EXPLORATION OF MYCELIAL PROTEINS AND SHIKONIN MEDIATED GROWTH INHIBITION OF *ASPERGILLUS TERREUS* USING PROTEOMICS APPROACH

Thesis submitted in fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

By

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JANUARY 2021

CERTIFICATE

This is to certify that the work reported in the Ph.D. thesis entitled **"Exploration of mycelial proteins and shikonin mediated growth inhibition of *Aspergillus terreus* using proteomics approach"** submitted at Jaypee University of Information Technology, Waknaghat, India, is submitted by **Sonia Kumari (Enrolment No. 166553)** at **Jaypee University of Information Technology, Waknaghat, India,** is a bona fide record of her original work carried out under my supervision. This work has not been submitted elsewhere for any other degree or diploma.

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ABSTRACT

Aspergillus terreus has emerged as an important opportunistic fungal pathogen. There has been a significant increase in cancer incidences, viral infections, and organ transplantation that lead to secondary fungal diseases. *A. terreus* causes mortality, high persistence, lack of early diagnosis, and inherent Amphotericin B (AmB) resistance. Morphological changes to the inhaled conidia are very crucial during invasive infections. Molecular methods help to explain active genes and their products at the various stages of development. Despite this very little has been studied regarding the determinants contributing to the pathogenesis, including AmB intrinsic resistance, and biomolecules associated with mycelia and biofilm formation. In documented work, we observed that the mycelia of *A. terreus* (NCCPF-860035) a clinical isolates rich in proteins from energy metabolism, ribosome biogenesis, oxidative homeostasis, cell wall, and structural components were revealed using nLC-ESI-MS/MS method. Majorly, important proteins (Catalase, superoxide dismutase, Hsp90, and Hsp70) may augment resistance against AmB in *A. terreus*. Additionally, SEM images and predicted biofilm-related secretory and adhesin proteins evident extracellular matrix (ECM) formation in *A. terreus*. Besides this being intrinsically resistant to the gold standard AmB therapy**,** the cure of these infections is a clinical threat now. Thus, improved therapeutics or new effective lead molecules are thus obviously the call of clinical professionals. Hence, in the present study, we have evaluated the phytochemicals (SHK, GA, CA, and QRT) against *A. terreus*. SHK showed higher efficacy (MIC_{50};2 µg/ml) among all the tested phytochemicals against planktonic as well as biofilm of *A. terreus*. The tested standard drugs (AmB, FLC, and ITC) were found less susceptible as showed higher MIC_{50}values for both planktonic and biofilm cultures of *A. terreus.* Differential proteome analysis was conducted to understand the inhibitory effect of SHK. The proteins/ enzymes from signaling pathways, oxidative stress, energy metabolism, and cytoskeleton organization were found differentially expressed. Further, relative gene expression analysis of important genes from the above-mentioned

pathways endorsed the proteome data. From these molecular studies, we have shown the crucial role of oxidative homeostasis and cytoskeleton dynamic in *A. terreus* embattled by

SHK. Thus, to corroborate we have also found elevated ROS using fluorescence assay and reduced catalase-peroxidase activity in SHK treated *A. terreus*. Moreover, defect in the germination of conidia, distorted hyphal structures, and depletion of ECM by SHK was also seen in preformed biofilms of *A. terreus* captured in SEM micrographs. Overall modulation of ROS homeostasis, metabolic shift, and cytoskeleton dynamics could be instrumental to the inhibitory mechanism of SHK in *A. terreus*. Our studies provided significant insight into the biology of *A. terreus* including the lead molecule, shikonin.

DECLARATION

I hereby declare that the work reported in the Ph.D. thesis entitled **"Exploration of mycelial proteins and shikonin mediated growth inhibition of *Aspergillus terreus* using proteomics approach"** submitted at **Jaypee University of Information Technology, Waknaghat, India,** is an authentic record of my work carried out under the supervision of **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 Ph.D. Thesis.

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ACKNOWLEDGMENT

Like many other aspirants, doing Ph.D. was penultimate goal of my life. It was a journey of mixed tides, which could not have been completed without sincere efforts of many people. Thus, I want to pay my highest gratitude to those, who helped me in carving out my dream and assisting immensely in the journey of my doctorate.

Prima facia, I am grateful to the God for giving me this opportunity in life to do my doctorate and write this thesis with all the good health and wellbeing that were necessary.

Words would definitely be short to pay my gratitude to my Guide, my mentor and the best Guru of my PhD journey, my research supervisor ***Dr. Jata Shankar,*** *for providing me the opportunity to do my research under his guidance. His tireless efforts and ever enduring support helped me out in every ups and downs of research. Thank you so much sir for showing such patience and bearing with me when it was most difficult. Your motivation, immense subjective knowledge was of great importance to me. He was robust and was always available when needed. It was indeed a great privilege working under him.*

Also, I am thankful to ***Prof. (Dr.) Sudhir Kumar****, Head of the Department of Biotechnology & Bioinformatics, for providing me all necessary facilities and platform in the department for carving out my research. His positive attitude and believe in my research were very encouraging for me to carry out my work.*

I am highly indebted and want to pay my sincere thanks to JUIT administration, Vice Chancellor, ***Prof. (Dr.) Vinod Kumar****, Director and Academic Head,* ***Prof. (Dr.) Samir Dev Gupta*** *and Registrar* ***Maj. Gen. (Retd.) Rakesh Bassi****, for providing all the facilities and infrastructure and thus helping me in realizing this work of mine. I would like to express my gratitude to my doctoral progress committee members (****Prof (Dr.) SP Ghrera, Dr. Tiratha Raj Singh****,* ***Dr. Rahul Shrivastava,*** *and* ***Dr. Hemraj Saini****) for their consistent support and valuable suggestions.*

I sincerely express my gratitude to ***Prof (Dr.) Rajinder Singh Chauhan,*** *(Erstwhile Head, Department of Biotechnology & Bioinformatics, JUIT) for giving me the opportunity to pursue my doctorate degree.*

I want to express my immense appreciation to other faculty members of the department of biotechnology and Bioinformatics for their continuous and everlasting cooperation and fruitful suggestions during my research work.

Laboratory staff are one's without which it is difficult to carry out your day-to-day tasks of research, they always go hand in hand and as such I would like to thank ***Mr. Ismail, Mr. Baleshwar, Mrs. Mamta Mishra, Mrs. Somlata Sharma and Mrs. Sonika Gupta*** *for their generous help in my research period.*

I am also highly beholden to ***Dr. Pooja Vijayraghavan*** *and lab members* ***(Dr. Shanu Huda and Lovely Gupta)*** *at Amity University of Information Technology, NOIDA, India, for helping me in conducting SEM and cytotoxicity experimentation.*

When all odds are against you, family members are the one's who are always by your stride. I want to pay my greatest gratitude to my parents ***Retd. (Er.) Gorakh Ram*** *and* ***Mrs. Nirmla Devi*** *as they believed in my dream of doing Phd and kick started this journey. I'm obliged to my parents for their constant blessing and never-ending love, you're my source of motivation and lifted me when it was needed the most. I am heartily thankful to my In-laws* ***Mr. Ashok Shishodia*** *and* ***late Mrs. Shanta Shishodia****, or their firm support and patience they have extended towards completion of my dreams.*

I express the deepest gratitude to my younger brothers ***Er. Vinay Kumar Bardhan and Dr. Manoj Kumar Bardhan****, for their unconditional love, encouragement, appreciation, support during my studies as well as research and lifting me uphill in this phase of life.*

Thanks, would be a small word for what I owe to my Husband ***Er. Aditya Shishodia****. He has paved the path before me and upon whose shoulders I stand today. He is the one who has profoundly motivated me in every way for opting and accomplishing the Ph.D. He is the one who have been offering all around support during the period of my studies and research both as a friend and as my life partner.*

I would like to extend my warm thanks to my beautiful Daughter ***Takshvi Shishodia****. She was born during my PhD tenure; her innocent smile always brings a smile on my face and cheerfulness even in odd times.*

Friends are inescapable part of one's life as they help you in achieving the best out of you and thus, I would like to thanks my dear friends ***Dr. Shardha Tiwari, Dr. Raman Thakur and Dr. Arjun Chauhan****, for their immense support and stand for me in these last five years. My heartfelt thanks to my lab mates* ***Tanvi Sharma, Deepak, Samiksha, and Shiuli Samaddar,*** *who were always there at the forefront of helping me when needed the most during my research. Also, my sincere thanks to all my seniors* ***Dr. Ira Vashistht, Dr. Ritika Verma, Dr. Nupur Munjal and Dr. Poonam Katoch,*** *for their continuous help and support to carry out my work efficiently and realize my dreams.*

Lastly, I would like to pay my gratitude and regards to anyone whom I missed to mention above and every one of those who directly or indirectly helped in my doctoral journey.

Blessed and proud to be a Doctorate.

(Sonia Kumari)

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LIST OF ABBREVIATIONS

µg	Microgram
µl	Microliter
µm	Micrometre
ABPA	Acute Bronchopulmonary Aspergillosis
AmB	Amphotericin B
AmBRS	Amphotericin B resistance
ART	Artemisinin
BAL	Bronchial Lavage
BLAST	Basic Local Alignment Search Tool
CA	p-Coumaric acid
cAMP	Cyclic adenosine mono phosphate
CDNA	Complementary DNA
CT	Threshold Cycle
DCFDA	2, 7-dichlorofluorescein diacetate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
DR	Drug resistance
ECM	Extracellular matrix
ELISA	Enzyme Linked Immunosorbent Assay
ESI	Electron spray ionisation
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
Fig.	Figure
FLC	Fluconazole

GA	Gallic acid
GO	Gene Ontology
HIV	Human Immunodeficiency Virus
HSP	Heat Shock Protein
IA	Invasive Aspergillosis
ITC	Itraconazole
ITRAQ	Isobaric Tag for Relative and Absolute Quantification
KEGG	Kyoto Encyclopaedia of Genes and Genomics
LC	Liquid Chromatography
MALDI	Matrix Assisted Laser Desorption Ionization
MAPK	Mitogen Activated Protein Kinase
mg	Milligram
MIC	Minimum Inhibitory Concentration
min	Minutes
mM	Millimolar
MS	Mass Spectrometry
MW	Molecular Weight
NaCl	Sodium Chloride
NCCPF	National Collection Centre of Pathogenic Fungi
ng	Nanogram
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PMSF	Phenyl methyl sulphonyl fluoride
QRT	Quercetin
qRT-PCR	Quantitative Real Time- Polymerase Chain Reaction
RNA	Ribonucleic Acid
ROS	Reactive oxygen species

RPMI	Roswell Park Memorial Institute medium
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SEM	Scanning electron microscopy
SHK	Shikonin
SNP	Single Nucleotide Polymorphisms
Th	T-Helper
Tm	Melting temperature
UniProt	Universal Protein Resource
VRC	Voriconazole

LIST OF SYMBOLS

%	Percentage
~	Approximately
≤	Less than equal to
≥	Greater than equal to
º C	Degree Celsius
g	Gram
h	Hour
L	Litre
β	Beta
Δ	Delta
µ	Micro

CHAPTER-1

THESIS PROLOGUE

Recently secondary fungal infections have emerged at an alarming rate due to an increase in patients with impaired immunity. All over the world among other mold infections, *Aspergilli*-mediated infections have increased in human-beings as well in domestic animals, and avians. *Aspergilli* are emitted into the atmosphere, soil, plant debris, food items, and indoor climate and responsible for major economic losses as they can have a detrimental effect on crops. The *fumigatus, flavus, terreus,* and *niger* are the prevalent subgenera of this *Aspergillus* being their significant impact as human opportunistic pathogens. These causes acute to chronic forms of infections in humans as well as multiple allergic reactions such as invasive aspergillosis allergic bronchopulmonary aspergillosis, allergic *Aspergillus* sinusitis, hypersensitivity pneumonia, and IgE-mediated asthma. *Aspergillus terreus* being sporophytic fungus ubiquitously present in the air can cause infection in patients with a weak immune system. *Aspergillus terreus* has importance amongst invasive pathogens [1, 2]. Ubiquitous small conidia of *A. terreus* is easily inhaled by humans and often cleared by innate and acquired immune responses. Whereas, in individuals with impaired immunity the alveolar and bronchial epithelial cells act as a critical barrier to the infection. There is a lack of phagocytic function of immune cells in these patients which enables conidia to germinate into hyphae and mycelia structures. Additionally, for people with respiratory disorders (asthmatic, allergic, and chronic obstructive respiratory disease), the lung epithelium cells are in direct contact with *Aspergilli* which makes them vulnerable to fungal manifestations. *A. terreus* conidia persist in antigen-presenting cells that allow more dissemination of conidia and lower efficacy against the drugs [3]. Thus, for the individual with a severely suppressed immune system and having respiratory disorders, inhaled conidia successfully germinate and form a hyphal and mycelial network. The transitions of conidia into hyphae in the host milieu represent a critical stage for the establishment of invasive/systemic infection. Also, the previous literature stated that immunological signals may rely on cell wall components of inhaled conidia due to the composition of glycoproteins, polysaccharides, and proteins that varies through transition events of conidia into the hyphae [4]. Despite the importance of the morphological transition

in *A. terreus*, the determinant contributes to hyphae and mycelium is yet to be established. Necessary knowledge of such reprogramming of conidia, hyphae, and mycelium may accelerate a better diagnostic and treatment strategy to control *A. terreus* infections. Thus, it is important to know protein/enzyme families of the pathogen in different morphotypes including mycelia. Several of these proteins may be crucial for the reprogramming of cellular machinery among morphotypes in *A. terreus*. Among various important species of Aspergillus, *A. terreus* is the one which should be more emphasized due to the high occurrence, inherent Amphotericin B resistant (AmBRS) nature, and high rate of invasive aspergillosis-related death [5]. Amphotericin B (AmB), is a broad-spectrum antifungal drug, ~ 98% *A. terreus* isolates were found not responding to AmB worldwide [5, 6]. Resistance to azole has also been acquired in *A. terreus*, ~ 5-10% isolates were azole-resistant [6]. The drug resistance (DR) mechanism in *A. terreus* is still not clear. Biofilm formation in *Aspergillus* species may defend pathogens against antifungal drugs and contributes to the mechanism of DR. Whereas, less is known about the development of biofilms in *A. terreus*. Thus, addressing the production of biofilm in *A. terreus* may be essential for the discovery of its inherent existence of resistance against AmB. Additionally, the occurrence of intrinsic drug resistant and evolving acquired DR due to repeated drug usage in *Aspergillus* isolates has additional clinical challenges. Moreover, antifungal drugs has hazardous effects like infusion-related infection, hypokalaemia, and nephrotoxicity in children suffering from aspergillosis (3-5 mg/kgAmB per day dosage) [7]. Several fungicides cause hazardous effects such as acute and chronic poisoning which become a major problem in various developing countries [8, 9]. Thus, there is an urgent need for the development of alternative antifungal approaches which will be less toxic and eco-friendlier. In this context, Phytochemicals (PhytoChem) with antifungal properties have acquired significance owing to their natural origin. PhotoChem derived from different parts of plants include tocopherol, phenolic, flavonoids, carotenoids, anthocyanin, and thiols have shown activities such as antimicrobial, anti-cancerous antioxidant, and anti-inflammatory [10, 11].

The genome sequence of *Aspergillus* species has made it much easier to understand the biology of these pathogenic fungi. Moreover, these studies rely primarily on the transcriptomic profile using high-throughput technologies (microarray and RNA-seq) to decode the essential factors involved in pathogenesis. However, there is a certain limitation to the transcriptomic methods, because transcriptomic studies reveal a part of the precise

mechanisms of events and rely also on post-transcriptional regulators. Consequently, proteomics studies are essential for advancing the science of microbiology. However, there are very limited proteomic studies available on medically important *Aspergillus* species at various morphological stages or during antifungal or phytochemical interactions. The availability of an annotated set of proteomes of many pathogenic and non-pathogenic *Aspergillus* species and limited experimental protein data allowed us to comprehend the molecular mechanisms involved in the biology of *Aspergilli.* Proteins expressed in morphotypes during morphological transitions and comparative protein mapping in response to drug/PhytoChem could be a robust method to add better insight into the mode of action as well as to provide a new lead molecule. Additionally, it shed light on the virulence, resistance mechanism, and invasive factors of the emerging fungal pathogen [12, 13]. The comprehension of drug-route contributing to its metabolism is also a significant prerequisite for understanding resistance mechanisms. In the case of clinically important fungi, adaptation to drugs, and mechanism to negate antifungal were beautifully documented [14]. Though the rapid advancements in technology and genomic data have facilitated mode of action drugs, however, the emergence of antifungal drug resistance against pathogens demands effective and safer molecules. Therefore, keeping in view these gaps existing in *A. terreus* morphogenesis and treatment regimen, the current work on "**Exploration of mycelial proteins and shikonin mediated growth inhibition of *Aspergillus terreus* using proteomics approach**" was undertaken with the following objectives.

Objectives:

1. Exploration of mycelial proteome in *Aspergillus terreus* to elucidate the protein/enzyme families associated with mycelial network and resistant mechanism.
2. To decipher the efficacy of phytochemicals (p-coumaric acid, gallic acid, shikonin and quercetin) against *Aspergillus terreus.*
3. Elucidation of mode of action of shikonin mediated inhibition of *Aspergillus terreus* using differential proteomic approach

CHAPTER-2

REVIEW OF *ASPERGILLUS*

2.1 *Aspergilli*

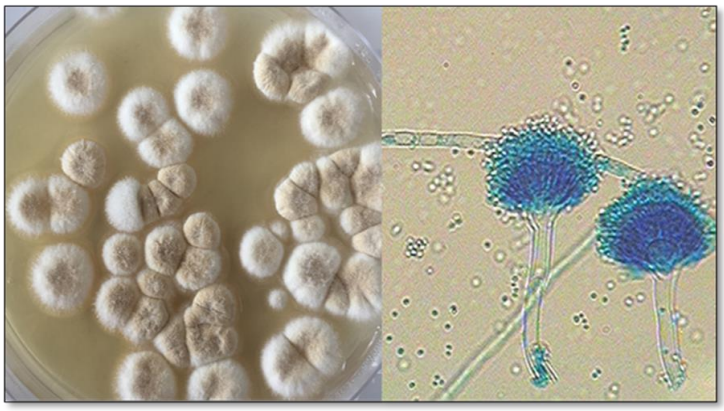
Fungi are the clinically and industrially relevant microorganisms and opportunistic fungal pathogens due to an increase in the immunosuppressed host [15, 16]. The *Aspergillus* genus comprises more than 350 species, and new species are growing each year. There are about 40 *Aspergilli* species all, which can lead to infection and human allergic disorders. Some of the *Aspergillus species* have acquired medical significance [17-19]. Among all *Aspergillus species* , *A. fumigatus* (67–73%), *A. flavus*(10–16%) and *A. terreus* (3–4%) cause lethal infections in human beings having impaired immunity as well as rarely to healthy persons [20].

The numbers of immunosuppressed people have risen over the past decade due to organ transplantation, cancer, and highly autoimmune disorders, which have made humans vulnerable to opportunistic fungal infections, and other diseases such as HIV [21, 22]. Also, the appearance of *Aspergilli* DR challenged the *Aspergillus* scenario worldwide [23, 24]. Besides, the inability to detect Aspergillus-related infections early poses a risk for vulnerable humans. Recently, *A. terreus* has become frequently cause IA in immune-suppressed cases, especially in cancer patients [25, 26]. Furthermore, the inherent resistance to AmB in *Aspergillus terreus* isolates makes it difficult to cure these infections and lead to major medical issues [27]. The genome sequencing of different *Aspergillus* species has allowed an understanding of the biology of these organisms. Also, high-throughput (microarray, RNA-seq) techniques can help to decipher the factors responsible for virulence as well as an invasion to host tissue by the pathogens [28]. While there are minimal studies on antifungal agents function with proteomic approaches to decode their mode of action, it is a step forward for the production of an effective antifungal molecule.

2.2 *Aspergillus terreus* morphology and distribution

Aspergillus terreus is found in soils warmer climates (tropical to subtropical regions) worldwide. It is generally habitat in soil, despite it has been located in different environments such as compost and dust. *A. terreus* is a thermotolerant species and tolerate temperate up to 45–48 °C. The production of two types of asexual spores i.e., phialoconidia (produced at the tip of vesicle present on conidiophores 2 μm in dia.) and aleurioconidia (produced directly on the hyphae size ranges 6–7 μm in dia.) and cinnamon-brown colony coloration make this species unique among others *Aspergillus* species. The brownish colour of conidia gets darker, as it grows dark on culture media [29-31]. The tiny conidia are globose-shaped, smooth-walled ubiquitously present in the environment, and easily inhaled by humans shown in Fig.2.1.

A **B** **C**



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

Fig. 2.1 (A) *Aspergillus terreus* culture on PDA media showing brown colour conidia **(B)** 100X magnification view of *Aspergillus terreus* showing conidia, phialides and conidiophore **(C)** Taxonomy of *Aspergillus terreus*

2.3 *Aspergillus terreus* an emerging opportunistic pathogen

Aspergillus terreus is a ubiquitous pathogen [32]. The common cause of aspergillosis was *A. terreus* documented in Austria (University and Hospital of Innsbrück), Houston (Texas) [25, 26]. It can cause opportunistic infections in individuals with weak immune systems such as (organ transplant HIV, cancer patients, etc). While *Aspergillus fumigatus* causes most of these cases, about 15% are caused by *Aspergillus terreus.* Invasive aspergillosis sufferers are now known to also have higher incidences of cancer and organ transplantation. Also, a 74 %-92 % increased risk of death was observed among transplant recipients suffering from invasive

aspergillosis [4, 33]. Although it is difficult to know the exact cause of death in immunocompromised patients clinically, approximately 60 .5% IA-related death risk was found in a report [34]. Only 59% of patients with IA among solid organ transplant recipients [35] and 25% among hematopoietic stem cell transplant recipients, were able to survive for a one-year time [36]. Approximately 30–50% of IA patients were killed due to delayed detection and emergence of drug-resistant isolates [37, 38]. The overall prevalence of *A. terreus* associated infections among all mold infections is found to be 5.2% [39]. In India, *A. terreus* related aspergillosis infections (6.6%) were found in a recent report from Chest Hospital (Delhi) [40]. *A. terreus* needs more attention compared to non-terreus *Aspergillus* species due to a persistent increase in the incidences with an elevated mortality rate (51 vs. 30), and inherent resistance to AmB [5]. AmB, is a commonly used antifungal with around 98% *A. terreus* isolates were observed AmB resistant around the world [5, 6]. According to another report, only 8% of *A. terreus* isolates has been observed sensitive to AmB (MICs; 0.5–1 mg/L) [5]. Resistance to azole has also been acquired in *A. terreus* isolates (~5-10%) [6], Azole drug therapy failure was reported in clinical sample from Danish of *A. terreus* stated by Arendrup *et al.* They documented ITC resistance may be due to M217I Cyp51A mutation in *A. terreus* isolates of *A. terreus* from Austria, Germany [41], and Great Britain were found highly (~10 percent) posaconazole resistant [6]. Hence, lack of AmB response and resistance to (VRC) possess additional clinical challenges to IA sufferer with a weak immune system [42]. Furthermore, conidia of *A. terreus* showed more persistence in the dendritic cell which contributes to high dispersal rate and low antifungal response when compared to *A. fumigatus* [3]. Another limitation stated in literature was conidia of *A. terreus* transferred from lungs to distant locations, such as the central nervous system [2, 43]. Thus, despite an increase in incidences of *A. terreus* related infections and the development of resistance against antifungal drugs this species is yet not well explored. Hence, *A. terreus* and emerging medical threats become an important fungus to take into consideration.

2.4 Diseases of manifestations caused by *Aspergillus terreus*

Aspergillus terreus has gained notoriety as an emerging opportunistic fungal pathogen due to an enormous rise in immunosuppressive conditions in individuals. It causes life-threatening IA in highly immunocompromised hosts. It is the leading cause of IA in patients (cancer cases) as stated by numerous medical centres around the world [25, 26]. The increased rate of mortality in *A. terreus* related infections due to immune suppression, resistance to AmB, and

virulent factors [25, 44, 45]. Additionally, the lack of diagnostic biomarkers against *A. terreus* adds on challenges to present scenarios. The inhaled conidia reside in immune cells (macrophage), transferred to secondary organs compared to other of *Aspergilli* [44]. In the case of an individual with a weak immune system, these persistent conidia begin to germinate and cause infection in those individuals. Another hyper-sensory reaction caused by the species *Aspergillus* is acute bronchopulmonary aspergillosis ABPA is one of the worst respiratory tract problems and occurs most commonly in people suffering from atopic asthma and cystic fibrosis [46]. *A. fumigatus* is the leading *Aspergilli* trigger but now *A. terreus* is an emerging causative agent of ABPA [15, 47, 48]. ABPA is characterized as eosinophilia, respiratory opacity, cystic fibrosis, and bronchial asthma. Complications with ABPA are along the same lines as classic asthma when T-cell's immune response causes pathophysiologic uniqueness [49]. When ABPA remains untreated, it causes respiratory arrest in infected patients [46]. Aspergilloma is not-invasive aspergillosis, often found in hosts; it is a ball of fungal mycelia in pre-existing lung cavities [50]. These cavities occur after treatment for TB, pulmonary disorders, and obstructive paranasal sinuses. It consists of fungal hyphae and sporulating fungal structure in the cavity of lungs embedded in a protein matrix. Aspergilloma had been known to be lethal occasionally after hemoptyosis [51].

