Temporal Transcriptional Analysis of Genes Encoding Heat Shock Protein Family in *Aspergillus flavus* Treated With and Without Shikonin

Thesis submitted in partial fulfillment of the degree of

MASTER OF SCIENCE

IN

MICROBIOLOGY

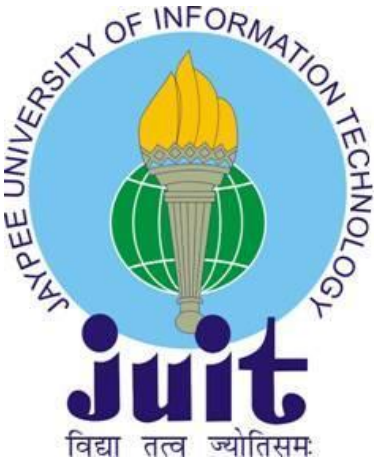
Submitted by:

MEGHA RANI

Enrolment number- 217853

Under the guidance of:

PROF. (DR.) JATA SHANKAR



JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY,

WAKNAGHAT, HIMACHAL PRADESH, INDIA

SELF - DECLARATION

I, **Megha Rani**, student of M.Sc. Microbiology, Jaypee University of Information Technology, Waknaghat, Solan, Himachal Pradesh do declare that work reported in the MSc. thesis entitled "**Temporal transcriptional analysis of genes encoding heat shock protein family in *Aspergillus flavus* treated with and without shikonin**" submitted at Jaypee University of Information Technology, Solan, India, is an authentic record of my work carried out under the supervision of **Prof. (Dr.) Jata Shankar**. I have not submitted this work elsewhere for any other degree or diploma. I am fully responsible for the contents of my MSc. thesis.

Megha Rani

Date:

Enrollment Number-217853

Department of Biotechnology and Bioinformatics

Jaypee University of Information Technology, India

CERTIFICATE

This is to certify that the work reported in the MSc thesis entitled "**Temporal transcriptional analysis of genes encoding heat shock protein family in *Aspergillus flavus* treated with and without shikonin**" submitted by Megha Rani at Jaypee University of Information Technology, Solan, India, is a bonafide record of her original work carried out under my supervision. This work has not been submitted elsewhere for any other degree or diploma.

Supervisor:

Prof. (Dr.) Jata Shankar Date -

Department of Biotechnology &Bioinformatics

Jaypee University of Information Technology, India

ACKNOWLEDGMENT

This thesis would not have been possible without the guidance and the help of several individuals who in one way or another contributed their valuable time, assistance & support in the preparation and completion of this study. It is pleasure to convey my gratitude to them all in my humble acknowledgement.

First and foremost, it gives me immense pleasure and privilege to express gratitude to my supervisor **Prof. (Dr.) Jata Shankar**. I would like to acknowledge him for the dedication, perseverance, trust and availability throughout the year. He has inspired me to become an independent researcher by allowing me the room to work in my own ways and made me realise the power and importance of critical thinking and logical reasoning in research. One simply could not wish for supportive and caring supervisor. I am indebted to him more than he may know.

I would like to thank **Prof. (Dr.) Sudhir Syal (HOD)**, Dept. of BT & BI, JUIT, Waknaghat (H.P) for allowing me to carry out this project. He's always been an immense support and guided us all through the right path for being a true researcher. He's extremely helpful and understanding.

Foremost, I want to express my deep sense of gratitude and devotion to **Dr. Sonia Kumari** (Assistant professor, Chandigarh University) for her continuous assistance and cooperation along the journey. I really appreciate how enthusiastically and calmly she shared her knowledge and spare some of her invaluable time for me. The guidance she has given me will always be remembered. I wish I had time to learn more from her.

I am deeply grateful to **Mrs. Sonika Gupta,** and **Mrs. Mamta Mishra** for their immediate availability to guide me in this work. They truly demonstrate what a brilliant and hard-working lab assistants can accomplish.

Last but not least, I would like to thank my family, specially my family & friends with all of my heart. Nothing I write can describe what they mean to me. I am eternally grateful for all the love, patience, blessings, encouragement and continuous kind support I got from them. I deeply appreciate their presence throughout my difficult times, for their advices and to believing me that it is possible to overcome all the challenges. They're my greatest strength and source of inspiration.

All may not be mentioned, but no one is forgotten

Contents

LIST OF FIGURES

List of tables

LIST OF ABBREVIATIONS

i. Da - Dalton
ii. CPA - Chronic pulmonary aspergillosis
iii. ABPA- Allergic bronchopulmonary aspergillosis
iv. AF - Acute aflatoxicosis
v. CF - Chronic aflatoxicosis
vi. SHK – Shikonin
vii. PBS – Phosphate Buffer Saline
viii. PBST – Phosphate Buffer Saline Tween 20
ix. EDTA – Ethylene Diamine Tetra-acetic acid
x. TAE – Tris-acetate-EDTA
xi. OD – Optical density
xii. µl - Microlitre
xiii. Ng – Nanogram
xiv. RT PCR – Real time Polymerase Chain Reaction
xv. NTC – Non Template Control
xvi. Δ – Delta
xvii. ΔΔ – Double delta

Abstract

Aspergillus flavus is one of the predominant species under *Aspergillus* genera. They grow in colonies and has yellowish and greenish spore. Three morphological stages of *Aspergillus flavus* are conidia, hyphae, and mycelia. They are saprophytic, ubiquitous, and mainly responsible for producing many mycotoxins. One such toxin is aflatoxin. Aflatoxin is highly carcinogenic substances that have the potential to contaminate food and feed crops, resulting in significant health implications for both humans and animals. Exposure to aflatoxin has been found to result in gastrointestinal disorders, including but not limited to aspergillosis, aflatoxicosis, cancer, and other diseases like diarrhea, dysentery, and colitis. Around 83% of deaths are due to hepatocellular carcinoma in East and sub- Sahara Africa. Nowadays, it is seen that fungi are becoming resistant to traditional drugs, so scientists are looking for new alternatives. Phytochemicals such as ascorbic acid, gallic acid, quercetin, and caffeine are reported to show positive results against various fungal species. Shikonin is one such phytochemical present in the roots of *Lithospermum erythrorhizon*. Interest of researchers has dramatically increased regarding the beneficial properties of shikonin over the past few years. In this project, we tried to see the effect on the expression of heat shock protein when A. flavus was treated with shikonin (MIC = 1.5µg/ml). Heat shock proteins, known as stress proteins, are molecular chaperones that help in protein folding or unfolding of protein aggregates. Heat shock proteins also participate in the morphogenesis of *A. flavus* and can act as new drug targets.

CHAPTER 1**: INTRODUCTION**

Aspergillus flavus belongs to the phylum Ascomycota. Ascomycota encompasses a vast array of over 64,000 species that have been identified on a global scale. *A. flavus* display various morphological structures, ranging from unicellular yeasts to complex fruiting bodies. They are characterised by the mycelial body composed of hyphae (branching filaments) that interconnect to form a complex network. The mycelium constitutes the fungal's vegetative component and is responsible for assimilating nutrients from the surroundings.

A. flavus exhibit a broad distribution across various habitats, encompassing terrestrial, aquatic, and biotic environments such as soil, plant, and animal tissues. Certain species, such as *A. terrus, A. fumigatus, A. parasiticus* have been identified as pathogens, inducing illnesses in flora and fauna, whereas others establish symbiotic associations with their hosts. *A. flavus* exhibit a global distribution and can thrive in diverse climatic regions. Certain species exhibit adaptations that enable them to thrive in harsh environments, including but not limited to arid deserts, geothermal hot springs, and Polar region. These organisms fulfil crucial ecological functions as decomposers (saprophytes) and facilitators of nutrient cycling. The life cycle of *A. flavus* is distinguished by the generation of both sexual and asexual spores, which serve significant functions in the propagation of the fungus. The production of conidia facilitates asexual reproduction synthesised within distinct conidiophores. Conidia are frequently dispersed through various mechanisms, including wind, water and can sprout and establish new colonies. The process of sexual reproduction in *A. flavus* involves the fusion of two haploid nuclei, resulting in the formation of a diploid zygote. This zygote then undergoes meiosis, leading to the production of four haploid nuclei. The nuclei undergo mitosis and subsequently divide into eight haploid ascospores. These ascospores are enclosed within the ascus and discharged into the surroundings, thereby instigating the formation of new colonies. *A. flavus* play a significant role as a plant pathogen, inducing various diseases in crops and other plant species such as maize, peanuts, and grains. *Aspergillus flavus* is a widely distributed filamentous fungus that can synthesise carcinogenic secondary metabolites, such as aflatoxin. The carcinogenic and mutagenic properties of these toxins, along with their capacity to induce liver damage and other health issues, render them a significant public health concern. Apart from producing aflatoxin, *Aspergillus flavus* is recognised for its ability to generate other secondary metabolites, including cyclopiazonic acid and aspergillic acid. The association of these metabolites with various health complications, such as neurological and gastrointestinal disorders, has been established. The capacity of *Aspergillus flavus* to synthesise aflatoxin has been thoroughly investigated owing

to their detrimental effects on human health. Aflatoxin is synthesised by *A. flavus* as a protective response to antagonistic microorganisms. However, they have the potential to contaminate food commodities and elicit morbidity in both humans and animals. Ingesting food items contaminated with aflatoxin has been associated with various health issues, such as liver cancer, hepatitis, and immune system suppression. Aflatoxin is a significant risk factor for compromised growth and cognitive development in children, rendering them particularly susceptible to its adverse effects. Various techniques have been devised to reduce the adverse consequences of *Aspergillus flavus* infestation, such as the application of fungicides, the implementation of biocontrol agents, and the genetic manipulation of crops. Identifying and quantifying *Aspergillus flavus* and its associated metabolites is paramount in guaranteeing the safety of food products. Since fungi are becoming resistant to azoles, echinocandins, and amphotericin B (mainly targeting the cell membrane and cell wall of the fungus), it is necessary to look for other options. Phytochemicals such as Gallic acid, Ascorbic acid, Quercetin, and Caffeine shows a promising decrease in the growth of *Aspergillus* species. Shikonin is one such phytochemical that shows promising results as an antifungus. Shikonin is derived from the roots of *Lithospermum erythrorhizon*. In fungi, Heat shock protein family plays a vital role in morphogenesis. Previously it is reported that Hsp70 and Hsp90 together or individually play important role filamentation of *Aspergillus* species. Hsp90, with the help of Hsp70, maintains the integrity of fungal cell walls as Hsp90 regulates calcineurin. Previously, it has been reported that inhibition of heat shock proteins reduces hyphal growth, spore count, and cell wall inhibition. Keeping this in view, inhibiting morphogenesis of *Aspergillus flavus* via heat-shock protein with shikonin needs further investigation.

