ROLE OF *CALCINEURIN* GENE AND ANTI-MICROBIAL COMPOUNDS IN CONIDIATION OF *Aspergillus flavus*

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May 2015

Submitted in Partial fulfilment of Degree of

Bachelor of Technology

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TABLE OF CONTENTS

CERTIFICATE

This is to certify that the work entitled, **"Role of calcineurin gene and anti-microbial compounds in conidiation of Aspergillus flavus"** submitted by **Ms Nupur Gupta (111804)** in partial fulfilment for the award of degree of Bachelor of Technology in Biotechnology of Jaypee University of Information Technology, Waknaghat, Solan has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

Signature of Supervisor:

Name of Supervisor : Dr. Jata Shankar

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ACKNOWLEDGEMENT

This thesis is the end of my journey in obtaining my B. Tech. Degree. This thesis has been kept on track and been seen through to completion with the support and encouragement of numerous people including my well-wishers, my friends, colleagues and various institutions.

Foremost, I would like to express my sincere gratitude to my supervisor, **Dr. Jata Shankar** for the continuous support of my study and research, for his patience, motivation, enthusiasm, and immense knowledge. His guidance helped me in all the time of research and writing of this thesis.

Besides my supervisor, I would like to thank PhD Scholars- **Ms. Shraddha Tiwari and Mr. Raman Thakur** for their encouragement, insightful comments, and never ending support.

My sincere thanks also goes to **Dr. R.S. Chauhan**, Dean, Head of Department, Biotechnology and Bioinformatics Department for providing me this opportunity of working on such a diverse project.

Last but not the least, I would like to thank my family: my parents **Mr. Sanjiv Kumar Gupta** and **Ms. Rajni Gupta** and my brother **Mr. Nitesh Gupta**, for being a constant source of encouragement and motivation through thick and thin. This project work would not have been possible without their support.

Signature of the Student :

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Date :

SUMMARY

This study was carried out on opportunistic pathogenic fungus *Aspergillus flavus*, one of the major cause of invasive Aspergillosis in immunocompromised humans and colonisation of cereal grains, legumes, and tree nuts, usually post-harvest. Role of calcineurin gene was established in conidiation of *A.flavus* strain MTCC-AF9367 grown at different concentrations of Calcium Chloride, using semi-quantitative PCR with primers specific to calcineurin. It was observed that the calcineurin gene expression increased with increase in concentration of calcium. This effectively leads us to believe that the calcineurin gene affects conidiation in *A. flavus* as it does in *A. fumigatus* along with affecting virulence, anti-fungal drug tolerance, hyphal development and phosphate transport and can therefore be an excellent target for therapeutic intervention.

In a separate study, methanol and water extracts of four phytochemicals- Ascorbic acid, caffeine, Gallic acid and Quercetin were evaluated for their anti-fungal properties using Poisoned Food Technique. Caffeine proved to be a promising candidate for an environment friendly antimicrobial compound since it shows complete inhibition at concentrations 1, 5 and 10 mg/ml. However, it showed only 35.21% inhibition in its 15 mg/ml concentration plate, the possible reason for which can be attributed to “Feedback Inhibition”. While gallic acid shows only 13.43% inhibition at 10 and 15mg/ml, quercetin seems to be a promising inhibitory phytochemical as it shows 31.34% inhibition at only 5mg/ml- comparable to Ascorbic acid’s 34.32% at 15 mg/ml. This study can be enhanced further to determine components of a natural, environmentally safe anti-microbial compound to replace conventional chemical pesticides.

LIST OF FIGURES

LIST OF TABLES

	Control (Average of Methanol and Water Controls). Diameter of each fungal inoculum containing 1x 10^6cells were measured on 1st, 2nd and 3rd day and %age inhibition was calculated	

ABBREVIATIONS

EDTA	Ethylene Diamine Tetra Acetic Acid
NaCl	Sodium Chloride
TE	Tris-EDTA
TAE	Tris-Acetate EDTA
EtBr	Ethidium Bromide
dNTPs	Deoxyribonucleotide Triphosphate
µl	Microliter
mL	Milli Litre
PCR	Polymerase Chain Reaction
IA	Invasive Aspergillosis
cDNA	Complementary DNA
AF	*Aspergillus flavus*
DEPC	DiEthyl Pyro Carbonate
HSP	Heat Shock Protein

1. INTRODUCTION

BACKGROUND

Aspergillus flavus is a facultative, plant parasitic pathogen, with the ability to infect several common crop species including corn, cotton, peanuts, and many other crops (Fountain et al., 2014). Economic losses due to the infection of grain crops such as maize by *A. flavus* is not only due to the expression of symptoms known as *Aspergillus* ear rot but also due to the subsequent contamination of the grain with mycotoxins called aflatoxin.