2.5 Diagnosis

The diagnosis of IA is problematic, and in immunosuppressed individuals, it is difficult to detect IA beforehand [52]. The gold standard techniques for diagnosing IA are the histopathological examination of biopsy samples from the lungs. However, the histological findings can differ from patients such as cancers or transplants [53]. Histopathology enables the identification of septate hyphae in positive samples (biopsy) for *Aspergillus* organisms. Positive samples (sputum) in immunocompetent are of no significance as *Aspergillus* species has often been observed with non-clinical symptoms in immunocompetent hosts [54]. Furthermore, positive sputum samples of immunocompromised individuals especially those with leukemia or stem cell transplantation, are considered to be invasive aspergillosis [55]. In addition to early IA diagnosis, chest radiographs are insignificant since similar chest x-rays and tuberculosis are used for other filamentous lung infections [56]. The recent progress has allowed *Aspergilli* antigens to be recognized in body fluids. Cell wall components of *Aspergilli*, β-glucan, and galactomannan are detected by sandwich ELISA more recently. FDA has set a threshold of 0.5 ng/ml for galactomannan detection by ELISA test for the

diagnosis of IA [57]. The key drawback of this test was not much sensitive (71%) and the presence of galactomannan in food and fungi (*Zygomycetes* and *Fusarium*) often results in false-positive [58, 59]. Another approach used in the diagnosis of IA is PCR that has detected *Aspergillus* DNA in suspected patients with BAL fluid [60, 61]. The biggest downside of this approach is that colonization and *Aspergilli* infection cannot be discriminated against. Besides, this procedure is restricted to limited labs. FDA has also recommended β-glucan detection in the serum of patients as it is more sensitive and productive to identify infections of invasive fungi. At the species level, however, aspergillosis diagnosis is challenging and would further improve therapy. Consequently, species-specific methods of diagnosis must be set out. Chest x-rays are used to diagnose aspergilloma along with the serological methods described earlier. The chest X-ray shows the fungal mass presence in known cavities [62]. However, serological diagnosis with chest x-rays are still advisable since such conditions are imitated with aspergilloma conditions such as neoplasms, granulomatosis with polyangiitis and hydatids cysts [63]. A modern pathogen-specific technique used to diagnose the precise microbial cause of *Aspergillus* infections, could now rely on a breath test to specifically detect the exogenous fungal signature metabolites [64].

2.6 Therapeutic strategies for invasive aspergillosis

It includes triazole, echinocandins, and polyenes. Isavuconazole was introduced in a new form against IA [65]. Echinocandins like AND, CAS, micafungin. These standard drugs acted on pathogens with a different mode of action. The major targets and known resistance determinant are tabulated in Table 2.1 below.

Amphotericin B tends to bind ergosterol and alters membrane functions [66]. AmB is no longer successful for *A. terreus* associated with IA and even other AmB-resistant *Aspergillus* species. Due to inherent AmBRS and high toxicity related problems, though combination therapies with synergistic response are effective in these situations [67, 68]. AmB related toxicity to humans includes adverse effects in children suffering from IA, majorly infusion-related infections, nephrotoxicity, and hypokalemia were observed due to 3 mg/kg to 5 mg/kg AmB doses [7]. However, liposomal AMB (L-AMB) has been shown to reduce toxicity in IA patients and persist longer against azole-resistant *Aspergillus* strains [69].

Table 2.1. List of standard antifungal drugs with targets and factors contributing to drug resistance

S. no.	Class of drugs	Resistant *Aspergilli* species	Targets of drug	Mechanism of resistance
1.	**Polyenes** Amphotericin B	*Aspergillus terreus, Aspergillus fumigatus, Aspergillus flavus*	Ergosterol and oxidative stress	Less or no membrane ergosterol content due to mutation in *ERG3* or *ERG6* Increased activity of catalase and heat shock proteins
2.	**Azoles** Fluconazole Itraconazole Voriconazole Posaconazole	*Aspergillus fumigatus, Aspergillus flavus*	Cyp51 proteins, block demethylation of C-14 of lanosterol	Mutation or overexpression of Cyp51gene
3.	**Echinocandin** Caspofungin Micafungin Anidulafungin	*C. glabrata*	β-1,3-glucan synthase protein	Mutations in fks1 and raised chitin levels

Triazole is another popular class of antifungals in clinical research. Azole also influences the biosynthesis of ergosterol that disrupts the structure and function of fungal cell membranes [70]. Currently, resistance to azole in *A. fumigatus* has been observed worldwide, with azole therapy risk against aspergillosis [71]. The suggested treatment for IA is VRC as mentioned in Aspergillosis Management Guidelines with other salvage drugs like ITC, AmB, and POS. Since voriconazole is the choice of treatment, there has been a substantial increase in azole-resistant isolates which leads to an increase in clinical failures. Thus, recent treatment approaches for azole-resistant and susceptible *A. fumigatus* isolates have focused on combinatorial drug therapy including azole (VRC) and echinocandins. Echinocandin is known to inhibit the biosynthesis of the cell wall by obstructing b-glucan syntheses [72]. In the case of combination therapy, COS and VRC were found effective against *A. niger* and *A. flavus* related IPA. However, this combination showed reduced efficacy for *A. fumigatus* associated IPA [73, 74]. According to another clinical report, the combinations of VRC and AND reduces mortality rate (19.3%) mortality when compared with monotherapy (27.5%) [75]. However, the efficacy of combination drugs for IA involves more clinical trials before clinical procedures. According to Denning, D.W. the AmB and azole-resistance be more frequent in clinical isolates of *Aspergillus* species, while azole is often first-line therapy, thus

these treatment choices for IA need amendments due to the advent of resistance against drug and also need to develop a better treatment regimen to combat the current situation [76]. Besides, appropriate treatment drug therapies depend on the host's immune responses, prognosis and organ function, and pathogenic species. Therefore, with the screening of new drug molecules, attention also needs to be paid to how drugs interact with host cells and pathogens. Worldwide, the major challenges are drug toxicity and high human fungicide exposure[8, 9]. PhytoChem as a potential candidate are becoming even more relevant than current drug discovery methods [77]. and we will be discussing the importance of PhytoChem as antifungals in the latter part of the review.

2.7 Novel therapeutics strategies against *Aspergillus* related infections

Fungal diseases in particular Aspergillus, are associated with limited antifungal medicines and toxicity to patients due to their high mortality and morbidity [78]. Furthermore, the detection of new drug goals for fungal pathogens is difficult because of the similarities between fungal cells and human cells. [79, 80]. Cell wall or membrane components along with transcription inhibition are the most common targets for antifungal medicines. New drug targets for fungal pathogens must also be investigated [78]. Furthermore, most of the pathogenic fungi gained a mechanism of resistance to established antifungal medicines through the processing, over-expression, and growth of fungal biofilm proteins [81, 82]. Through the production of a new formulation for existing medicines, the discovery of new pharmaceuticals, and improved support molecules, such as nanoparticles, it is thus important to improve the treatment of antifungals in sites of infection. The new anti-*Aspergillus* target was the signaling pathway of calcineurin. The protein of calcineurin is connected with an activation of the calcineurin signaling process in *Aspergilli* or other fungal pathogens such as Candida, which is linked to different biologic processes [83]. It governs the morphogenesis of the fungal cells and tolerance to antifungals, besides regulates ergosterol biosynthesis, chitin, and β-glucan. Moreover, triphenylethylene has emerged as a new compound that blocks the calcineurin pathway by activating calmodulin [84, 85]. Further, inhibition of microtubule synthesis, and heat shock proteins (Hsp90 and Hsp70) has emerged as new antifungal targets [86]. Besides, novel antimicrobial formulations and structural improvements have been used in the past decade for the development of less harmful antifungals like *N*-methyl-*N*-D-fructose AmB methylester is an AmB derivative [87]. The current licensed antifungal medicinal products are

echinocandins. This drug class prevents cell wall glucose biosynthesis from pathogens in fungi [88]. Additionally, new azole compounds for addressing aspergillosis and countering resistance were synthesized. Against *A. fumigatus* strains (PC945 and PC1244) [89]. These compounds are being used to suppress invasive aspergillosis through inhalation. CD101 novel echinocandin was designed to treat severe fungal infections such as invasive aspergillosis. However, the drug has grown clinically and has shown promising results against candidemia [90]. Furthermore, olorofim (F901318) a novel compound developed against fatal *Aspergillus* infections [91]. Despite considerable attempts to monitor the antifungal resistance or to produce new antifungal compounds and to improve existing drugs with high toxicity are inadequate. Thus, alternative therapies like immunotherapy with antifungals are also needed [92, 93].

2.8 Phytochemicals as an alternative

There is a continuous search of novel chemical and their mode of action do develop antifungals with high efficacy. To achieve this, plants are the major producers of promising natural products of therapeutic interests in the control of fungal diseases. It's the need of the hour to identify new phytochemicals, the development of drugs with them having less toxicity, and more effectiveness. In this way, the combination of phytochemicals with traditional drugs is an apt solution for the above-mentioned drawbacks of the drugs. Although, all the discoveries in alternative therapy, PhytoChem gained lots of interest. But still, these compounds are not available for practice in clinical use. Therefore, effective PhytoChem needs to be tested for their anti-*Aspergillus* activity and mechanism of action so that they can be used as key drugs with or without standard drugs. PhytoChem such as tocopherol phenolic, thiols, flavonoids, anthocyanin, and carotenoids showed promising [10, 11]. Recently, the use of quercetin (QRT) to limits the development of *A. parasiticus* and *A. flavus* were studied and proven to be a promising biological agent to control the aflatoxin food crops [94]. Whereas, another group of researchers showed shikonin activity against *C. albicans* isolates as well as Fluconazole resistant *C. albicans* [95]. Artemisinin (ART) was more explored PhytoChem and also investigated for its inhibitory effect against *A. fumigatus* [96]. Additionally, a synthetic coumarin-derivatives (SCD-1) was explored against in *A. fumigatus* [97]. Thus, to overcome the DR in *A. terreus* our study is focused on screening effective and potent phytochemicals that could be alone or in combination used to improve the existing therapeutic approaches.

2.9 Drug resistance mechanism

Excessive use of antifungals is one of the main causes of DR, which could be due to long, incomplete doses. It is also depending on *Aspergillus* species, antifungal molecule, and the geographic location [98, 99]. Also, some *Aspergillus* species are intrinsically resistant to certain antifungal Although the data for drug-resistant genes and genome mutations are available, not sufficient to drawn exact mechanisms involved in molecular mechanisms involved in DR. Thus, it is very difficult to combat invasive fungal secondary infections by limited therapeutic choices in Resistant isolates. In the case of *A. fumigatus* leading cause of aspergillosis, the molecular aspects related to DR is more explored in comparison to other species. The principal categories of mechanisms for DR in *Aspergilli* include (1) alteration of targets (mutations which reduce binding of the drug), (2) lack of efficacy of the drug, increasing drug efflux, and drug-targets overexpression or sequesters of antifungal agents [100]. Recently various resilience patterns, including inherent resistance in *Aspergillus* species, were observed using new mechanisms and the rise of resistance to more than one drug class at the same time. *A. terreus* and *A. fumigatus* have been studied in the clinical setting or frequently found resistant isolates compared to other species of Aspergillus. The major factor contributing to DR of *A. fumigatus* (Azole-resistant) is mutation/overexpress of cyp51A gene (encode for 14-sterol-demethylase) major enzymes involved in the biosynthesis of ergosterol [101]. In addition, mutation in cyp51 genes (other location) or in promoter region (TR34 / L98H and TR56 / Y121F / T289A) was observed [102]. Azole resistance is predominant in the environmental route with cyp51A (TR34 / L98H) mutations stated in resistant and susceptible strains of *A. fumigatus* genome sequencing [103]. Also, from mutational studies on biofilms of *A. fumigatus*, ‘cspA’ (glycophosphatidylinositol-anchored cell wall protein) was observed critical for the formation of biofilm and integrity of cell-wall, and in drug response. Furthermore, in *A. fumigatus* the substitution of S678P in Fks1p, contributes to resistance to echinocandin [104]. Furthermore, in biofilms, *A. fumigatus* high level of gliotoxin was found to provide DR [105]. In contrast with other *Aspergilli*, *A. terreus* is intrinsically immune to AmB, but the mechanism is less unclear. It has been proposed that the upregulation of ERG5, ERG6, and ERG25 (ergosterol biosynthesis genes) contributes to resistance against AmB [106-108]. In contrast to this, another study stated that better protection management for oxidative damages in resistant isolates of *A. terreus* may attribute to the resistance mechanism instead of ergosterol content

[27]. In AmBRS strains of *A. terreus* SOD and CAT activity were greater in contrast to sensitive isolates [109]. Besides, Hsp70 and Hsp90 inhibitors could substantially improve the effectiveness of azole medicines and AmB for resistance in *A. terreus* [86, 110]. However, the exact role of heat shock proteins is not known yet known thus it is of great interest to thoroughly examine the mechanisms by which they contribute to AmBRS in *A. terreus*.

2.10 Progress and relevance of study in *Aspergilli*

The genome sequencing study of *Aspergilli* allows the understanding of the pathogenesis and mycotoxin biosynthetic pathways. Besides, this comparative genomic study culminated in an appreciation of the variations of virulence and morphology of *Aspergillus* species. The genomic data should be extended to mRNA or protein expression levels. The *Aspergilli* mRNA studies may enable the response of the organism to external stimuli including antifungal or morphology mechanisms. They also allow predicting the interactions between host pathogens in *in-vitro* or *in-vivo* situations. Such mRNA-level studies can, however, not provide a clear picture without functional studies including proteomics. Because of the importance of the *Aspergilli* species, proteomic studies have been conducted mostly on *A. fumigatus* to explore the set of proteins in morphotypes and under stress conditions. In addition to this, the differential proteome profiling of different morphotypes of *Aspergillus* species shows the primary proteins/enzymes of different cellular processes or biochemical, such as the transfer of conidia-to-mycelial stage including secondary metabolite [12]. Different research studies are currently being carried out to classify *Aspergillus* species. in different developmental stages [111-114]. During the transition of *Aspergilli* morphotypes, the relative protein profile of conidia, mycelia added knowledge on cellular as well as biochemical cycles. [12, 111, 113-116]. However, little proteomic studies on *A. terreus*, and due to the increased medical importance of *A. terreus* it is now necessary to study its morphological events. Germinations of conidia involve virulent proteins high-level transcripts (TER and DHO genes) and (MPKC and Hog1) as suggested by a previous proteome study on *A. terreus* [114]. To date, a proteomic analysis of the interaction of host pathogens has provided information on the establishment of infection in *Aspergilli* [112, 117]. According to a previous report from our lab we have now understanding of various regulators of immune responses in human lung epithelial cells in response to *A. terreus* and the cytoskeleton organizational change during

internalizations of conidia by human lung cells were depicted, also involved nuclear factor-kB pathway of host immune response. This pathway induces IL-8 development and could therefore be further explored for biomarker targets to regulate inflammation during lung infections [118]. RNA-seq study on the interaction of *A. fumigatus* during IA infection mice and profiled differential transcript levels in murine kidneys discovered different immune reactions including (Th-1 and Th-17-type) at different stages of infection [119]. Currently, scientists have been able to establish better drug and diagnostic markers for different fungi through proteomic studies [120, 121]. Secondary metabolites may also evaluate/improve a non-invasive diagnostic method for *Aspergillus* infection. Therefore, it is necessary to learn about secondary intermediate metabolites in *Aspergilli*, thereby contributing to morphology and pathogenesis. Although, this area is not explored few reports provided knowledge about morphotype specific secondary metabolites having a potential role in the pathogenesis of *Aspergillus* related infections are well documented in a review by Shankar *et al.*.[12, 122] . These intermediates are main drivers for evaluating the impact of toxigenicity and virulence against the host system [123]. Thus, using proteomics approaches the biosynthesis of a few mycotoxins was traced in the case of *A. fumigatus* biosynthesis of mycotoxins like pseurotin A in conidia and mycelia gliotoxin, fumagillin, and fumitremorgins in mycelia were reported [124]. While in *A. flavus* aflatoxin B1/B2 known to cause cute aflatoxicosis its biosynthesis observed during germinating conidia and supposed to have a role on *A. flavus* growth and development [113]. In contrast to this, the germinating conidia of *A. terreus* showed the expression of intermediates of geodin and terretonin mycotoxin [114]. Thus, these metabolites could be beneficial for progress in non-invasive diagnostic markers for *Aspergillus* infections [125]. Overall, the activation of mycotoxin biosynthesis pathways varies from species to species and active under different morphological conditions and stresses in *Aspergilli*. The cells of the host immune system interact with fungal moieties (chitin, mannan, galactomannan, and β-glucan). There are different soluble (pentraxins and lung collectin) and cell-based immune receptors (dectin-1, Pentraxin, and TLR receptors) known to better recognize *Aspergilli* [126-128]. Any changes in PRRs have therefore an effect on the structure and function and defects host immune signaling. Thus, the study of these SNPs or mutations which may apply to people prone to fungal infections would also be an area of interest. In this context, the immunoinformatic *A. terreus* study conducted and indicates that non-synonymic SNPs of the Dectin- 1 and pentraxin receptor genes and their effect on protein structure may be

considered for the risk assessment of fungal infections [129, 130]. Further, the importance of proteomics to characterize the antifungal response is discussed below.

2.11 Antifungal response using a proteomic approach in *Aspergilli*

Proteomics advances allow researchers to examine resistance pathway facilitators in DRS and DSS, also classify possible morphotypes specific biomarkers in *Aspergilli* [131]. There is far less information available on the differential proteome profile under antifungal exposure in *Aspergilli*, so to obtain a detailed picture of the drug/PhytoChem response in *Aspergillus* species, there have been limited studies available in the response of antifungals such as AmB, CAS, and azole (ITC and VRC) in *Aspergilli* [132-135].

Also, potent PhytoChem such as ART, coumarin-derivative, and QRT were explored for their made of action /new antifungal targets using proteomics approaches [96, 97, 136]. Protein profiling underAmB exposure in *A. fumigatus* showed down-regulation of the energy metabolism and ergosterol biosynthesis protein Erg13 (AFUA_3G10660) and, translational machinery was upregulated Gautam P *et al.*, (2008) using MALDI[133]. Additionally, AmB exposure increases the abundance of a heme biosynthetic protein (Hem13 ;AFUA_1G07480) that implicates the requirement for more heme-molecules. Iron molecules act as a co-factor and requirement for ergosterol biosynthesis. Under iron-deficiency, iron enzymes lose their activities [137].Also, the up-regulation of antioxidant enzymes (manganese superoxide dismutase, Prx1/ LsfA, and catalase) during treatment with AmB documented the oxidative stress-mediated cell damage in *A.fumigatus* [133]. Further, ITC differentially regulates 54 proteins (12 proteins showed high expression and 42 proteins showed low expression) in *A. fumigatus* [134]. Also, the catalases were found in high abundance suggesting oxidative response. On the other hand, a ribosomal reshuffling was observed in the protein profiling in *A. fumigatus* under exposure CAS using iTRAQ, [132, 138]. In previous studies, it was speculated that mitochondrial hypoxia response domain protein was downregulated up to 16-fold (24 and 48 h) in DSS and was relatively not modulated in the DRS under CAS but not found this during treatment of AmB and VRC to *A. fumigatus*, proposed as a CAS- specific biomarker [132, 133, 135]. As well as, biofilms in *Aspergillus* species are very less explored yet and found that biofilm formation-related protein showed an abundance of translational regulatory proteins which requirement of a new set of proteins during biofilm establishment in *A. fumigatus*

[139]. Intracellular protein analysis using 2D-PAGE and MS-TOF in *A. niger* showed the presence of proteins involved in cell-to-cell adhesion, which help in surface adhesion and establishment of biofilm [140]. Thus, in *Aspergilli* antifungal drugs, majorly influence the energy processes, oxidation stress, biosynthesis of cell-wall, and ribosomal reprogramming.

The safe and efficient alternative treatment of aspergillosis may be given by natural plant products with antimicrobial properties. The antimicrobial properties of these molecules, which have been extensively studied and still, are not available for clinical use. PhytoChem like ART and p-Coumaric acid (CA) have been tested for anti-*Aspergillus* agents [96, 97]. There are few studies conducted in *Aspergilli* for the elucidation of the mode of action of PhytoChem molecules using proteomics approaches [136]. It has been demonstrated by proteomics study that QRT inhibitory action involves oxidative stress and cell wall-related protein and also switching of MAPK to cAMP / PKA signaling to overcome the stress in *A. flavus* [136]. Exposure of ART to *A. fumigatus* leads to remodeling of the cell wall as proteins such as thaumatin domain protein, conidial hydrophobin, galactomannan were found in low abundance in differential proteomics study [133]. Also, oxidative phosphorylation related genes were found down-regulated in microarray and absent in the protein data, indicating NADH dehydrogenase may be ARTtarget and may disrupt the membrane potential in *A. fumigatus* [96]. In contrast to this, standard antifungal treatment has not significantly affected the oxidative phosphorylation process in *A. fumigatus*, *C. albicans*, and *S. cerevisiae* [133, 141-145]. Subsequently, SCD-1 was found an efficient inhibitor of *Aspergilli,* and the mechanism of action includes novel targets from proteins related to riboflavin biosynthesis as showed low abundance [97]. Thus, to understand the inhibitory mechanism and identification of novel drug targets proteomics could be a robust technique. Furthermore, information gained on protein/enzyme influence under the antifungal molecule could be explored to enhance current antifungal therapies.

2.12 Biofilm formation in *Aspergilli* and role in drug resistance

Microorganisms grow on a plethora of surfaces viz., sediment, skin, bark, rock, and mucosal tissues [146, 147]. The majority of bacterial and fungal pathogens are present in the human bodies in the form of biofilms. This results in a reduction of the drug response and host immunity. Similarly, biofilm having characteristic parallel-packed hyphae has been witnessed in the case of *A. fumigatus* [148]. Also, the formation of a self-produced ECM has been well documented [149]. In a study conducted by [150], aspergilloma specimens were found to have hyphae surrounded by ECM which further endorsed the concept of biofilm formation by *A. fumigatus*. Still, the exact mechanism of the biofilm formation in *Aspergilli* is not well studied and devised. However, while studying the mechanism of biofilm formation in *C. albicans*, it suggested that the cell wall proteins viz., Hwp1, and Als3, are associated with this [151].

A perusal of literature hence suggests that proteins from cell wall will surely play a vital role in *A. fumigatus* biofilm formation. Moreover, a study conducted by [152] revealed that cspA, a cell wall protein is associated with Biofilm Formation and DR in *A. fumigatus*. *Aspergillus fumigatus* biofilms consisting of monosaccharides, α-1,3-glucans, Galactomannan, polyols, melanins, and proteins (hydrophobins and DNA) having hyphal cells embedded in ECM have been found on Dialysis catheters [150, 153, 154] *et al.* 2013; [147]. Transcription factors are also involved in biofilm formation. This has been endorsed by studying the involvement of agglutinin-like sequence (ALS) proteins, proteins resembling Hyr1, and common in fungal extracellular membranes (CFEM) proteins in biofilm formation (Nobile and [147, 155]. Additionally, modulations in metabolic activities that could be associated with virulence have been observed during biofilm formation [139]. There is a very high death risk and it is difficult to eradicate biofilm-associated infections with current drug therapies, so more intense experiments are required to decipher the exact function of biofilm in the drug-resistance mechanism in *Aspergilli*.

CHAPTER -3

EXPLORATION OF THE MYCELIAL PROTEOME IN *ASPERGILLUS TERREUS* TO ELUCIDATE THE PROTEIN/ENZYME FAMILIES ASSOCIATED WITH THE MYCELIAL NETWORK AND RESISTANT MECHANISM.

3.1INTRODUCTION

Aspergillus terreus requires more attention among other *Aspergilli* due to the high incidence of IA associated causality (51 vs. 30%) as well as inappropriate prognosis (21v/s 38%) [5]. *A. terreus* produces numerous amount of small conidia (~2-5µm in dia. in size) spreads ubiquitously [156]. These tiny conidia were easily inhaled by humans and if not removed by (acquired or innate) immune responses specifically in patients with impaired immunity. These conidia-initiated germination and invade inside the lungs to germinate into hyphae to cause systemic infection [12, 19]. *Aspergillus terreus* is known to have intrinsic AmBRS, all over the world, ~98% *A. terreus* isolates were found resistant against AmB [5, 6]. Also, it is acquiring azole resistance ~ 5-10% isolates were found to be azole-resistant particularly for voriconazole [6]. Proteins/enzymes involved in ROS detoxification, as well as membrane-fluidity imbalance, may add to the resistance mechanism of AmB in *A. terreus* [13]. AmBRS strains showed higher expression of sod2 and cat transcripts upon AmB treatment that suggested better oxidative damage management in AmBRS strains of *A. terreus* [27, 106, 108, 109]. Thus the knowledge of these variable consequences of the drug resistance or susceptible *A. terreus* isolates for the antifungal drug stresses is critical for understanding of their role in DR and Biofilm formation in *Aspergilli* is an another factor for antifungal resistance, which is the result of fungal cell adhesion due to environmental factors[13]. Biofilm provides temporary resistance against the drug in *Aspergillus* species by protecting pathogen in the hostile environment [140, 148, 157-159]. In *A. terreus*, biofilm formation is less investigated, thus insight into biofilm formation is required. The morphological transitions are very crucial for the initiation and establishment of invasive infections. The conidia of *A. fumigatus* expand isotopically leads to germination than elongate to become

hyphae. Further, the branching of hyphae leads to the formation of a mycelial network (Suh *et al.*, 2012). Different morphotypes have variable contents of proteins, glycoproteins, and polysaccharides which leads to different immunological response during the transition of conidia into the hyphae/mycelia[4]. The activation of Th1 within 3 days of post-infection and Th2 and Th17 immune responses within 5day of post-infection of *Aspergillus fumigatus* has been observed in transcriptome profiling [119]. Also, in *A. terreus*a rearrangement in proteins/enzymes of cytoskeleton components described the assimilation of *A. terreus* conidia during interaction with lung epithelial cells in humans [118]. Shankar *et al.* briefly reviewed the proteins/enzymes and signaling cascade that is crucial during different development stages of *Aspergilli* [12]. The conidia of *A. terreus* start germination after 16h of inoculation in the DMEM medium. Also, their proteomics study shed light on virulence factors and biosynthesis pathways of secondary metabolites during germinating conidia [114]. Despite the importance of the morphological transition in *A. terreus*, the determinants contribute to hyphae and mycelium are yet to be established. Necessary knowledge of such reprogramming of conidia, hyphae, and mycelium may accelerate the formulation of a precise diagnostic as well as treatment strategy to control *A. terreus* infections. Thus, it is required to elucidate enzymes (proteins families) present in mycelia morphotypes. Synthesis of new proteins/modification of protein activity may be crucial for the reprogramming of cellular machinery among morphotypes in *A. terreus*. In this context studies were undertaken to profile mycelial proteins of *A. terreus*(NCCPF860035) at 48h to analyse the relevant biochemical and cellular pathways contributing to the pathogenesis and DR.

3.2MATERIAL AND METHOD

3.2.1 Culture conditions: *Aspergillus terreus* (NCCPF-860035) was procured from the National Culture collection of pathogenic fungi (NCCPF), PGIMER Chandigarh India. PDA media was used to maintain *A. terreus* cultures at 37°C for 4-5 days. Conidia were harvested in phosphate buffer saline (pH 7.4) with 0.05 % Twin 20. Further, a haemocytometer was used to count CFU, and conidia suspension (1×10^{6}) was prepared in PBS (pH 7.4) and stored at 4°C for further experimentation.