\

CHAPTER 2: **REVIEW OF LITERATURE**

2.1 An opportunistic pathogen

Aspergillus genera have around 500 species and are saprophytic and ubiquitous [1]. In 1809, Link reported that after *Aspergillus fumigatus*, *Aspergillus flavus* is the predominant species under *Aspergillus* genera. *Aspergillus flavus* is a filamentous fungus. They grow mainly in soil and decomposing botanical matter and have been observed to generate diverse mycotoxins, such as aflatoxin [2]. Aflatoxin is highly carcinogenic substances that have the potential to contaminate food and feed crops, resulting in significant health implications for both humans and animals. *Aspergillus flavus* is mainly responsible for causing rot in fruits like peaches and crops such as cottonseed, peanut, and maize [3].

Table 2.1 Biological nomenclatures

Binomial name	*Aspergillus flavus*
Kingdom	Fungi
Division	Ascomycota
Class	Eurotiomycetes
Order	Eurotiales
Family	Trichocomaceae
Genus	*Aspergillus*
Species	*A. flavus*

Aspergillus flavus is classified as an opportunistic pathogen, indicating its propensity to induce illness in individuals with compromised immune function. Exposure to *A. flavus* generally does not elicit any symptoms in individuals who are in good health. Nonetheless, *A. flavus* can potentially induce various infections, such as invasive aspergillosis, chronic pulmonary aspergillosis, and aspergilloma, in immunocompromised individuals, such as those with HIV/AIDS or cancer [4]. The most severe manifestation of *Aspergillus flavus* infection is invasive aspergillosis, which exhibits a significant mortality rate. The disease is commonly observed in individuals who have undergone organ transplantation, chemotherapy, or other forms of immunosuppressive therapy [4].

2.2 Geographical distribution and ecology

Aspergillus flavus are distributed worldwide. This is due to the fact that conidia of *Aspergillus flavus* with the aid of air easily disperse in the environment [5]. *Aspergillus flavus* frequently present in terrestrial substrates, decomposing organic matter, and enclosed spaces such as residential and commercial structures. *Aspergillus* species such as *A. flavus and A. paraciticus* are frequently linked to food spoilage and produce mycotoxin like aflatoxin that pose a threat to both plants and humans [6]. Certain species exhibit a higher prevalence in tropical and subtropical regions, whereas others are predominantly distributed in temperate regions. For its optimum growth, they require hot and humid environment with water activity of 0.86 to 0.92. *Aspergillus flavus* is frequently detected in regions with elevated temperatures and humidity levels, whereas *Aspergillus niger* is predominantly present in moderate climatic zones [6]. *Aspergillus* spp has the ability to endure diverse environmental conditions, such as extreme pH levels, elevated salinity, and high temperatures. *A. fumigatus*, being a prevalent *Aspergillus* species is frequently detected in soil, compost, and deteriorating organic matter.

Due to climatic variation in India like hot climate (50°C) in Rajasthan, humid and hot weather in Uttar Pradesh and Punjab makes a perfect environment for the growth of *Aspergillus* species [7]. In 1974, *Aspergillus flavus* contaminated the corn which resulted in hepatitis in large population of tribes in Rajasthan and Gujarat. Around 106 people died in 105 villages due to hepatitis and all because of the consumption of corn contaminated with aflatoxin, produced by *Aspergillus flavus* [8]. Apart from western part of India, aflatoxin outbreaks are also seen in Andhra Pradesh. In year 1994, around 2 billion chicken died in Andhra Pradesh [8,9]. *A. flavus* has developed adaptations to thrive in environments characterised by high temperatures and humidity, enabling it to proliferate at temperatures as high as 45°C.

2.3 Morphology

The morphological features of *Aspergillus* are known to exhibit variations across different species, apart from certain fundamental features that are commonly observed within this taxonomic group [10]. *Aspergillus* is characterised by a fundamental structure comprising of a multicellular mycelium, which is comprised of hyphae. Hyphae are thread like structure exhibit both vertical and horizontal growth, thereby establishing an interconnected network

capable of spreading across a given surface. Certain species of *Aspergillus* have the capability to generate ascospores, which are sexual spores, in addition to conidia [11]. *Aspergillus* species has the capacity to generate diverse spores, which can serve as a means of distinguishing between the various species. Conidia are the predominant type of spores produce by *Aspergillus*. The morphological characteristics of conidia, including variations in colour and size, are frequently employed for the purpose of distinguishing between various *Aspergillus* species [10]. *Aspergillus flavus* grow in colonies and has yellowish and greenish spore. Three morphological stage of *Aspergillus flavus* are conidia, hyphae and mycelia.

2.3.1 Macroscopic morphology:

Colonies of *A. flavus* exhibit a characteristic greenish-yellow to yellow-green hue and surface of the colony exhibit a powdery appearance. The diameter of the colony may vary between 1-10 cm, depend upon the availability of nutrients, temperature, and other environmental factors [12].

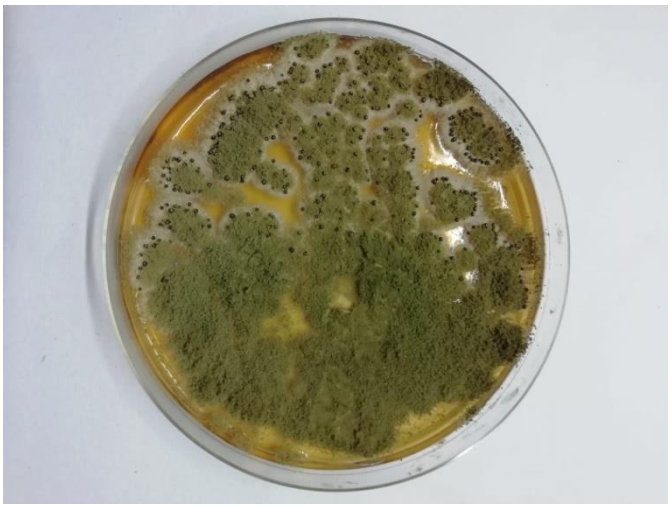


Figure 2.1 *Aspergillus flavus* MTCC 9367

2.3.2 Microscopic morphology:

A. flavus is a filamentous fungus comprised of branching hyphae that form a mycelium. The diameter of the hyphae is within the range of 3-5 µm, and they have the potential to extend to a length of several centimetres. Hyphae may exhibit either septate or aseptate characteristics, indicating the presence or absence of cross walls [12].

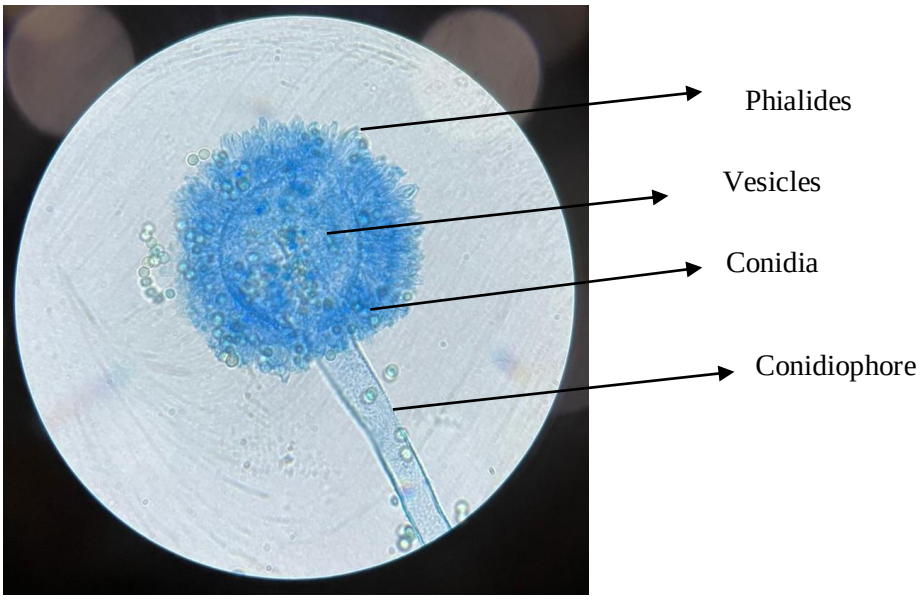


Figure 2.1 Microscopic view of *Aspergillus flavus* MTCC 9367 at 100x

2.4 Aflatoxin production and prevention

Mycotoxins are a class of poisonous secondary metabolites that are synthesised by specific fungi and have the potential to contaminate food and feed crops. Aflatoxin is one such mycotoxin produced by *Aspergillus* species which is highly toxic and carcinogenic. It is majorly produced by *Aspergillus flavus* and *Aspergillus paraciticus*. Other species like *Aspergillus terrus* and *Aspergillus fumigatus* also produce aflatoxin [13]. They pose a great threat to human and animal health due to their potential to induce unfavourable outcomes such as acute toxicity, cancer, immunosuppression, and neurotoxicity. Presence of mycotoxins in agricultural products can result in diminished crop yield and quality, thereby causing economic losses. Aflatoxin is categorised into four categories B1, B2, G1, and G2. They are categorised on the basis of different fluorescence they produce. The potency of mutagenicity, carcinogenicity, and toxicity of aflatoxin are as follows: B1> G1>B2>G2. It is

mainly determined by LD50 [14]. After formation, aflatoxins can covalently attach to DNA and other macromolecules within the organism, ultimately damaging cellular and tissue. The harm caused by this can result in the development of liver cancer, hepatitis, and various other health consequences in humans and animals. Several frequently encountered mycotoxins are aflatoxins, ochratoxins, fumonisins, and deoxynivalenol. Ochratoxins are known mycotoxins synthesised mainly by *Aspergillus* and *Penicillium* fungi and are commonly detected in cereals, coffee, and wine [15]. Molecular characterization of all four aflatoxins is presented in Table 2.2.

