients back to the soil. Some fungi also parasitized arthropods and thus used in integrated pest management (IPM) [12]. For the longest times, fungi are used in food industry for the production of food such as, bread, cheese, soy sauce, miso, tempeh, etc., and beverages such as beer, wines and spirits. Apart from the usefulness of fungi some fungi are also proved to be pathogenic for living organisms and known to cause infections in humans, animals and plants.

Fungi consists of various structures which can be explained as follows, almost all fungi have filamentous part except the yeast cells, and they can be unicellular or multicellular in nature. Fungal cells consists of long thread-like structures known as hyphae which together forms the mesh like-structure known as mycelium. They possess call wall made of polysaccharides and chitin.

Fungi can be classified based on their mode of nutrition as well as on type of spore formation.

- ❖ Based on mode of nutrition:

1) Saprophytic: Such fungi obtain their nutrition by feeding on dead organic materials.
   Examples- *Aspergillus, Penicillium* and *Rhizopus*.
2) Parasitic: Such fungi obtain their nutrition by living on other living organisms which are denoted as hosts and absorbing their nutrients for their own survival.
   Examples- *Puccinia* and *Taphrina*
3) Symbiotic: Survival of such fungal species depends on other organisms with which they are in symbiotic relation with. In such relation both the parties are mutually benefitted.
   Examples- *Mycorrhiza* and *Lichen*

- Based on spore formation:
    1) Zygomycetes: Such fungal cells are formed by the fusion of two different cells. Their sexual spores are known as zygospores and asexual spores are known as sporangiospores. Their hyphae doses not contains septa.
    Example- *Mucor*
    2) Ascomycetes: Fungi of this group are also known as sac fungi. Their sexual spores are known as ascospores and asexual spores are known as conidiospores.
    Example- *Saccharomyces*
    3) Basidiomycetes: This group contains mushrooms. Sexual spores known as basidiospores and asexual reproduction done by conidia, budding or fragmentation.
    Example- *Agaricus*
    4) Deuteromycetes: Fungi of this group are also known as imperfect fungi as they do not follow the regular reproduction cycle like other member of fungi kingdom. Asexual reproduction one by conidia and they do not reproduce sexually.

Due to advances in medical care in the at-risk immunocompromised patients there is increased in opportunistic fungal infections. Such infections are caused by opportunistic fungal pathogens that are the group of fungi that do not cause infections in healthy people but can cause infection in immunocompromised people [13]. *Aspergillus* is a genus of kingdom fungi that belongs to the family Aspergillaceae and is part of order Eurotiales. Fungi of *Aspergillus* genus are ubiquitous in nature and tend to grow in any possible space from outside environment soil to indoor surfaces like walls, etc. They are known to cause infection in humans, particularly those with compromised immune system. Not all members of this genus causes infections, some are also used in production of medicines and food items. Such as *A. terreus* is used for the production of various secondary metabolites such as lovastatin, a cholesterol-lowering drug [14]. The most common species of Aspergillus that causes infections in humans are *Aspergillus flavus* it produces mycotoxin known as aflatoxins which can contaminate food and cause liver damage in humans [15], *Aspergillus fumigatus* which can be associated with respiratory infections, including aspergillosis, which can get severe in people with compromised immune systems. *Aspergillus niger* is also associated with aspergillosis [16] and *Aspergillus terreus* produce tremorgenic toxins that are known as territrems that can cause neurological symptoms such as tremors, convulsions, and paralysis other toxins produced by *A. terreus* are citrinin, gliotoxins etc [17].

Due to the rise in anti-microbial resistance in microorganisms it is quite a task to treat such infections. In the last few years, *A. terreus* known to be leading cause of invasive aspergillosis in the patients with weak immunity especially in cancer patients. Therefore, there is requirement of more therapeutic options and drugs to beat such infectious demons. For that understanding these organisms, pathways involves in their infectious stage and genes related with that is crucial.

2.2 Taxonomic classification of *Aspergillus terreus*

Kingdom: Fungi
Phylum: Ascomycota
Order: Eurotiales
Family: Trichocomaceae
Genus: *Aspergillus*
Species: *terreus*

2.3 Morphology and Distribution of *Aspergillus terreus*

Aspergillus terreus is ubiquitous in nature. It is often found in warm and humid regions and also been found in tropical and sub-tropical regions around the world. It can also survive in extreme environmental conditions such as high salinity, alkalinity and temperature. Its colonies grows at fast rate and appears to be white cotton like at initial stages followed by brownish-grey as it get old and the reverse side of the colony appears to be yellowish-brown in colour with wrinkled texture. Its hyphae consists of septa and is typically measure as 2-4 µm in diameter. For asexual reproduction it produces conidiophores that contains small and round conidia. These conidiophores are unbranched, smooth-walled and are around 200-500 µm in length. Conidia are spherical in shape, smooth walled and around 2-4 µm in diameter. They are produced in large numbers and dispersed in air which makes *A. terreus* and airborne contaminant. [5, 17]

2.4 Life cycle of *Aspergillus terreus*

There are four stages that comprise the life cycle of *A. terreus:*

Spore germination: The first stage is the germination of spore which will then give rise to hypha that will grow and form mycelium web

Vegetative growth: During this stage the mycelium continues to grow and tries to obtain nutrients from surrounding environment and produces enzymes to break down complex substrates into simpler ones.

Asexual reproduction: *A. terreus* reproduces asexually by forming spores known as conidia. These spores are formed at the tips of specialized hyphae called conidiophores. When the conidia are mature, they are released into the environment and can germinate to start a new life cycle. [18]

Sexual reproduction: *A. 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. [19, 20]

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

2.5 Ecological Role of *Aspergillus terreus*

Aspergillus terreus is a saprophytic fungus that means it obtains its nutrients by breaking down dead and decaying organic matter. They helps in recycling the nutrients and energy in ecosystem, thereby play crucial role in ecological environment. It is important decomposer of cellulose, lignin and hemicellulose which are complex polymers that are found in plant cell wall, by breaking those *A. terreus* releases various nutrients to the soil which are then used up by other organisms and plants itself. It also produces some secondary metabolites that have anti-microbial activity and protect plants from disease causing pathogens. Hence, it is and important member of soil microbial community [21].

2.6 Industrial Uses of *Aspergillus terreus*

Aspergillus terreus is a versatile industrial microorganism. Some of its important industrial uses are:

Lovastatin production: Lovastatin is a cholesterol-lowering drug produced by the fungus as a secondary metabolite, and it inhibits an enzyme involved in cholesterol synthesis in humans [22]

Itaconic acid production: Itaconic acid is a platform chemical used in the production of various chemicals is synthesis using *A. terreus* [23].

Secondary metabolites production: Secondary metabolites such as terrain, terretonin and butyrolactones can be produced by *A. terreus*. They have potential application in agricultural, pharmaceutical and food industries [24].

2.7 Germination in *Aspergillus terreus*

Germination is the initial step in the life cycle of any filamentous fungus and so in *A. terreus*. After the dispersion of its spores in the environment they begin to germinate when comes in suitable condition [25]. Various steps of germination is:

1. **Activation**: Spores get activated when comes in suitable environmental conditions, such as adequate moisture, temperature and availability of nutrients.
2. **Hydration**: Once activated, spores takes up a lot of water causing it to swell and rupture its outer wall which is the hardest.
3. **Germ tube formation**: From the site of rupture a single extension of cytoplasm appears which is known as germ tube. This germ tube grows in polarised manner, elongates in one direction and forming a cell at the tip end.
4. **Branching**: Later, the tip cell begins to branch and produces the network of hyphae. This hyphae penetrates the substrate and absorbs nutrients for growth.
5. **Colonization**: The hyphae forms the mycelium that spreads over the substrate surface and allows fungus to form colonies [26].

2.8 Diseases caused by *Aspergillus terreus*

Due to increase in number of immunosuppressant patients the *A. 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 reside in the immune cells of the host of long period of time and get transferred to other secondary organs via macrophages. When the immune system of the host gets compromised the spores starts to germinate and initiates their life-cycle. One such study on how epithelialcells responds on interaction with *A. terreus* conidia shows various proteins

involvement from pathways such as, Jak/Stat, TLR signalling, NOD receptor signalling, NF-kB which shows strongs immune response against fungal infections. [10]. A range of diseases can be caused by *A. terreus*, such as, invasive aspergillosis (IA), Allergic bronchopulmonary aspergillosis (ABPA), aspergilloma [17]. *A. terreus* type can cause a range of diseases such as invasive aspergillosis which is the serious infection that affects people with undergoing chemotherapy or organ transplantation and can affect various organs such as lungs, brain and skin, otomycosis which is an ear infection and can lead to hearing loss, keratitis which is the infection of cornea can lead to vision loss and sinusitis. Allergic bronchopulmonary aspergillosis (ABPA) is a rare condition in which the body mounts an allergic response to *Aspergillus terreus* spores. ABPA can cause symptoms such as wheezing, coughing, and shortness of breath. Therefore, it become essential to understand the cellular mechanisms and biology of *A. terreus*.