3.2.2 Large scale culture: Large scale mycelial culture preparation was performed using conidial suspension (1 x 106 conidia/ml) in PBS of *A. terreus* as inoculum in DMEM (pH 7.4) with glucose and supplemented with 10% FBS to mimic the host

environment [114]. To determine the transition of morphotypes in *A. terreus* cultures were monitored at each hour of incubation using a light microscope (Olympus, India). The different morphotypes of *A. terreus* conidia, germinating conidia, hyphae, and mycelia were observed at different time points. More than 90% of mycelia growth of *A. terreus* was observed under a light microscope. Further, mycelia were harvested after 48h, followed by washing of sample thrice with chilled PBS (pH 7.4).

3.2.3 Protein extraction from the mycelial extract of *Aspergillus terreus*: Protein isolation from the mycelial mat (1g) in two independent biological replicates were carried out by grinding with liquid nitrogen in pastel and mortal. Further cells were lysed with buffer consisting sodium phosphate (50 mM) , EDTA (2 mM), DTT (0.2 mM) and PMSF (1 mM) (pH 7.4). The sample was then incubated for 4h at 4°C with continuous stirring followed by centrifugation at 15000 rpm at 4°C for 20 minutes. Precipitation of collected supernatant was carried out using the TCA acetone method, for which 5% trichloroacetic acid (TCA) was used at -20°C for 24h. After 24h of incubation precipitates of protein were washed 4-5 times with chilled acetone to remove the traces of TCA [114]. The pellets of proteins were dried and dissolved in 6M Gn-HCL plus 0.1 M Tris (pH 8.5) buffer for nLC-ESI-MS/MS analysis. The protein concentration was estimated using the Bradford method [160].

3.2.4 Mass Spectrometry using nLC-ESI-MS/MS

3.2.4.1 Sample preparation: Using two independent-biological samples, protein data were generated at Vproteomics, Delhi (India). An amount of 50µl of protein sample (~0.75µg/ml) has been reduced using 5 mM tris. It was further alkylation with 50 mM iodoacetamide followed by trypsin digestion (1:50, trypsin/lysate ratio) for 16h at 37°C. Using EASY-nLC 1000 system coupled with QExactive MS spectrometer (Thermo Fisher Scientific) analysis was carried out for protein pellets re-suspended in 5% acetonitrile, 0.1% formic acid (Buffer A) [161].

3.2.4.2 MS Analysis: The peptide mixture (1.6 µg) was resolved using a 25 cm PicoFrit column and 1.9 µm of C18-resin was used to fill the column (Dr. Maeisch, Germany). Followed by loading of peptide mixture in buffer A and further, 0–40% gradient of 95% acetonitrile, 0.1% formic acid (buffer B) was used to elute at a flow rate of 300 nl/min for 70 min.

3.2.4.3 Data processing: The data generated in two raw spectral files were processed against the *Aspergillus terreus* UniProt (reference database) using Proteome Discoverer software (V/2.2). The criteria for precursor (10 ppm) and fragment mass tolerances (0.5 Da) have considered for search using Sequest. Additionally, both Protein false discovery rate and peptide-spectrum match were set to 0.01 FDR.

3.2.4.4 GO analyses: The gene ontology (GO) term was defined as predicted proteins using Blast2GO V.5 (https://www.blast2go.com). Further, the UniProt database was used for the prediction of a few proteins participating in different metabolic-pathways [114, 162].

3.2.4.5 *In-silico* prediction of Secretory proteins: *Aspergillus terreus* proteins were subjected to SECRETOOL a web tool, integrated for secretome analysis of fungi [163] that enable secretory protein predictions out of amino acid sequence files based on signal peptides in one step and this is available at http://genomics.cicbiogune.es/SECRETOOL/Secretool.php.

3.2.4.6 Detection of Protein-Protein interactions: STRING (ver.10.5) analysis was carried out for protein-protein interactions (PPIs) network analysis. Protein interaction network analysis for proteins from major biosynthetic pathways (cell wall and cytoskeleton, signal transduction proteins, heat shock proteins, and predicted secretory proteins). A total of 85 shortlisted proteins were subjected to the STRING database using different modes (action view, confidence, interactive, and confirmation or evidence view) [164].

3.2.4.7 Relative transcript expression profiling using Quantitative RT-PCR: To carry out the relative gene expression analysis of important proteins in *A. terreus* morphotypes (germinating conidia and mycelia), total RNA from2-independent biological replicates was extracted. Cultures of *A. terreus* were prepared from an inoculum of conidial suspension (1 x 10^6 conidia/ml) in PBS in DMEM (pH 7.4) with glucose and supplemented with 10% FBS. The germinating conidia and mycelia were harvested at 16h and 48h respectively. RNA extraction was carried out using the TRIzol method (TRIzol-Invitrogen, US), and DNase I (Thermo-Scientific, US) was used to remove the genomic-DNA contamination. Nanodrop spectrophotometer (Thermo Scientific, US) was used to assess the quantity and quality of RNA at A260nm/A280nm. Verso cDNA kit (Thermo-Scientific, US) was used to prepared cDNA from extracted RNA samples (1µgRNA) following

manufactures instructions [165]. NCBI based tool; Primer-Blast was used to design primers for selected genes [166], enlisted in **Table 3.1** Further to carry out qRT-PCR; the CFX96 machine (BIORAD, USA) set up with SYBER green dye (Biorad) was used. Each PCR reaction was performed using cDNA 100ng. The PCR conditions were; initial denaturation at 95°C (3 minutes), 39-cycles of 95°C (10 seconds), melting temp. (Tm) 49 -58°C (45 seconds.), elongation at 72°C (30 seconds.). Concurrently, a Tm analysis was conducted and 40S ribosomal gene was used as a reference gene [114]. The expression data analysis was carried out using the "comparative ΔΔCt" method [167].

3.2.4.8 Biofilm formation and scanning electron microscopy: To establish biofilm in *A. terreus* 1 x 10^6 conidia/ml was used to inoculate in each well, following cultivation at 28°C for 48h in Czapekdox broth with no stirring [168]. The non-adherent cells were removed by aspirating medium after 24h and replaced with fresh media. Further, grow biofilm till 48h and non-adherent cells were removed followed washing with PBS three-time thoroughly. Biofilm was dried by in microtiter plate onto tissue paper and visualized using SEM. For the SEM, the dried biofilms were fixed with 2% glutaraldehyde for 2h then the samples were subjected to ethanol for 10 min to dehydrate the biofilms and again dried and then mounted on an aluminum sheet. Gold-palladium alloy was used to coat the biofilms. Scanning electron microscope (Zeiss SEM, MA EVO -18 Special Edition) was used to observe the samples using different magnifications.

3.3 RESULTS

3.3.1 Morphotypes in *Aspergillus terreus*: Cultures of *A. terreus* were examined under a light microscope to track the morphological transitions every 12 hrs, we have found that the germination occurs around after 12-16h. The germinating conidia lead to form hyphae around 24h in continuous growth. Excessive hyphal growth in forms of mycelial networks (approximately 90-95%) at 48h. The microscopic images of various morphotypes in *A. terreus* shown in Fig. 3.1

Table 3.1 List of primers of selected genes for gene expression analysis.

S.NO	Name	Primer
1	Terrelysin	Forward 5'-ATCCATATCCGCGACCGT-3'
		Reverse 5'-GTCAGGGCTTTCTTCTCATCC-3'
2	Translation factor G (tif35)	Forward 5'-GTCGTGTCACCAGAGTATTCC-3'
		Reverse 5'-GGATAAGGTGGCGATAACCG-3'
3	40S ribosomal protein S1 (40s), reference gene	Forward 5'-CATTGGCCGTGAGATCGAG-3'
		Reverse 5'-CCCTTGTCATCGGTGGTAGA-3'
4	Dicer-like protein 1 (dcr1)	Forward 5'-AAATTCCTCGGTGCGTTGG-3'
		Reverse 5'-CGGCTACATCGCTCTGACTA-3'
5	Mitogen-activated protein kinase (mpkC)	Forward 5'-ACGATCCTACCGATGAGCC-3'
		Reverse 5'-GCGTCATATAACCCGACGAG-3'
6	Heat shock protein 70 (HSP70)	Forward 5'-GACCACGGAAATCGAGCAGA-3'
		Reverse 5'-CATGGTGGGGTCGGAAATGA-3'
7	Heat shock protein 90 (HSP90)	Forward 5'-CTCGCCAAGAGCCTCAAGAA-3'
		Reverse 5'-GCTCCTTGATGATGGGGGAC-3'
8	Catalase (Cat)	Forward 5'-ACCTCTTCGCCTTTGACTGG-3'
		Reverse 5'-TCTTCTCATAGGCCGGGTCA-3'
9	Superoxide dismutase(sod)	Forward 5'-GGCTGTTGTGGTTTTGCCTG-3'
		Reverse 5'-AGGAGATGGTGGTGAGGGAG-3'

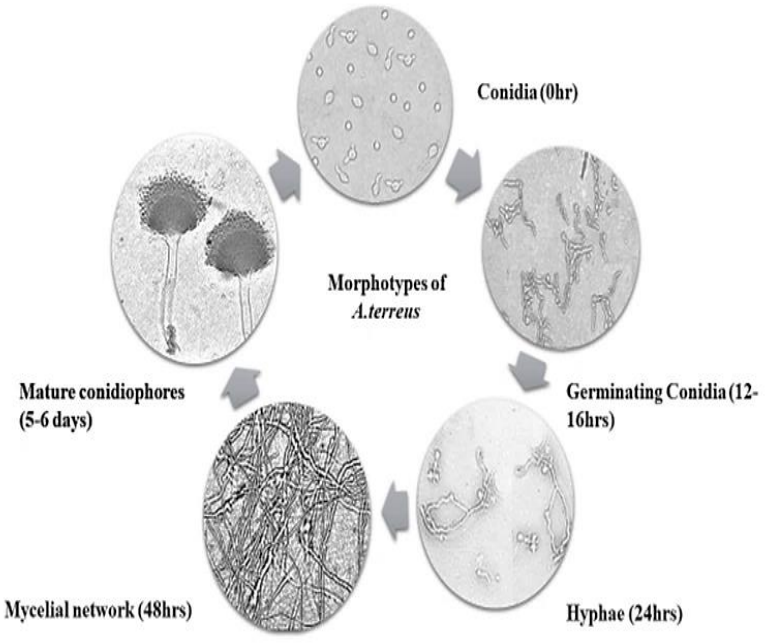


Fig. 3.1 Morphological transition of *A. terreus* from conidia to mycelium at different time points.

3.3.2 Mycelial proteins profiling of *Aspergillus terreus:* We have obtained mycelial proteome raw data in spectral files using nLC-ESI-MS/MS and *in-silico* data analysis was carried out using the following pipeline represented in Fig.3.2 (Spectral files abstained attached in Appendix-1)

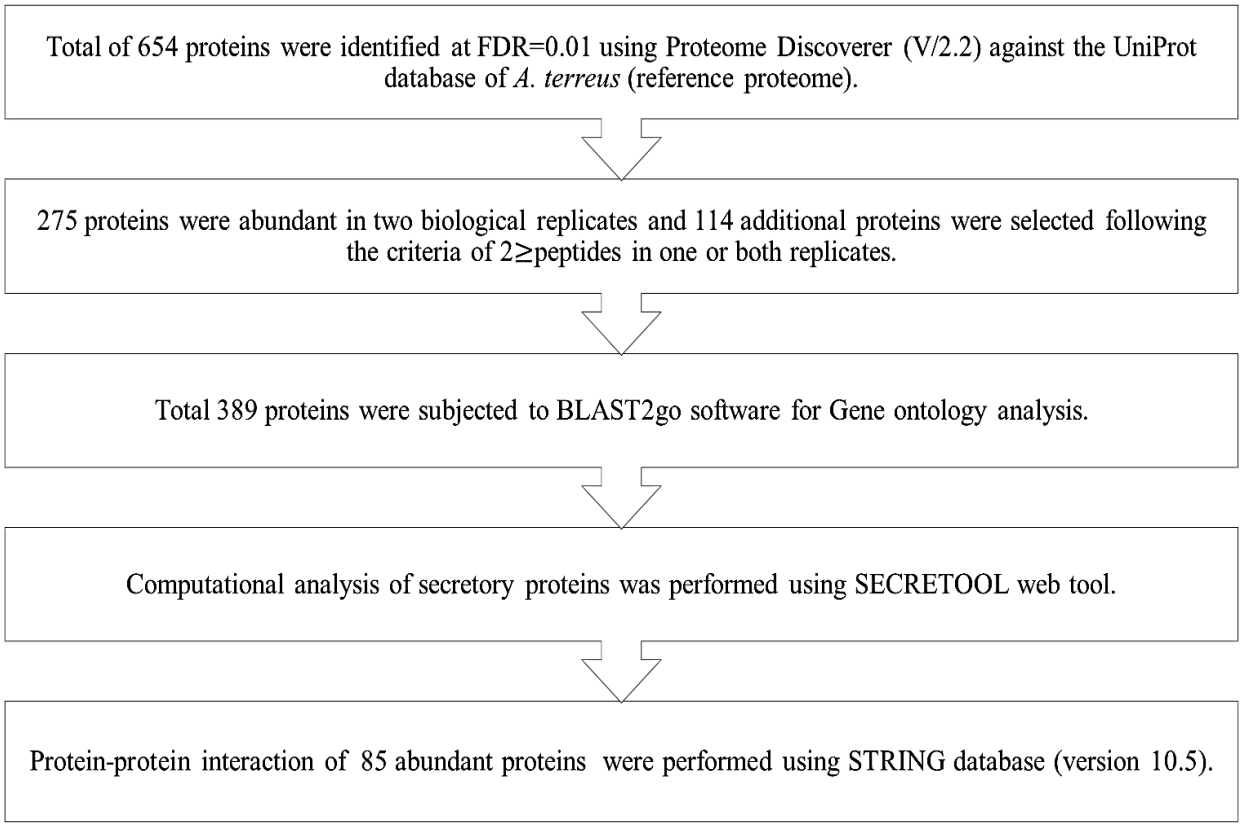


Fig.3.2 Flow chart of pipeline used for *in silico* analysis of mycelial proteome data in *Aspergillus terreus.*

3.3.3 Gene ontology results

Gene ontology terms are defined for biological processes, molecular processes, and cellular processes for identified proteins. Biological processes under 14 GO slim categories showed proteins from ribosomal biogenesis (18%), cell/stress homeostasis (14%), cellular respiration (11%), transport (13%), protein metabolism (12%), and 8% protein were related to carbohydrate metabolism, cytoskeleton, and cell wall organization represented in pie chart Fig.3.3 (Details of Important proteins are given in Appendix -2)

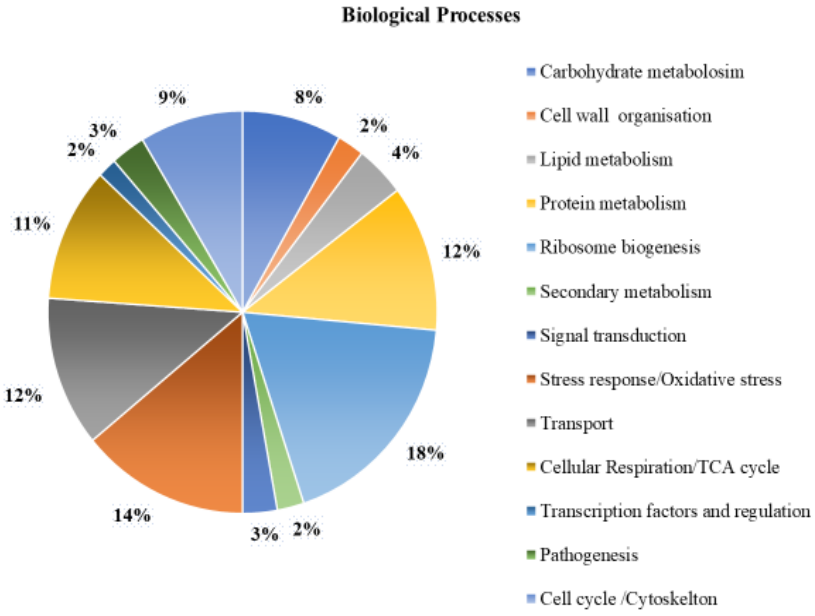


Fig.3.3 Biological processes predicted by Blast2Go proteins in mycelia of *Aspergillus terreus*. Data represented in the pie chart with percentages. Different colours representing different GO slim categories.

Molecular functions to predicted protein were categorized in 11 GO slim categories represented in pie chart Fig.3.4. (Details are given in Appendix -3) Mostly the proteins have RNA /DNA binding (22%), oxidoreductase activity (17%), protein binding (16%), transferase activity (14%), hydrolase activity (11%).

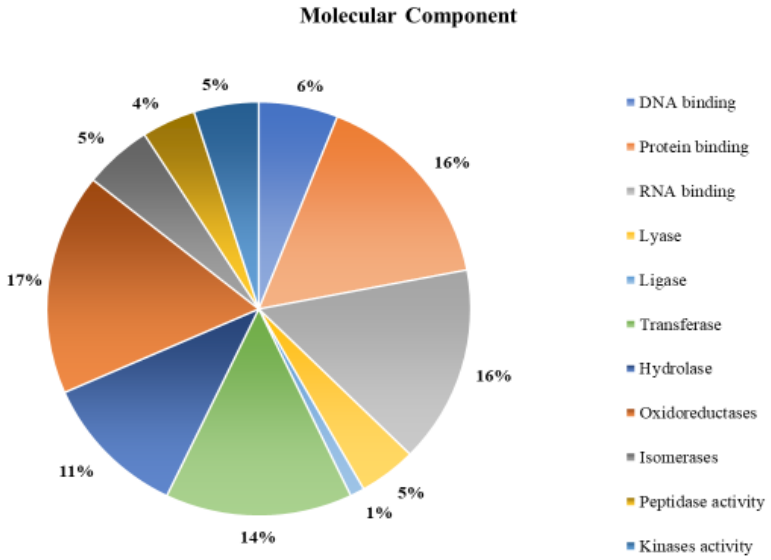


Fig.3.4 Molecular processes for predicted proteins in mycelia of *Aspergillus terreus*. Data represented in the pie chart with percentages. Different colours representing different GO slim categories

Further, the cellular component was predicted in GO analysis showed that most abundant proteins were from nucleus (17%), ribosome (17%), membrane (13%), cytosol (12%), and mitochondria (12%) and others are given in Fig. 3.5 (Details are given in Appendix -3)

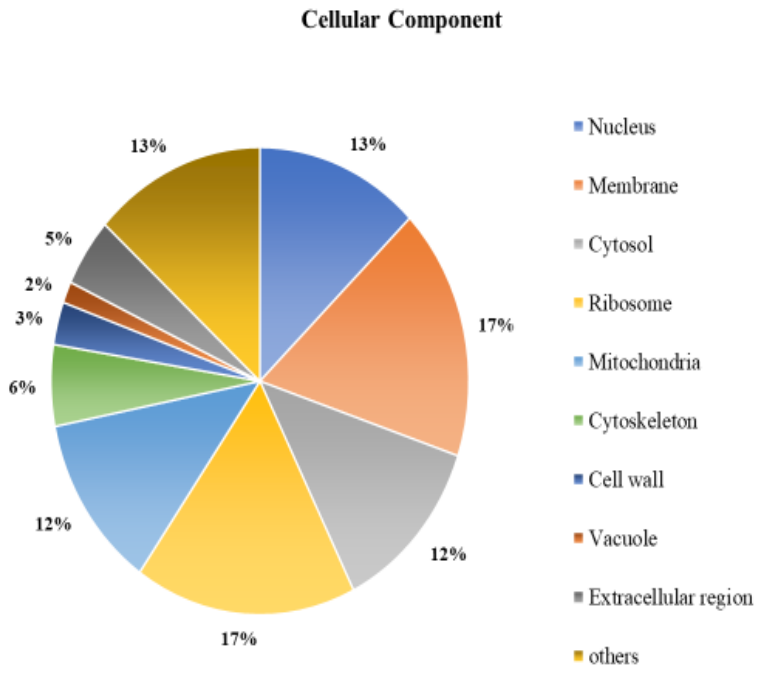


Fig.3.5 Cellular processes for predicted proteins in mycelia of *Aspergillus terreus*. Data represented in the pie chart with percentages. Different colours representing different GO slim categories.

Proteins from cellular homeostasis, energy metabolism, ribosome biogenesis, cell wall, and structural components were observed in GO analysis under the cellular component. These pathways may be crucial for hyphal and mycelial growth in *A. terreus*. Thus, considered for further analysis. Also, the probable secretory proteins were predicted using SECRETOOL analysis and we have found 8 secretory proteins that are enlisted in Table 3.2 below with biological functions.

3.3.4 Mapping of protein interactions

We have selected 85 abundant proteins out of a total of 389 for PPIs using the STRING database. These proteins belong to signaling pathways, secretory, heat shock, cell wall/ cytoskeleton, etc. Only 54 proteins showed significant enrichment in INTERPRO and PFAM Protein Domains.

Table 3.2 List of secretory proteins predicted by using SECRETOOL in *Aspergillus terreus*

S.NO.	Protein ID	Protein Name	Biological function
1	Q0CM99	Extracellular cell wall glucanaseCrf1/allergen	Cell wall and carbohydrate metabolism
2	Q0CYJ2	Allergen Asp f 15	Hypersensitive immune response
3	Q0CFR6	Uncharacterized protein	Integral membrane component
4	Q0CW86	Protein disulfide-isomerase (tigA)	Cell redox homeostasis
5	Q0CMQ3	1,3-beta-glucanosyltransferase	Cell wall elongation
6	Q0D1R1	Beta-hexosaminidase	Carbohydrate metabolism
7	Q0CWQ0	Uncharacterized protein	NA
8	Q0CW27	Uncharacterized protein	Calcium ion binding (predicted)

Most of the interacting proteins were belonging to the signaling cascades, energy metabolism, and cell wall component modulation. Interactomes represented in Fig.3.6 and the proteins that didn't show any interaction with other proteins were excluded.

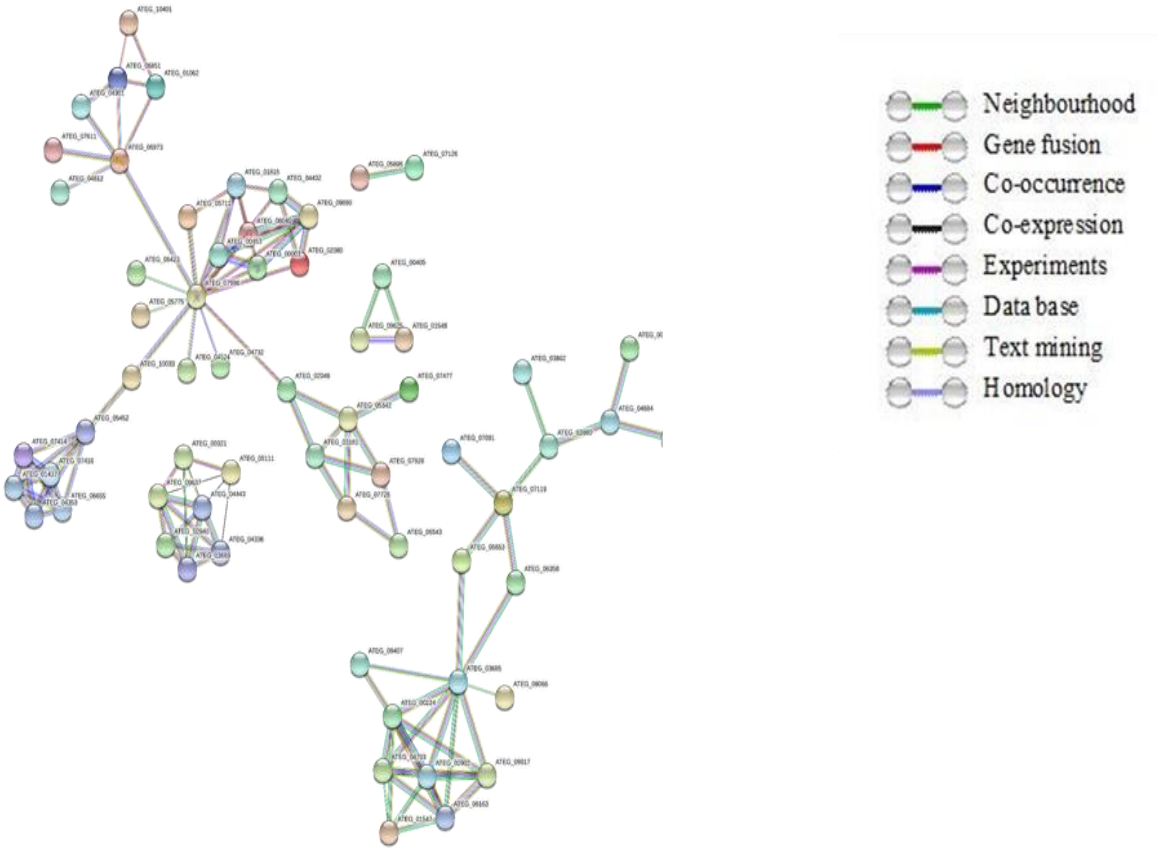


Fig.3.6 Protein-protein interacting network predicted by STRING. Details are given in Appendix-3

3.3.5 Categorization of mycelial proteins in *A. terreus*

The abundant proteins/enzymes shortlisted in mycelial proteome data were further categorization according to their functional relevance. These proteins were classified based on GO terms, homology with other *Aspergillus* species, and literature-based evidence. We have categorized these proteins into different groups such as proteins associated with mycelial growth and establishment, proteins related to biofilm establishment and resistance, proteins related to invasion and pathogenesis.