Although Link first described this species in 1809, biology and pathogenicity of *A. flavus* was not studied extensively until the mid-1960s when the incidence of Turkey X disease which killed over 100,000 turkey poults due to aflatoxin contaminated feed associated with *A. flavus* infected peanuts. This resulted in broad screening of feed and food. Chemical structures of the major aflatoxins were elucidated, and research was conducted to prevent post-harvest contamination of grain crops through the modulation of storage conditions. However, during a particularly severe outbreak of aflatoxin contamination in maize in the late 1970s in the U.S. it was found that *A. flavus* could both, colonize and produce aflatoxin on developing maize kernels prior to harvest. (Fountain et al., 2014)

Since then, research efforts have been focused on determining the source of host plant resistance to prevent *A. flavus* infection and subsequent aflatoxin production pre-harvest and before transportation to storage. Numerous techniques and approaches have been employed including modern plant breeding and genetics tools such as proteomic, transcriptomic, and biochemical analyses in order to discover the mechanism responsible for host plant resistance and the interaction between the two organisms (Fountain et al., 2014). Till date, it has been established that resistance is inherited quantitatively with a strong genotype by environmental influence (Fountain et al., 2014). It is a complex interaction with a high environmentally induced variability and abiotic and biotic stress strongly influencing resistance or susceptibility.

Mycotoxins are a group of structurally diverse secondary metabolites produced by various fungal species and can contaminate various foodstuffs, crops or human foods (Maggon et al., 1977). The ingestion of these contaminated materials may lead to serious health problems in animals and humans, such as liver, kidney or nervous system damage, immunosuppression and carcinogenesis. They comprise a group of several hundreds of

chemically different toxic compounds. Aflatoxins (AFs) are a group of mycotoxins produced by the spoilage of fungi *Aspergillus*, particularly *Aspergillus flavus* and *Aspergillus parasiticus*.

They are most commonly known for causing acute or chronic liver disease, but they are also considered immunosuppressive, hepatotoxic, mutagenic, teratogenic, and carcinogenic (Mohammedi et al, 2013). There are four major aflatoxins- B1, B2, G1 and G2. They are named so due to their ability to fluoresce under ultraviolet light: B- blue and G- green). The International Agency for Research on Cancer (IARC) has classified B1, B2, G1 and G2 in the group I as human carcinogen. *A. flavus* generally produces only B1 and B2. B1 is the most toxic form. Its genotoxicity results from liver cytochrome P-450 epoxidation of B1 to B1 *exo*-8,9epoxide (BO) which reacts with DNA at the guanyl N7 atom after intercalation, forming a genotoxic DNA adduct (Mohammedi et al, 2013).

Invasive Aspergillosis (IA) caused by the opportunistic pathogenic fungus *Aspergillus fumigatus* is a leading cause of mortality in immunocompromised patients. While the mortality from invasive Candidiasis caused by *Candida albicans* has decreased by 50% over the last two decades, the mortality associated with IA has increased 357% over the same period of time (Steinbach et al., 2006). Unfortunately, the optimal treatment for IA remains unknown yet. No study has examined the specific role of calcineurin in the pathogenicity of this increasingly important fungal pathogen *A. flavus*.

Chronic Pulmonary Aspergillosis and Aspergillomas is caused by *Aspergillus* infecting the body and growing in cavities in the lungs which would usually been created by a previous health problem such as tuberculosis. Once the *Aspergillus* has infects the lungs, it starts to grow as a fungal ball yielding allergens or toxins that is put into the person's body. Symptoms of Aspergilloma breathing problems, chronic cough, coughing out blood, weight loss, tiredness and fatigue appear after long periods of time. Aspergillomas can be detected through X-ray or blood tests. Drugs such as itraconazole or voriconazole can be administered to the patients for Apergilloma but are not known to be fully effective. Anti-fungal drugs may even be injected into the cavity where the Aspergilloma is growing. Surgery is recommended in severe cases where the size of Aspergillomas gets bigger.

OBJECTIVE

Different strategies have been established for preventing AFs contamination of susceptible plants and crops. The application of chemicals compounds in the agricultural fields has led to a number of environmental and health problems due to their residual toxicity, carcinogenicity, hormonal imbalance and spermatotoxicity (Fountain et al., 2014). There is a need to design new and environmentally safe methods of reducing infection by aflatoxigenic *aspergilli* and to inhibit aflatoxin biosynthesis. Plants are considered as sources of useful metabolites. Plants contain a wide variety of secondary metabolites such as tannins, terpenoids, alkaloids, and flavonoids, reported to have in vitro antifungal properties (Fountain et al., 2014).