1) Invasive aspergillosis

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. Combination of these drugs are 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 [3]

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 triggers 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. Over the time if infection left untreated, severe damage to lungs can occur which can also leads 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 [27, 28].

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 [29].

2.9 Immune response towards aspergilli

Firstly the fungal conidia has to cross the first line defence of the immune system that are skin, mucous membrane, tears, etc. Once the conidia enters the body of the host, immune cells such as macrophages and neutrophils recognise the fungal cells and are capable of engulfing them and alerting the immune system regarding the presence of the infection. Recognition of fungal cells is carried on by recognising PAMPs using PRRs present on immune cells, such as toll like receptors, dectin-1, C-reactive protein [30]. If the innate response is not sufficient adaptive response activates to eradicate the infection. Adaptive immune response involves T cells and B cells that produce antibodies against the fungal cells. But in some cases the infection

through Aspergillus species become difficult to treat and cause severe illness especially in people with compromised immune system [30].

2.10 Newly discovered antifungal compounds and targets for Aspergillus species

Aspergillus terreus is not very much studied but it needs to be studied due to the high mortality rate of its infections. Anti-microbial resistance and toxicity of drugs in some patients are also the reason that there is need to develop new drugs and discovered new compounds with anti-fungal properties. But it is challenging to find the new drug candidates due to the similarity fungal and human cells share. Talking about the targets, calcineurin pathway is trending nowadays as it is associated with various biological mechanisms of *Aspergilli.* Triphenylethylene is a newly discovered compound that is suggested for fungal infections as it blocks the calcineurin pathways by acting on calmodulin which basically activates calcineurin [31]. According to some studies heat shock protein (HSP) 90 can also prove to be the promising target.

2.11 Heat shock protein

Heat shock proteins (HSPs) are the group of proteins that are produced in stress conditions of cell such as high temperature, exposure to toxins, etc. They were first identified in the cell which was in extreme stress condition hence got their name "heat shock" from there. These proteins are involved in protein folding, protein trafficking and regulation of immune system, protects cells from damage caused by stress and plays role in repairing damaged proteins. They acts as molecular chaperons and assists folding and refolding of proteins to maintain them in their functional conformation. These proteins are found in all living organisms from bacteria to human cells. HSPs are classified into various classes based on their molecular weight. The major families of HSPs are, HSP90, HSP70 and HSP60 [32].

In *A. terreus* HSP90 is well studied and involved in folding and activation of variety of other proteins other than that it has also been shown to play role in biosynthesis of secondary metabolites such as lovastatin. HSP70 is also important HSP in *A. terreus* as is seems to play role in conidiation that is formation of conidia. HSP40 acts as co-chaperone that helps HSP70 in protein folding [33]. Understanding role of HSPs in *A. terreus* or any other *Aspergillus* species is necessary for the development of novel strategies to control the infection and production of valuable secondary metabolites [8].

These proteins are involved in various pathways of protein degradation such as ubiquitin-proteasome system and autophagy. The prior one is responsible for the degradation of misfolded or short-lived proteins whereas the later responsible for degradation of damaged organelles and larger protein aggregates. HSP90 and 70 are known to involve in degradation of misfolded and damaged proteins by binding to them and allow their ubiquitination followed by degradation by proteasome. HSPs also help in presenting peptides and antigens over the cell surface for T-cell recognition, this mechanism is known as cross-presentation [34].

Dysfunction of HSPs can leads to accumulation of misfolded and damaged proteins which can ultimately leads to cell death or the development of various diseases including cancer in case of human or animal cells. Therefore, inhibition of these proteins can increase the susceptibility of *Aspergillus* species to stress and drugs and reduce the pathogenesis. Several studies have suggested that HSP70 and 90 inhibitors seems to show anti-fungal activity as *Aspergillus* species although the mechanism for the same is not well understood. Some studies have also investigated that use of phytochemicals and natural compounds such as curcumin are resveratrol has been seen to inhibit HSPs activity and hence they can be used as potential anti-fungal agents for treatment of *Aspergillus* infections. However, deep research is required to fully understand the mechanism of action of HSP inhibitors and optimize their use in the treatment of infections.

2.12 Shikonin as an anti-fungal agent

Shikonin is a phytochemical derived from the roots of the Chinese medicinal herb *Lithospermum erythrorhizon.* It has been known to possess anti-inflammatory, antioxidant and antimicrobial properties, other natural plant products with such properties are also present [35]. Studies suggest that shikonin can be a potent anti-fungal agent as its anti-fungal activity has been seen in *Candida albicans, A. fumigatus and Cryptococcus neoformans* [36]**.** It acts on fungal cell by disrupting their cell membrane and inhibiting its growth and proliferation. One such study suggested that it disrupts the cell wall and cell membrane of Candida albicans and leads to the leakage of intracellular contents of the cell ultimately leads to cell death. Shikonin is known for its ability to generate reactive oxygen species (ROS) which can cause oxidative damage to the cell membrane [37]. Also, Shikonin has been seen in enhance the efficacy of existing anti-fungal agents such as, fluconazole and amphotericin B [38] which indicates that

it could be used in combination with such anti-fungal agents to improve the efficiency of the treatment. However the mechanism of action of shikonin as an anti-fungal agent is still not fully understood. Nevertheless, it is a promising compound that possess the potential to be developed into a novel antifungal agent.

2.13 Amphotericin B as an anti-fungal agent

Amphotericin B or AmB is one of the most effective, broad-spectrum anti-fungal agent [39], but it causes high toxicity in some patients. It function by binding to the ergosterol, a component of fungal cell membrane that is absent in animal cells, thus disrupting cell membrane's structure by forming pores in it which leads to leakage of intracellular components and ultimately cell death. It is used to treat various fungal infections such as IA, cryptococcal meningitis, candidemia and mucormycosis [40]. AmB is administered intravenously in the patient's body and its dose depends on the body weight of the patient and infection's severity. There are number of side effects that are associated with AmB such as, fever, chills, nausea, headache and even damage of vital organs such as kidney. In order to minimise the side effects it is often prescribed with other drugs such as corticosteroids, diphenhydramine, and acetaminophen [41, 42].

The current work on "**Temporal transcriptional analysis of genes encoding heat shock protein family of *Aspergillus terreus* with and without Shikonin and Amphotericin B**" was performed with following objectives:

1. To assess the transcripts of HSP 70 and 90 genes at 16 hour, 24 hour and 48 hour in *Aspergillus terreus*
2. Response of HSPs with and without shikonin and amphotericin B drug in combination

3. CHAPTER 3- MATERIAL AND METHODOLOGY

4. 3.1 Materials

5. 3.1.1 Chemicals

Chemical	Manufacturer
Potato Dextrose Agar (PDA)	HIMEDIA
RPMI 1640 medium	HIMEDIA
Sodium chloride	Vetec
Potassium chloride	HIMEDIA
Sodium phosphate dibasic	HIMEDIA
Potassium phosphate monobasic	HIMEDIA
Tween 20	Merck
Lactophenol cotton blue	HIMEDIA
Trizol reagent	Takara
Chloroform	HIMEDIA
Isopropyl alcohol	Loba Chemie
Ethanol	HIMEDIA
Nuclease free water	Affymetrix
Agarose (1.2%)	HIMEDIA
Tris	HIMEDIA
Glacial acetic acid	Merck
EDTA (0.5M)	Sigma-Aldrich
Diethyl pyrocarbonate	Sigma
EtBr	HIMEDIA
RT PCR kit	Agpath RT PCR kit
cDNA synthesis kit	Verso cDNA Synthesis kit
6X DNA loading dye	Thermo Scientific
1kbp DNA ladder	Gene Ruler
Amphotericin B	HIMEDIA
Shikonin	HIMEDIA
Fetal bovin serum	Gibco

3.1.2 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'

3.1.3 Instrumentation and equipment

Instrument	Manufacturer
Laminar air flow	Microsil India
Incubator with shaker	Labnet
Weighing balance	Denver
Centrifuge	HITACHI, Eppendorf
Autoclave	SANYO
Agarose gel electrophoresis setup	Bio-RAD
Gel doc. System	BIO-RAD
Freezer (4°C, -20°C, -80°C)	Celfrost/Vestfrost solutions/New Brunswick Scientific, resp.
Spectrophotometer	Thermo Fisher scientific
Thermocycler (PCR, RT-PCR)	BioRad, Applied biosystems

Equipment	Manufacturer
Micropipettes	Eppendorf/Thermo Scientific/ AXYPET
Microtips	Tarson
Conical flasks	JSGW
Microcentrifuge tubes (1.5ml and 2ml)	Eppendorf
Falcon tubes (50ml, 15ml)	Tarson
Petriplates	Genaxy
Reagent bottles (1 litre and 100 ml)	JSGW
Mortar and pestle	JSGW

3.1.4 Biological material

Aspergillus terreus nccpf strain 860035

3.2 Methodology

3.2.1 Revival of *A. terreus* culture

Revival of *A. terreus* nccpf strain 860035 was done by streaking the master culture on petri plates containing PDA media using sterilised inoculating loop inside LAFH. After inoculation the plates were sealed using parafilm and placed in incubator at 25°C (this optimised temperature suggested by nccpf catalogue). Full growth can be seen within 4-5 days.