Table 2.2 Molecular characterization of four types of aflatoxin

Aflatoxin	**Molecular weight**	**Fluorescence (365 nm)**
AFBI ($C1_7H_{12}O_6$)	312 Da	Blue
AFG1 **(**$C1_7H_{12}O_7$)	328 Da	Yellow green
AFB2 ($C_{17}H_{14}O_6$)	314 Da	Blue
AFG2 ($C_{17}H_{14}O_7$)	330 Da	Yellow green

2.5 Diseases caused by *A. flavus*

Spores of *Aspergillus flavus* are widely distributed in the environment and can contaminate crops at all production stages, from pre-harvest to post-harvest [16]. Aflatoxin contamination is commonly observed in crops such as maize, peanuts, cottonseed, and tree nuts. However, it can also contaminate crops, including spices, figs, and dried fruits [17]. Aflatoxin is synthesised by fungal organisms during crop cultivation and preservation, resulting in contamination. Following the harvest of crops, fungi have the potential to persist and generate aflatoxins throughout the storage phase. [18]. Aflatoxins undergo hepatic metabolism, leading to hepatocellular injury and elevated susceptibility to hepatocellular

carcinoma. The prolonged exposure to aflatoxins has been associated with the emergence of hepatocellular carcinoma, a variant of liver cancer, among individuals. Aflatoxin B1, the most potent and widely distributed type of aflatoxin, has been categorised as a Group 1 carcinogen by the International Agency for Research on Cancer (IARC) [14].

Exposure to aflatoxins has been found to potentially suppress the immune system potentially, thereby increasing an individual's susceptibility to infections. Aflatoxin has been observed to exhibit immunosuppressive properties in both animals and humans, which can result in heightened vulnerability to infectious ailments. Aflatoxin has the potential to hinder the growth and development of children. Research has indicated that prenatal exposure to aflatoxins may result in adverse outcomes such as reduced birth weight, impaired growth, and developmental setbacks in offspring. Aflatoxins can potentially hinder the growth and development of juvenile animals [19,20]

2.5.1 Aspergillosis

Aspergillosis mainly related to lungs and affect respiratory and central nervous systems. It is classified as allergic, invasive, saprophytic and colonizing and result in bronchopulmonary aspergillosis, allergic alveolitis and asthma. It affects individuals with chronic diseases, immune compromised system, HIV/AIDS, organ transplant recipients and chemotherapy patients. Spores enter human body mainly through breathing and cuts on body and infection. Recent studies showed that Aspergillosis is mainly caused by *Aspergillus flavus* in 65% of the children. [21]. Aspergilloma sometimes also result in thoracic discomfort, dyspnea and hemoptysis, [22]. Aspergillosis when not treated initially, it can spread over to other parts of body including brain, kidney, liver and may cause death of the patient [23].

2.5.2 Cancer

Aflatoxin is carcinogenic in nature and one of the most toxic mycotoxin produce by *Aspergillus* species. Not only human but animals are also affected by this mycotoxin. AFB1 out of other four aflatoxin is most carcinogenic in nature and mainly responsible for causing hepatocarcinogen and also induces tumor formation in colon, kidney and lung in human as well as in animals. Liu and Wu reported that around 4.6% - 28.2% of hepatocellular carcinoma in world is because of aflatoxin exposure [24]. Countries like China and East

Africa are majorly affected by hepatocellular carcinoma. 83% of deaths are due to hepatocellular carcinoma in East Africa and sub- Sahara Africa [25].

2.5.3 Aflatoxicosis

Aflatoxicosis is mainly related to hepatic disorder. It majorly affects the liver of human as well as animals. Aromatic amino acids (phenylalanine, tyrosine and tryptophan) and branch chain amino acid (valine, leucine and isoleucine) are two important factors which mediate the neurotransmission, which play a very important role in proper performance of liver. Over consumption of aflatoxin leads to disturbance in the balance of aromativ amino acid and branch chain amino acid [26]. This cause hepatic diseases, bleeding, oedema, mental changes, degradation of heme and jaundice. Aflatoxicosis are majorly categorised into two types: acute aflatoxicosis and chronic aflatoxicosis [27].

2.5.3.1 Acute aflatoxicosis

Acute aflatoxicosis (AF) is mainly caused by exposure to high concentration of aflatoxin in animals and human. AF leads to acute liver damage, oedema hemorrhage and even death. Mutation in liver DNA is the major cause behind AF. AF also leads to protein inactivation in liver [28]. Approx 12 people died in Kenya due to AF. Around 100 calves were died in 1981 in Australia because of AF. Death due to AF also reported in Argentina [29].

2.5.3.2 Chronic aflatoxicosis

Chronic aflatoxicosis (CA) is mainly caused due to exposure low concentration of aflatoxin for prolonged period of time. CA majorly leads to hepatocellular carcinoma. Individual suffereing from hepatitis B are more at risk. Various studies reported that exposure to aflatoxin for long period of time leads to impaired reproductivity, low immunity, jaundice, high mortality rate, growth inhibition and anemia [30]. Around 90,000 deaths due to CF reported every year worldwide.

2.6 Methods to control aflatoxin

Management of aflatoxin contamination is very important to control various diseases. There are various strategies are implemented in latest years to control aflatoxin. Some are mentioned are below.

2.6.1 Chemical method

It is a very efficient method to degrade aflatoxin (AFB1). Various acids are used for treating aflatoxin such as citric acid, lactic acid, propionic acid, boric acid, salicylic acid, oxalic acid etc. Citric acid hydrolysis the AFB1 and transform it into AFB2a. Lactic acid convert AFB1 into AFB2a, AFB2 and AFD1 [31]. When treated with acid at high temperature AFB1 shows complete degradation [32].

2.6.2 Physical method

There are various physical methods to control aflatoxin in nature. Some of the physical treatments are irradiation, roasting, microwave, extrusion, cooking, heating, etc. However, mycotoxins are heat-stable so heat treatment is not a very effective method to degrade aflatoxin [33]. Only heating at 200℃ for prolonged period of time shows some positive result. Ozone treatment is an effective method to degrade aflatoxin [34].

2.6.3 Enzymatic treatment

Enzymatic treatment is one such efficient method to treat aflatoxin. Various fungal enzymes are reported by researches which can efficiently degrade aflatoxin. One such example is *Armellaria tabescens*. Multienzyme produced by this fungi inhibit and degrade AFB1 [35]. Enzyme such as peroxidise produced by *Aspergillus flavus* and *Aspergillus paraciticus* shows some property to degrade aflatoxin (AFG1 and AGB1). Horseradish peroxidise is a plant specific enzyme also reported to have AFB1 degradation activity [36].

2.7 Antifungal compounds and their target

Fungal infections can potentially cause death in human beings, especially in those with compromised immune systems. Fungal infections and the development of resistant strains persist as an obstacle for medical practitioners [37]. The significance of creating novel antifungal agents is paramount. Antifungal agents can selectively target different structural elements of the fungal cell, including the cell wall, cell membrane, and intracellular components. The cellular wall is an essential component for the survival of fungi and is comprised of diverse polysaccharides, such as chitin, β-glucan, and mannan. Echinocandins, a category of antifungal agents, are designed to target β-glucan synthase specifically. This enzyme plays a crucial role in the synthesis of β-glucan, a vital constituent of the fungal cell wall. Echinocandins, namely caspofungin, micafungin, and anidulafungin, have been granted clinical approval. The efficacy of these agents is particularly notable

against *Candida* and *Aspergillus* species. Antifungal agents can also target the cell membrane. Ergosterol, a specific sterol found exclusively in fungi, plays a crucial role as a constituent of the fungal cell membrane. Azoles are one type of antifungal agent that functions by inhibiting the activity of lanosterol 14α-demethylase, an enzyme responsible for converting lanosterol to ergosterol [38]. This inhibition leads to the suppression of ergosterol synthesis. Azoles, namely fluconazole, itraconazole, and voriconazole, exhibit a wide range of efficacy against numerous fungal strains, encompassing *Candida* and *Aspergillus species*. Fungal cytoplasmic membranes have many transporters that facilitate the absorption of nutrients and the expulsion of hazardous substances. Efflux pumps can remove antifungal agents from the cell, diminishing their effectiveness [39]. Enzymes and metabolic pathways are fundamental components of biochemical processes. Antifungal drugs can target various enzymes and metabolic pathways [40,41].

2.8.1 Role of Hsps in *Aspergillus* Morphogenesis:

The process of germination is a crucial stage in the life cycle of *Aspergillus* fungi, as it marks the initiation of their growth from spores. Heat shock protein are crucial in the process of spore germination as they are involved in the expression of genes that are associated with spore germination [42]. The regulation of hyphal growth and branching involves the participation of Hsp70 and Hsp90 [43].

2.8.2 Shikonin as an anti-fungal agent

Phytochemicals such as ascorbic acid, gallic acid [44], quercetin [45], and caffeine are reported to show positive results against various fungal species. Shikonin is known to be the active principles of Zicao. It is mostly present in roots of *Lithospermum erythrorhizon.* But it is also reported to be present in the roots of *Onosma, Echium, Arnebia, Eritrichium.* Shikonin mostly used in ointments and cream because of its high lipophilicity. Studies has proven that shikonin possess properties such as anti-inflammatory, antioxidant, antimicrobial, antithrombotic. Interests of researchers have dramatically increased regarding the beneficial properties of shikonin over the past few years. When it comes to antibacterial properties, shikonin shows positive result. Shikonin reported to be active mostly against gram positive bacteria including *Bacillus subtilis, Enterococcus faecium* and *Staphylococcus aureus* (MIC ranging between 0.30 and 6.25 mg/ml). On the other hand shikonin shows negative result against *Pseudomonas aeruginosa, Escherichia coli*, and *Micrococcus luteus.* Shikonin possesses bactericidal properties. 200µM inhibit *Stenotrophomonas maltophilia* and *P.*

aeruginosa from forming biofilm. Antifungal properties of shikonin were tested against *Trichophyton rubrum, T. tonsulans* var. *sulfureum T. mentagrophytes, Saccharomyces sake Epidermophyton fluccosum* and *Microsporum gypseum.* Sasaki et al. reported that shikonin shows two times higher antifungal activity (MIC = 4 mg/ml) against *Saccharomyces cerevisiae* and four times against *Candida krusei* [46]. Shikonin also shows positive result against *Aspergillus terrus*. In this study we will see the effect of shikonin at MIC 1.5 µg/ml on various heat shock proteins of *Aspergillus flavus.*