3.3.5.1 Proteins associated with mycelial growth and establishment

In present mycelial proteome data, we observed an abundance of ribosomal proteins that have been observed in our data, with a total of 79 ribosomal proteins operating in transcription /translation, ribosomal biogenesis, or in the regulation of other biological processes. Hyphae and mycelia are the rapidly growing structures of fungi that require more ribosomes for translational machinery to complete the demand for new proteins. Thus, the important ribosomal proteins having a function in ribosome biogenesis and morphogenesis in *A. terreus* enlisted in the given Table.3.3 **(**more details of the proteins given in Appendix-2)

Fig. 3.3 List of ribosomal proteins with important role in morphogenesis in *A. terreus*

S.No.	Uniprot IDs	Protein Name	Biological Functions
1	Q0CTP4	Ribosomal protein S26e	Morphogenesis
2	Q0C8F1	Cell division cycle protein 48	Ribosome biogenesis
3	Q0CAG3	Zinc finger protein gcs1	Ribosome biogenesis
4	Q0C9J7	Translational activator GCN1	Ribosome biogenesis and morphogenesis
5	Q0CNW8	Telomere and ribosome-associated protein Stm1	Protein synthesis
6	Q0CJV0	RNA binding effector protein Scp160	Mating response pathway
7	Q0CUP5	G-protein complex gamma subunit Ste18/GpgA	Signaling pathway

The abundance of enzymes/proteins from energy metabolisms (carbohydrate, lipid, and cellular respiration, the glycolytic cycle was observed in mycelia of *A. terreus*. The conidia of *A. terreus* are metabolically less active but when germinates to hyphae and mycelia it requires high energy for growth and development. Also, earlier stated the cellular respiration plays a critical in the pathogenesis of invasive pulmonary aspergillosis caused by *A. fumigatus*. Thus,

we have identified important proteins/enzymes from cellular respiration and another energy metabolism that were enlisted in Table 3.4. Also, we have identified several proteins from the cytoskeleton organization and cell wall that may provide stability to the mycelial structure. Major of gene products (proteins) from the cell wall and cytoskeleton rearrangement have been known to perform structural stability along with the adhesin characteristics may contribute to biofilm establishment, Table3.5

Table 3.4 List of energy metabolism-related proteins in mycelia of *Aspergillus terreus*

S.NO.	Uniprot ID	Protein Name	Biological function
1.	Q0CNN1	Fructose-bisphosphate aldolase	Carbohydrate metabolism
2.	Q0CR22	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex	Carbohydrate metabolism
3.	Q0CNN1	Fructose-bisphosphate aldolase	Carbohydrate metabolism
4.	Q0D1G0	Phosphoglycerate kinase	Carbohydrate metabolism
5.	Q0C917	Glyceraldehyde-3-phosphate dehydrogenase	Carbohydrate metabolism/adhesion molecule against host
6.	Q0CGS3	Citrate synthase	TCA cycle
7.	Q0CA77	Alcohol dehydrogenase I	Energy metabolism
8.	Q0CGR6	Malate synthase	Carbohydrate metabolism
9.	Q0CSV2	Isocitrate lyase	Carbohydrate metabolism
10.	Q0CTT2	Enolase/allergen Asp F 22	Carbohydrate metabolism
11.	Q0CRJ9	Pyruvate kinase	Carbohydrate metabolism
12.	Q0CKY1	Malate dehydrogenase	Carbohydrate metabolism

3.3.5.2 Proteins related oxidative homeostasis and drug resistance mechanism

Mycelia of *A. terreus* showed an abundance of various proteins/enzymes of oxidative homeostasis which may be involved in the DR mechanism in *A. terreus*. Previously is known that cellular antioxidant systems of fungi. Major protein was enlisted in Table.3.6

Table 3.5 List of proteins associated with cytoskeleton and cell wall components in mycelia of *Aspergillus terreus*

S.No.	Uniprot ID	Protein Name	Biological function
1.	Q0CNF3	Cell wall serine-threonine-rich Galactomannoprotein (Mp1)	An antigenic cell wall mannoprotein role in pathogenesis.
2.	Q0CWT3	UDP-N-acetylglucosamine pyrophosphorylase	Cell wall /Chitin synthesis
3.	Q0CP60	cAMP-dependent protein kinase regulatory subunit	Morphogenesis and Signaling pathway
4.	Q0D152	Cell wall biogenesis protein phosphatase Ssd1	Cell wall organization
5.	Q0CNY5	Arp2/3 complex subunit Arc16	Cytoskeleton organization
6.	Q0CHN5	Actin cortical patch component	Cytoskeleton organization
7.	Q0C7D3	Actin binding protein	Cytoskeleton organization
8.	Q0CL85	GPI-anchored cell wall organization protein (Ecm33)	Extracellular matrix adhesin
9.	Q0CMQ3	1,3-beta-glucanosyltransferase (gel2)	Cell wall organisation
10.	Q0D1R1	Beta-hexosaminidase	Carbohydrate metabolism
11.	Q0CZ22	Coronin-like protein crn1	Cytoskeleton organization and polarized growth
12.	Q0CNC2	Profilin/allergen	Cytoskeleton organization and polarized growth
13.	Q0CIR1	Septin AspB	Cytoskeleton organization and polarized growth
14.	Q0CH69	Actin, gamma	Cytoskeleton organization and polarized growth
15.	Q0CPT3	Tropomyosin	Cytoskeleton organization and polarized growth

1.3.5.3 Proteins related to invasion and pathogenesis

Mycelia of *A. terreus* showed few proteins associated with invasion and pathogenesis such as Q0CIR1 (Septin), Q0CNC2 (Profilin/allergen), Q0CTT2 (Enolase/allergen Asp f 22), Q0CYJ2 (Allergen Asp f 15), Q0CNF3 (Cell wall serine-threonine-rich Galactomannoprotein) Q0CM15 (Extracellular dipeptidyl-peptidase Dpp4) and Q0CL85

(GPI-anchored cell-wall protein). Thus there could be further explored for their role in *A. terreus*. Also, we observed several uncharacterized proteins, further classification of these will provide *A. terreus* specific mycelial proteins.

Table 3.6 List of proteins related to oxidative homeostasis and role in drug resistance

S.NO.	Uniprot ID	Protein Name	Biological function
1.	Q0CFC3	Cofilin	Oxidative stress
2.	Q0CEF6	Thioredoxin-domain-containing protein	Antioxidant enzymes/Oxidative stress
3.	Q0CFQ7	Catalase (cat1)	Antioxidant enzymes /Oxidative stress
4.	Q0CW86	Protein disulfide-isomerase tigA	Cell redox homeostasis
5.	Q0CLU2	mitochondrial peroxiredoxin PRX1	Antioxidant enzymes /Oxidative stress
6.	Q0CIE1	Superoxide dismutase [Cu-Zn]	Antioxidant enzymes /Oxidative stress
7.	Q0CE88	Heat shock90	Morphogenesis, biofilm resistance
8.	Q0D231	Heat shock70	Cell wall integrity and Signaling pathways
9.	Q0CGH0	Allergen Asp F3	Cellular homeostasis
10.	Q0CNX3	Glutathione S-transferase	Cell redox homeostasis
11.	Q0CKS9	Dihydrolipoyl dehydrogenase	Cell redox homeostasis
12.	Q0CT03	Thioredoxin reductase	Removal of superoxide redicals

3.3.6 Relative gene expression analysis in germinating conidia and mycelia

We have shortlisted few proteins from abundant pathways from proteome data for their transcript expression using qRT-PCR analysis. The selected genes enlisted in Table.3.1 for expression analysis belong to the signaling pathway, virulence factor, antioxidant enzymes, heat shock proteins, and translation process. The expression of genes studied in mycelia extracts in comparison to the germinating stage conidia of *A. terreus*. Genes *sod, cat, Hsp90, tif35, mpkc* were found to be upregulated in mycelia morphotypes of *A. terreus* in comparison to the germinating stage conidia whereas *Hsp70* showed no static change and gene encoding for terrelysin showed lesser expression in mycelia. Thus, gene expression data observed

incoherence with our proteome data which confirms the abundance of respective proteins. The bar diagram representing expression values shown in Fig. 3.7

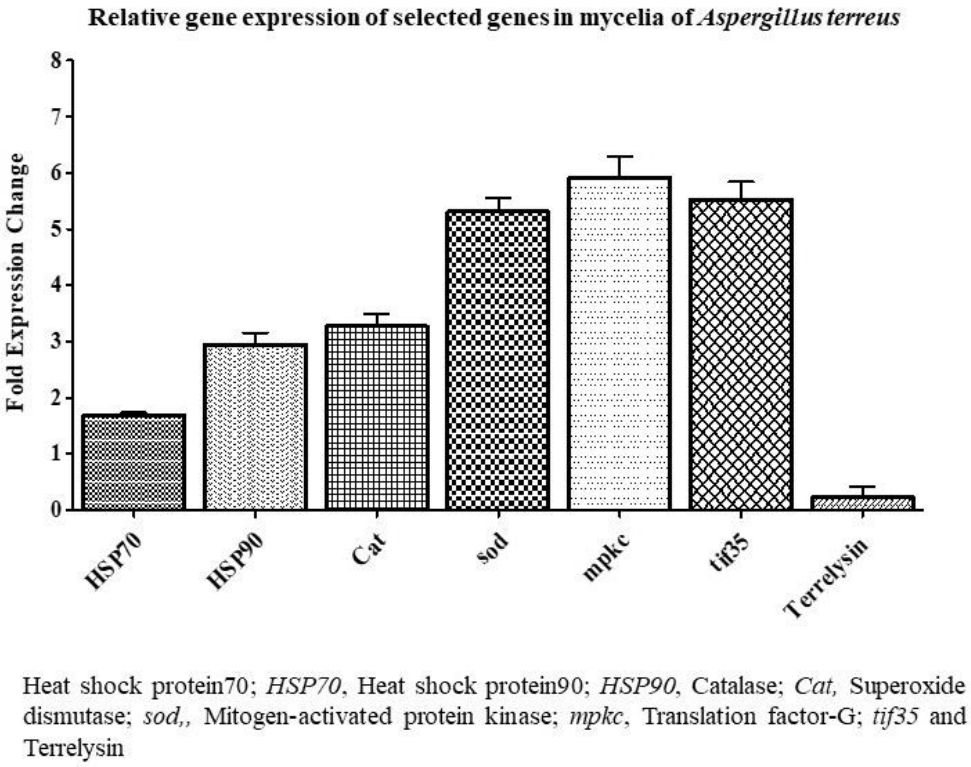


Fig. 3.7 Relative gene expression analysis (fold change) (mycelia versus germination conidia)

3.3.7 Comparison of mycelial proteins with conidia and germinating conidia proteins in *Aspergillus terreus*

On comparison of mycelial proteins with the limited number of available experimentally derived proteins observed during germination [114], approximately 1-2% protein was observed commonly in both conidia and mycelia. Proteins such as 40S ribosomal protein, Elongation factor 2, ATP synthase subunit β, Cell division control protein, Serine/threonine-protein kinase srk1, Succinate dehydrogenase, Actin-related protein-subunit 5, and Adenylate kinase were might play an important role in both conidia and mycelia forms. Most of them belonged to conserved proteins or essential cell metabolism pathways. Further, gene ontology of other proteins observed was related to ROS homeostasis, ribosomal biogenesis, and cell wall and cytoskeleton organization. Also, we observed hypothetical proteins or proteins with

unknown functions in the database. These proteins would be further explored as mycelial-specific proteins in *A. terreus*

3.3.8 Comparative analysis of mycelial proteins to other *Aspergilli*

Predicted proteins in our data set were compared with the available mycelial protein dataset observed in other *Aspergillus* species (*A. flavus, A. niger, A. fumigatus*) [12, 112, 115, 116, 138, 169-171]. In present mycelial proteome data in *A. terreus* total of 65 proteins were common in one or more than one in other species of *Aspergillus* and among 65-proteins,14-proteins enlisted in Table 3.7 were co-found in *A. flavus* and *A. fumigatus*. Most of these proteins were contributing to the central biological processes according to GO analysis thus these processes are crucial during mycelial development in *Aspergilli*.

Table 3.7 Mycelial proteins of *A. terreus* co-detected in *A. flavus* and *A. fumigatus*

S.No.	Uniprot IDs	Protein Name	Biological Functions
1.	Q0CL85	GPI-anchored cell wall organization protein (Ecm33)	Extracellular matrix adhesin
2.	Q0CW79	Ran-specific GTPase-activating protein	Signaling pathways
3.	Q0D1G0	Phosphoglycerate kinase	Carbohydrate metabolism
4.	Q0CKS3	Peptidyl-prolyl cis-trans isomerase	Cellular protein homeostasis
5.	Q0CE18	Nucleoside diphosphate kinase	Lipid metabolism and Morphology
6.	Q0CTT2	Enolase/allergen Asp f 22	Mycelial glycoprotein involves in adhesion
7.	Q0CNN1	Fructose-bisphosphate aldolase	Carbohydrate metabolism
8.	Q0CR22	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex	Carbohydrate metabolism
9.	Q0CGS3	Citrate synthase	TCA cycle
10.	Q0CA77	Alcohol dehydrogenase I	Energy metabolism
11.	Q0CH69	Actin, gamma	Cytoskeleton component
12.	Q0C917	Glyceraldehyde-3-phosphate dehydrogenase	Carbohydrate metabolism/adhesion molecule against host
13.	Q0CMQ3	1,3-beta-glucanosyltransferase (gel2)	Cell wall organisation
14.	Q0D231	Heat shock70	Signaling pathways

3.3.8 SEM Imagining of biofilms in *A. terreus*

We observed biofilm-like structures in *A. terreus* cultures after 48h of incubation. Biofilm consists of a matrix enclosing cells adhered to each other and the surface [172]. SEM imaging at different magnifications depicted the presence of ECM and the network of embedded mycelia in *A. terreus* Fig. 3.8 Images captured show compact structures adhered together and encased in the ECM. We also observed porous ECM.in between loose and dense hyphal structures. Thus, these observations evident the biofilm formation in *A. terreus* and may contributes to the failure of antifungal therapies in *A. terreus* isolates.

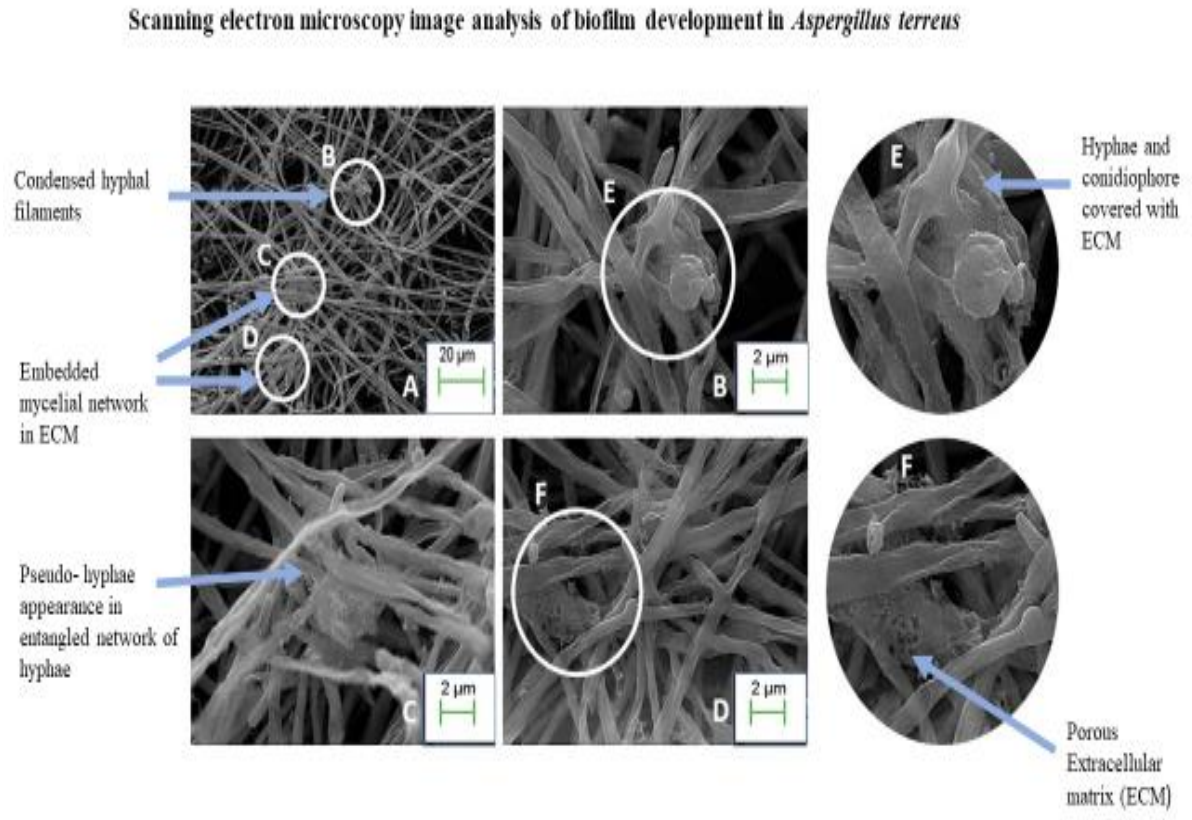


Fig.3.8 Scanning electronic microscopy (SEM) images of biofilm formation in *Aspergillus terreus* at 48h (inoculum concentration 1×10^6 conidia/mL,) (A) mycelium networks embedded in ECM (extracellular matrix), 1000X (B-C) Hyphae covered with ECM showing superficial appearance, 5000X (D) *Aspergillus terreus* biofilm showing porous ECM, 5000X (e-F) Zoom out view of C and D showed clear image biofilm formation.

3.4 Discussion

A. terreus causes opportunistic infection mostly in immune-deficient patients worldwide. The clinical isolates are found to have resistance against standard antifungal drugs. Morphological transition is crucial in invasive infections. Thus, to acquire the knowledge of biological events during the morphological transition in *A. terreus* we have explored mycelial proteome using nLC-ESI-MS/MS. We have identified 389 proteins and biological processes showed that mycelia of *A. terreus* have an abundance of proteins related to ribosomal biogenesis, energy metabolism, cell wall, and cytoskeleton components. Also showed expression of in enzymes from the antioxidant system. The abundance of the protein associated with ribosome biogenesis suggested active translational machinery and a large number of ribosomes are required by growing mycelia. Rapidly growing mycelia demands a new set of proteins. Previously, a steady increase in ribosomal proteins was observed in *A. fumigatus* and depicted that translational events are necessary for vegetative growth. Their iTRAQ and microarray data presented an abundance of ribosomal protein (70.5%) at early developmental stages [138]. Additionally, identified a ribosomal protein (S26) with a translational activator (GCN)1 was found [173, 174]. Also, the compared data have shown less similarity in proteins of conidia, germinating conidia, and mycelia of *A. terreus* [114]. Thus, biological machinery during the transition of mycelia of *A. terreus* from conidia involves a different set of proteins.

The proteins from carbohydrate or energy metabolism identified in our data are similar to other *Aspergillus* species that may suggest less variation in key metabolism in *Aspergilli*. As earlier known that processes like carbohydrate and energy metabolism remain conserved in different fungal species. When we compared our data among other *Aspergillus* species 14 proteins were co-detected in the mycelial stage. Most proteins belong to essential metabolic processes. Thus, the present data showed protein diversity in *Aspergilli*. In this context, as stated previously the in *Aspergilli* central biological process remains conserved, whereas disparities were observed in secondary metabolism, utilization of carbon, and various stress response that suggested genomic and functional diversity within *Aspergillus* species [175].

However, fewer proteins in *A. terreus* showed energy metabolism was observed during germination processes but in mycelia of *A. terreus* more of enzymes from the carbohydrate/TCA cycle suggested that proper growth and development of hyphae/ mycelia

sufficient ATP supply is required. PPIs analysis revealed that carbohydrate metabolizing enzymes could also be related to signal transduction and stress pathways in *A. terreus*.

The antioxidant system involves major enzymes (thioredoxin, catalase, superoxide dismutase, glutathione S-transferase, peroxiredoxin which contributes to oxidative stress in *C. albicans* [176]. In addition to this, it was stated earlier that in *C. albican* ROS generated through H_2O_2 induces filamentous growth [177] and also hyperpolarized bud growth in *C. albican* related by antioxidant enzyme thioredoxin [178]. Interestingly, the expression of superoxide dismutase enzyme subunits depends on morphotypes of *C. albicans*, yeast cells showed Sod4 expression, while hyphae showed expression of Sod5 [179]. Similarly, *A. fumigatus* (Sod1 and Sod2) are highly active during germination while AfSod3 induced during hyphal/ mycelia development [180-182]. Additionally, the AmBRS mechanism in *A. terreus* may involve SODs and CATs [109]. Thus, present data suggested that mycelial of *A. terreus* being rich in antioxidant enzymes enables them to grow under antifungal stress conditions and protects from host immune. A key axis of the host-pathogen interaction is the equilibrium between host-produced ROS and the stress responses induce in fungi, beautifully documented in a review by Warris *et al.* [183]

The abundance of proteins contributing to re-organization and cell-wall biogenesis in current data suggested the crucial role these processes in *Aspergillus* species. Important proteins like extracellular cell wall glucanase, 1, 3-β-glucanosyl transferase, and GPI anchored cell-wall organization protein are involved in cell-wall modulation during infection as stated previously [184, 185]. In contrast, the data with germinating conidia in *A. terreus* showed an abundance of enzymes of cell-wall degradation which contributes to the germination processes [114]. Also, the modulation of the immune responses in mice/humans during invasive infections by *A. fumigatus* involved GPI-anchored proteins like 1, 3-beta-glucanosyltransferase, extracellular cell wall glucanase /Crf1 [4]. Interestingly, identified UDP N-acetylglucosamine pyro-phosphorylase is an important component of the cell-wall in fungus. In *A. fumigatus,* it is a lead antifungal molecule [186]. Identified cytoskeleton related proteins such as fimbrin, actin-binding protein, Arp2/3 complex subunit Arc16, dynactin subunit, cofilin, and actin cortical patch component) were involved in hyphal-growth and instability of mycelial structure [184, 187]. As stated previously identified proteins cofilin also contributes to oxidative stress response in *A fumigatus* [188]. Also, other proteins like coronin-like protein (crn1) play polarised growth during germination role and hyphal morphology and septin

contribute to morphology transitions during invasion and pathogenesis of *Neurospora crassa* [189] and [190]. Therefore, the abundance of these proteins in mycelial proteome data suggested that structural stability is provided by cytoskeleton components of *A. terreus.*

Additionally, we observed signaling pathway regulators (regulators of G-proteins, calmodulin with other regulatory motifs, cAMP). G-protein plays an important role during morphogenesis, and in stress in *A. fumigatus* [191]. Identification of the G-protein complex gamma subunit (GpgA) may be involved in morphological transitions in *A. terreus* as similar has been observed previously in *A. fumigatus* and *A. nidulans* [192, 193]. According to previous reports, cAMP-PKA signaling regulators involves in morphogenesis and pathogenicity in *A. fumigatus* [194]. Also, Mpkc pathway in *A. flavus* contributes to the germination of conidia [113]. Thus, the abundance of these signaling regulators in *A. terreus* may be associated with morphogenic transitions and cellular stress.

We have shortlisted few genes for qRT-PCR studies from abundant pathways such as carbohydrate metabolism, antioxidant enzymes, signaling pathway, terrelysin, and translational factor. We have found a correlation of transcript data with the protein data thus suggested a crucial role of these pathways in mycelial network establishment. We observed low genes as well as protein expression of terrelysin in our data which correlates with previous reports suggesting high terrelysin expression in germinating conidia of *A. terreus* [114]. Also, stated in another report [195] that higher terrelysin expression during germinating conidia, however, the expression level lowers in hyphae and mycelium or disperses to extracellular environment/medium [196].

Heat shock proteins play a role in normal biological activities in fungi and are highly expressed in stressful situations, including antifungal drug treatments [197]. Also, inhibitors of Hsp90 and Hsp70 have been found to increase the potency of drugs AmB and azole drugs in *A. terreus*, although the particular mechanism of AmBRS is still uncovered [110]. The Hsp90 and Hsp70 played crucial role under the influence of antifungals in different fungal species [198]. Thus, a wealth of heat shock proteins (Hsp90, Hsp60 and Hsp70) appear vital during *A. terreus* mediated infections and may also be accompanying AmBRS. Currently, Hsp90 in conjunction with the formation of biofilms controls several signals in *C. albican* [199] And often known in *Aspergilli* and *Candida* spp. for DR and dispersion of biofilm [200].

In fungal pathogens, the extracellular proteins are of clinical significance and also substantially essential for the ECM [201]. We observed that cell wall glucanase Crf1/allergen (Asp f 9), 1, 3-β-glucanosyltransferase, Asp f 15, and β-hexosaminidase are predicted secretory-proteins in our data and Asp F15 was also identified as extracellular by Mee-Jung Han *et al*. on glucose supplemented medium in *A. terreus* [202]. Asp F15 protein was found to have hypersensitive responses during infection [93].

The biofilm-forming tendency is a crucial mechanism for resistance against drugs [201]. Although, the biofilms in *A. terreus,* unprecedentedly less examined during the chronic form of aspergillosis the fungal balls/aspergilloma in the lung-cavity were reported. Mostly, in the case of *A. fumigatus* the invasive pulmonary aspergillosis is caused by the ECM. These are occasionally dense hyphae structures with conical heads that grow into a biofilm encapsulated in an adhesive ECM. Analysis of SEM images (Fig. 3) results in the condensed structure of the mycelium embedded in the ECM also pseudo hyphae network covered with a porous ECM layer. SEM imagining of *A. fumigatus* showed porous and condensed types of ECM. In *A. fumigatus*, the formation of biofilm as well as its structure can be influenced by temperature and growth conditions [203]. These built-in mycelial networks can be coordinated through biochemical signaling molecules via an intracellular communication system. The ECM of *A. fumigatus* consists of 40% of proteins, 43% of carbohydrates, 14% of lipids, and 3% of aromatic compounds as well as extracellular DNA [204]. The proteome of biofilm is less explored yet but in *A. fumigatus* low expression of glycolytic-pathway and the high expression of end steps of TCA was observed and low expression of Asp-hemolysin protein was observed during biofilm [139].

Thus, in the case of *A. terreus* the abundance of TCA cycle intermediates, various antigenic proteins with oxidative phosphorylation in our data added their role in the development of ECM, which needs further validation in future work. Also, polysaccharides of ECM are responsible for the cohesive property and may protect the host immune response [172]. Current data on SEM showed ECM formation in *A. terreus* that could further be explored for their role during host-pathogen interaction as well as during drug treatment.

Therefore, conclusively, the data generated in this objective provided the annotated collection of mycelial proteins in *A. terreus* with experimental evidence. Due to the limited data available on the experimental proteome in *A. terreus*, the Aspergillus-induced vaccine

/immunodominant allergens are less explored. Thus, our mycelial protein data can be further explored for immunogenic properties by mapping epitopic areas of possible peptides that elicit both immune responses (T-cells and the B-cell) by immunoinformatic techniques [205]. Also, during mycelium growth, the abundance of ribosome biogenesis, antioxidant enzymes, etc plays an important role. Knowledge of the AmBRS mechanism in *A. terreus* has been provided by SOD, CAT, and Hsp70/ Hsp90. Overall, our data indicate that mycelium of *A. terreus* contains heaps of different proteins/enzymes that help organisms to survive under stress conditions.