3.2.2 Cell count

For counting the cells, 2-3ml of PBST was added to the fungal culture plate, left for few minutes and cells were gently picked using pipette after slight rubbing, 100µl of cell suspension added to microcentrifuge tube along with 100µl of lactophenol cotton blue dye making the total volume of cell suspension 200 µl, then mixed using pipette and the mixture left for few minutes.

Cell counting was done using hemocytometer, which is a chamber device designed for counting cells.

3.2.3 Growth of culture in broth

3.2.3.1 For control cultures

After four days growth of *A. terreus* on plates, inoculation in 45ml RPMI-1640 medium + 5ml FBS (total 50ml media) was done. For preparation of the inoculum, the plates with fungal culture were dispensed with 10ml of PBST (0.05% Tween 20) and left for 5 minutes. Afterwards, the surface was slightly rubbed using the tip of 1ml pipette and the suspension was picked and 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 after removal of supernatant and mixed with hands. Once the cell pellet mixed in PBS, 1ml of it was pulled using pipette and added to the broth. After inoculation flask covered with cotton plug and aluminium foil and placed in incubator shaker at 25°C at 100rpm shaking for required time (16 hour, 24 hour, and 48 hour).

3.2.3.2 For treated cultures

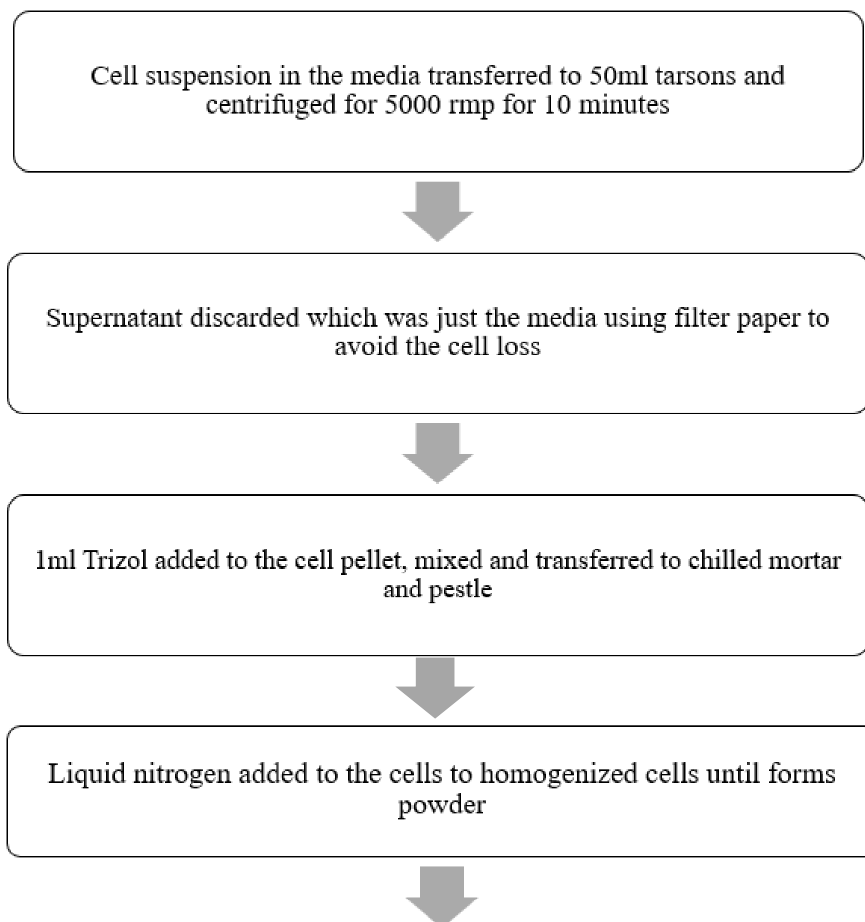
For treated culture of *A. terreus* in combination of Shikonin+ AmB, 50ml media containing 45ml RPMI-1640 media and 5ml FBS was prepared by adding 20μl of SHK (MIC value= 2μg/ml of SHK) and 38μl of AmB (MIC value= 3.8μg/ml) anti-fungal agents [34].

Once the media is prepared with anti-fungal agents same process as above followed for inoculation. Flasks then placed in incubator at 25°C at 100 rpm shaking for required time points (16hour, 24 hour, and 48 hour).

3.2.4 RNA extraction

RNA extraction was performed using TRIZOL-CHOLOROFORM method at different time points such as at 16 hour, 24hour and 48 hour both for treated and control samples.

The process for the same is as follows-



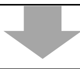
2ml of the trizol added slowly to the mortar mix using pestle and incubate for 5 minutes at room temperature to allow complete dissociation of nucleoprotein complexes

It will convert in liquid form, transfer the liquid using pipette to the microcentrifuge tubes

Add 500µl of chloroform to them and shake vigorously by hands for 15 seconds and incubate at room temperature for 2-3 minutes

Centrifuge the sample at 12000 rpm for 15 minutes at 2-8°C

After centrifugation mixture separates into lower red organic phase, interphase and upper colorless aqueous phase, RNA resides in the upper aqueous phase



Aqueous phase is transferred to fresh microcentrifuge tubes and 1ml of isopropyl alcohol was added

Incubate at RT for 10 minutes and centrifuge at 12000 rpm for 10 minutes at 2-8°C

RNA will precipitate and forms a gel like pellet on the side and bottom of the tube

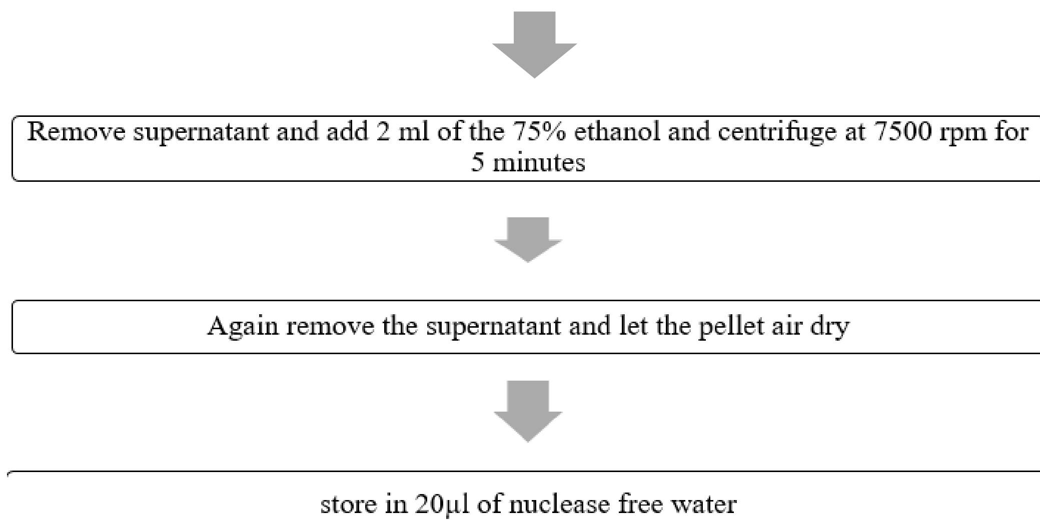


Figure3: Upper colourless phase that contains RNA, interphase and lower organic phase

3.2.5 Agarose gel electrophoresis

Once the RNA is pulled gel electrophoresis was run to check its quality and yield to some extent. For that 700 ml of 1X TAE buffer working was prepared in DEPC water from 10X TAE buffer stock. For 1.2% agarose, 1.2 grams of agarose was weighed and mixed in 100 ml of 1X TAE buffer, heated till become transparent followed by addition of 1μl of EtBr. Gel was poured in the casting tray with comb in and left to get set for 30 minutes. Once the gel is set, it was put in the buffer chamber and remaining 600ml of the buffer poured in.

For sample loading, in first well 6X DNA ladder was loaded and in the remaining wells RNA samples were loaded. Setup was ran at 100V for 45 minutes followed by visualisation of the RNA bands in gel doc. System.

Therefore, total of six different RNA were pulled, which are as follows-

- 16 hour control
- 16 hour treated with combination of SHK and AmB
- 24 hour control
- 24 hour treated with combination of SHK and AmB
- 48 hour control
- 48 hour treated with combination of SHK and AmB

- **Sample preparation-**

DNA ladder preparation- 2μl loading dye and 3μl ladder

RNA sample preparation- 2μl loading dye and 3μl RNA sample

Table1: 1 litre of 10X TAE buffer preparation (stock)

Components	Quantity
Tris	48.5 grams
Glacial acetic acid	11.4 ml
0.5 M EDTA	20 ml
DEPC water	Up to 1lt

- **700 ml of 1X TAE buffer preparation from stock-**

For preparation of 700ml of 1X working from 10X stock of TAE buffer, 70ml of the stock was added to 630ml of DEPC water.