CHAPTER 3: **MATERIALS AND METHODS**

3.1 Materials

3.1.1 Biological material

1. *Aspergillus flavus* MTCC 9367

3.1.2 For culture growth

1. Potato dextrose broth (potato infusion 20%, agar 2%, dextrose 2%, ph5.6), Himedia, Mumbai, India

2. Phosphate buffer saline

i. sodium chloride

ii. potassium chloride

iii. sodium phosphate dibasic

iv. potassium phosphate monobasic

3. Phosphate buffer saline with 0.05% tween 20

3.1.3 For RNA extraction from control and treated sample

1. Shikonin (MIC_{50} 1.525)

2. TRIzol reagent

3. Chloroform

4. Isopropyl alcohol

5. 75% Ethanol

6. Nuclease free water

3.1.4 For gel electrophoresis

1. Agarose

2. DEPC water

3. TAE buffer

4. Ethidium bromide

5. Bromophenol blue

6. 1 kb DNA ladder

3.1.5 For cDNA synthesis

1. 5X Synthesis buffer

2. dNTPs

3. Oligo dT

4. Hexamer

5. RT enhancer

6. Enzyme mix

7. Template

8. Nuclease free water

3.1.6 For RT-PCR reactions

1. Green master mix (Taq polymerase, dNTPs, $MgCl_2$, reaction buffer and two types of dye)

2. Primers (table 3.1)

Selected Hsps	Forward sequence	Reverse sequence
Hsp70	5′-CCTACTCCCTCAAGAACACC-3′	5′-GAGACTCGTACTCCTCCTTG-3’
Hsp90	5′-CGTCAAGTCCATCACTCAGC-3′	5′-GCTTGTGGATGCGCTCGGC-3’
Hsp60	5′-GGTTTGACAGCTCCAAGG-3′	5′-GTGGTACCAAGGAGAGAGG-3’
Hsp98	5′-GAGAGATGAGGCAGAACG-3′	5′-TCCACCTCGAGTCTTTCG-3’
Tubulin	5′-GGAATGGATCTGACGGCAAG-3′	5′-GGTCAGGAGTTGCAAAGCG-3’

3. DNA template

4. Nuclease free water

3.1.7 Instruments

1. Haemocytometer

2. Microscope

3. Centrifuge

4. Weighing balance

5. Autoclave

6. Laminar air flow

7. Freezer (4℃, -20℃, -80℃)

8. Gel doc. System

9. Nanodrop

10. Agarose gel electrophoresis setup

11. Incubator with shaker

12. Spectrophotometer

13. Thermocycler (PCR, RT-PCR)

3.2 Methodology

3.2.1 Revival of *Aspergillus flavus* MTCC 9367

Aspergillus flavus MTCC 9367 (toxigenic strain) was cultured on potato dextrose agar plate and incubated at 30 ℃ for 72h. After 72h spores were harvested using PBS (phosphate buffer saline) with tween 20 (0.05%). Spores were further washed with PBS twice. Later viability of the cells was checked using haemocytometer. 1×10^6 spores were for further experiments [47].

3.2.2 Growth of culture in broth

3.2.2.1 For control sample

3 flasks of 50 ml of potato dextrose were prepared and 1×10^6 spores which were previously harvested used as inoculum. After inoculation, the flasks were kept at 30℃.

3.2.2.2 For treated sample

3 flasks of 50 ml potato dextrose broth with 15 µl shikonin (MIC value= 1.5 µg/ml) were prepared. 1×10^6 spores were used to inoculate the media and incubated at 30℃.

3.2.3 RNA extraction

After incubation, RNA was extracted at 12h and 24h for both control and treated sample with TRIzol method.

RNA extraction process is as follows-

1. *Aspergillus flavus* MTCC 9367 was crushed into powder form using liquid nitrogen. 2-3 ml of TRIzol reagent is used for homogenisation.
2. The homogenised sample was incubated for 5 min at room temperature.
3. 0.2 ml of chloroform was added per 1 ml of TRIzol reagent.
4. The tubes were shaken vigorously for 15 seconds and then incubated for 2-3 min at room temperature.
5. The samples were centrifuged at 12000 ×g for 15 min at 2- 8℃.
6. After centrifugation supernatant (aqueous phase) was carefully transferred into fresh tube.
7. 0.5 ml of isopropyl alcohol was added to the freshly transferred sample per 1 ml of TRIzol reagent. Then sample was incubated for 10 min at room temperature.
8. Sample was centrifuged at 12000 ×g at 2-8℃ for 10 min.
9. The RNA gets precipitated.
10. Supernatant was removed and pellet was washed with 75% ethanol. 1 ml of 75% ethanol was added per 1ml of TRIzol reagent.
11. Sample were mixed using vortex and centrifuged at 7500 ×g at 2-8℃ for 5 min.
12. RNA pellet was air dried and stored in 20 µl of nuclease free water.

3.2.4 Agarose gel electrophoresis

To check the integrity and yield of isolated RNA, agarose gel electrophoresis was performed.

1. 10X TAE was prepared using DEPC (diethyl pyrocarbonate) water (table 3.2).

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

2. 1X TAE was prepared as running buffer from 10X TAE buffer.
3. 1.2 g of agarose was weighed and mixed with 100 ml of 1X TAE buffer. To mix it properly, the solution was heated until the solution become completely transparent.
4. 1 µl of ethidium bromide was added in the solution and then was poured in the casting tray. Comb was inserted to make the wells and left to get solidify.
5. Once the is set, comb was carefully removed, and 2 µl of loading dye (bromophenol blue) and 3 µl RNA sample was used for loading in the well.
6. Running buffer (1X TAE buffer) was poured in the chamber.
7. Gel was run for 45 min.
8. Gel was observed under UV at wavelength of 302 nm.

3.2.5 RNA quantification

Quality and quantity of RNA was estimated using A_{260}/A_{280} nm with Nanodrop spectrophotometer (Thermo Scientific, USA). RNA was diluted 100 times for quantification. RNA quantity was calculated using the equation:

$$40\ g/ml \times OD_{260} \times \text{dilution factor.}$$

3.2.6 cDNA synthesis

After RNA was quantified cDNA was synthesised using Verso cDNA synthesis kit. All the components were added carefully in the PCR vials according to the manual. Protocol was also set according to the manual.

Table 3.3 20 µl Reaction mixture

Components	Volume (µl)
5X synthesis buffer	4
dNTPs	2
RNA primer	1
RT enhancer	1
Enzyme mix	1
Template	1-5
Nuclease free water	To 20

Table 3.4 Cyclic program for cDNA synthesis

Steps	Temperature	Time	No. of cycles
cDNA synthesis	42°C	30 min	1 cycle
Inactivation	95°C	2 min	1 cycle

3.2.7 qRT-PCR

First all the primers were diluted 100 times (10 µl stock primer and 990 µl nuclease free water). Then qRT- PCR was performed for selected genes i.e *tubulin, Hsp90, Hsp70, Hsp98,* and *Hsp60*. Tubulin is a house keeping gene and used as reference gene. Total 12 reaction mixture was prepared (3 NTCs, 3 positive controls, 6 samples). All the components were carefully were added in the PCR vials according to the manual. iQ™ SYBR® Green supermix was used. Protocols were set according to the manual.

Table 3.5 20 µl qRT-PCR reaction mixture concentration

Components	Volume (µl)
Green master mix 2X	10
Forward primer	1
Reverse primer	1
DNA template	2
Nuclease free water	6

Table 3.6 Cyclic program for qRT-PCR

Steps	Temperature (°C)	Time
Initial denaturation	95	3 min
Denaturation	95	10 sec
Annealing	54-60	30 sec
Extension	72	30 sec
Melt curve	65-95	0.05 sec

Upto 40 cycles

Table 3.7 Annealing temperature for selected genes

Selected genes	Annealing temperature
Tubulin (Positive control)	56℃
Hsp90	60℃
Hsp70	56℃
Hsp98	54℃
Hsp60	54℃

Table 3.8 RT-PCR condition for *tubulin*

Steps	Temperature	Time
Initial denaturation	95℃	3 min
Denaturation	95℃	10 sec
Annealing	60℃	30 sec
Extension	72℃	30 sec
Melt curve	65℃ to 95℃	0.05-0.5 sec

Table 3.9 RT-PCR condition for *Hsp90*

Steps	Temperature	Time
Initial denaturation	95℃	3 min
Denaturation	95℃	10 sec
Annealing	60℃	30 sec
Extension	72℃	30 sec
Melt curve	65℃ to 95℃	0.05-0.5 sec

Table 3.10 RT-PCR condition for *Hsp70*

Steps	Temperature	Time
Initial denaturation	95℃	3 min
Denaturation	95℃	10 sec
Annealing	56℃	30 sec
Extension	72℃	30 sec
Melt curve	65℃ to 95℃	0.05-0.5 sec

Table 3.11 RT-PCR condition for *Hsp98*

Steps	Temperature	Time
Initial denaturation	95°C	3 min
Denaturation	95°C	10 sec
Annealing	54°C	30 sec
Extension	72°C	30 sec
Melt curve	65°C to 95°C	0.05-0.5 sec