CHAPTER-4

TO DECIPHER THE EFFICACY OF PHYTOCHEMICALS (P-COUMARIC ACID, GALLIC ACID, SHIKONIN AND QUERCETIN) AGAINST *ASPERGILLUS TERREUS*

INTRODUCTION

Due to the steady rise in immunocompromised patients, *A. terreus* received more attention in the last couple of years. It is intrinsically resistant to standard drug AmB and also acquiring resistance to azoles. The choice of antifungal therapy has been limited in *A. terreus* diagnosed invasive aspergillosis. Also due to a higher rate (51%) of IA-associated mortality [5] it is a major clinical problem these days. AmB is a commonly used broad-spectrum antifungal drug. High frequency (98%) of AmBRS was observed worldwide [5, 6]. Also, found to acquire azole resistance with approximately 5-10% frequency in *A. terreus* [6]. The DR mechanism in *A. terreus* is still not explored much needs more attention. Standard antifungal drugs showed an opposing effect on children suffering from aspergilloses like hypokalaemia, nephrotoxicity, and infusion-related infection (3-5 mg/kg of AmB per day) [7]. In developing countries, acute and chronic poisoning was observed due to the use of fungicides and pesticides which become a major problem [8, 9]. Approximately, 5.7 billion people have been measured country by country on the burden of serious fungal infections documented by the Leading International Fungal Education (LIFE) platform since 2013. Also, per year, 1.5 million deaths are estimated [206]. Thus, the present scenario needs alternative methods of treatment approaches against DR isolates, which will be safe, efficacious, and eco-friendlier. In this context, PhytoChem is the best alternative for having antifungal activity and are of natural origin.

Plant products (anthocyanin, thiols, phenolic, flavonoids, tocopherol and carotenoids) were derived from diverse parts of plants. These have a wide range of biological properties like anti-cancerous, antioxidant, antimicrobial, and anti-inflammatory actions [10, 11]. It has been found from the literate perusal that plant extracts from herbs (*Pyrostegiavenusta, Lonicera japonica, Piper betle, Vicia faba, Terminalia catappa*, and *Carya illinoensis*) were explored for their antifungal activity various Candida species [207-213]. The inhibition was possibly

induced by phenolic acids (GA and EA), rutin, and tannins [214]. Recently, the antifungal activity of PhytoChem like coumarin [97], ART [96], QRT [94], eugenol, and thymol [215] was studied. ART was well explored having antimalarial activities and its molecular pharmacology very well summarized in a recent report [216], though studied extensively, but still not any of the PhytoChem has been able to practice at the commercial level. Thus, our study aimed to investigate the inhibitory activity of four PhytoChem (QRT, SHK, GA, and CA,) against *A. terreus*-NCCPF860035. All the PhytoChem tested was screened with detailed, perlustrated literatures and found to have specific biological characteristics (antioxidants, antimicrobials and anticancer). We have therefore chosen these four phytochemicals to reap their use in therapeutics. SHK is a naphthoquinone derivative and has been thoroughly reviewed for pharmacological properties (anti-inflammatory, antioxidant, anti-cancerous, antimicrobial, and antithrombotic) by Andujar *et al.* 2013 [217]. QRT belongs to a group of flavonoids in plants and is ubiquitous in photosynthetic cells. It has been explored for its antifungal activity extensively by various researchers. QRT limits the growth of *A. flavus* and *A. parasiticus* and proven to be a promising biological agent to control the aflatoxin contamination in food crops [218]. One of the most common phenolic acids in the plant kingdom is gallic acid (GA). This crystalline compound is colorless or quite yellow with widespread use in the pharmaceutical and food processing industries. The biological properties with a clear view of therapeutic implications are very well documented in the recent review [219]. Also, the antifungal GA against *C. albicans* and its effects on ergosterol biosynthesis in *Trichophyton rubrum* has been studied earlier [220]. The p- coumaric acid is a hydroxy derivative of cinnamic acid has antioxidant and antimicrobial activities [221]. Also, its antifungal activity was stated earlier as its effects germination of conidia and mycelial growth in *Botrytis cinerea* [222]. To date, limited researches have been performed into the action mechanism of phytochemicals, its cytotoxicity, its synergies, and the anti-virulence potential. Thus, in the present objective, we have analysed selected potential PhytoChem to reap further their therapeutics implementations. We observed that tested PhytoChem showed an inhibitory effect against *A. terreus* and among all SHK showed higher efficacy. Additionally, we have conducted computational prediction of ADMET properties of SHK and *in vitro* cytotoxicity to check the drug-likeliness and toxicity of this lead molecule.

4.1 MATERIAL AND MATERIALS

4.1.1 Stock preparation: A clinical isolate of *Aspergillus terreus* [NCCPF-860035] was used to carry out this experiment with similar culturing conditions documented in material and methods of Chapter section 3.2.1. The inoculum 1×10^6 spores/ml was used in all antifungal assays. To accomplish this objective, we used AmB, Fluconazole (FLC), and Itraconazole (ITC). PhytoChem e. g, GA, CA, QRT, and SHK to test their inhibitory effect against *A. terreus.* Stock solutions of 1mg/l or 5mg/ml of tested drugs **(**FLC, ITC and AmB,) and photochemical (QRT, GA, CA, and SHK) were prepared in recommended solvents details are given in Appendix-4

4.1.2 Antifungal assays

4.1.2.1 The poisoned-food technique for mycelial inhibition: The primarily antifungal susceptibility testing of antifungal drugs (FLC , ITC and AmB) and screened PhytoChem (CA, QRT, SHK and GA, and) were examined using the Poisoned-food protocol (Grover and Moore(1962). We have prepared various concentration ranges (0, 5, 10, 20, 40, 80) µg/ml for standard drugs and (0, 50, 100, 200, 400) µg/ml for PhytoChem against 1×10^6conidia of *A. terreus* as inoculum size from previous literature for screening drugs and PhytoChem. Further, a 5 mm dia. disc was inoculated in the middle of PDA plates containing PhytoChem/drugs at various concentrations following the incubation of the plates at 37 ºC in dark including control plates (drug/PhytoChem-free) [218]. The dia. of mycelial of *A. terreus* was measured after 24h, 48h, and 72h, then mycelial growth inhibition calculates was analysed using the following formula.

$$Mycelial\ Inhibition\ (\%) = \frac{(Control - Test)}{Control} \times 100$$

4.1.2.2 MIC_{50} calculations: We have calculated MIC_{50} (minimal inhibitory concentration required inhibits 50% of growth) by performing MTT assay in planktonic as well as biofilms of *A. terreus*, under optimum growth conditions. The working concentrations of tested drugs ITC, FLC and

AmB,) ranges 0-32 µg/ml, phytochemicals (CA, GA, and QRT) ranges, 0-400µg/ml and for SHK ranges, 0-32 µg/ml. The micro dilutions were prepared in was prepared in RPMI 1640 medium. *A. terreus* spores (1×10^6 spores/ml) were inoculated in RPMI 1640 medium alone or along with standard drugs or PhytoChem separately at 37°C for 24 h in 96-well flat-bottom microtiter plates. After 24h, 10 µl (5 mg/ml) of MTT was added into each well followed by incubation at 37°C for 3-4 h followed by removal of the supernatant. Using 100 µl of DMSO fungal spores was lysed [133]. MTT assay was performed at 570nm using (Multiskan) spectrophotometer. The percentage of growth inhibition was determined from the standard curve and then MIC_{50} values determined for each tested compound [218, 223].

4.1.3 ***In-vitro*** **combinatorial effect of shikonin**: A checkerboard microdilution method was used to determine the combined effect of SHK and the antifungal drug AmB, and the kind of interaction was calculated based on fractional inhibitory concentration index (FICI) [224]. Deferent concentrations of SHK and AmB were prepared (0.5, 1, 2, 4, 8 µg/ml). The two-fold dilutions were made for SHK on vertical orientation, while the AmB was horizontal orientation in 96-well microtiter plate using RPMI medium as shown in **Fig. 4.1.**MTT assay was performed as described above for MIC calculation. The interaction was calculated following formula:

$$FICI = FIC\ A + FIC\ B$$

where, FIC A is the MIC of SHK in the combination/MIC of SHK alone, and FIC B is the MIC of AmpB in the combination/MIC of AmB alone

The observed FIC index is then assessed according to literature based cut-off i.e. FICI=0.5 is synergy, FICI>0.5–1 is additive, FICI>1 to <2 is indifference and FICI=2 is antagonism [224].

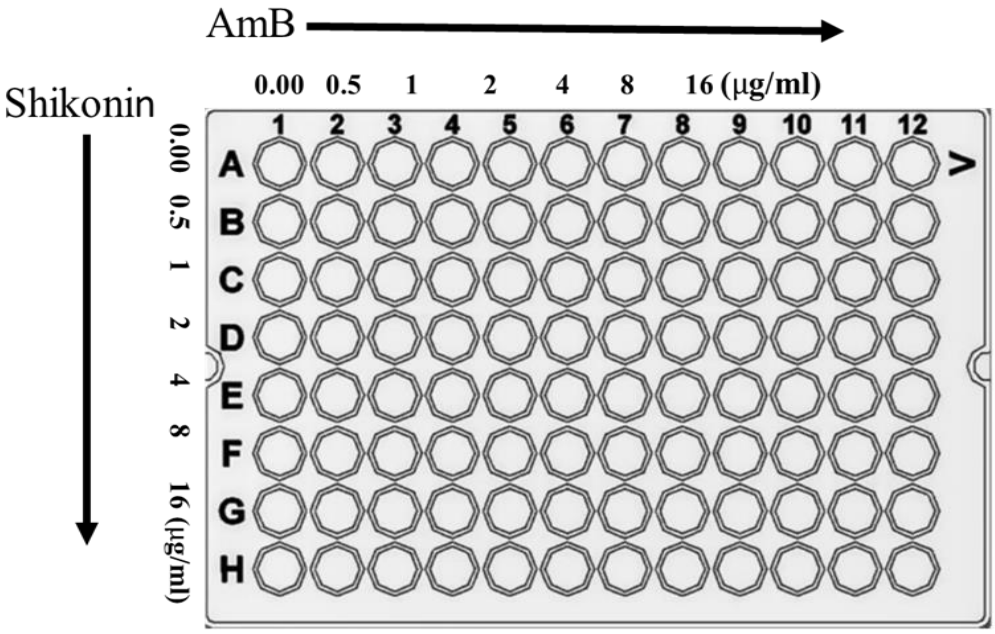


Fig.4.1 Pattern of distribution of different concentrations of SHK and AmBfo conduct Checkerboard assay.

4.1.4 ***In vitro*** **cytotoxicity and drug-likeness of shikonin**: Shikonin was evaluated for *in vitro* cytotoxicity against normal lung epithelial normal cell line L-132 using the MTT assay described above. Furthermore, Absorption, Distribution, Metabolism, Excretion, and Toxicity (ADMET) properties were predicted using computational approaches. The compound structure was obtained from Pubchem and pharmacokinetics, drug-likeness and toxicity of SHK was predicted by using the free web-based tools i.e. SwissADME(http://swissadme.ch)[225] and pkCSM(http://biosig.unimelb.edu.au/pkcsm/prediction) [226]

4.1.5 **Statistical analysis:** All data sets were analysed by column analysis by paired t-test (a nonparametric test), was used to assess significant differences in the group in three experiments. Differences were considered significant at $p<0.05$ using GraphPad Prism version 5.0 software. All data presented in mean ± SD.

4.2 RESULTS

4.2.1 Mycelial inhibition: Significant inhibition of mycelial growth of *Aspergillus terreus* was seen by tested PhytoChem and standard drugs. Standard drugs showing significant inhibition at 20µg/ml **Fig.4.2**. However, PhytoChem (QRT, GA, and CA) at 200 µg/ml and SHK at 20 µg/ml concentration showed notable mycelial inhibition at 24 and 48h **Fig. 4.3.** The results depicted PhytoChem SHK at low concentration showed high efficacy against *A. terreus*. Further, for MIC_{50} determination, MTT assay was performed for drugs orPhytoChem.

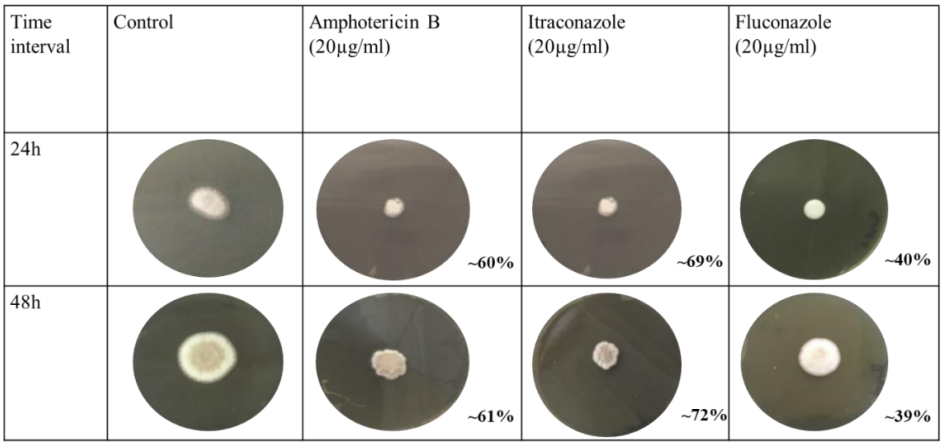


Fig.4.2 Mycelial diameter of *A. terreus* with the percentage of growth inhibition by tested drugs (AmB, ITC, and FLC) after 24h and 48h

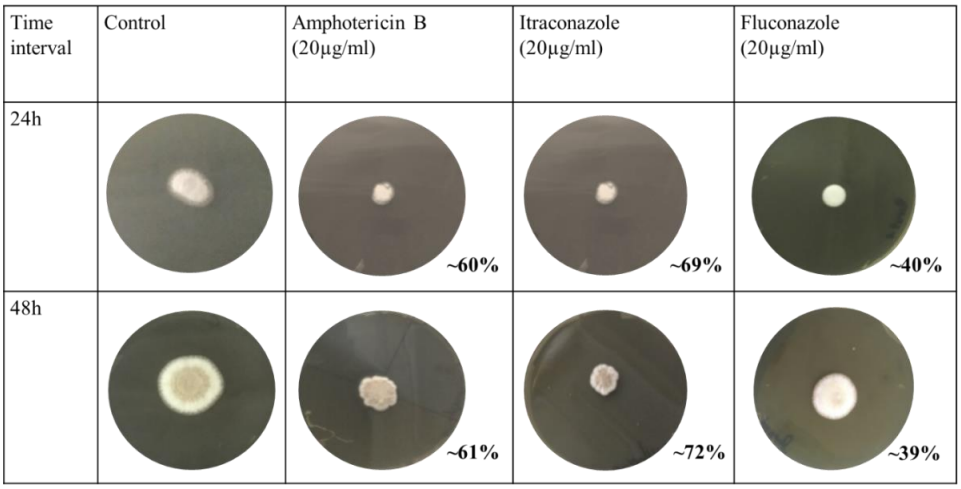


Fig.4.3 Mycelial diameter of *A. terreus* with the percentage of growth inhibition by tested PhytoChemical (CA, SHK, GA, and QRT) after 24h and 48h

4.2.2 **MIC_{50} value calculation**: We have plotted the OD values obtained in MTT assay and calculated the percentage of growth inhibitions and considered the 50% minimal inhibitory concentration of tests compounds for further studies. The plotted graphs for tested phytochemicals/ drugs shown in Fig. 4.4 and Fig.4.5. Also, the percentage of biofilm inhibition by SHK was calculated and plotted in graph in compassion to the biofilm inhibition by tested standard drugs Fig. 4.6. The calculated the MIC_{50} values of planktonic and biofilm cultures of *A. terreus* for drugs and PhytoChem enlisted in Table 4.1. Among tested PhytoChem SHK showed low MIC_{50}; 2µg/ml for planktonic and 4µg/ml for biofilm. Further, SHK was evaluated for the combinatorial effect with AmB. The calculated FIC; 0.828 classified as additive interaction with AmB (0.5 < FICI £1.0) [227]. MIC values were decreases in a combination of SHK and AmB Table 4.2. The *in vitro* prediction of interactions among two drugs is crucial for determining exact doses in combination therapies [227]. Thus, SHK may be explored in combination therapy with existing drugs.

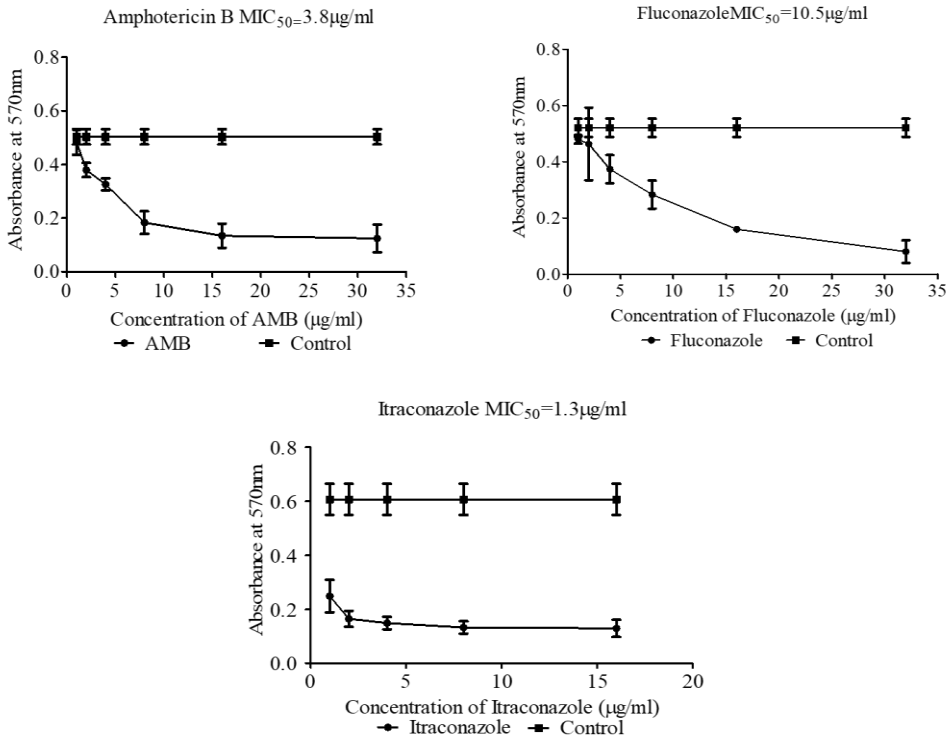


Fig.4.4 Graphical representation of the inhibitory activity of tested drugs in *Aspergillus terreus* (mean ± SD (n=3, per condition) and p -value is < 0.05)

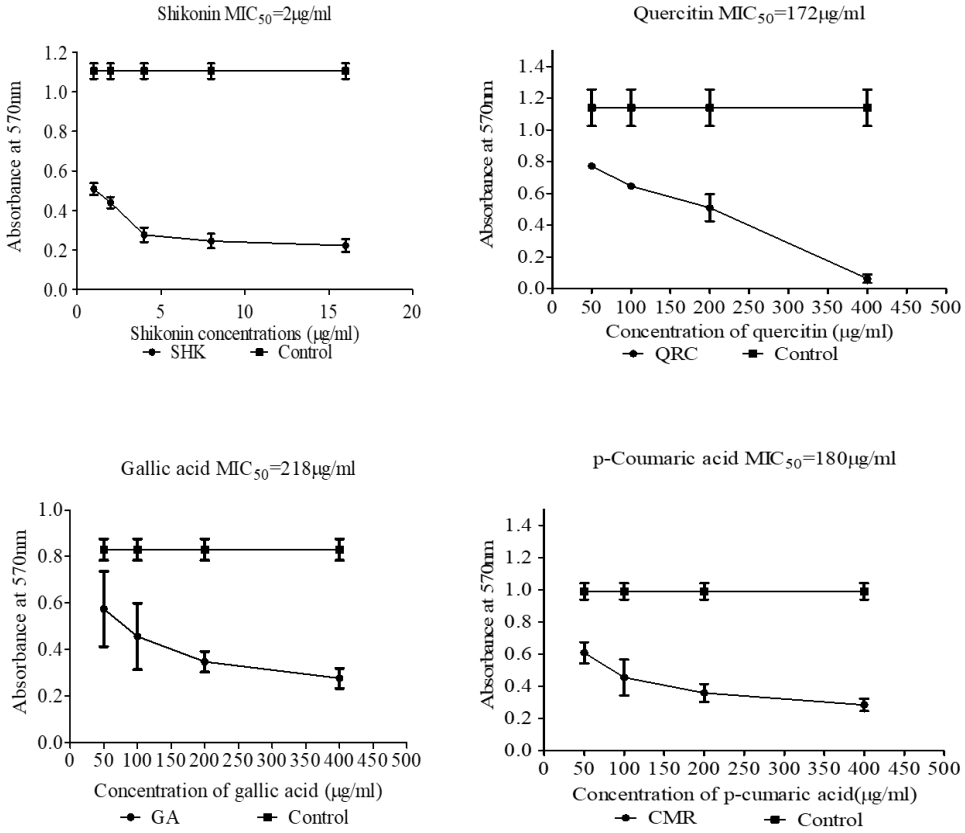


Fig.4.5 Graphical representation of the inhibitory activity of tested phytochemicals in *Aspergillus terreus* (mean ± SD (n=3, per condition) and p-value is < 0.05)

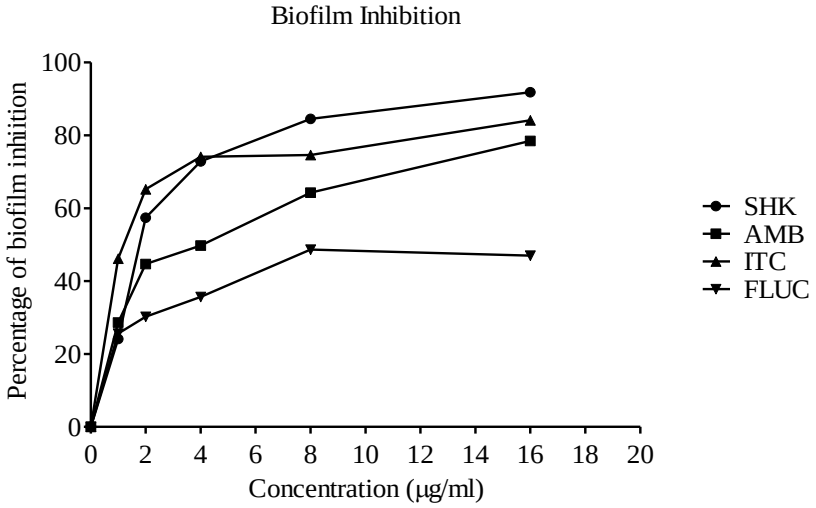


Fig. 4.6 Graphical representation of biofilm inhibition tested drugs and PhytoChem in *Aspergillus terreus* graph plotted to represent percentage inhibition of biofilm (mean ± SD (n=3, per condition) and the p-value is < 0.05

Table 4.1 MIC_{50} values of Phytochemicals and standard antifungal drugs calculate for planktonic cells and biofilm of *Aspergillus terreus*

SNo.	Drug/ PhytoChem	MIC $_{50}$ values for planktonic (µg/ml	MIC $_{50}$ values for biofilm (µg/ml)
1	Amphotericin B	3.8	6.6
2	Fluconazole	10.5	13.6
3	Itraconazole	1.3	3
3	p-Coumaric	180	-
4	Gallic acid	218	-
5	Shikonin	2	4
6	Quercetin	172	-

Table 4.2 *In vitro* combinatorial effect of shikonin and amphotericin B in *Aspergillus terreus*

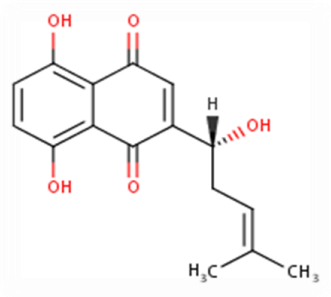
Strain	Agent	MIC values (µg/ml)		FICI	Outcome
		Alone	Combination		
A. terreus (NCCPF860035)	Shikonin	2.30	1.50	0.828	**Additive interaction**
	Amphotericin B	3.86	0.90		

4.2.3 *In Vitro* cytotoxicity and drug-likeness of shikonin

In silico prediction of ADME properties to select and rationalize the biological activity of natural compounds refines the drug discovery process [228]. These methods are less expensive than *in-vivo* preliminary screening tests. The *in-silico* approach ensures a high-performance approach that avoids costly late-stage failure [229]. The predicted Physico-chemical property of SHK is summarized in Table 4.3. The result of the drug-likeness

evaluation of SHK concluded that it accomplishes all the acceptable range of Lipinski, Ghose, Veber, Egan, and Muegge [230-234] parameters and also predicted orally bioavailable Fig.4.7 (details are documented in Appendix-5).

Computational predictions of ADMET properties of shikonin using SwissADME



Shikonin

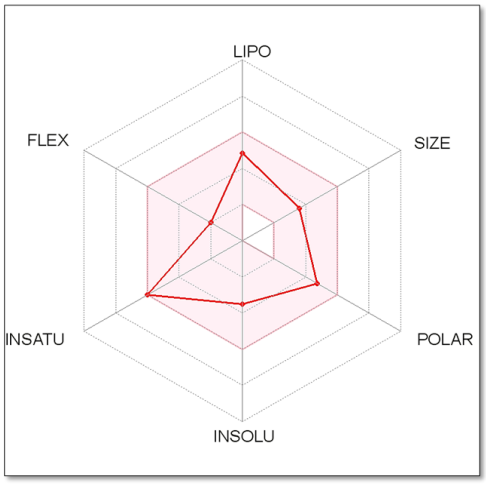


Fig.4.7 The Bioavailability Radar of drug-likeness of shikonin using SwissADME (http://swissadme.ch). The highlighted area in the pink showing the optimal range for each property.