3.2.6 cDNA synthesis

cDNA or copy DNA or complementary DNA is synthetic DNA which is transcribed from mRNA which does not contains the non-coding part of the DNA. Using enzyme named reverse transcriptase cDNA is prepared, due to that it does not contains the intronic part of the DNA. To synthesise cDNA, isolated RNA was first quantified and later cDNA was synthesise of various temporal points of treated as well as control samples using verso cDNA synthesis kit. The reaction mixture was prepared in PCR tubes.

Table2: 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 depends on the amount of RNA (in ng) present per microliter and based on that different volume of template RNA was added to the reaction mixture as cDNA is synthesised using 100 nanograms of RNA.

Table3: Cyclic program for cDNA synthesis

Steps	Temperature	Time	Number of Cycles
cDNA synthesis	42°C	30 minutes	1
Inactivation	95°C	2 minutes	1

3.2.7 Real time PCR (RT PCR)-

RT PCR is commonly used to measure the fold of gene expression. Here in this study we analysed the expression of HSP70 and HSP 90 genes of *A. terreus* at different time points of control and treated samples. 40s ribosomal protein was used as positive control as it is a house keeping gene and therefore, express all the time in the cell [34, 43]. Autoclaved distilled water was used as non-template control or NTC.

Reaction mixture was prepared in PCR vials and the total volume kept was 20μl. For each sample 40 cycles ran which took around 1 hour 32 minutes.

Reaction mixture was prepared in total of 12 PCR vials containing, 3 NTCs, 3 positive controls and 6 samples.

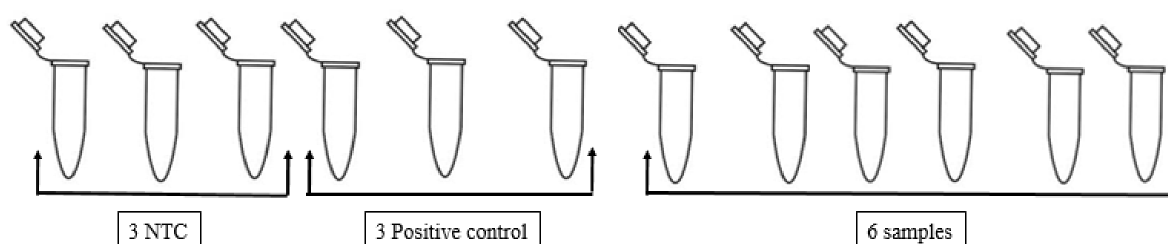


Table4: Reaction mixture concentration of different components

For 20μl of total volume.

Components	Quantity (in μl)
Green master mix	10
cDNA template	2
Primers	1 each forward and reverse

Nuclease free water	**6**

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.

Table5: Cyclic program for qRT PCR-

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

Table6: Annealing temperature of the selected primers

Primer	**Annealing temperature (in °C)**
HSP 70	65.1
HSP 90	65.1
40s Ribosomal protein	64

(Thermo Fisher Scientific)

CHAPTER 4: RESULTS AND DISCUSSION

4.1 *A. terreus* culture revival-

Culture of *A. terreus* was revived from the master culture by plating onto the PDA plates. Incubation was done at 25°C for 4-5days.

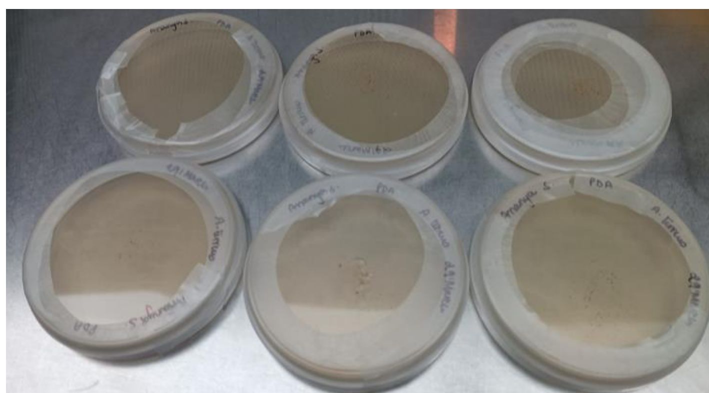


Figure4.1.1: Petri-plates with PDA inoculated with A. terreus inside LAFH

Figure4.1.2: *A. terreus* growth on 4th day

4.2 Cell counting-

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

- **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)

Total number of cells- 24 cells

Total number of viable cells- 21 cells

$$\text{Percentage of viable cells} = \frac{\text{No. of viable cells}}{\text{Total no. of cells}} \text{X100}$$

$$\text{Average number of cells per square} = \frac{\text{No. of viable cells}}{\text{No. of squares}}$$

$$\text{Dilution factor} = \frac{\text{Final volume}}{\text{Volume of cells}}$$

$$\text{Concentration (viable cells/ml)} = \text{Average number of cells per square} * \text{Dilution factor} * 10^4$$

Total cell count in 200μl appear to be **10.5 X 10^4 cells/200 μl** and **0.525 X 10^6 cells/ml.**

Once the concentration of cells known, *A. terreus* was grown in 50 ml of media containing 45 ml RPMI 1640 and 5 ml of FBS at different time points in normal conditions and in presence of shikonin and amphotericin B.

4.3 RNA extraction and band visualisation on agarose gel after electrophoresis-

RNA was extracted from normal and SHK+AmB treated fungal broth culture at three different time points (16, 24 and 48 hour).

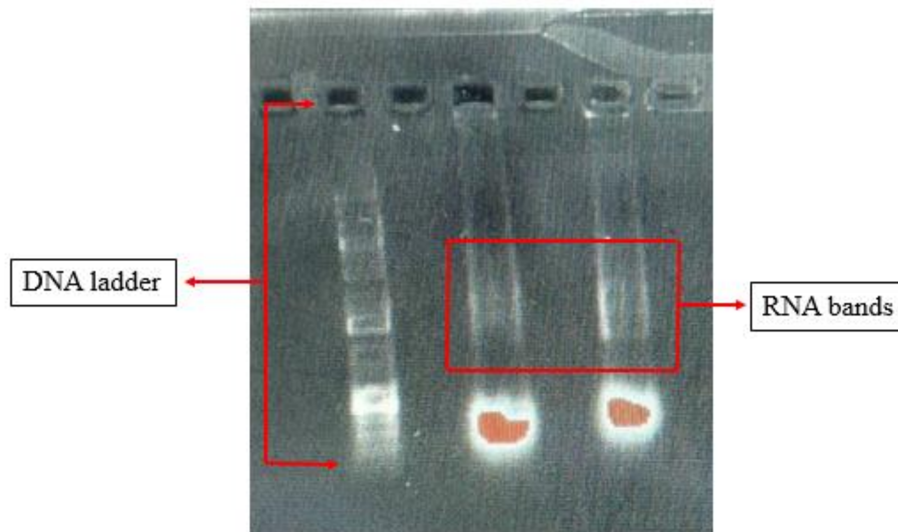
Figure4.3.1: Upper colourless phase that contains RNA, interphase and lower organic phase

- **RNA bands visualisation on 1.2% agarose gel**

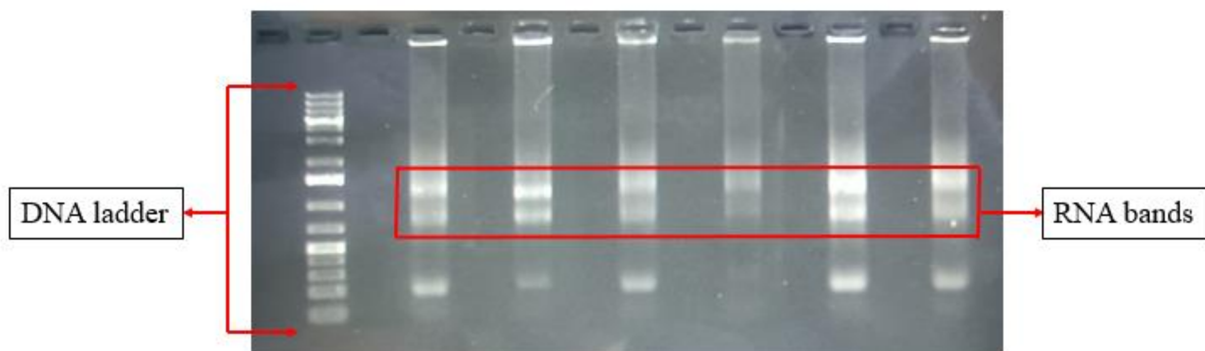
Gel electrophoresis is the most crucial technique for fractionation of RNA. As RNA is polyanion polymer thus it will migrate from negative terminal (black wired) towards the positive terminal (red wired) on the application of electric field.

1.2% 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.