Table 3.12 RT-PCR condition for *Hsp60*

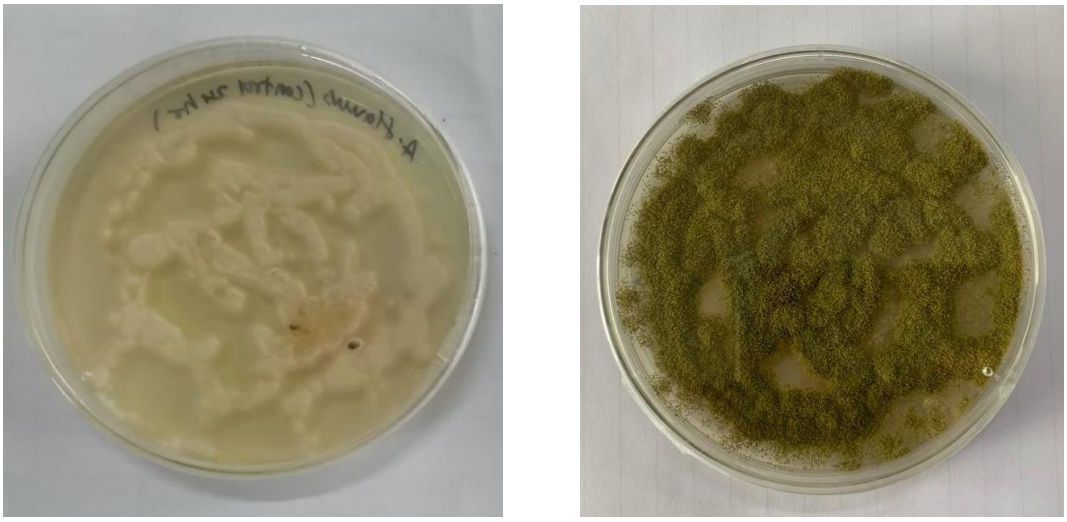
Steps	Temperature	Time
Initial denaturation	95°C	3 min
Denaturation	95°C	10 sec
Annealing	54°C	30 sec
Extension	72°C	30 sec
Melt curve	65°C to 95°C	0.05-0.5 sec

Tubulin was considered as reference gene for data analysis and to check the primer specificity melting curve analysis was done. $2^{-\Delta\Delta CT}$ method was used to calculate the relative gene expression of selected genes and to quantify the expression of genes.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Results and discussion

4.1.1 Revival of *Aspergillus flavus* MTCC 9367



(a) (b)

Figure 4.1 Revival of the culture (a): Growth after 24h of inoculation, (b): Growth after 72h of inoculation.

4.1.2 Spore harvest

Figure 4.2 Spores were harvested using PBST and washed with PBS.

4.1.3 RNA band visualisation under UV

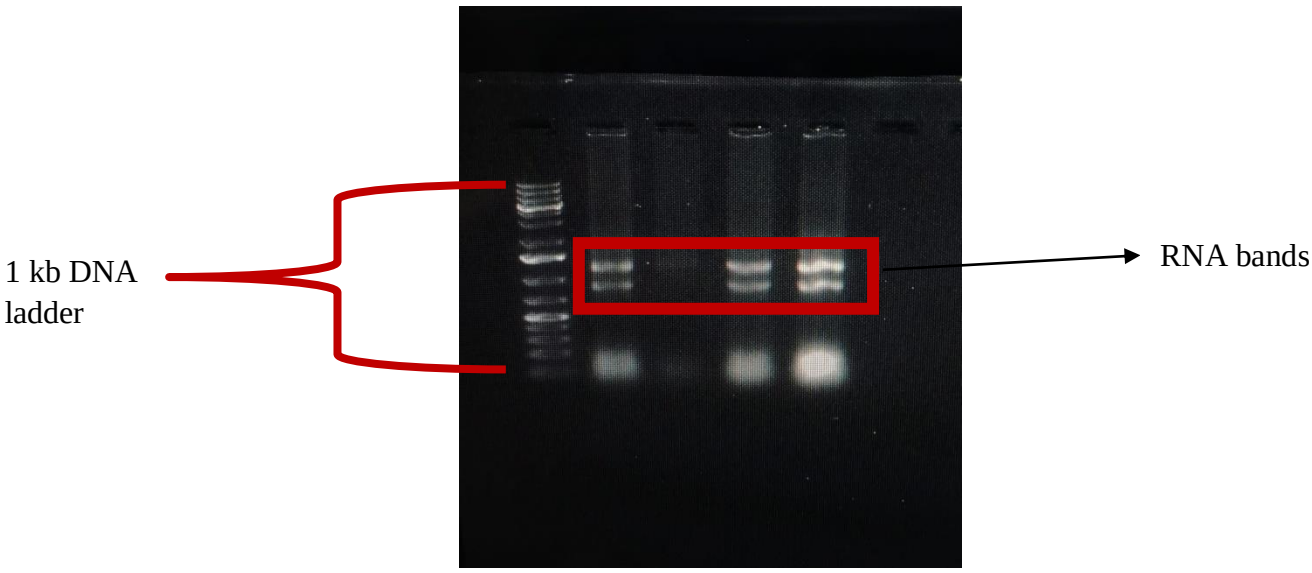


Figure 4.3 RNA bands at 12h of control sample

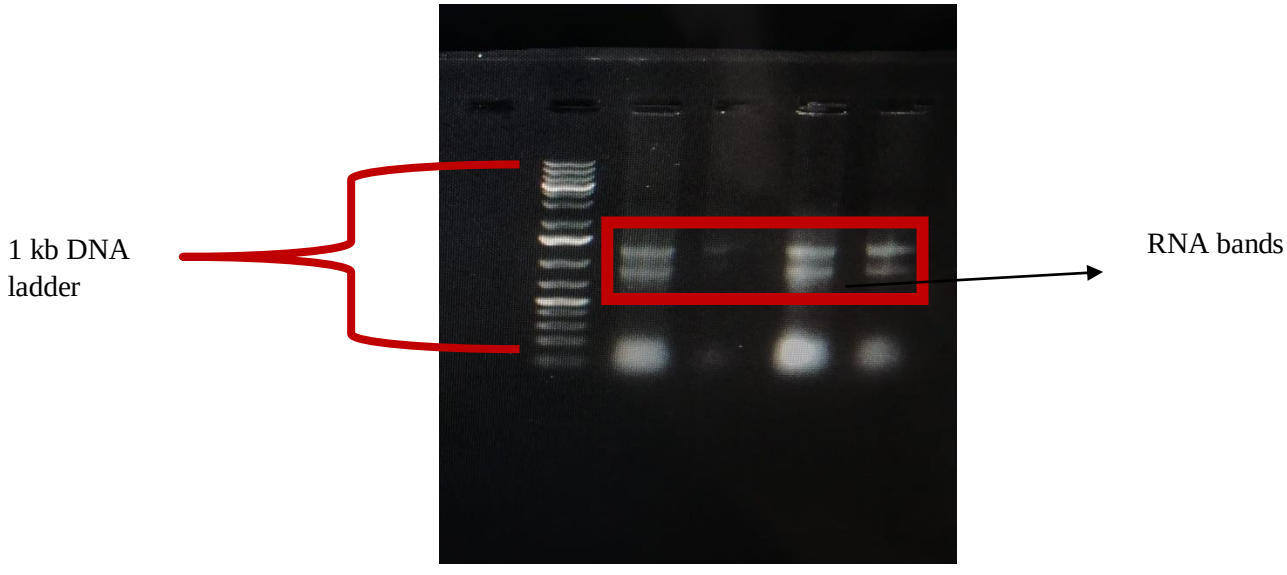


Figure 4.4 RNA bands at 12h of treated sample

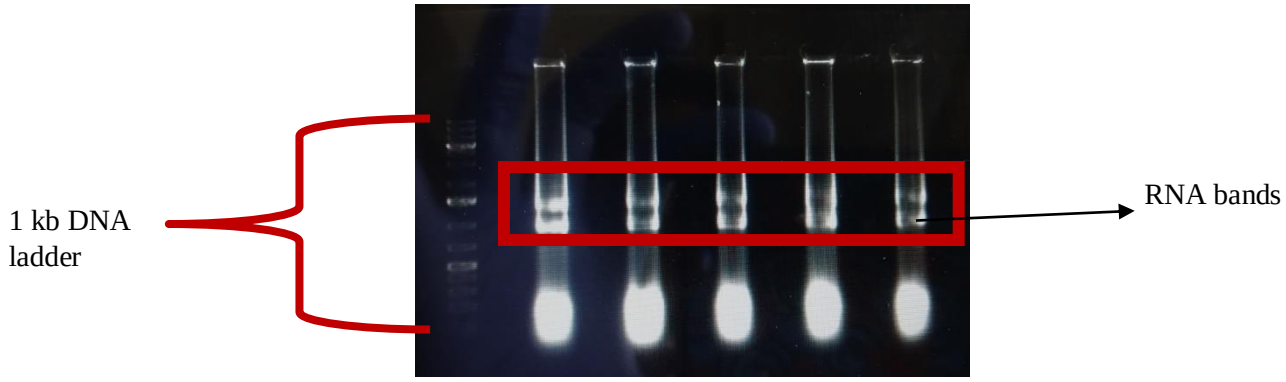


Figure 4.5 RNA bands at 24h of control sample

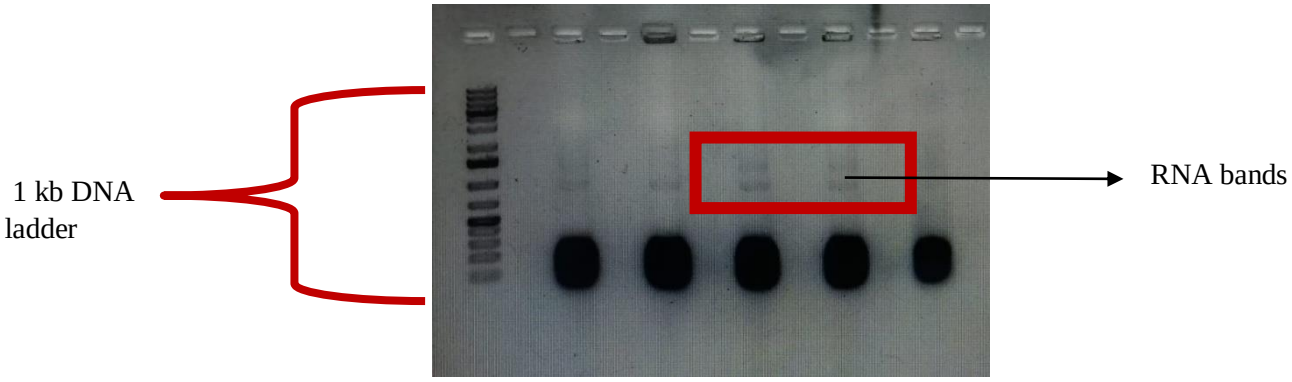


Figure 4.6 RNA bands at 24h of treated sample

4.1.4 RT-PCR results

4.1.4.1 C_T value of housekeeping gene (*tubulin*) at 12h

Sample	C_T1	C_T 2	C_T 3	Avg. C_T
Control	20	19.96	19.8	19.92
Treated	25.09	23.65	25.13	24.62333