Exploration of parameters of bioavailability for metabolically designing stable drugs, and to avoid drug-drug interactions is important. Accordingly, the ADMET parameters of SHK were calculated using the pkCSM and SwissADME online tools enlisted Table.4.3. These results depicted that SHK has high biological activities and promising ADMET properties, thus a potentially interesting candidate for further studies. Compared to *in vivo* studies, cytotoxicity studies using cell lines are quite easier to sustain and inexpensive [235]. In the present study, a normal lung epithelial cell line (L-132) was used. Cytotoxicity discovered that the compound has less inhibition of the cell line (L-132) proliferation, 50% inhibition was shown at a concentration of 24.72µg/ml. And we have found very low concentration MIC_{50}; 2 µg/ml is effective against *A. terreus.* Thus, the effective concentration of SHK has no/less cytotoxic on normal lung epithelial cell lines. However, earlier studies also showed that SHK has minimal toxicity to normal cells and selective inhibition of various cancer cell lines[236-238]

Table 4.3 *In silico* prediction of physico-chemical properties of shikonin

S.No.	Property	Criterion	*In-silico* analysis
1	Molecular weight	< 500	288.3
2	Heavy atoms	-	21
3	Aromatic heavy atoms	-	6
4	Fraction Csp3	=0.25	0.25
5	Molar Refractivity	= 130	77.82
6	Hydrogen donor	= 5	3
7	Hydrogen acceptor	= 10	5
8	Rotational bonds	< 10.0	3
9	Total Polar Surface Area	< 150	94.83
10	Log P (lipophilicity)	< 5	2.11
11	Log S (Solubility)	=6	-3.24
12	Bioavailability	–	0.55

4.3DISCUSSION

Phytochemicals have caught the eyes of researchers over recent years, PhytoChem may be derived from plant components (leaves, roots, bulbs, etc.), these extracts have proved a promising, non-toxic, antimicrobial agent [239]. The influence of PhytoChem is dependent on fungal organisms as stated by Leal *et al.*, which may vary between groups of fungi. *In-vitro* antifungal assays are primarily in determining the antifungal activity of novel compounds [240]. According to Hamza et al., only those natural products that display MIC ≤0.5 mg / ml will be known as potent fungal growth inhibitors [241]. Thus, in our study, we have interpreted data accordingly and observed a substantial inhibitory effect of the inspected molecules, and calculated

MIC values are given in Table 4.1 showed the tested PhytoChem differed greatly in their activity against fungi. Previously, GA and QRT. were explored for their antifungal activity against *Aspergillus* species and we observed variation in MIC values suggested the effect of photochemical varies within fungal species [94]. Among all SHK showed high efficacy against planktonic (MIC_{50} value 2 µg/mL) as well as biofilms (MIC_{50} value 4 µg/mL) of *A. terreus*. These results correlated the previously stated antifungal activity against *C. albicans* (MIC_{80} 4 µg/mL) and showed higher (>16 times) efficacy in FLC-resistant *C. albicans* [95]. We have also observed the antibiofilm activity of SHK which is higher than the standard tested drugs (AmB, FLC, and ITC). The tested isolate of *A. terreus* showed higher MIC_{50} values for planktonic as well as biofilm of *A. terreus,* which indicates low drug susceptibly as CLSI guidelines, the tested *A. terreus* [NCCPF860035] isolates could be resistant against ITC, FLC and AmB [242-244]. Additionally, we observed a higher MIC_{50} value in the case of biofilm compared to planktonic cultures of *A. terreus,* it has been known that. Higher MIC is needed to disrupt the biofilm structures that could be responsible for DR in *A. terreus* against AmB and azole. Previously in *A. fumigatus* biofilms the polyene, azole, and echinocandin were found less effective which contributes to DR [82] . *In vitro* combinatorial effect, SHK with AmB showed additive interaction and reduces the MIC_{50} value of AmB ~4 times. Thus, suggested that SHK may increase the efficacy of AmB in combination with SHK. Similarly, in the case of *A. fumigatus* Cis-9-hexadecenal showed an additive effect in combination with AmB and enhanced the drug efficacy reported by [168]. A combination of molecules that interact synergistically and raise the amount of antifungal activity is significant. Scientists presently attempting to test a cocktail of therapeutic phytochemicals and/or phytochemicals with existing therapeutics have demonstrated its huge potential in progress in antifungal therapies. To assess the adequacy, time, and risks associated with combinatorial therapy, the literature still lacks well-controlled clinical trials. Thus it is a need of an hour to focus on such emerging salvage therapies to overcome DR in *Aspergilli*.

Thus, from this objective, we have suggested that the tested isolate of *A. terreus*[NCCPF-860035] showed low drug (FLC, AmB, and ITC) susceptibility. SHK significantly reduces the growth of planktonic cultures and biofilm formation of *A. terreus*. The *in-vitro* combinatorial evaluation showed the additive interaction of SHK with AmB. Thus, due to the more effectiveness of SHK against *A. terreus*, it could be explored as a potential PhytoChem against DR isolate of *A. terreus*. SHK showed high biological properties and promising ADMET parameters with less toxicity for normal human lung cell lines. Thus, it could be explored as a drug-like antifungal candidate. Further, the mechanism of the inhibitory action of SHK will be investigated in the next objective.

CHAPTER 5

ELUCIDATION OF MODE OF ACTION OF SHIKONIN MEDIATED INHIBITION OF *ASPERGILLUS TERREUS* USING DIFFERENTIAL PROTEOMIC APPROACH

5.1. INTRODUCTION

The prevalence of secondary invasive fungal infections rapidly increases worldwide. Latest reports have documented approximately 3,000,000 chronic pulmonary aspergillosis cases and approximately 250,000 IA cases occur annually worldwide [206]. *A. terreus* is an occasional cause of invasive aspergillosis (~4% of all) [42]. Treatment of *A. terreus* related infections is now burdensome, due to the rise in individuals with the impaired immune system and also the emergence of DR strains. However, the facilitators of pathogenesis are poorly known, leading to a significant clinical concern. The failure of gold-standard antifungal agents in *A. terreus* resulted in the recurrence of infections. Therefore, as an urgent necessity, new antifungals that are more potent and sensitive than conventional drugs must be developed. Despite the introduction of novel antifungals, their production and effects are sluggish, To produce safe and commercially viable antifungals, the emergence of DR has prompted researchers to shift their attention to herbal products (PhytoChem). Extensive attempts are currently underway to discover new biologically active compounds with novel structures for the production of new potent antifungals.

A safe and efficient alternative treatment strategy against aspergilloses may be established by PhytoChem with antifungal activity. The antimicrobial activities of these compounds have been extensively researched and yet none of them are available for clinical

practice [13]. In this context, our previous objective was focused on primarily screening and testing the efficacy of four PhytoChem, and among them, SHK showed effective inhibition of *A. terreus* isolate. Also, in silico prediction of biological properties and promising ADMET parameters It is a potent pharmaceutical substance with a well-known and extensive range of anticancer, antimicrobial, anti-inflammatory, antioxidant and wound healing activity previous literature shikonin. From our first objective, we could derive the crucial processes in hyphal/ mycelium establishment in *A. terreus,* as well as gain information about pathogenesis and the inherent AmB resistance mechanism. The proteomic data generated could be applied to draw significant inferences in the present objective.

Shikonin is a naphthoquinone extracted from the dried roots of plants belonging to the family Boraginaceae, *Lithospermum erythrorhizon, Arnebiaeuchrom*a, and *Arnebia guttata*. SHK exhibits an anti-oxidant, anti-inflammatory, antitumor, antimicrobial properties [217, 245]. Anti-candida and anti-biofilm activity of SHK reported against FLC-resistant *Candida albicans* isolates. And also, it is known to have anti-cancerous activity [236, 246]. Specifically, in non-small cell lung cancer A549 cells the substantial inhibition of cell proliferation by SHK therapy at 8 μM for 24 hours was observed. Also, their study stated substantial suppression of cell adhesion, ECM as well as the invasion was also observed by a reduced dose of SHK (2.0 μM for 24 h) [246]. From, previous studies we have little understanding of how the existing antifungal work. Thus, it is necessary to elucidate the mode of the inhibitory effect of SHK in *A. terreus*. In general, this molecule is known to tend to accept electrons to generate highly redox-active molecules and generates reactive oxygen species (ROS) when reacts to molecular oxygen. High ROS accumulation changes the cell's redox balance by creating oxidized macromolecules including lipids, proteins, and DNA [247]. Proteomic and transcriptomic advances facilitated the study of molecular aspects during drug effects. The differential proteome profile of antifungal exposure was found to be a promising technique to elucidate the mode of action and also to discover new drug targets. In the case of PhytoChem exposure, there are very fewer proteomics reports in *Aspergilli* but previous studies have gained knowledge about the role of the signaling cascade (MAPK /cAMP/PKA) in *A. flavus* under QRT treatment [19]. Also, in *A. fumigatus* the exposure of ART may modulate cell wall-related proteins, oxidative phosphorylation enzymes, and genes from the ergosterol biosynthesis [14]. In another study proteome, investigation underexposure of synthetic coumarin-derivatives (SCD-1) in *A. fumigatus* showed less abundance of

riboflavin biosynthesis proteins [97]. Thus. in the present objective, we have performed differential proteome profiling to understand the mode of the inhibitory action of SHK (MIC_{50}=2µg/ml for 24h) in *A. terreus* using nLC-ESI-MS/MS. We have obtained proteins and enzymes from various important metabolic pathways under SHK exposure in *A. terreus* and also validated our results using qRT-PCR and biochemical assays. We have also analysed the morphological effects exhibited by SHK on *A. terreus* morphotypes using SEM.

5.2MATERIAL AND METHODS

5.2.1 Culture conditions and procedure for shikonin treatment

Aspergillus terreus clinical isolate (NCCPF 860035) was used to carry out this experiment with similar culturing conditions documented in Material and Methods of Chapter 3 section 3.2.1. To carry out differential proteome studies we have prepared two different cultures one is controlled and another is treated. The control sample is without any treatment and the treated sample was prepared by giving SHK treatment at MIC_{50} =2µgml to *A, terreus*. The conidia (1×10^6) of *A. terreus* was used as inoculum in DMEM (containing glucose) with 10% FBS. SHK treatment was given for 24h at 37ºC at continuous shaking (100rpm). Samples (control and treated) were harvested after 24h by Centrifugation at 1800g for 5 minutes and washed thrice with PBS to remove media traces. The experiment was performed in three independent biological replicates.

5.2.2 Differential proteome profiling

Protein extraction from harvested samples (1g mat) was carryout by following the procedure documented in material and methods of Chapter 3 section 3.2.2. afterward, Protein samples 200 µl (~1µg/µ) of control and SHK-treated were provided to Vproteomics, (New Delhi-India) for MS analysis. The differential proteome profiling of SHK-treated and untreated samples was performed using the protocols thoroughly documented in material and methods of Chapter 3 section 3.2.3. Further, data generated in spectral files were subjected to bioinformatic analysis. The overall workflow to conduct differential proteome profiling is given in Fig. 5.1

5.2.3 Differential gene expression study: Quantitative Real-Time PCR was performed to conduct differential gene expression of SHK treated and untreated samples of *A.*

terreus. We have prepared the treated and untreated samples using a protocol as explained above in material methods section 5.2.1 in three independent biological replicates. Further, RNA isolation was performed using the protocol documented in material and methods of Chapter 3 section 3.2.2. The NCBI based tool was used to design primers (Primer-Blast) for shortlisted genes [166] from proteome data. The list of primers is given below in Table.5.1. the stepwise flowchart of the methodology used to carry out qRT-PCR is given in Fig.5.2.

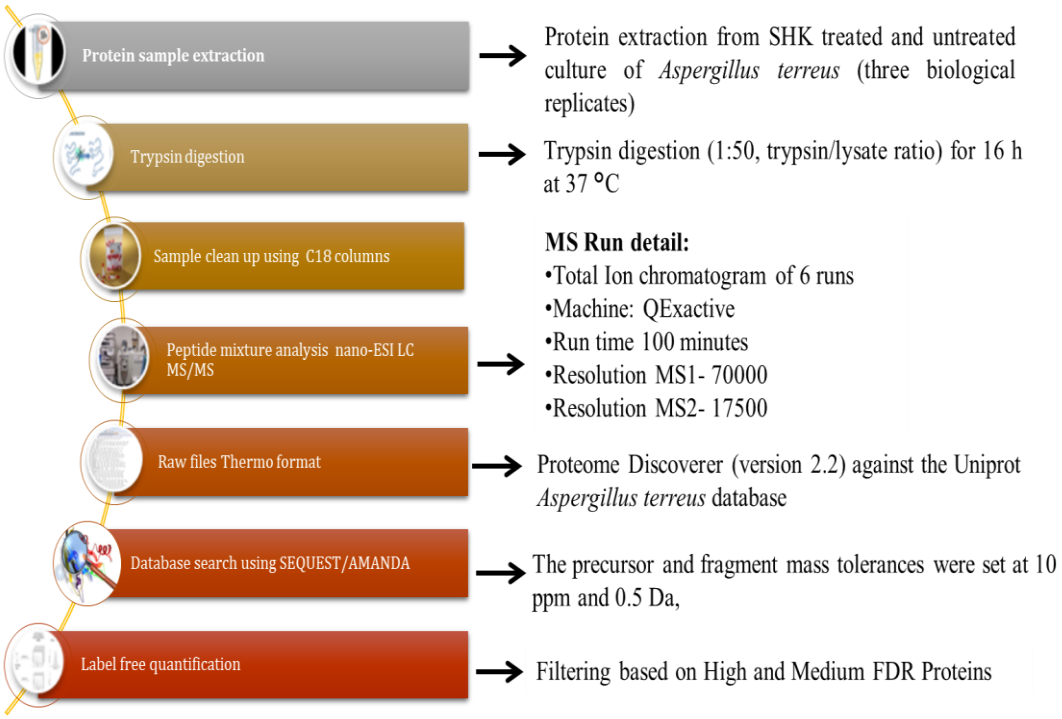


Fig.5.1 Flowchart representation of overall methodology used to carry out differential proteome profiling of shikonin treated and untreated samples in *Aspergillus terreus*

5.2.4 Catalase assay: To validate the effect of SHK on the antioxidant system of *A. terreus*, we have estimated catalase activity in control and SHK treated samples of *A. terreus*. Samples were prepared in a buffer [KPi containing 0.1 mM PMSF] using the previously discussed procedures of culturing and SHK-treatment. The catalase assay kit (Cayman Chemicals co.) was used to assess the catalase activity in soluble protein extracts as per manufactures instructions. Catalase activity was calculated in terms of the formation of formaldehyde (nmol/min/ml) from the

formaldehyde standard curve.The stepwise procedure of catalase assay is documented in Appendix-7.

Table 5.1 List of primers for shortlisted gene for relative expression analysis in shikonin treated and control sample of *A. terreus*

	Uniprot IDs	**Gene name**		**Sequence**
1	Q0CSL9	NADH-ubiquinone oxidoreductase (NADH)	FP	GGTTCTCGTCTTTCGGTCGT
			RP	CAGACGGACTTGGGGATGAC
2	Q0CD12	Catalase-peroxidase (cat)	FP	ACCTCTTCGCCTTTGACTGG
			RP	TCTTCTCATAGGCCGGGTCA
3	Q0CIE1	Superoxide dismutase [Cu-Zn](sod)	FP	GGCTGTTGTGGTTTTGCCTG
			RP	AGGAGATGGTGGTGAGGGAG
4	Q0CMQ3	1,3-beta-glucanosyltransferase	FP	GAACAACGGGCTCGAGTACA
			RP	TAGTTGAAGTTGGGCGTGCT
5	Q0D1R1	Beta-hexosaminidase	FP	GAACAACGGGCTCGAGTACA
			RP	TAGTTGAAGTTGGGCGTGCT
6	Q0CST2	Mitogen-activated protein kinase spm1	FP	AGCTCAACCAGATCCTGCAC
			RP	GGGGATCTTCGGCATGTAGG
7	Q0CP60	cAMP-dependent protein kinase (cAMP)	FP	ACGGCATGGGCAACAAAATC
			RP	GAGAGTGCTCTTGGGATCGG
8	Q0CTR9	Protein rho-1	FP	TCTCTTACCCCGACTCCCAC
			RP	CCCTGGCAGAAGTGAAGGAC
9	Q0CW56	Protein kinase C(pkc)	FP	GCAACAGGTTGCTGTCAAGG
			RP	AGACACGCTTCTCGGACTTG
10	Q0D231	Heat shock 70(Hsp70)	FP	GACCACGGAAATCGAGCAGA
			RP	CATGGTGGGGTCGGAAATGA
11	Q0CE88	Heat shock protein 90 (Hsp90)	FP	CTCGCCAAGAGCCTCAAGAA
			RP	GCTCCTTGATGATGGGGGAC
12	Q0CFE2	Succinate dehydrogenase (SDH)	FP	CCCGAGGATGGTCTGGAGTA
			RP	GTACTCCTCGCTGTTCCACC
13	Q0CF25	Protein ras-1(ras-1)	FP	ATCTGGAAAAGGAGCGGGTG
			RP	TCCTTGTTGTAGCGGCGAAT
14	Q0CST2	Mitogen-activated protein kinase (mpkC)	FP	ACGATCCTACCGATGAGCC
			RP	GCGTCATATAACCCGACGAG

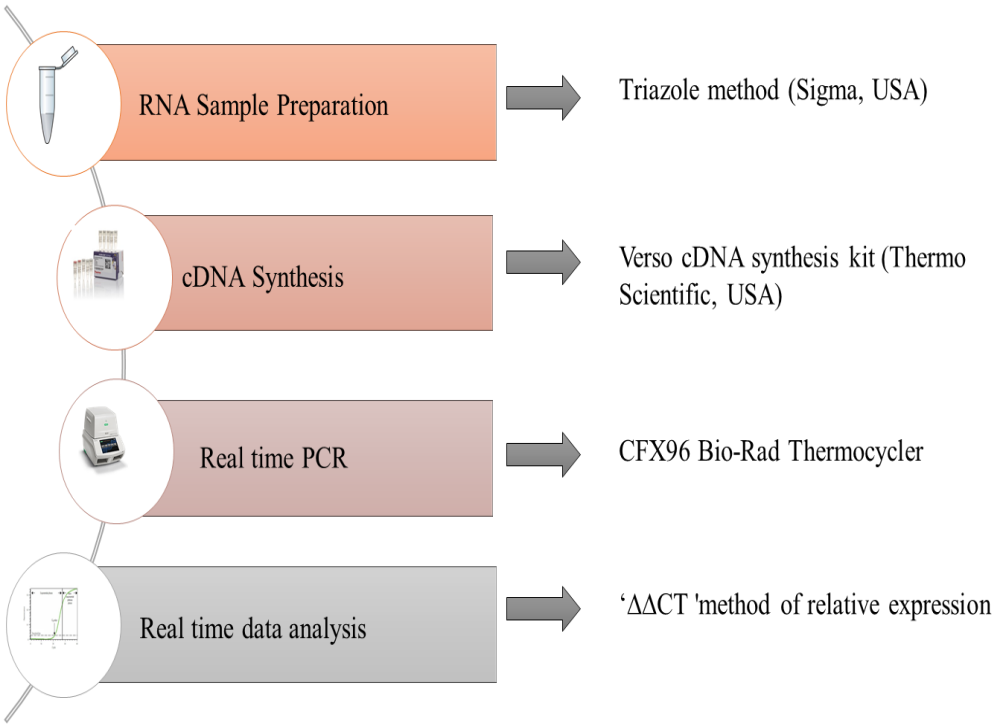


Fig.5.2 Flowchart representation of the overall methodology used to carry out differential gene expression of shikonin treated and untreated samples in *Aspergillus terreus*

5.2.5 Cellular ROS estimation: To determine the impact of SHK on intracellular ROS levels in *A. terreus*, ROS estimation was conducted using the DCFDA (2, 7-dichlorofluorescein diacetate) fluorescent dye. SHK treatment (MIC_{50} of SHK) and control samples of *A. terreus* was prepared by inoculating (1×10^6 cells/mL) spores following incubation at 37°C for 24h with constant agitation (100 rpm), cell was then treated at 37°C with DCFDA (20 µg/ mL) for 45 min. Afterward, the fluorescence intensity (excitation at 485 nm and emission at 520 nm) was observed under a fluorescence microscope using100 µL cell suspensions at a constant set of parameters. ImageJ software [248] was used to analyse the fluorescence intensities. The stepwise flowchart of protocol is given in Appendix-6

5.2.6 Effect of shikonin on morphogenesis of *A. terreus* using SEM: Antifungal agents are known to alter the normal morphology of pathogens which assist in their inhibitory action. Thus, to study the impact of SHK on the morphology of *A. terreus*. We have prepared the planktonic culture of *A. terreus*(1×10^6conidia) for SHK-treated (MIC_{50}; 2 µg/ml)and control samples. Planktonic cells were harvested

in PBS by centrifugation at 2700 rpm after 12 and 24h. Besides this, the pre-formed biofilm of *A. terreus* (48h) was treated with SHK at MIC_{50}; 4 µg/ml for 6h. Afterward, samples were prepared for SEM imaging following the protocol documented in Material and Method Chapter-3. Then microscopic images were captured on a Zeiss SEM (MA EVO -18 Special Edition) at different magnifications. A detailed procedure is given in the Appendix.

5.2.7 Statistical analysis: Mass spectrometric experiments were carried out in three different biological replicates and data were analysed by Welch's T-test ($p-\leq 0.05$) using Perseus Software. The statistical examination was done with GraphPad Prism software version 5.0. for other data using paired t-test (non-parametric test) and the mean differences at ($p \leq 0.05$) were considered to be significant and represented as mean (± SD) results.

5.3RESULTS

5.3.1 Differential protein profiling: The spectral data generated using nLC-ESI-MS-MS for SHK treated and untreated samples of *A. terreus* have been deposited to the ProteomeXchange Consortium via the PRIDE [81] partner repository with the dataset identifier PXD01679. The data was documented using Proteome Discoverer software and identified a total of 1715 proteins (FDR 0.01). The overall pipeline used for differential proteome data is given in Fig.5.3. **(**spectral files given in Appendix-). Further, statistical analysis (Welch's T-test) observed 105 proteins with significance change (p-value ≤ 0.05), a heat map diagram showing a pattern of differential expression of proteins given in Fig. 5.4. We have enlisted the 22 proteins solely abundant in control and 14 proteins exclusively present in SHK-treated samples in Table 5.2 and Table 5.3 respectively, further to carryout *insilco* data analysis to frame out the modulation of molecular events in *A. terreus* under SHK treatment. We have further increased the value of cut-off expression to 2 and 1.5-fold change, and most of the findings were derived from statistically relevant proteins and 2-fold cut-off, but 1.5-fold protein / enzymes were also considered

where it was considered necessary to understand the functional or biological mechanism. All groups were then subjected to BLAST2go and Uniport database for GO analysis.

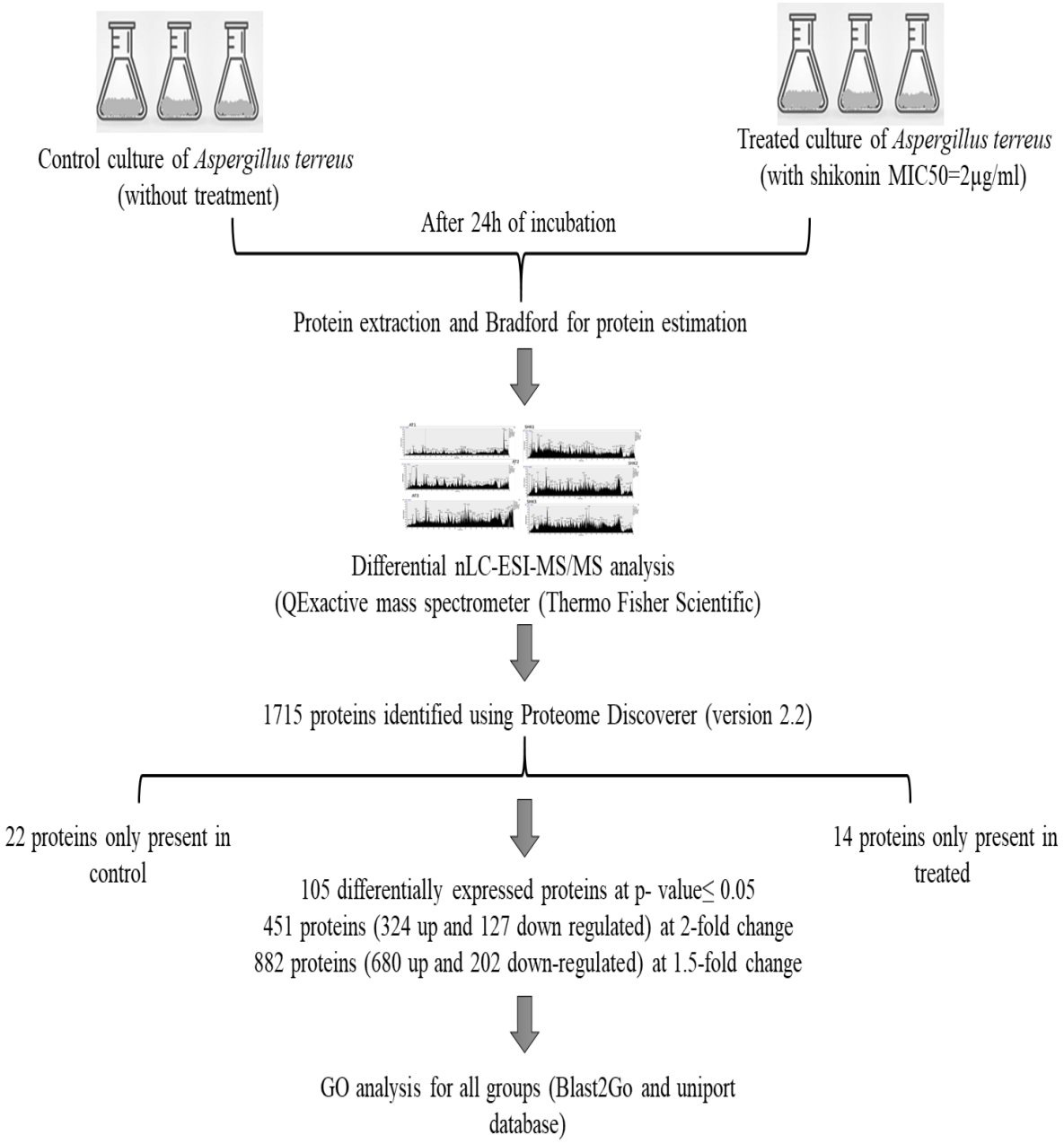


Fig.5.3 Flowchart representation of the overall pipeline used for differential Mass Spectrometric data processing in *A. terreus*

Table 5.2 List of proteins with predicted biological functions only present in control samples of *Aspergillus terreus*