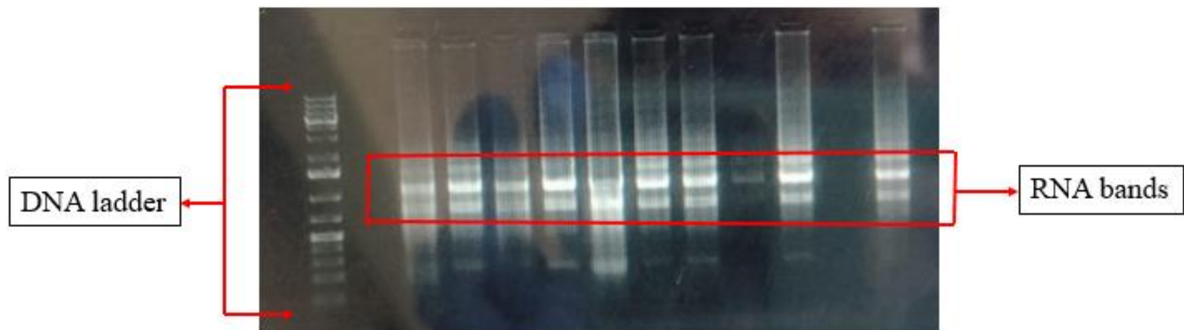
4.3.1 For 16 hour control-



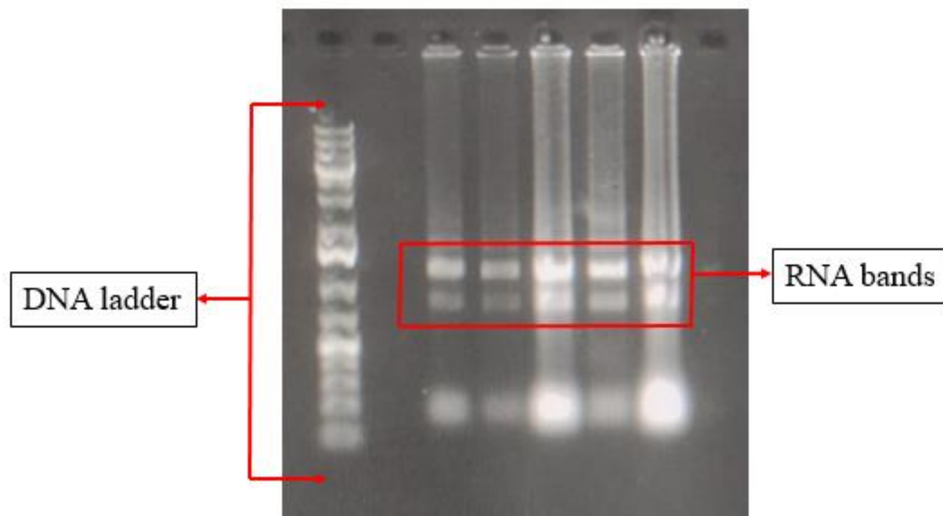
4.3.2 For 16 hour treated-



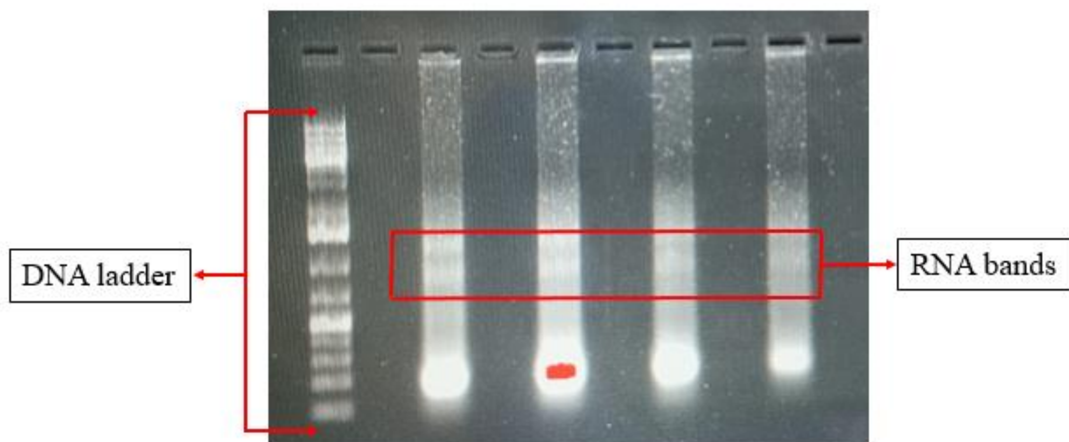
4.3.3 For 24 hour control-



4.3.4 For 24 hour treated-



4.3.5 For 48 hour control-



4.3.6 For 48 hour treated-

- **RNA quantification using spectrophotometer-**

After seeing the visible bands of the RNA, its quantification was done using spectrophotometer. Spectrophotometer analysis provide the average concentrations of the nucleic acids (DNA or RNA) present in a mixture as well as determine their purity. The analysis is based on the fact that nucleic acids absorb UV light in specific pattern, in case of DNA and RNA the absorbance take place at 260 nm. Beer-Lambert law predicts the linear change in absorbance with concentration, 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, 10µl of RNA sample to that added 990µl of distilled water was added and the optical density was determined at 260nm and 280 nm using spectrophotometer machine.

Once the OD was known, 260/280 ratio was calculated. Ratio approximately or near to 1.8 indicates the purity for DNA and ratio near to 2.0 indicates purity for RNA. If the ratio appears lower than or equal to 1.6 that may indicate presence of protein contamination.

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

$$\mathbf{40\mu g/ml * OD_{260} * Dilution\ Factor}$$

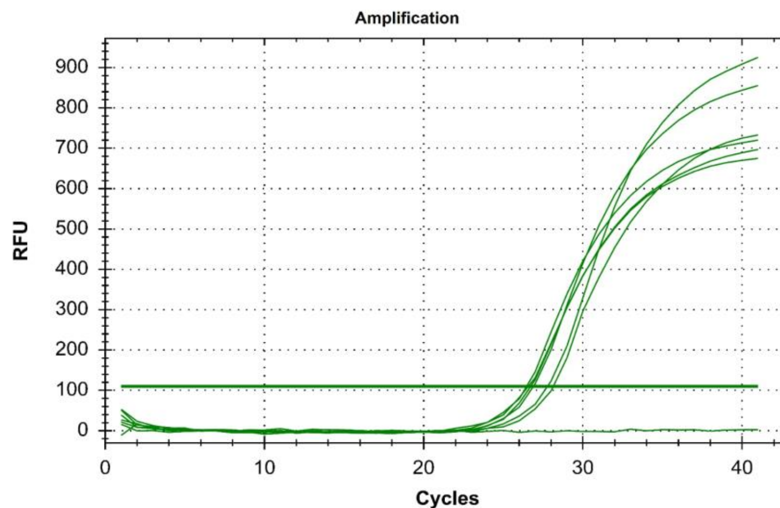
After that cDNA synthesis was carried on, based on the amount of RNA obtained as cDNA is synthesised using 100ng of the RNA.

4.4 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 (C_t) values. The reaction mixture was prepared in 12 PCR vials, containing 3 NTC, 3 positive control (40S ribosomal protein) and 6 sample vials containing samples of different time points of normal conditions as well as treated conditions.

Below are the amplification plots and C_t values for time point and condition.

4.4.1 16 hour control (HSP70)-

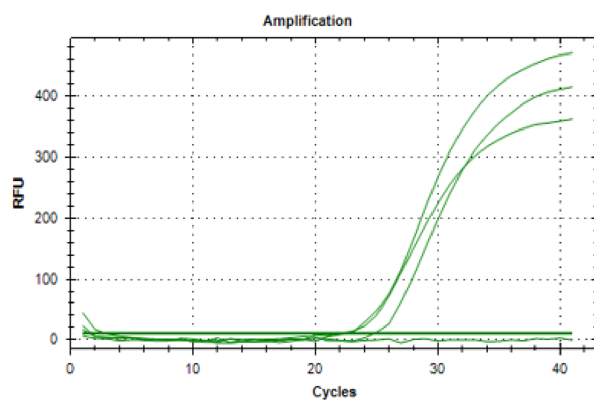


Amplification plot for 16 hour control HSP70 gene

Samples	Ct value	Ct mean
NTC	N/A	0.00
Positive control (40s RP)	26.57	27.09
Positive control (40s RP)	27.77	27.09
Positive control (40s RP)	26.89	27.09
Sample	21.64	21.86
Sample	21.28	21.86
Sample	21.78	21.86
Sample	22.12	21.86
Sample	22.31	21.86
Sample	22.02	21.86

C_t value and C_t mean of 16 hour control HSP70 gene

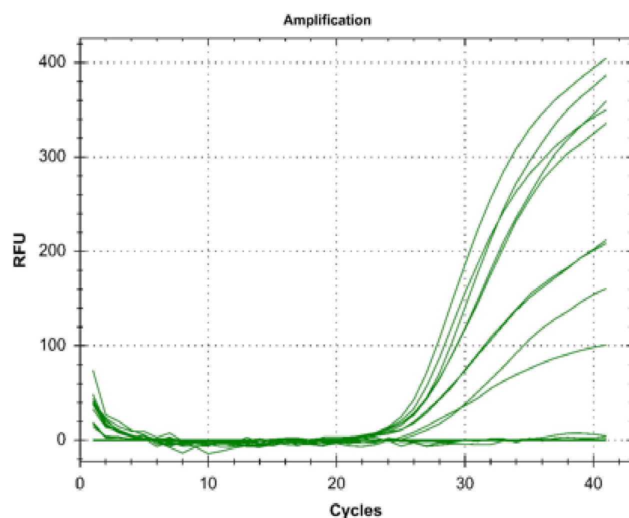
4.4.2 16 hour control (HSP 90)-



Samples	Cq	Cq mean
NTC	N/A	0
NTC	N/A	0
NTC	N/A	0
Positive control (40s RP)	26.71	27.09
Positive control (40s RP)	26.46	27.09
Positive control (40s RP)	28.14	27.09
Sample	22.38	23.09
Sample	23.61	23.09
Sample	22.75	23.09
Sample	23.9	23.09
Sample	23.56	23.09
Sample	22.37	23.09

Left shows the amplification plot and right shows the C_t values and C_t mean of 16 hour control sample for HSP 90 gene.