4.1.4.2 C_T value of *Hsp90* at 12h

Sample	C_T 1	C_T 2	C_T 3	C_T 4	Avg. C_T
Control	19.91	19.6	19.55	19.8	19.715
Treated	23.35	23.32	23.21	23.15	23.2575

4.1.4.3 C_T value of *Hsp70* at 12h

Sample	C_T 1	C_T 2	C_T 3	C_T 4	C_T 5	C_T 6	Avg. C_T
Control	19	19.22	8.74				18.98667
Treated	27.67	28.04	28.02	27	24.51	24	26.54

4.1.4.4 C_T value of *Hsp98* at 12h

Sample	C_T 1	C_T 2	C_T 3	C_T 4	C_T 5	C_T 6	Avg. C_T
Control	29.26	30.6	8.51	28.35			29.18
Treated	21.49	21.7	21.37	22.82	22.49	1.43	21.88333

4.1.4.5 C_T value of *Hsp60* at 12h

Sample	C_T 1	C_T 2	C_T 3	C_T 4	C_T 5	C_T 6	Avg. C_T
Control	21.66	21.33	20.87				21.28667
Treated	24.53	23.9	23.81	25.05	24.62	24.39	24.38333

4.1.4.6 C_T value of Housekeeping gene (*tubulin*) at 24h

Sample	C_T 1	C_T 2	C_T 3	C_T 4	Avg. C_T
Control	20.12	20.08	19.95	12.8	19.9875
Treated	27.59	27.03	27.86		27.49333

4.1.4.7 C_T value of *Hsp90* at 24h

Sample	C_T 1	C_T 2	C_T 3	C_T 4	C_T 5	C_T 6	Avg. C_T
Control	19.3	19.35	20.21	19.61			19.6175
Treated	30.28	29.73	29.64	29.41	29.37	29.34	29.62833

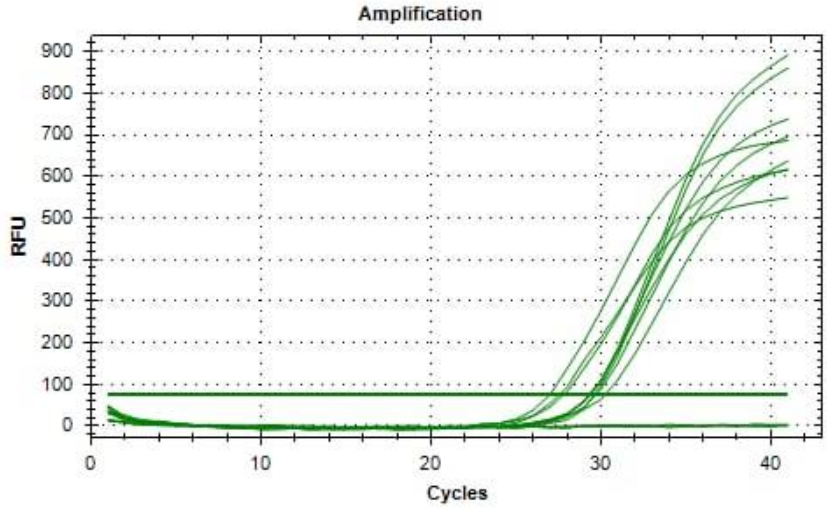


Figure 4.7 Amplification plot of *Hsp90* at 24 hr.

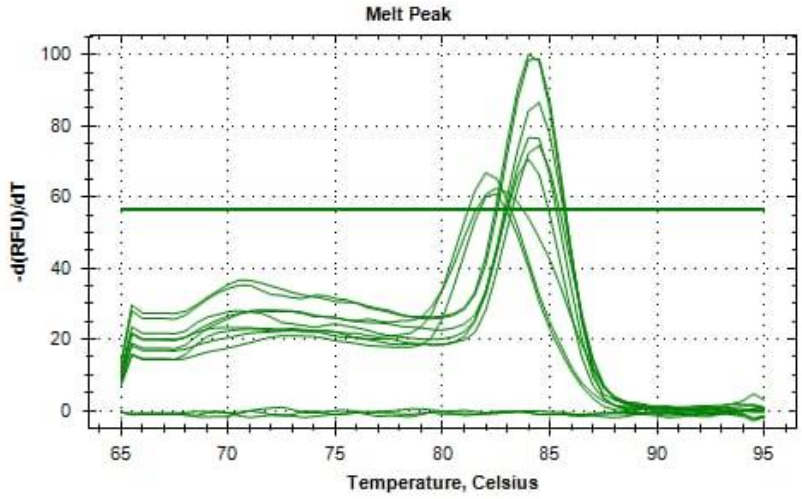


Figure 4.8 Melt peak of *Hsp90* at 24 hr.

4.1.4.8 C_T value of *Hsp70* at 24h

Sample	C_T 1	C_T 2	C_T 3	C_T 4	C_T 5	C_T 6	Avg. C_T
Control	25.57	25.66	25.19	24.65			25.2675
Treated	31.69	32.03	32.06	32.22	30.87	30.44	31.55167

4.1.4.9 C_T value of *Hsp98* at 24h

Sample	C_T 1	C_T 2	C_T 3	C_T 4	C_T 5	Avg. C_T
Control	19.3	19	19.07	18.94		19.0775
Treated	22.44	25.35	22.06	25.96	23.38	23.838

4.1.4.10 C_T value of *Hsp60* at 24h

Sample	C_T 1	C_T 2	C_T 3	C_T 4	C_T 5	C_T 6	Avg. C_T
Control	17.6	17.78	18.68	16.36			17.605
Treated	31.98	34.84	32.94	33.75	33.96	32.02	33.24833

4.1.5 Data analysis

4.1.5.1 ΔC_T, $\Delta\Delta C_T$ and $2^{-\Delta\Delta CT}$ calculations

To see the change in the expression of heat shock proteins when treated with shikonin, $2^{-\Delta\Delta CT}$ (Livak) method was used. For that, first ΔC_T and $\Delta\Delta C_T$ were calculated.

$$\Delta C_T = \text{Avg. } C_T \text{ (gene of interest)} - \text{Avg. } C_T \text{ (housekeeping gene)}$$

$$\Delta\Delta C_T = \Delta C_T \text{ (treated)} - \Delta C_T \text{ (control)}$$

4.1.5.2 ΔC_T, $\Delta\Delta C_T$ and $2^{-\Delta\Delta CT}$ values for *Hsp90* at 12h

Sample	Average CT value	ΔC_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta CT}$
Control	19.715	-0.205	-1.16083	2.235865
Treated	23.2575	-1.36583		

4.1.5.3 ΔC_T, $\Delta\Delta C_T$ and $2^{-\Delta\Delta CT}$ values for *Hsp70* at 12h

Sample	Average CT value	ΔC_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta CT}$
Control	18.98667	-0.93333	2.85	0.138696
Treated	26.54	1.916667		

4.1.5.4 ΔC_T, $\Delta\Delta C_T$ and $2^{-\Delta\Delta CT}$ values for *Hsp98* at 12h

Sample	Average CT value	ΔC_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta CT}$
Control	29.18	9.26	-12	4096
Treated	21.88333	-2.74		

4.1.5.5 ΔC_T, $\Delta\Delta C_T$ and $2^{-\Delta\Delta CT}$ values for *Hsp60* at 12h

Sample	Average CT value	ΔC_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta CT}$
Control	21.28667	1.366667	-1.60667	3.045474
Treated	24.38333	-0.24		

4.1.5.6 ΔC_T, $\Delta\Delta C_T$ and $2^{-\Delta\Delta CT}$ values for *Hsp90* at 24h

Sample	Average CT value	ΔC_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta CT}$
Control	19.6175	-0.37	2.505	0.176165
Treated	29.62833	2.135		

4.1.5.7 ΔC_T, $\Delta\Delta C_T$ and $2^{-\Delta\Delta CT}$ values for *Hsp70* at 24h

Sample	Average CT value	ΔC_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta CT}$
Control	25.2675	5.28	-1.22167	2.33216
Treated	31.55167	4.058333		

4.1.5.8 ΔC_T, $\Delta\Delta C_T$ and $2^{-\Delta\Delta CT}$ values for *Hsp98* at 24h

Sample	Average CT value	ΔC_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta CT}$
Control	19.077	-0.91	-2.74533	6.705446
Treated	23.838	-3.65533		

4.1.5.9 ΔC_T, $\Delta\Delta C_T$ and $2^{-\Delta\Delta CT}$ values for *Hsp60* at 24h

Sample	Average CT value	ΔC_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta CT}$
Control	17.605	-2.3825	8.1375	0.003551
Treated	33.24833	5.755		

After calculating the $\Delta\Delta C_T$ value of all target genes, graph of relative change in expression was plotted.