SNo	Uniprot ID	Protein Name	Biological functions
1.	Q0C7L3	Steroid monooxygenase	Oxidoreductase activity and ion binding
2.	Q0CD12	Bifunctional catalase-peroxidase Cat2	Oxidoreductase activity and response to stress
3.	Q0CFE2	Putative iron-sulfur protein subunit of succinate dehydrogenase Sdh2	Oxidoreductase activity and generation of precursor metabolites and energy
4.	Q0C9S4	Uncharacterized protein (nucleoporin Nsp1)	Nucleocytoplasmic transport and protein transport
5.	Q0CA85	Chromatin structure-remodeling complex subunit snf2	Cellular nitrogen compound metabolic process
6.	Q0CAG0	Uncharacterized protein (nucleoside-diphosphate-sugar epimerase)	Nucleocytoplasmic transport, oxidoreductase
7.	Q0CBR3	S-adenosyl-L-methionine-dependent methyltransferase	Methyltransferase activity
8.	Q0CGF5	Peptidyl-prolyl cis-trans isomerase-like 1	Cellular protein modification process and protein folding
9.	Q0CGI5	40S ribosomal protein S30	Structural constituent of ribosome, translation
10.	Q0CGN3	Synaptobrevin	Vesicle-mediated transport
11.	Q0CGZ7	FAD/ NAD binding oxidoreductase	Oxidoreductase activity and cellular nitrogen compound metabolic process
12.	Q0CJH4	RNA-binding domain-containing protein	mRNA processing
13.	Q0CLE8	Mitochondrial 37S ribosomal protein RSM25	Structural constituent of ribosome
14.	Q0CLM2	Oxidoreductase	Oxidoreductase activity
15.	Q0CVH2	Probable alpha-galactosidase C	Carbohydrate metabolic process
16.	Q0D0K4	Uncharacterized protein (SAGA complex component)	Protein-containing complex
17.	Q0CRB3	Uncharacterized protein, proline utilization protein predicted	-
18.	Q0C8T8	Uncharacterized protein	-
19.	Q0C9B9	Uncharacterized protein	-
20.	Q0CIX4	Predicted protein	-
21.	Q0CNV4	Uncharacterized protein	-
22.	Q0CT82	Uncharacterized protein	-

Table 5.3 List of proteins with predicted biological functions only present in SHK treated samples of *Aspergillus terreus*

S. No.	Uniprot ID	Protein Name	Biological functions
1.	Q0C8W1	DNA helicase	ATPase activity / response to stress
2.	Q0CA10	Uncharacterized protein, probable myosin	Cytoskeleton organization
3.	Q0CBN7	Uncharacterized protein	-
4.	Q0CBR7	Ubiquitin-conjugating enzyme	Cellular protein modification
5.	Q0CD13	Siderophore iron transporter mirB	Transmembrane transporter activity
6.	Q0CE61	Oxidoreductase	Oxidoreductase activity
7.	Q0CEN1	Uncharacterized protein	Signal transduction, kinase activity
8.	Q0CEX3	OPA3 domain protein	Cytoskeletal protein binding
9.	Q0CJJ4	Cytochrome P450 55A3	Oxidoreductase activity, ion binding
10.	Q0CKQ1	Low molecular weight phosphotyrosine protein phosphatase	Cellular protein modification process
11.	Q0CLM9	Uncharacterized protein	-
12.	Q0CLV5	Uncharacterized protein	-
13.	Q0CQJ3	Glutamate-cysteine ligase Gcs1	Sulfur compound metabolic process
14.	Q0CV20	Importin β-5 subunit	Nucleus; protein transporter

5.3.2 Gene ontology results

Gene ontology was defined in biological process, molecular process, and cellular process at 2-fold change and shown in (Fig. 5.5., Fig 5.6 and Fig.5.7) and the overall results showed that differentially expressed proteins are typically from oxidative homeostasis, signaling, and energy pathways. Also GO analysis of 105 proteins showed a significant change in protein expression of oxidative pathways (26%), signaling pathways (14%), energy metabolism (11%) (details of proteins were given in the Appendix-8). Thus, our results suggested modulation in these important pathways under SHK exposure in *A. terreus* which may contribute to its mode of action. Further. the major enzymes/ proteins from these pathways were shortlisted to enhance the data.

Heat map of 105 significant proteins expressed differentially in both samples (p-value≤. 0.05)

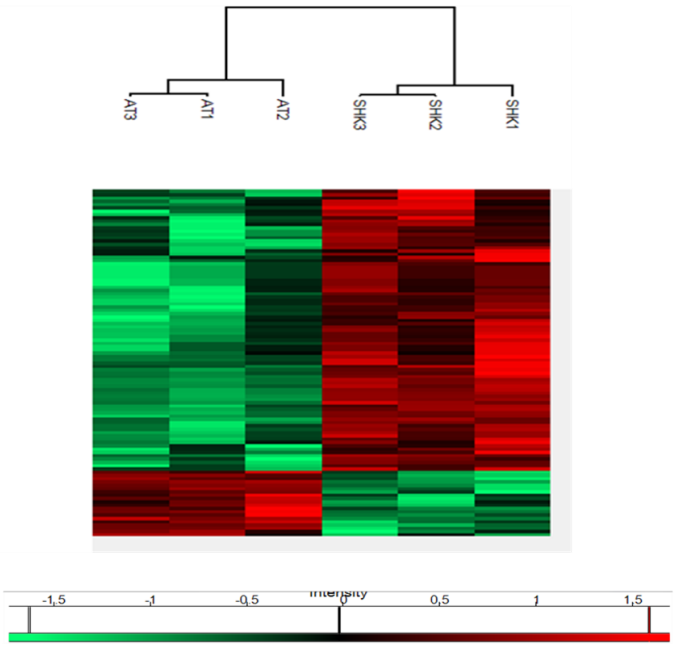


Fig.5.4 Heat map showing differential expression of 105 significant proteins (control and SHK-treated) samples of *Aspergillus terreus*. (Red color -upregulated and green color down regulated proteins) The detailed information of these proteins given in Appendix-9

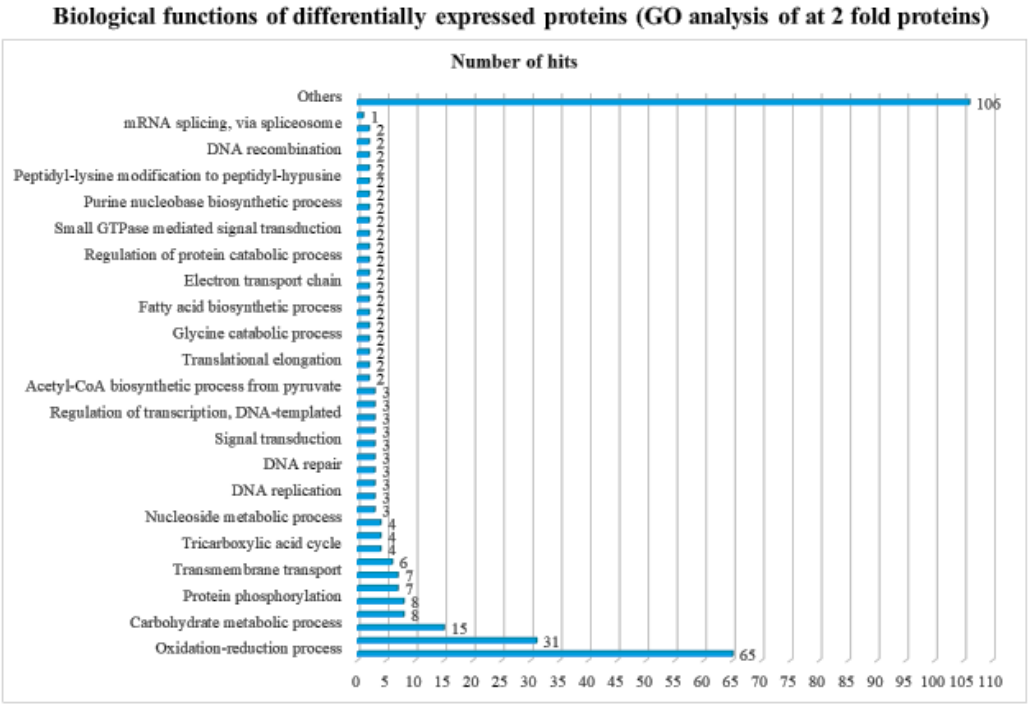


Fig.5.5. Biological functions of differentially expressed proteins (GO analysis of proteins with = 2-fold change)

Molecular functions of differentially expressed proteins (GO analysis of at 2 fold proteins)

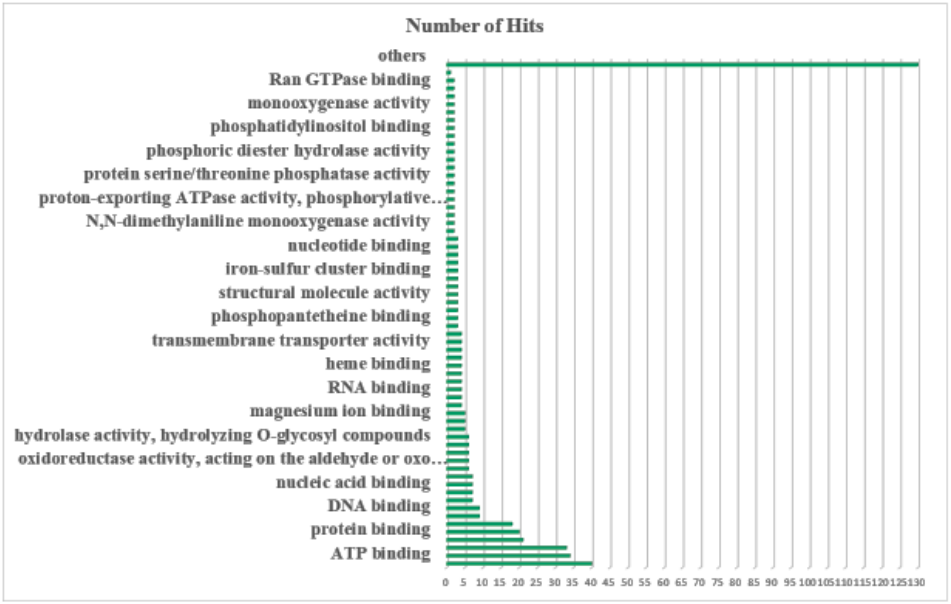


Fig.5.6. Molecular functions of differentially expressed proteins (GO analysis of proteins with = 2-fold change)

Cellular Component of differentially expressed proteins (GO analysis of at 2 fold proteins)

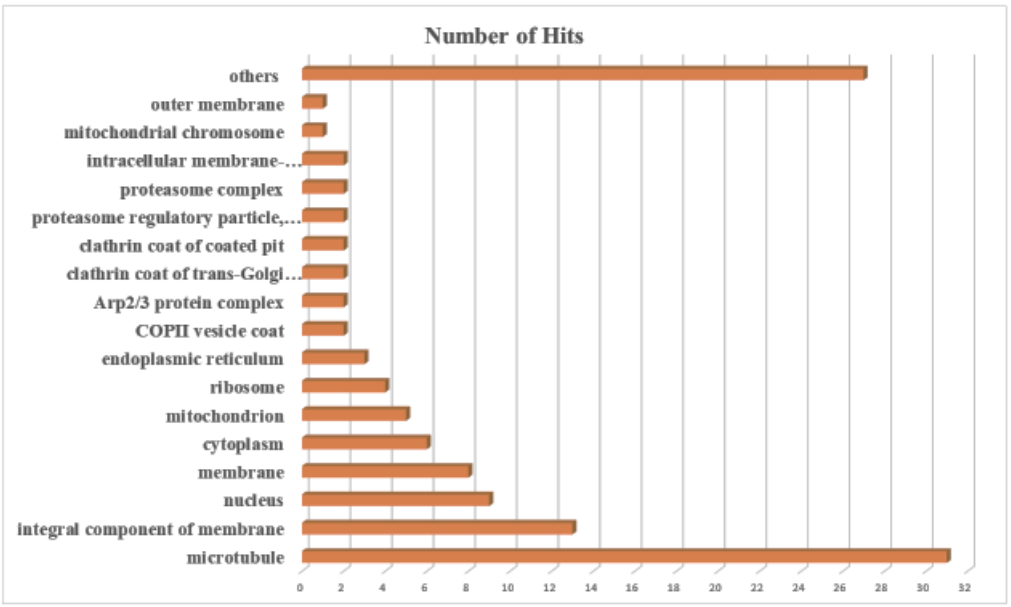


Fig.5.7. Cellular functions of differentially expressed proteins (GO analysis of proteins with = 2-fold change)

5.3.3 Effect of shikonin on molecular events in *Aspergillus terreus*

Antifungal agents have primary and secondary drug targets that contribute to its mode of action. These targets modulate various molecular and cellular processes in the pathogen. The information of these molecular event helps in attaining knowledge of the mechanism of inhibitory action of antifungals and also gain insight into the resistance mechanism. Thus from our proteome data analysis, we have documented various important enzyme/proteins from differentially expressed processes categorized based on GO results.

5.3.3.1 Proteins associated with carbohydrate/energy metabolism: Most carbohydrate proteins displayed higher expression but were shown to be more expressive in treated samples are pyruvate carboxylase, pyruvate dehydrogenase, and glyceraldehyde-3-phosphate dehydrogenase. In SHK treated samples, significantly low enzyme expression, including isocitrate lyase and malate synthase (glyoxylate pathway key proteins), succinyl-co-A transferase, and TCA-cycle malate dehydrogenase was seen. Few enzymes in the pentose pathways were up-regulated such as L-xylulose reductase, glucose-6-phosphate 1-dehydrogenase, transketolase, and transaldolase.

5.3.3.2 Oxidative homeostasis related proteins: Redox cycle enzymes have been modulated by SHK in *A. terreus*. The SHK-treated sample showed up-regulation in oxidoreductase, 2OG-Fe (II) oxygenase, ubiquinol-cytochrome c iron-sulfur reductase, FAD-binding domain enzyme, oxidoreductase, NADH-ubiquinone oxidoreductase 51 kDa subunit, mitochondrial precursor, and cytochrome-p450. Catalase-peroxidase major antioxidant is not present after SHK-treatment. However, there was no significant change in the expression of thioredoxin reductase and superoxide dismutase (Mn) by SHK.

5.3.3.3 Signaling pathway regulators: In stress environments, signaling mechanisms are critical, major signaling proteins in response to the SHK treatment in *A. terreus* were differentially expressed. We observed upregulation in Mpkc, spm1, kinase protein (Pkc-c), kinase protein (dsk1), and kinase serine/threonine protein under SHK treatment in *A. terreus*. Also, in response to

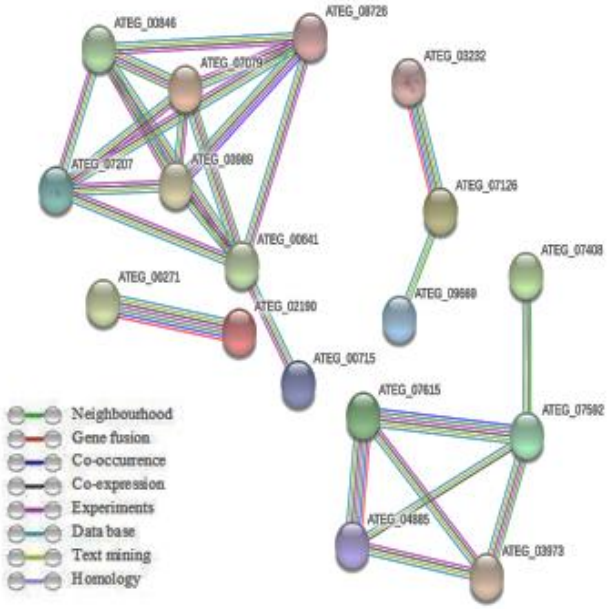
SHK therapy, small GTPase such as ras1, rho1, and rab have been upregulated. Other important signaling components are enlisted in the Appendix.

5.3.3.4 Cytoskeleton organization components: Rearrangement of cytoskeleton has been observed under SHK exposure in *A. terreus*. Regulatory cytoskeleton-associated proteins have been upregulated with microtubular processes. Other cytoskeleton dynamics are upregulated, such as adenyl-cyclase-associated protein, Tubulin subunit B, Tubulin alpha-1 subunit, numbers of β-tubulin subunits, and the kinesin-like protein.

5.3.3.5 Other salient proteins: Upregulation of the few ergosterol-pathway proteins (C-14sterol-reductase, hydroxymethyl glutaryl-CoA synthase, and probable 14-α sterol demethylase), the cell wall-related proteins (mannose-1 phosphate guanylyl, SUN domain protein, putative, cell wall glucanase) was found in our data. Whereas, heat-shock proteins (Hsp60, Hsp70, Hsp90, and Hsp98) have shown a small change in expression in contrast to samples from the controls. Also transport protein such as ABC transporters, MFS sugar transporters, ATP synthase unit d and SHK monosaccharide transporters have also been upregulated in SHK samples.

5.3.4 Detection of Protein-protein interactions

The network of interactive proteins was obtained using the STRING database for a total of 141 proteins (105 proteins statistically important, 22 proteins solely in control, and 14 proteins exclusively in SHK treated samples). Only 27 protein showed significant interacting network Fig. Substantial enrichment has been observed in terms of the UNIPROT, the INTERPRO protein domain. Most of these interacting proteins are associated with mitochondria, ETC, oxidoreductase, and ribosomal proteins more details of these proteins are given in Fig. 5.8, details are given in the Appendix-10



Protein -Protein interaction results using STRING v.10.5(http://string-db.org/), Interaction studied between 85 proteins of abundant proteins with the following parameters: species-*Aspergillus terreus*, high confidence level—0.700, active prediction methods, all input—UniProt accession numbers of detected proteins. Only proteins with more than two interacting nodes represented in Figure

Fig.5.8 String results showing interacting proteins in evaluated groups.

5.3.5 Relative gene expression analysis

qRT-PCR results for selected genes from the oxidative cycle, cell wall components, and signaling pathways about proteome data. The relative gene expression in fold change for selected genes under SHK treatment in comparison to control samples of *A. terreus* is represented in bar graphs (≥ 2-fold significance level) Fig.5.9 (A and B). We observed a significant change in gene expression of selected genes under SHK treatment the relative expression changes. Results have shown that higher transcription expression of signaling cascade genes [Pkc, cAMP, Mpkc, and ras-1] suggested active signaling processes the under SHK stress. Genes such as [hxeb, gel, rho-1, and spm1] relevant to cell wall organization have been up-regulated indicating regulation of cell wall integrity. NADH and sod encoding transcripts were up-regulated indicating an active redox cycle, although down-regulation for catalase gene was observed. As a consequence, our findings are consistent with our proteome evidence, it has been suggested that modulation in ROS homeostasis, signaling, and cell wall organization pathways play a key role in SHK mode of action in *A. terreus*. The table indicates the expression in fold change in the chosen

protein with the respective SHK-treated transcripts. Further, these results were validated at the biochemical level.

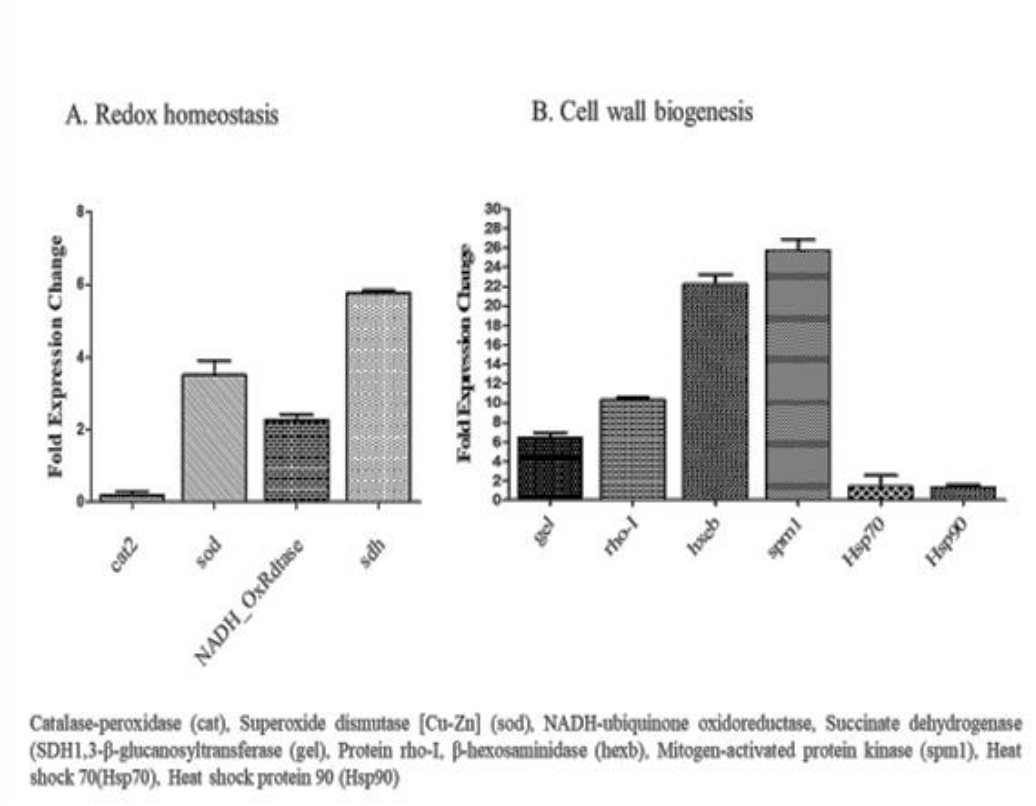


Fig.5.9 Relative gene expression of various pathway genes in SHK-treated vs control *A. terreus* determined by qRT-PCR. Bar graph (A) showing expression of genes encoding for redox homeostasis, Bar graph (B) showing expression of genes encoding for signaling pathway and Bar graph (C) showing expression of genes encoding for cell wall biogenesis.

Table 5.4 List of important differentially expressed proteins under SHK treatment in *A. terreus* with protein expression vs transcript expression in Fold Change

SNo.	Uniprot ID	Protein Name	Transcript expression (Fold change)	Protein expression (Fold change)
1	Q0CD12	Catalase-peroxidase (cat)	0.184347	-
2	Q0CIE1	Superoxide dismutase [Cu-Zn](sod)	3.499983	0.948366474
3	Q0CSL9	NADH-ubiquinone oxidoreductase (NADH)	2.258266	3.207464708
4	Q0CFE2	Succinate dehydrogenase (SDH)	5.757064	-
5	Q0CW56	Protein kinase C(pkc)	5.402648	1.5730956
6	Q0CST2	Mitogen-activated protein kinase (mPKC)	4.052091	3.329881038
7	Q0CP60	cAMP-dependent protein kinase (cAMP)	8.207825	1.017217244
8	Q0CF25	Protein ras-1(ras-1)	14.49014	13.58117285
9	Q0D231	Heat shock 70(Hsp70)	1.404769	2.069146799
10	Q0CE88	Heat shock protein 90 (Hsp90)	1.360057	1.126898164
11	Q0CLK1	Pyruvate carboxylase (PCY)	1.021248	19.28742836
12	Q0CRX8	Terrelysin (Terr)	5.052509	23.53236042
13	Q0CPF9	Eukaryotic translation initiation factor 3 (TEF-3)	13.51978	2.414132373
14	Q0CM19	Calcium-ATPase	0.2114419	0.33829737
15	Q0CH42	Calcineurin	0.7589096	1.096423518
16	Q0CY81	Calmodulin (cmd)	2.297928	0.829372976
17	Q0D1R1	Beta-hexosaminidase	25.75297	2.598311
18	Q0CMQ3	1,3-beta-glucanosyltransferase	6.535662	2.564672
19	Q0CTR9	Protein rho-1	22.23869	2.873572

5.3.6 ROS estimation

As the modulation in several redox homeostasis enzymes shown in our proteomic and transcript data indicates endogenous oxidative stress production. Thus, to validate ROS production we have estimate intracellular ROS levels in SHK-treated and untreated *A. terreus* cultures. The result showed that SHK treatment significantly increases the ROS levels in *A. terreus*. Fig.5.10 depicting the greater intensity of fluorescence due to ROS in *A. terreus* in comparison to control (untreated samples). These results validate high ROS generation during SHK metabolism in *A. terreus*. Hence, it has been evident from the catalase and ROS assay that these processes have played a key role in the inhibitory mechanism of SHK in *A. terreus*

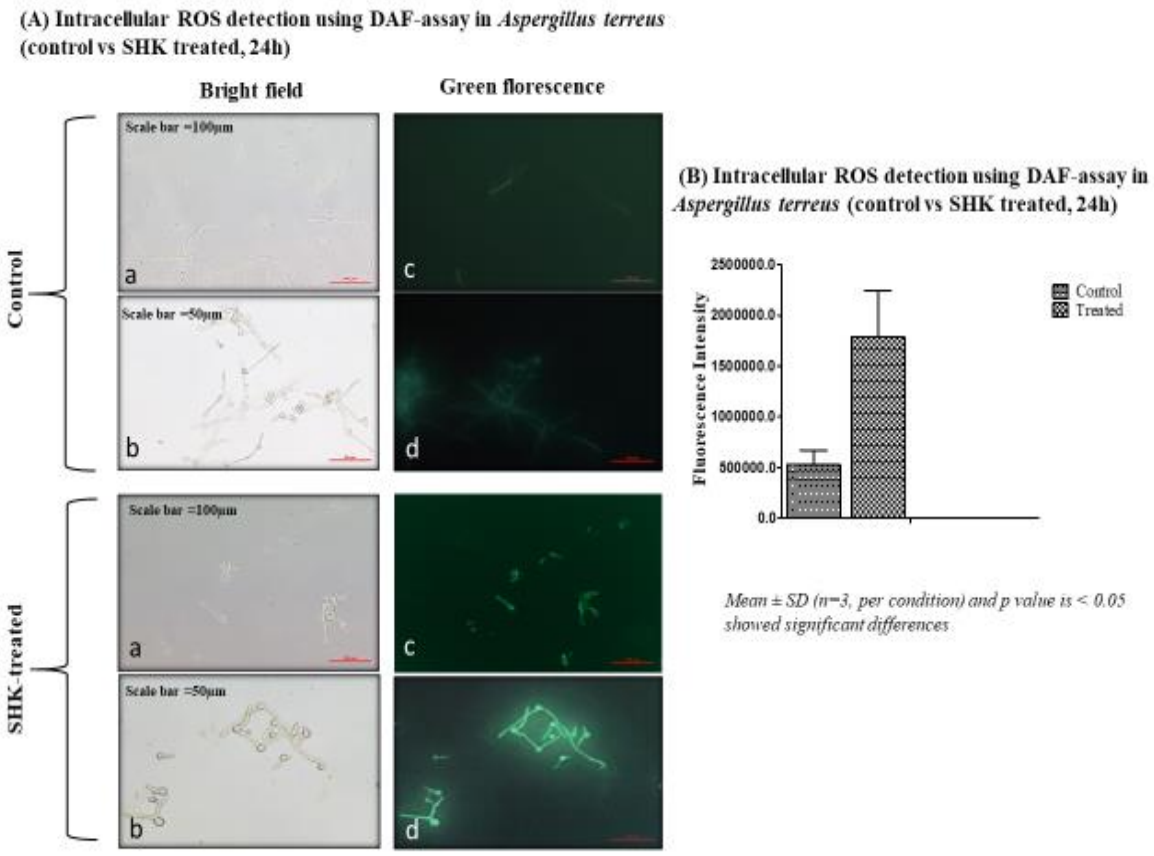


Fig. 5.10 (A) Fluorescence microscope images showing intracellular ROS accumulation using DAF-assay in *Aspergillus terreus* (control vs SHK -treated, 24h) (B) Bar graph showing intracellular ROS intensities in *A. terreus* (control vs SHK treated, 24h) calculated by using ImageJ.