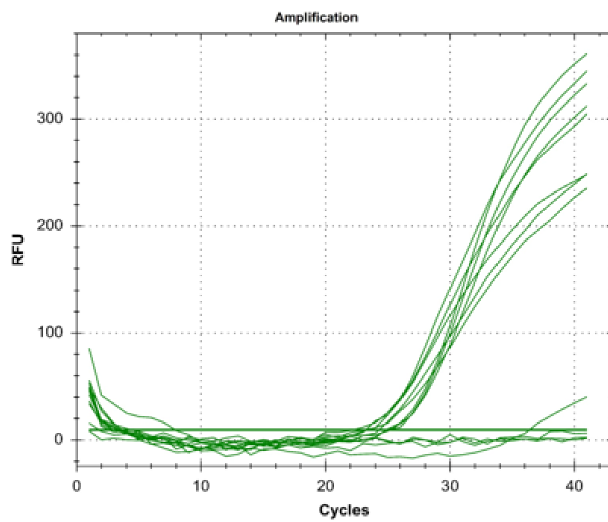
4.4.3 16 hour treated (HSP 70)-



Samples	Cq	Cq mean
NTC	N/A	0
NTC	N/A	0
NTC	N/A	0
Positive control (40s RP)	23.55	22.13
Positive control (40s RP)	18.55	22.13
Positive control (40s RP)	24.29	22.13
Sample	16.76	20.07
Sample	20.7	20.07
Sample	22.09	20.07
Sample	22.06	20.07
Sample	20.49	20.07
Sample	18.34	20.07

Left shows the amplification plot and right shows the C_t values and C_t mean of 16 hour treated sample for HSP 70 gene.

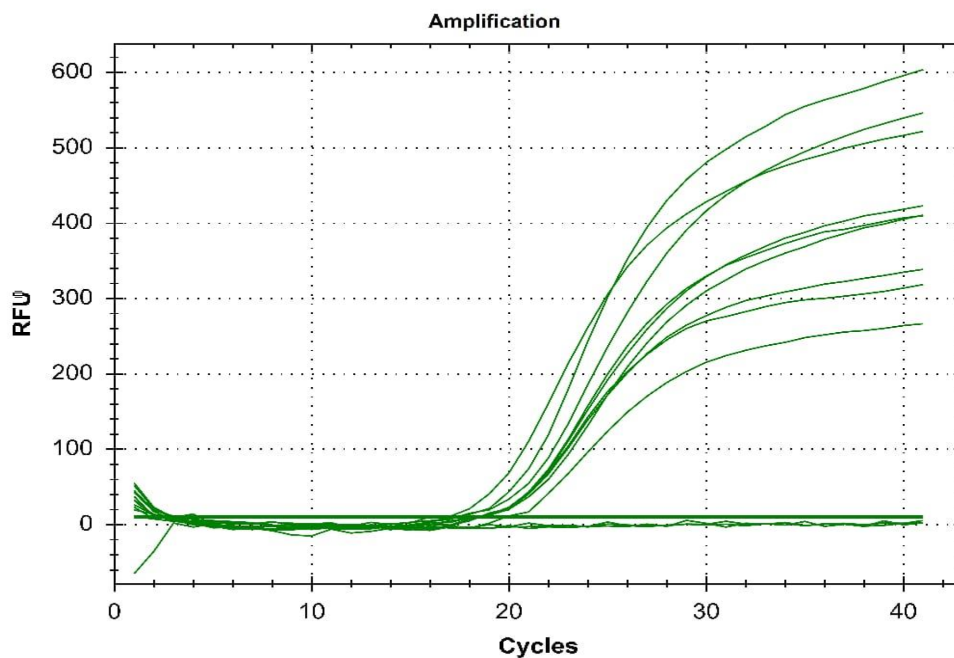
4.4.4 16 hour treated (HSP 90)-



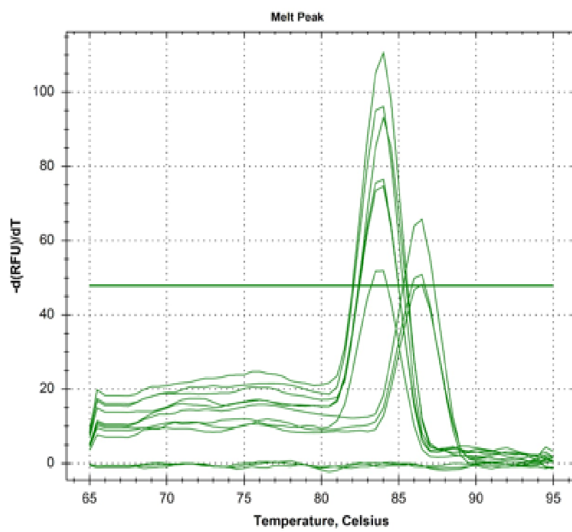
Samples	Cq	Cq mean
NTC	N/A	0
NTC	N/A	0
NTC	N/A	0
Positive control (40s RP)	25.18	24.99
Positive control (40s RP)	24.9	24.99
Positive control (40s RP)	24.9	24.99
Sample	23.09	23.2
Sample	23.23	23.2
Sample	23.34	23.2
Sample	22.2	23.2
Sample	24.3	23.2

Left shows the amplification plot and right shows the C_t values and C_t mean of 16 hour treated sample for HSP 90 gene.

4.4.5 24 hour control (HSP 70)-



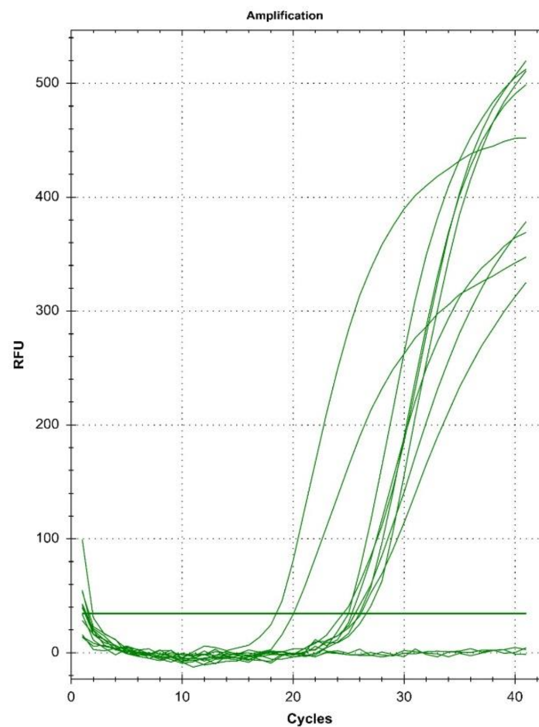
Amplification plot of 24 hour control sample for HSP 70 gene



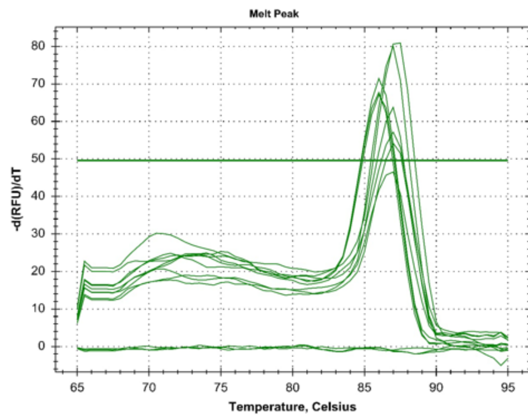
Samples	Cq	Cq mean
NTC	N/A	0
NTC	N/A	0
NTC	N/A	0
Positive control (40s RP)	18.53	18.56
Positive control (40s RP)	18.55	18.56
Positive control (40s RP)	18.61	18.56
Sample	16.37	17.98
Sample	19.89	17.98
Sample	18.4	17.98
Sample	17.42	17.98
Sample	18.39	17.98
Sample	17.43	17.98

Left shows the melt plot and right shows the C_t values and C_t mean of 24 hour control sample for HSP 70 gene.