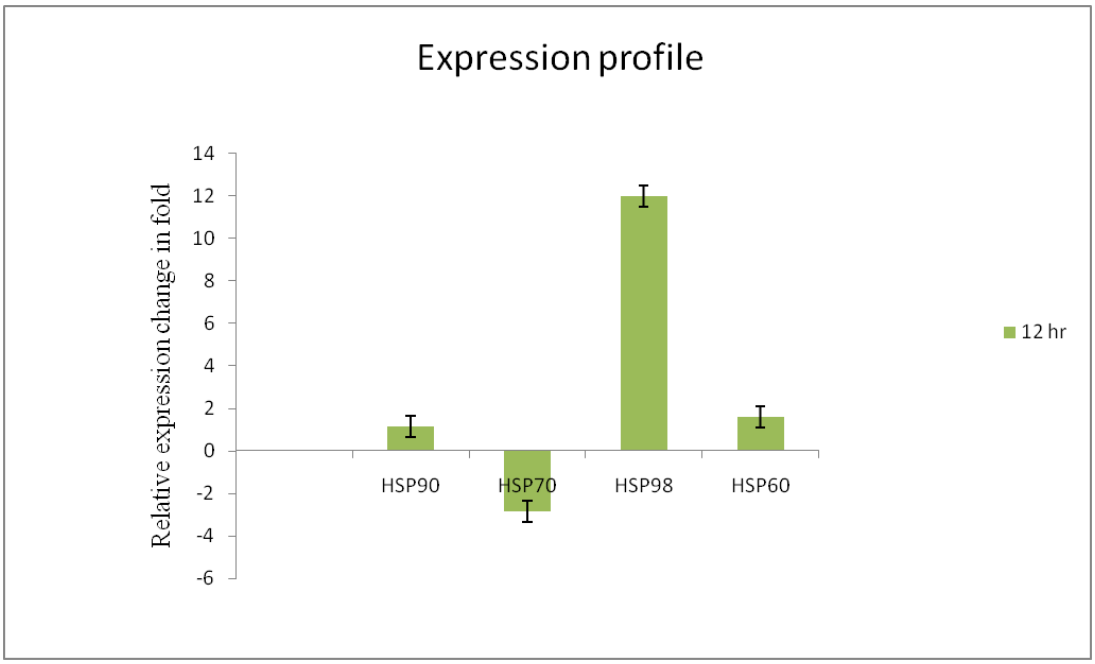


Figure 4.9 Relative expression change in fold at 12h

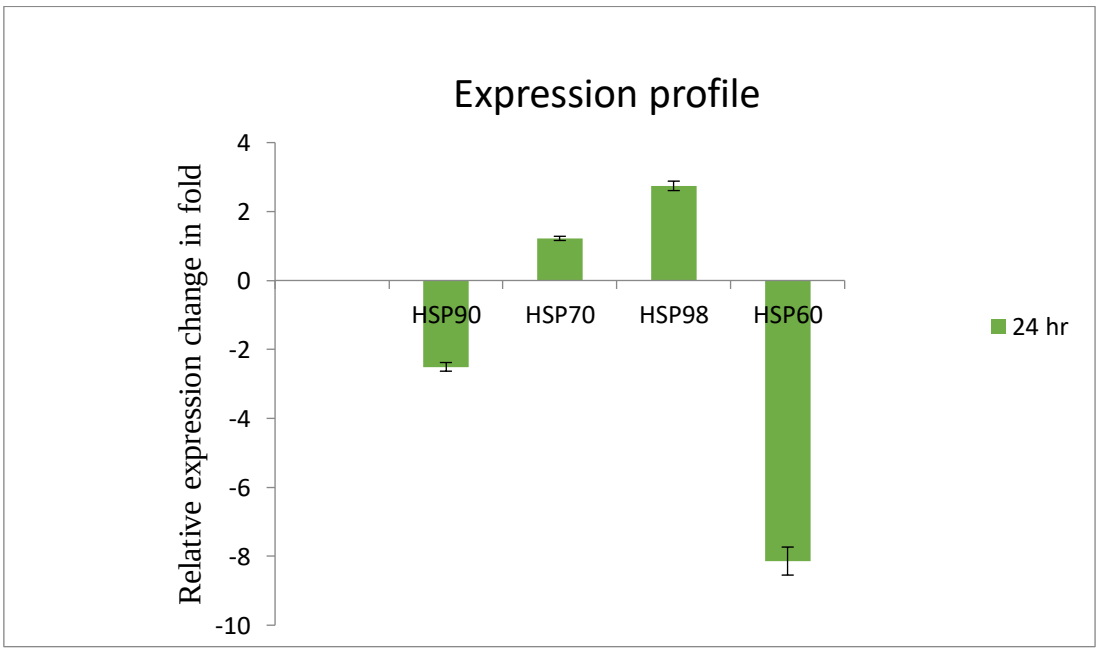


Figure 4.10 Relative expression change in fold at 24h

After analysis we can conclude that, upon treatment with shikonin, *Hsp98* was up regulated at both the hour (12 hr and 24 hr), but the fold change in expression was more at 12 hr compared to 24 hr. *Hsp60* was slightly up regulated at 12 hr and highly down regulated at 24 hr. *Hsp90* was slightly up regulated at 12 hr and slightly down regulated at 24 hr, where as *Hsp70* was slightly down regulated at 12 hr and slightly up regulated at 24 hr.

4.1.6 Effect of shikonin on the morphology of *A. flavus*

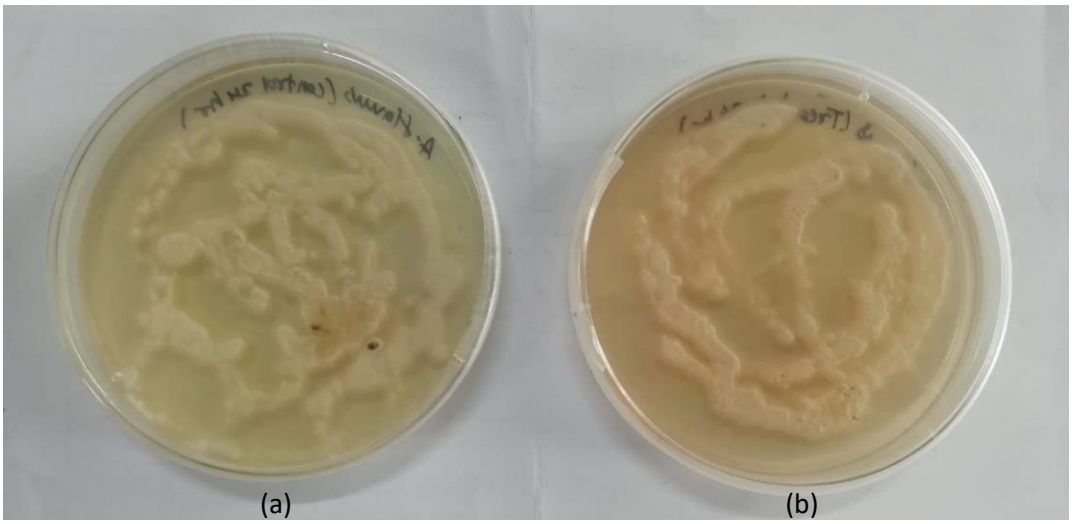


Figure 4.11 (a) growth of *A. flavus* after 24h on PDA (b) growth of *A. flavus* after 24h on PDA containg shikonin

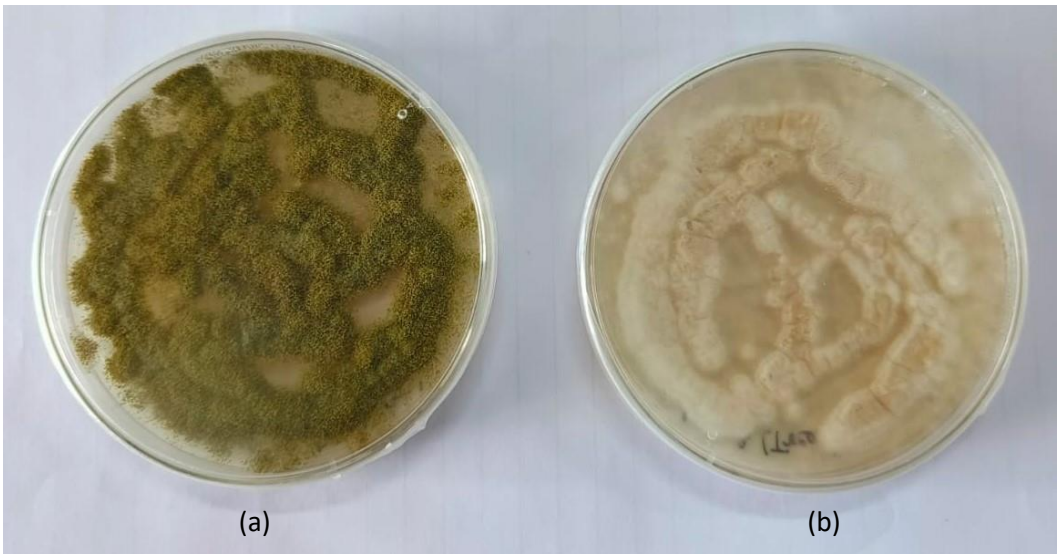


Figure 4.12 (a) growth of *A. flavus* after 72h on PDA (b) growth of *A. flavus* after 72h on PDA containg shikonin

When *A. flavus* was cultured on PDA containg shikonin, growth of A. flavus was inhibited. From this observation it has been concluded that, apart from heat shock proteins shikonin also modulate the expression of other genes involved in the morphogenesis of *A. flavus.* Effect of shikonin on genes involved in calcium signalling pathways which play a major role in morphogenesis, cell survival and virulence need further studies.

Conclusion

Aspergillus flavus exists in four forms in nature: dormant conidia, active conidia, hyphae and mycelia. Transition from dormant conidia to mycelia is a very important stage for *Aspergillus flavus* because mycelium is the only stage able to produce aflatoxin. So drugs which can prevent this transition will be very useful to eradicate the fungi. Studies have shown that heat shock proteins, also known as stress protein, plays a critical role in morphogenesis of *A. flavus* and play a major role in activation of various signalling pathways. Hsp90, Hsp70 and Hsp60 are dominant in fungal kingdom, and play a key role in morphogenesis, antifungal resistance and stress adaptation. Hsp70 is also proven to be linked with calcium signalling pathway. Calcium signalling pathway also reported to play a major role in morphogenesis, cell survival and virulent activity. Traditional drugs such as azoles and echinocandins majorly target the cell membrane and cell wall of the fungus. Heat shock proteins can also serve as drug target as fungus becoming resistance to traditional drugs. Phytochemicals are shows promising results against various fungal species. My studies shows that shikonin which is a phytochemical extracted from the roots of *Lithospermum erythrorhizon* able to modulate the expression of Hsp90, Hsp70, Hsp98 and Hsp60 and shikonin may able to prevent the growth of *A. flavus.*