5.3.7 Catalase activity

The lower expression of the catalase-peroxidase enzyme has been shown in SHK-treated samples through the protein and transcript analysis. We have therefore carried out an enzymatic assay of catalase-peroxidase to see the effect of SHK on the function of the catalase enzyme. Catalase peroxidase is involved in the catalyzation of H2O2 with peroxidatic activity in molecular oxygen and water. Under SHK-treatment in *A. terreus*, the catalase activity was measured in terms of nmol/min/ml. The results showed substantial low activity of catalase in *A. terreus* shown in the bar graph under SHK treatment Fig. 5.11

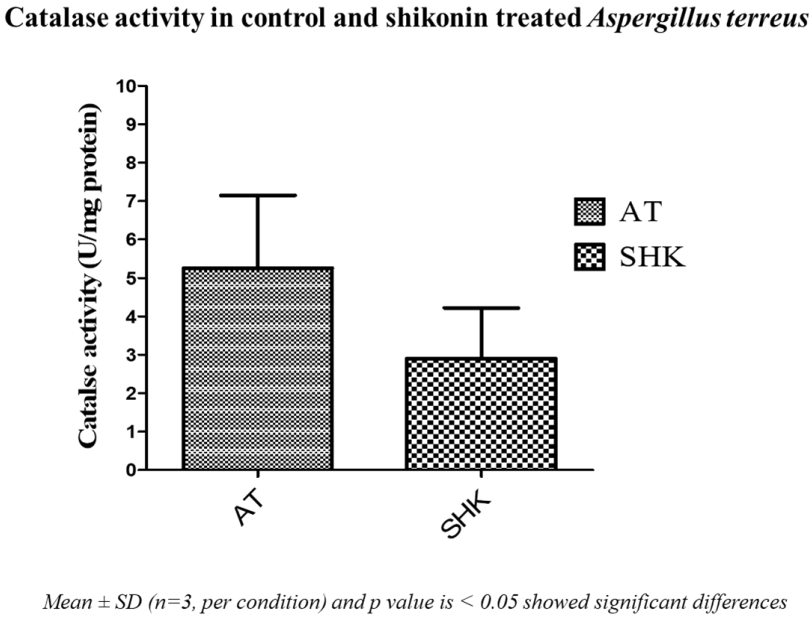
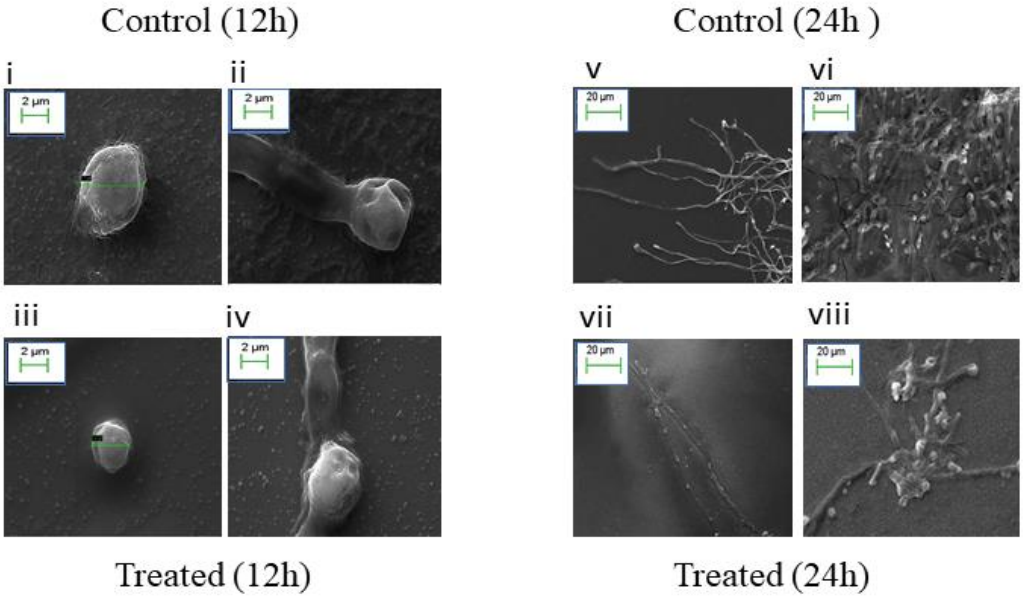


Fig.5.11 Catalase activity (U/mg protein concentration) in control and SHK-treated *A. terreus*

5.3.8 Effect of Shikonin on morphogenesis and biofilm of *Aspergillus terreus*

SEM was performed at two separate times (12 and 24h) to demonstrate the effect of SHK on conidial cell walls, germination, and hyphal growth in *A. terreus* Fig.5.11(A). We have found untreated *A. terreus* conidia was 5.7 µm in dia. compared to 3.4µm in SHK-treated *A. terreus* at 12h). Less protuberance on the conidial cell surface, as well as collapsed germinating conidia unable to polarise hyphae growth, have been found in treated conidia. After 24 h, normal hyphae formation was observed in control but thin and distorted hyphae were seen under SHK treatment.

A. Effect of shikonin on morphology of *Aspergillus terreus*



B. Effect of shikonin on biofilm eradication in *Aspergillus terreus*

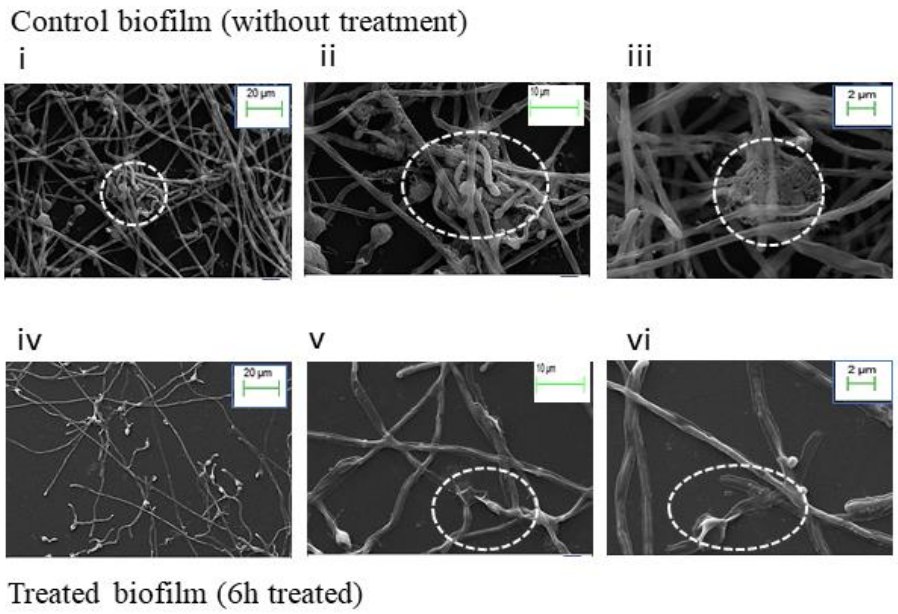


Fig.5.12 (A) Scanning electron microscope images showing effect of SHK on morphology of *A. terreus* at different time points i.e. 12h control (i, ii) Magnification (M);15K×, SHK-treated (iii, iv) M;15K×, and 24h control (v, vi) M;1K×, SHK-treated (vii) M;1.5 K× (viii); M; 2K×. (B) Scanning electron microscope images showing effect of SHK on biofilm eradication (6h treatment) of *A. terreus.* (i) M; 2K×, ii; M; 4K×, (iii) M; 5K× control biofilm and (iv); M; 3K×, (v) M; 1K×, (vi) M; 5K×) SHK-treated biofilm at different magnifications.

5.3.9 Biofilm eradication by shikonin in *Aspergillus terreus*

The biofilm inhibition by SHK was observed in our previous objective using an antifungal test. Thus, here we have validated the ECM eradication by SHK in pre-formed biofilms of *A. terreus* using SEM. SHK treatment significantly eliminates the biofilm structures of *A. terreus* depicted by SEM images captured. The results are shown in Fig. In control samples, well-formed ECM was seen at various magnifications (Fig.5.11(B). Whereas, no biofilm was developed after SHK treatment, and even distorted ECM was observed. Shrunken hyphae were also observed with structural changes. Therefore, SEM imaging indicated remarkable eradication of *A. terreus* biofilm by SHK.

5.4 DISCUSSION

Certain antifungal agents have intrinsic resistance due to prolonged incomplete dosage and widespread use of fungicides based on azole within agriculture in *Aspergillus species* [13]. Thus, PhytoChem can be used to avoid the transfer of antifungal resistance from the atmosphere to clinical isolates as an alternative antifungal agent. From our previous objective, we observed that SHK efficiently inhibits the *A. terreus* growth at low MIC_{50}; 2ug/ml, further we have performed differential proteome analysis to understand its mode of action. From our study, we observed differential expression of proteins/ enzymes of various important pathways under influence of SHK.

The previous literature review suggested that the cell membrane and the germination of *Aspergillus* conidia are affected by antifungal agents. Conidia morphogenesis is also accomplished by the modulation and activation of different signaling pathways in cytoskeletal components [249]. The SEM results have led to the depiction of morphological changes in antifungal therapy. Our findings have shown that SHK limited conidial swelling, which could lead to structural defects in conidium germination and polarised hyphal growth. Also, after 24 hours aberrant hyphal structures that are shrunken and distorted were captured in *A. terreus*. Similarly, SEM has been used to detect the apoptotic effects of *C. albicans* during QRT treatment [250]. Moreover, QRT was found to suppress the swelling of conidia in *A. flavus* at early developmental stages using SEM imaging [251]. In *A. fumigatus* cis-9-hexadecenal a plant product leads to alteration in cell surface organizational and fewer protrusions on the conidial surface using electron microscopy [252]. Also, simvastatin and AgNPs exposure alter

the germination of spore and vegetative growth of *Aspergillus species* using SEM imaging [253]. Besides this, in our differential protein analysis, we have seen modulation in the cytoskeleton, signaling, and cell wall-associated proteins which may be related to polarized growth and morphological changes in *A. terreus.* Thus, interference of SHK during morphological transitions are evidenced in *A. terreus.* Similarly, the role of calcium signaling related genes during germination under QRT exposures supported by SEM imaging has been reported in the recent report on *A. flavus* [251].

The formation of biofilms also protects fungal pathogens from antifungal drugs [201]. SHK demonstrated biofilm eradication in *A. terreus* observed in SEM. A thick hyphal network covered by a porous ECM layer was observed under the usual biofilm-forming conditions. SHK prevents the development of ECM and hyphae contour depletion. Incoherence with our study SHK was proposed recently as a desirable molecule having antibiofilm activity in *C. albicans* [254]. SHK may therefore be further explored in drug-resistant isolates of *Aspergilli* for anti-biofilm activity.

The differential proteome presented a molecular aspect of SHK-mediated inhibition of *A. terreus*, which involves significant modulations in biological and cellular events such as energy metabolism regulation, cellular respiration, signaling, cell walls, and organization of cytoskeletons. In *A. terreus* carbohydrate metabolism-related proteins/enzymes have been increased whereas the TCA-enzymes conjugating the ETC have been reduced by SHK treatment. Besides, the decline in glyoxylate pathway key enzymes (isocitrate lyase and malate synthase) was also observed. Overall, energy scarcity during SHK treatment has been observed in *A. terreus*. Previous studies have shown that antifungal stress has triggered energy crises and modulated energy metabolic pathways in cells to resolve stress. It is known that the isocitrate lyase key gene from the glyoxylate cycle in *C. glabrata* is essential for metabolic versatility [255]. Also, the stated role of the glyoxylate cycle in the tolerance of oxidative stress in earlier studies [256]. Higher expression of enzymes from the pentose pathway involved in the development of NADPH has also been observed. NADPH enters the redox cycle directly to counteract the ROS [257]. Present data corroborates that, probably, *A. terreus* retains the cellular redox potential by the production of NADPH in response to the oxidative stress produced by SHK. Thus, the down-regulation of the glyoxylate cycle and the up-regulation of pentose pathway enzymes jointly indicated the production of oxidative stress by SHK.

Also, the active oxidative phosphorylation components that suggest that the metabolism of SHK triggers redox reactions in *A. terreus*. major proteins of oxidative homeostasis (GPX, APX, SODS, CAT) have also been expressed differently, supporting high SHK exposure ROS production. ROS plays a critical role in the morphogenesis of fungi and oxidative burst mediated apoptosis [258]. Furthermore, in developing fungi, this ROS plays a dual role. Its biological effect depends not only on the ROS levels produced but also on the balance of its antioxidants. ROS may serve as regulators for the development of fungal growth (germination and cellular communications). However, very high ROS build-up induces metabolic changes, signal transduction, and cell subsurface activity in fungi [259]. Antioxidant components (SODS, GPX, APX and CAT) usually nullifies the ROS under steady-state, and SODs serve as the first line of defense against oxidative stress. [258]. Intriguingly, in our research, the effectiveness of SHK against *A. terreus* is highlighted by the less catalase-peroxide expression, low enzyme activity in the SHK-treated samples. As it has been previously reported that AmB resistant isolates *A. terreus* have a high activity of catalase compared with susceptible [27, 109]. ATR isolates were able to withstand oxidative stress generated by AmB compared to the production of high levels of ROS in ATS. Similarly, as referred from the previous literature we have also found an abundance of CAT, SODS, and other antioxidants enzymes in mycelial protein data (first objective) [260]. Thus, evident that mitochondrial ROS and antioxidant system plays a significant role in overcoming the internal AmBRS in *A. terreus.* Besides, AmB with pro-oxidant combat with the AmBRS in *A. terreus* [261]. Whereas, in *C. albicans* antioxidant N-acetylcysteine (NAC) and glutathione (GSH) have significantly reduced the antifungal effect of SHK [95]. Thus, it can be inferred that SHK may interfere with the antioxidant system of *A. terreus* and disturb the redox potential. We, therefore, hypothesized that the development of ROS and mitochondrial dysfunctions may be contributing to the probable inhibitory mechanism of SHK. To corroborate this, ROS level estimation in the SHK-treated and untreated samples were conducted and observed significantly high ROS accumulation to verify oxidative stress. On the other hand, after SHK therapy, decreased catalase-peroxidase enzyme activity was observed that is supporting our hypothesis. Various studies have already reported high ROS accumulation because of standard antifungal agents in *C. albicans, C. neoformans* and *A. fumigatus* [262]. AmB standard drug showed ROS production in *A. fumigatus* and causes oxidative damage, as depicted through proteomics and microarray data [133]. In coherence to our results a recent

report on *C. albicans* showed that high endogenous NO and ROS involved into antifungal action of SHK [263]. Posch *et al.*, found role of mitochondrial activity (ROS, SOD, and CAT), signaling pathways and alterations to ATR strains in coping with the oxidative burst produced by AmB [264]. To combat stress, it is necessary to trigger signaling paths under antifungal exposure [136]. Stress-related signaling pathway component activation was also observed in our data. (PkA, cAMP and Mpkc), Also small G-proteins (Rab, Ras-1, Rho1and) were also found to be active in our data, indicates that that Ras1, PKC, and CAMP signaling cascades can be linked to the SHK inhibition mechanism. The role of RAS / PKA signaling regulates by antifungal agents and leads to the production of ROS by mitochondria has been previously stated in literature [265]. Also contributes to AmBRS mechanism by suppressing the activation of Ras-signaling which leads to high ROS development and elevated AmB susceptibility in resistant strain [266]. SHK may modulate cytoskeleton dynamics by regulating the cytoskeleton and microtubule-based processes. It is important to note that SHK regulates the adenyl cyclase-associated proteins which control actin reorganization with the Ras signaling. Ras activation was previously observed in *S. cerevisiae* by adenyl cyclase dependent actin stabilisation which leads to a rise in cAMP, triggering ROS build-up and apoptosis [267]. In contrast to this, remarkably high Ras activity also underlies the maintenance of actin polarity in *Aspergillus nidulans* [268]. Thus, our data suggested that high ROS accumulation in *A. terreus*by SHK may also be linked to Ras-signaling and cytoskeleton dynamics. Other proteins such as heat shock proteins play a key role in the response to morphogenesis and antifungal treatment drug therapy [197]. The Hsp70 and Hsp90 proteins were found regulated by SHK in our data. These heat shock proteins will exemplify the stress of SHK. Previous studies showed that Hsp70 and Hsp90 blockers may overcome the AmBRS in *A. terreus* and suppose to play a role in the response of antifungal stress in *Aspergilli* [45, 110]. The schematic representation of major proteins/enzymes and pathways of *A. terreus* embattled by shikonin is given in Fig. 5.13. Broad spectrum antifungal agents mostly target ergosterol biosynthesis or cell wall, which limits therapeutic options, due to the emergence of resistance even when used in combination. However, modulation of few proteins related to cell-wall and ergosterol were also seen, which can counteract the stress generated by SHK [96].

The overall conclusion from this objective suggested that SHK alters the normal morphology of *A. terreus* and eradicates the ECM in pre-formed biofilms. Modulation in proteins and

genes expression of ROS homeostasis, signaling, and energy metabolism pathways may suggest their role is SHK action. High ROS accumulation and low catalase activity during treatment with SHK in *A. terreus* induce oxidative burst inhibition the probable inhibitory mechanism. Thus, SHK could be a lead antifungal molecule with a known mechanism of action and its clinical applications of SHK could be a suitable alternative that can open new avenues in antifungals against DR isolates.

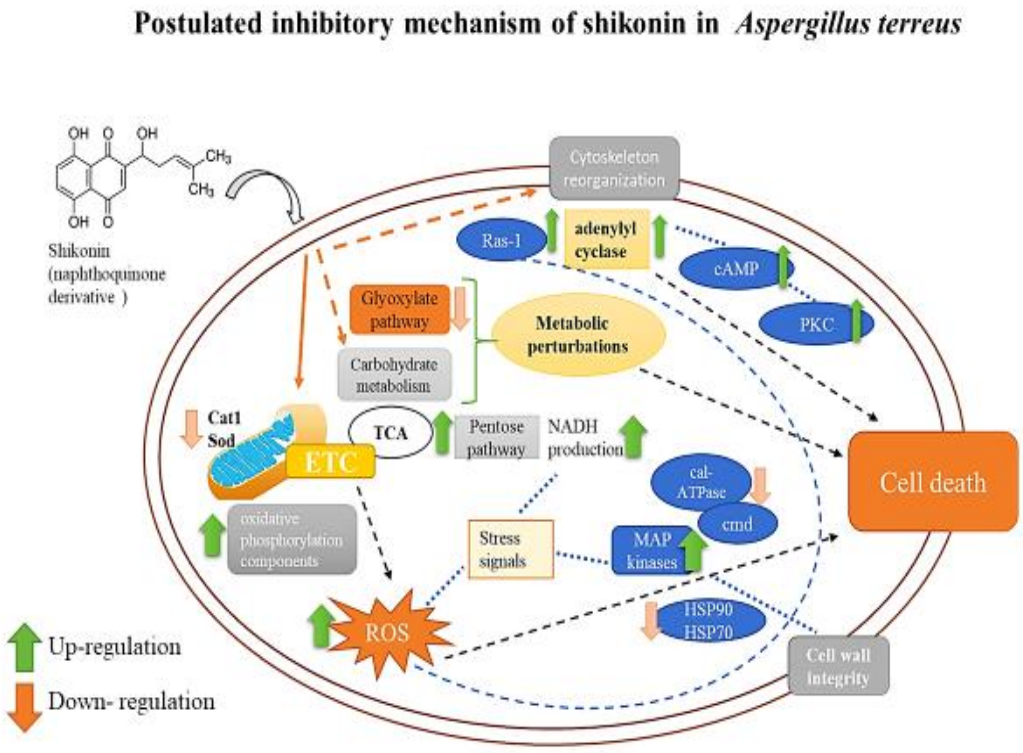


Fig.5.13 Schematic representation of major proteins and pathways of *Aspergillus terreus* embattled by shikonin.

CHAPTER -6

CONCLUSION AND FUTURE PROSPECTS

Our study showed that antioxidant enzymes, ribosome biogenesis, and reshuffling of structural components contribute to the biological and cellular processes of the mycelial network in *A. terreus*. Antioxidants enzyme (SOD and CAT) and heat shock proteins (Hsp70, Hsp90) can be further tested to increase our knowledge of *A. terreus* inherent AmBRS. Also, an abundance of biofilm-associated secretory proteins (glucanase Crf1/allergen, 1, 3-β-glucanosyltransferase, and β-hexosaminidase), adhesins, and SEM images evident the biofilm formation and could be further explored for contribution to DR mechanism in *A. terreus*. Mycelia of *A. terreus* found rich in diverse proteins/enzymes which help organisms develop under stresses. Thus, the overall mycelial proteome set enriched the experimental data in *A. terreus* and further *in-silico* analysis at the peptide level will increase the information of allergenic proteins. And pave the way for the single peptide or multi peptide-based vaccine or allergen-specific to *A. terreus*. Photochemical can be one of the best ways to overcome DR to *A. terreus*. SHK significantly suppresses the growth of planktonic cells as well as biofilms of *A. terreus*. SHK modulates germination and morphogenesis, and significantly eliminate the ECM in *A. terreus*. On the other hand, proteome analysis showed that several proteins from redox homeostasis, signaling cascades, and cytoskeleton and cell wall components are crucial during SHK metabolism within *A. terreus*. SHK exhibits overall energy deprivation, modulation in redox homeostasis, and interference with cytoskeleton dynamics which may assist in the inhibitory action of SHK. The high ROS generation and the low activity of CAT have inhibited the growth of *A. terreus.* SHK showed promising drug-like properties thus could be further investigated for its medical significance in the future. Additionally, the antifungal effect of SHK against *A. terreus* clinical isolates provides an additional benefit to cancer victims as it also has anti-cancer properties. Therefore, acting as a single drug that can be efficacious in fighting cancer whereas thwarting secondary fungal infections is very beneficial.

For future perspective, further SHK as a potent PhytoChem can is tested for its complementary or synergist effect with available antifungals to enhance current medical regimes. Also, to establish SHK as a new antifungal molecule with new drug targets against DR isolates of *A. terreus*, it is essential to conduct the *in-vivo* studies using a mice model of IA and clinical studies in the coming future. Furthermore, SHK from different natural plant sources for large-scale production can be implemented.

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LIST OF PUBLICATIONS

1. **Sonia Kumari Shishodia** and Jata Shankar. Proteomic analysis revealed ROS-mediated growth inhibition of *Aspergillus terreus* by shikonin," *Journal of Proteomics*, vol. 224, p. 103849, 2020. https://doi.org/10.1016/j.jprot.2020.103849 **(IF 3.6 (JCR), cite score 7.2, SCOPUS,)**

2. **Sonia Kumari Shishodia** and Jata Shankar. Proteome Exploration of Mycelial Proteins from *Aspergillus terreus* Revealed Ribosome Biogenesis and Antioxidant Enzymes. *Current proteomics.* Vol. 17, No. 5, 2020. http://dx.doi.org/10.2174/1570164617666191004163734 **(IF-0.7, Cite score 1.4, SCOPUS)**

3. **Sonia Kumari Shishodia**, Shraddha Tiwari & Jata Shankar (2019) Resistance mechanism and proteins in Aspergillus species against antifungal agents, *Mycology*, 10:3, 151-165. https://doi.org/10.1080/21501203.2019.1574927. (**Cite score 2.3 Scopus)**

4. **Sonia K Shishodia**, Shraddha Tiwari, Shanu Hoda, Pooja Vijayaraghavan and Jata Shankar (2020) SEM and qRT-PCR revealed quercetin inhibits morphogenesis of *Aspergillus flavus* conidia via modulating calcineurin-Crz1 signaling pathway, *Mycology.*11:2, 118-125. https://doi.org/10.1080/21501203.2020.1711826**. (Cite score 2.3 Scopus)**

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1. **Sonia Kumari Shishodia** and Jata Shankar. MYCOCON - 2018" held on "22nd to 23rd September, 2018 at Hotel Pride Plaza, Aerocity, New Delhi, India abstract entitled: Proteome analysis of mycelial extracts from opportunistic pathogen *Aspergillus terreus* revealed underlying molecular pathways involved in pathogen development and establishment.

2. **Sonia Kumari Shishodia** and Jata Shankar. Poster presented in '3 rd Himachal Pradesh Science Congress 2018' held on 22-23rd October 2018 at IIT Mandi, HP (Jointly organized by HIMCOSTE & IIT Mandi), entitled; Scanning Electron Microscope and Proteomic Analysis of *Aspergillus terreus*; Revealed Biofilm Associated Protein in Mycelial Morphotypes.

3. **Sonia Kumari Shishodia** and Jata Shankar (2019). Deciphering the efficacy of phytochemicals against amphotericin B resistant *Aspergillus terreus*. International Conference on Recent Trends in [Jaypee University of Information Technology, Waknaghat : 1-3 August, 2019], pp.44.

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1. **Sonia Kumari Shishodia** and Jata Shankar. Proteomic analysis revealed ROS-mediated growth inhibition of *Aspergillus terreus* by shikonin," *Journal of Proteomics*, vol. 224, p. 103849, 2020. https://doi.org/10.1016/j.jprot.2020.103849 **(IF 3.6 (JCR), cite score 7.2, SCOPUS,)**

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