4.4.6 24 hour control (HSP 90)



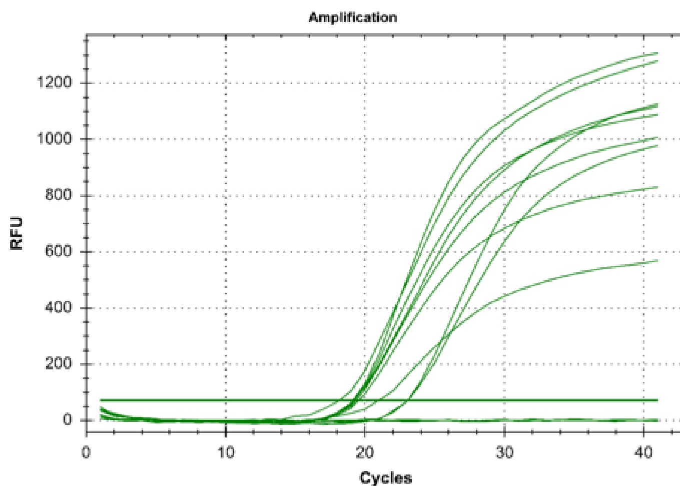
Amplification plot of 24 hour control sample for HSP 90 gene



Samples	Cq	Cq mean
NTC	N/A	0
NTC	N/A	0
NTC	N/A	0
Positive control (40s RP)	26.59	25.69
Positive control (40s RP)	24.5	25.69
Positive control (40s RP)	25.98	25.69
Sample	26.11	23.4
Sample	25.29	23.4
Sample	25	23.4
Sample	25.59	23.4
Sample	20.04	23.4
Sample	18.47	23.4

Left shows the melt plot and right shows the C_t values and C_t mean of 24 hour control sample for HSP 90 gene.

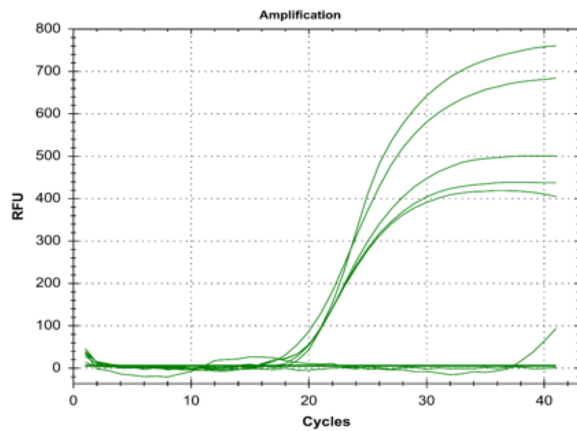
4.4.7 24 hour treated (HSP 70)-



Samples	Cq	Cq mean
NTC	N/A	0
NTC	N/A	0
NTC	N/A	0
Positive control (40s RP)	20.98	19.03
Positive control (40s RP)	13.05	19.03
Positive control (40s RP)	23.06	19.03
Sample	19.55	19.1
Sample	18.16	19.1
Sample	19.35	19.1
Sample	19.17	19.1
Sample	19.14	19.1
Sample	19.23	19.1

Left shows the amplification plot and right shows the C_t values and C_t mean of 24 hour treated sample for HSP 70 gene.

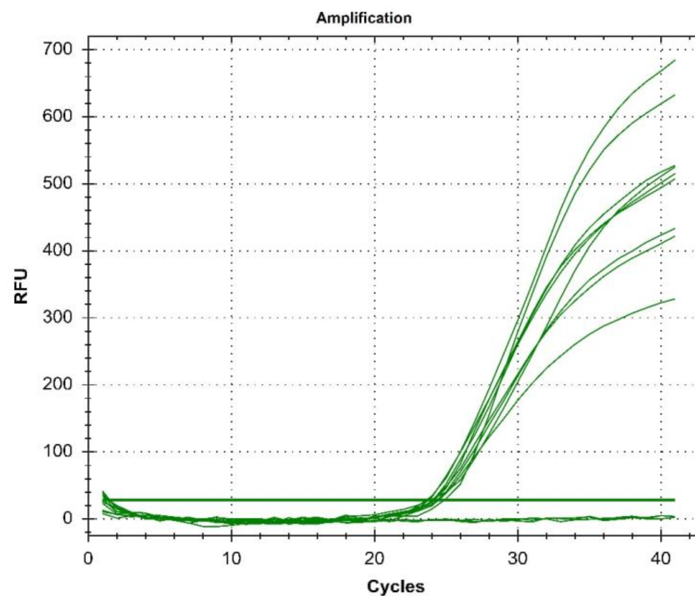
4.4.8 24 hour treated (HSP 90)-



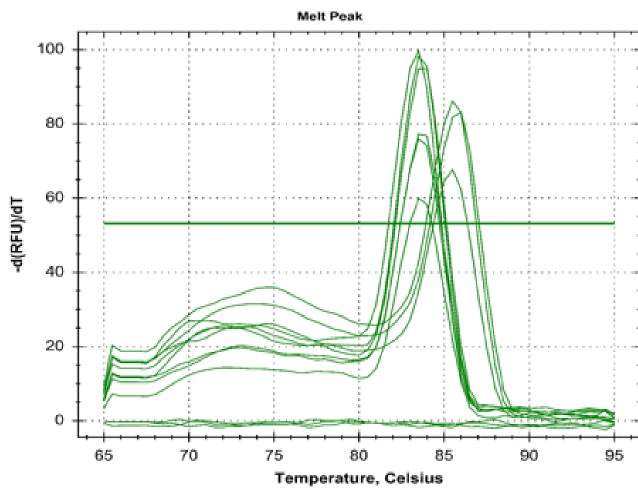
Samples	Cq	Cq mean
NTC	N/A	0
NTC	N/A	0
NTC	N/A	0
Positive control (40s RP)	20.98	19.03
Positive control (40s RP)	13.05	19.03
Positive control (40s RP)	23.06	19.03
Sample	15.6	16.326
Sample	15.69	16.326
Sample	15.51	16.326
Sample	18.08	16.326
Sample	16.75	16.326

Left shows the amplification plot and right shows the C_t values and C_t mean of 24 hour treated sample for HSP 90 gene

4.4.9 48 hour control (HSP 70)-



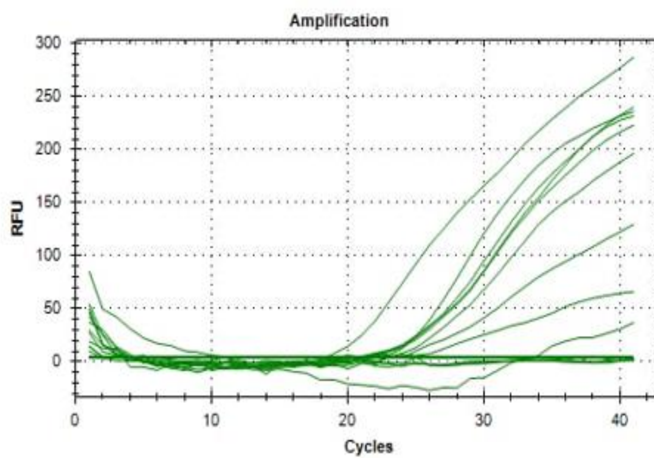
Amplification plot of 48 hour control sample for HSP 70 gene



Samples	Cq	Cq mean
NTC	N/A	0
NTC	N/A	0
NTC	N/A	0
Positive control (40s RP)	24.88	24.28
Positive control (40s RP)	23.62	24.28
Positive control (40s RP)	24.36	24.28
Sample	24.11	24.08
Sample	24.47	24.08
Sample	23.77	24.08
Sample	24.19	24.08
Sample	23.92	24.08
Sample	24.07	24.08

Left shows the melt peak and right shows the C_t values and C_t mean of 48 hour control sample for HSP 70 gene