References

1. B. Mousavi, M. T. Hedayati, N. Hedayati, M. Ilkit, and S. Syedmousavi, "Aspergillus species in indoor environments and their possible occupational and public health hazards," Current Medical Mycology, vol. 2, no. 1, pp. 36-42, 11/23/received.
2. A. Bansal, M. Sharma, A. Pandey, and J. Shankar, “Aflatoxins: Occurrence, Biosynthesis Pathway, Management, and Impact on Health,” in Fungal Resources for Sustainable Economy, Singapore: Springer Nature Singapore, 2023, pp. 565–594.
3. T. Michailides and T. Thomidis, "First report of *Aspergillus flavus* causing fruit rots of peaches in Greece," *Plant pathology,* vol. 56, no. 2, pp. 352-352, 2007.
4. S. Zhang, B. J. Monahan, J. S. Tkacz, and B. Scott, "Indole-diterpene gene cluster from *Aspergillus flavus* ," *Applied and environmental microbiology,* vol. 70, no. 11, pp. 6875-6883, 2004
5. H. Abbas *et al.*, "Ecology of *Aspergillus flavus* , regulation of aflatoxin production, and management strategies to reduce aflatoxin contamination of corn," *Toxin Reviews,* vol. 28, no. 2-3, pp. 142-153, 2009
6. D. Hagiwara, A. Watanabe, K. Kamei, and G. H. Goldman, "Epidemiological and Genomic Landscape of Azole Resistance Mechanisms in *Aspergillus* Fungi," *Frontiers in Microbiology,* vol. 7, p. 1382, 09/21
7. R. K. Sharma and S. Parisi, "Aflatoxins in Indian Food Products," in *Toxins and Contaminants in Indian Food Products*: Springer, 2017, pp. 13-24.
8. T. Gopal and S. Zaki, "Aflatoxicosis in fowls," *Indian veterinary journal,* 1969
9. J. Guinea, T. Pelaez, L. Alcalá, M. Ruiz-Serrano, and E. Bouza, "Antifungal susceptibility of 596 *Aspergillus fumigatus* strains isolated from outdoor air, hospital air, and clinical samples: analysis by site of isolation," *Antimicrobial agents and chemotherapy,* vol. 49, no. 8, pp. 3495-3497, 2005
10. P. Rodrigues, C. Soares, Z. Kozakiewicz, R. Paterson, N. Lima, and A. Venâncio, "Identification and characterization of *Aspergillus flavus* and aflatoxins," 2007.
11. B. W. Horn *et al.*, "Sexual reproduction in *Aspergillus flavus* sclerotia naturally produced in corn," *Phytopathology,* vol. 104, no. 1, pp. 75-85, 2014
12. M. A. Klich, *Indentification of common Aspergillus species*. Centraalbureauvoor Schimmelcultures, 2002.

13. J. Shankar et al., “Molecular insights into development and virulence determinants of Aspergilli: A proteomic perspective,” Front. Cell. Infect. Microbiol., vol. 8, p. 180, 2018.
14. T. K. Patel, R. Anand, A. P. Singh, J. Shankar, and B. N. Tiwary, “Evaluation of aflatoxin B1 biosynthesis in A. flavus isolates from central india and identification of atoxigenic isolates,” Biotechnol. Bioprocess Eng., vol. 19, no. 6, pp. 1105–1113, 2014.
15. W. Ellis, J. Smith, B. Simpson, J. Oldham, and P. M. Scott, "Aflatoxins in food: occurrence, biosynthesis, effects on organisms, detection, and methods of control," *Critical Reviews in Food Science & Nutrition,* vol. 30, no. 4, pp. 403-439, 1991.
16. R. R. M. Paterson and N. Lima, "How will climate change affect mycotoxins in food?," *Food Research International,* vol. 43, no. 7, pp. 1902-1914, 2010.
17. G. A. Payne, "Process of contamination by aflatoxin-producing fungi and their impact on crops," *Mycotoxins in agriculture and food safety,* vol. 9, pp. 279-306, 1998.
18. K. Campbell and D. White, "Evaluation of corn genotypes for resistance to *Aspergillus* ear rot, kernel infection, and aflatoxin production," *Plant Disease,* 1995.
19. Heinemann, et al. "Environmental investigations and molecular typing of Aspergillus flavus during an outbreak of postoperative infections." *Journal of Hospital infection* 57.2: 149-155, 2004.
20. W. J. Steinbach, "Pediatric aspergillosis: disease and treatment differences in children," *The Pediatric infectious disease journal,* vol. 24, no. 4, pp. 358-364, 2005.
21. T. Walsh and D. Dixon, "Nosocomial aspergillosis: environmental microbiology, hospital epidemiology, diagnosis and treatment," European journal of epidemiology, vol. 5, no. 2, pp. 131-142, 1989.
22. J. Shankar, G. C. Cerqueira, J. R. Wortman, K. V. Clemons, and D. A. Stevens, “RNA-Seq profile reveals Th-1 and Th-17-type of immune responses in mice infected systemically with Aspergillus fumigatus,” Mycopathologia, vol. 183, no. 4, pp. 645–658, 2018.
23. R. Anand, J. Shankar, B. N. Tiwary, and A. P. Singh, “Aspergillus flavus induces granulomatous cerebral aspergillosis in mice with display of distinct cytokine profile,” Cytokine, vol. 72, no. 2, pp. 166–172, 2015.
24. Y. Liu and F. Wu, "Global burden of aflatoxin-induced hepatocellular carcinoma: a risk assessment," Environmental health perspectives, vol. 118, no. 6, p. 818, 2010.

25. G. D. Kirk, E. Bah, and R. Montesano, "Molecular epidemiology of human liver cancer: insights into etiology, pathogenesis and prevention from The Gambia, West Africa," Carcinogenesis, vol. 27, no. 10, pp. 2070-2082, 2006.
26. F. Aguilar, S. P. Hussain, and P. Cerutti, "Aflatoxin B1 induces the transversion of G--> T in codon 249 of the p53 tumor suppressor gene in human hepatocytes," Proceedings of the National Academy of Sciences, vol. 90, no. 18, pp. 8586-8590, 1993
27. J. H. Williams, T. D. Phillips, P. E. Jolly, J. K. Stiles, C. M. Jolly, and D. Aggarwal, "Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences, and interventions," The American journal of clinical nutrition, vol. 80, no. 5, pp. 1106-1122, 2004.
28. E. A. Bailey, R. S. Iyer, M. P. Stone, T. M. Harris, and J. M. Essigmann, "Mutational properties of the primary aflatoxin B1-DNA adduct," Proceedings of the National Academy of Sciences, vol. 93, no. 4, pp. 1535-1539, 1996.
29. M. L. G. Pereyra et al., "An outbreak of acute aflatoxicosis on a chinchilla (Chinchilla lanigera) farm in Argentina," Journal of veterinary diagnostic investigation, vol. 20, no. 6, pp. 853-856, 2008.
30. J. D. Groopman, T. W. Kensler, and C. P. Wild, "Protective interventions to prevent aflatoxin-induced carcinogenesis in developing countries," Annu. Rev. Public Health, vol. 29, pp. 187-203, 2008.
31. R. S. Shukla, R. J. Verma, and D. N. Mehta, "Kinetic and mechanistic investigations on reductions of aflatoxins by lactic acid," Bioorganic & medicinal chemistry letters, vol. 12, no. 19, pp. 2737-2741, 2002.
32. K. R. WILLIAMS and M. F. DUTTON, "Destruction of Aflatoxin during the production of hydrolysed vegetable protein," Journal of Food Protection, vol. 51, no. 11, pp. 887-891, 1988.
33. L. B. Bullerman and A. Bianchini, "Stability of mycotoxins during food processing," International Journal of Food Microbiology, vol. 119, no. 1, pp. 140-146, 2007.
34. G. E. Mann, L. Codifer, and F. G. Dollear, "Effect of heat on aflatoxins in oilseed meals," Journal of Agricultural and Food Chemistry, vol. 15, no. 6, pp. 1090-1092, 1967.
35. D.-L. Liu, D.-S. Yao, R. Liang, L. Ma, W.-Q. Cheng, and L.-Q. Gu, "Detoxification of aflatoxin B 1 by enzymes isolated from Armillariellatabescens," Food and Chemical Toxicology, vol. 36, no. 7, pp. 563-574, 1998.

36. C. Das and H. Mishra, "In vitro Degradation of Aflatoxin B1in Groundnut (Arachis hypogea) Meal by Horse Radish Peroxidase," LWT-Food Science and Technology, vol. 33, no. 4, pp. 308-312, 2000
37. S. K. Shishodia, S. Tiwari, and J. Shankar, “Resistance mechanism and proteins in Aspergillus species against antifungal agents,” Mycology, vol. 10, no. 3, pp. 151–165, 2019.
38. S. Harari, "Current strategies in the treatment of invasive Aspergillus infections in immunocompromised patients," Drugs, vol. 58, no. 4, pp. 621-631, 1999.
39. L. Scorzoni et al., "Antifungal Therapy: New Advances in the Understanding and Treatment of Mycosis," Frontiers in microbiology, vol. 8, 2017.
40. J. Shankar, T. D. Wu, K. V. Clemons, J. P. Monteiro, L. F. Mirels, and D. A. Stevens, "Influence of 17β-estradiol on gene expression of Paracoccidioides during myceliato-yeast transition," PLoS One, vol. 6, no. 12, p. e28402, 2011.
41. K. Reddy, C. Reddy, and K. Muralidharan, "Potential of botanicals and biocontrol agents on growth and aflatoxin production by Aspergillus flavus infecting rice grains," Food control, vol. 20, no. 2, pp. 173-178, 2009.
42. S. Tiwari, R. Thakur, and J. Shankar, “Role of heat-shock proteins in cellular function and in the biology of fungi,” Biotechnol. Res. Int., vol. 2015, p. 132635, 2015.
43. C. Thakur, A. S. Malhotra, and J. Shankar, “Integrated bioinformatics analysis to study Gallic acid-mediated inhibition of polyketide synthase A from aflatoxin biosynthesis pathway of Aspergillus flavus,” Chem. Afr., 2023.
44. S. Tiwari and J. Shankar, “Integrated proteome and HPLC analysis revealed quercetin-mediated inhibition of aflatoxin B1 biosynthesis in Aspergillus flavus,” 3 Biotech, vol. 8, no. 1, p. 47, 2018.
45. I. Andújar, J. L. Ríos, R. M. Giner, and M. C. Recio, “Pharmacological properties of shikonin - a review of literature since 2002,” Planta Med., vol. 79, no. 18, pp. 1685–1697, 2013.
46. S. K. Shishodia and J. Shankar, “Proteomic analysis revealed ROS-mediated growth inhibition of Aspergillus terreus by shikonin,” J. Proteomics, vol. 224, no. 103849, p. 103849, 2020.
47. S. K. Shishodia, S. Tiwari, S. Hoda, P. Vijayaraghavan, and J. Shankar, “SEM and qRT-PCR revealed quercetin inhibits morphogenesis of Aspergillus flavus conidia via modulating calcineurin-Crz1 signalling pathway,” Mycology, vol. 11, no. 2, pp. 118–125, 2020.