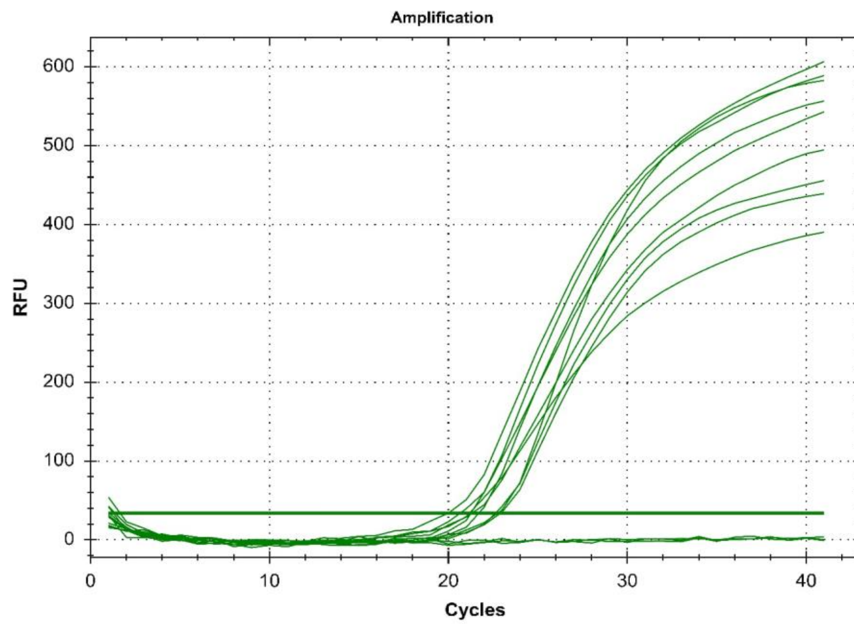
4.4.10 48 hour control (HSP 90)-



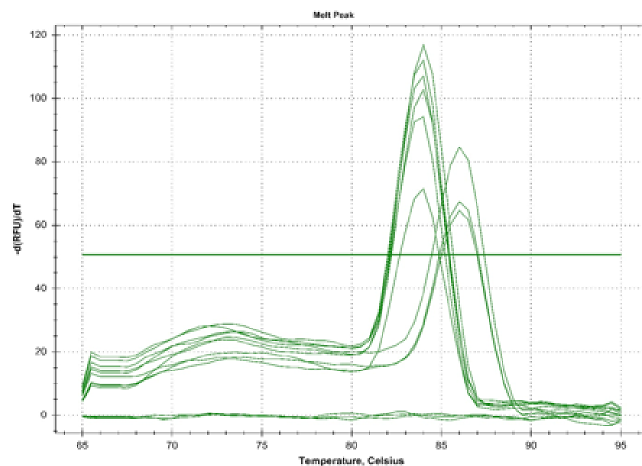
Samples	Cq	Cq mean
NTC	N/A	0
NTC	N/A	0
NTC	N/A	0
Positive control (40s RP)	18.3	20.81
Positive control (40s RP)	22.98	20.81
Positive control (40s RP)	21.17	20.81
Sample	24.04	21.4
Sample	20.84	21.4
Sample	19.13	21.4
Sample	22.89	21.4
Sample	20.42	21.4

Left shows the amplification plot and right shows the C_t values and C_t mean of 48 hour control sample for HSP 90 gene

4.4.11 48 hour treated (HSP 70)-



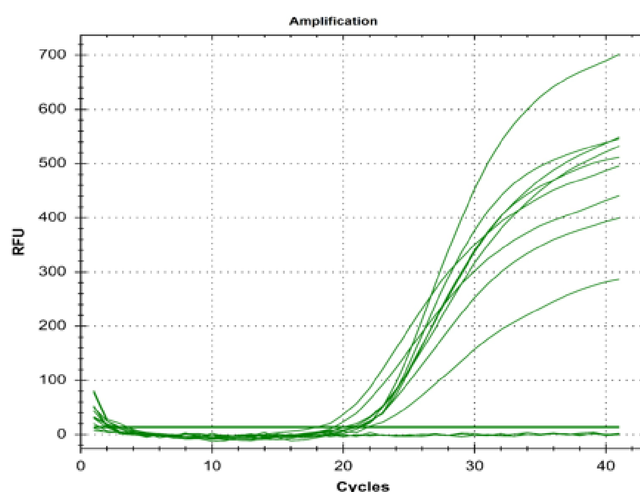
Amplification plot of 48 hour treated sample for HSP 70 gene



Samples	Cq	Cq mean
NTC	N/A	0
NTC	N/A	0
NTC	N/A	0
Positive control (40s RP)	22.92	25.62
Positive control (40s RP)	22.75	25.62
Positive control (40s RP)	22.62	25.62
Sample	21.32	21.02
Sample	21.18	21.02
Sample	21.26	21.02
Sample	20.66	21.02
Sample	21.7	21.02
Sample	20	21.02

Left shows the melt peak and right shows the C_t values and C_t mean of 48 hour treated sample for HSP 70 gene

4.4.12 48 hour treated (HSP 90)-



Samples	Cq	Cq mean
NTC	N/A	0
NTC	N/A	0
NTC	N/A	0
Positive control (40s RP)	21.34	21.13
Positive control (40s RP)	21.05	21.13
Positive control (40s RP)	21	21.13
Sample	21.01	20.11
Sample	21.84	20.11
Sample	19.99	20.11
Sample	19.92	20.11
Sample	19.95	20.11
Sample	17.98	20.11

Left shows the amplification plot and right shows the C_t values and C_t mean of 48 hour treated sample for HSP 90 gene

4.5 C_t value calculation using double delta Ct method-

To obtain the expression profile, expression fold and standard deviation of HSP 70 and HSP 90 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:

ΔCt value = Average Ct value of gene of interest – Average Ct value of House keeping gene

ΔΔCt value = ΔCt value of treated – ΔCt value of control

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

Table 7: Double delta Ct values for untreated and treated samples at different time points-

16 hour

HSP70				
Sample	**Average Ct value**	**ΔCt value**	**ΔΔCt value**	**2^-(ΔΔCt)**
Control (untreated)	21.85	-5.231	1.743	0.298
Treated	20.07	-3.488		
HSP90				
Sample	**Average Ct value**	**ΔCt value**	**ΔΔCt value**	**2^-(ΔΔCt)**
Control (untreated)	23.09	-3.995	3.665	0.078
Treated	23.23	0.329		

24 hour

HSP70				
Sample	**Average Ct value**	**ΔCt value**	**ΔΔCt value**	**2^-(ΔΔCt)**
Control (untreated)	17.983	-2.064	-0.855	1.809
Treated	19.1	-2.92		
HSP90				
Sample	**Average Ct value**	**ΔCt value**	**ΔΔCt value**	**2^-(ΔΔCt)**
Control (untreated)	25.497	5.45	-11.144	2262.967
Treated	16.326	-5.694		

48 hour

HSP70				
Sample	**Average Ct value**	**ΔCt value**	**ΔΔCt value**	**2^-(ΔΔCt)**
Control (untreated)	24.088	1.536	-2.463	5.514
Treated	21.02	-0.926		
HSP90				
Sample	**Average Ct value**	**ΔCt value**	**ΔΔCt value**	**2^-(ΔΔCt)**
Control (untreated)	21.464	-1.087	-0.744	1.674
Treated	20.115	-1.831		

After the calculations of $\Delta\Delta C_t$ values for all the samples, the graph for relative expression fold for HSP70 and 90at all three time points was plotted.

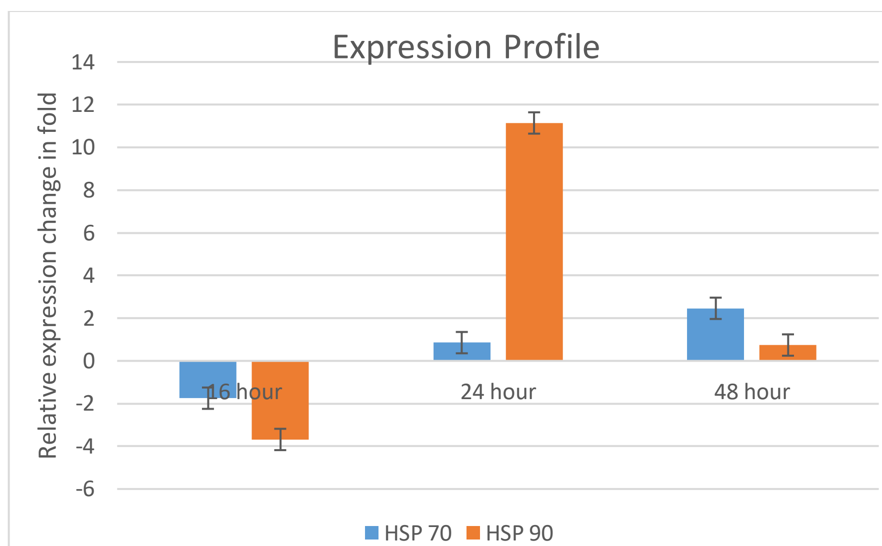


Figure 4.5.1: Relative gene expression of HSP70 and 90 at different time points. (This data represent treated (SHK+AmB) versus control (normal condition) values

It was seen that expression fold for HSP90 was highest at 24 hour and the expression of HSP70 was highest at 48 hour. Expression fold for both the genes HSP70 and 90 was seen downregulated at 16 hour time point, which suggested that at 16 hour time point they were slightly expressed.

CHAPTER 5: CONCLUSION

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 which belongs to polyene class of antifungals and have been used for almost 50 years to treat fungal infection. But *A. terreus* shows intrinsic resistance towards this. On the other hand shikonin which is a plant derived anti-fungal agent shown to increase the efficiency of other antifungal drugs.

Therefore, in this study the expression of heat shock protein 70 and heat shock protein 90 of *A. terreus* was compared between normally grown cells and cells that are grown in combination of shikonin and amphotericin B to determine the effect of shikonin on amphotericin B.

Data generated from RT PCR suggested that at 16 hour there is not much difference between the expressions of HSP70 and 90 may be due to the initiation of germination stage of the fungal cells. At 24 hour there is a decrease in HSP90 gene of drug treated condition may be due the drug action. Although at 48 hour expression of both HSP90 and 70 is less as most concentration of the drug has been consumed till the time. But increase in the expression of HSP90 at 24 hour suggested that this drug combination is working. The clear data can be obtained by using the HSP90 inhibitor geldanamycin for future approach as extension of this study.

CHAPTER 6: REFERENCES

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