The objective of this study was to establish the role of calcineurin gene in conidiation of *Aspergillus flavus*, which is well established in other species *Aspergillus fumigatus*. Since substantially decreasing fungal growth can prevent disease establishment and decrease mortality rate in case of Invasive Aspergillosis, revealing that calcineurin plays a globally conserved role in the conidiation of *Aspergillus flavus*, it can be an excellent target for therapeutic intervention.

Also, by evaluating the fungitoxic effect of various phytochemicals against growth and conidiation *Aspergillus flavus*, one can determine components of a natural, environmentally safe anti-microbial compound to replace conventional chemical pesticides.

2. REVIEW OF LITERATURE

Calcineurin is a Ca^{+2}-calmodulin-dependent serine/threonine phosphatase consisting of a catalytic A subunit and a regulatory B subunit (Steinbach et al., 2006). Its structure and activation pathway are highly conserved from yeast to high eukaryotes. Studies in *Saccharomyces cerevisiae* have provided a thorough understanding of the calcineurin signalling pathway and its function (Karababa et al., 2006). This pathway is first activated by an increase in intracellular calcium due to external stimuli. In *S. cerevisiae,* exposure of a haploid strain to the opposite mating pheromone or exposure to ions (Na^{+}, Mg^{2+}, OH^{-} and Mn^{2+}) leads to a rise of intracellular calcium. The plasma membrane Ca^{2+} channel made up of the proteins encoded by MID1 and CCH1 is one mechanism of calcium entry that activates the calcineurin signalling pathway. Calcium may also be released from the vacuole via a vacuolar Ca^{2+} channel (Yvc1p) under other conditions. Cytosolic Calcium binds to calmodulin at EF-hand motif domains and is activated by conformational changes. Activated calmodulin then interacts to specific domains on calcineurin A subunit and leads to the activation of the phosphatase activity by conformational changes. Calcineurin specifically regulates some proteins by dephosphorylation. Recently, it was shown that HPH1 encodes a tail-anchored integral membrane protein localized to the endoplasmic reticulum (ER) and is activated and distributed within the ER in a calcineurin-dependent manner and involved in growth under stress conditions (Karababa et al., 2006).

Calcineurin also dephosphorylates Czr1p and regulates its localization (Steinbach et al., 2006). The phosphorylated form of Crz1p accumulates in the cytosol under resting conditions. Elevated Ca^{+2} in turn leads to calcineurin dephosphorylating Crz1p and its nuclear localization. Crz1p contains a C_2H_2 zinc finger motif that binds to a specific element on the promoter of genes called CDRE (Calcineurin-Dependent Response Element) which is shown to be sufficient for Ca^{2+}- and calcineurin-dependent gene expression. Using DNA microarrays, calcineurin activation has been established to induce 153 genes involved in cell wall biosynthesis, ion homeostasis, vesicle trafficking, lipid synthesis, and protein degradation (Karababa et al., 2006). The vast majority of calcineurin-dependent genes have a Crz1p-dependent expression, thus suggesting that Crz1p is a major mediator of calcineurin-activated gene expression in yeast and can be a potential antifungal drug target.

Other studies performed in pathogenic yeasts such as *Cryptococcus neoformans* showed that calcineurin is essential for morphogenesis, for growth at 37°C and virulence. In *Neurospora crassa*, calcineurin plays a role in hyphal growth and cell morphology. In *Aspergillus nidulans*, calcineurin catalytic subunit gene disruption (CNA1) results in growth arrest, thus suggesting that it is required for cell cycle progression (Steinbach et al., 2006). In *C. albicans*, involvement of calcineurin in antifungal tolerance, cell morphogenesis and virulence has recently been reported. Loss of tolerance to several antifungal agents (fluconazole, terbinafine, caspofungin), altered growth against agents perturbing cell wall structures (Congo Red, calcofluor white) and other growth-inhibiting agents (e.g. fluphenazine, caffeine) has been reported on deletion of genes encoding the calcineurin regulatory subunit (CNB1) or the catalytic subunit (CMP2/CMP1/CNA) (Steinbach et al., 2006). This demonstrates that calcineurin is involved in cell survival under different stress conditions. The cnaΔ/Δ mutant strain is affected but not blocked in its ability to undergo filamentation as compared with the wild type mice infected with the cnaΔ/Δ or cnb1Δ/Δ when subjected to conditions known to induce dimorphic switch (Steinbach et al., 2006). Moreover, mice with Δcna mutant strain survived when compared to mice with a wild type strain. Despite some differences in the role of calcineurin among pathogenic yeast, a common characteristic of calcineurin among fungal pathogens is its involvement in virulence.

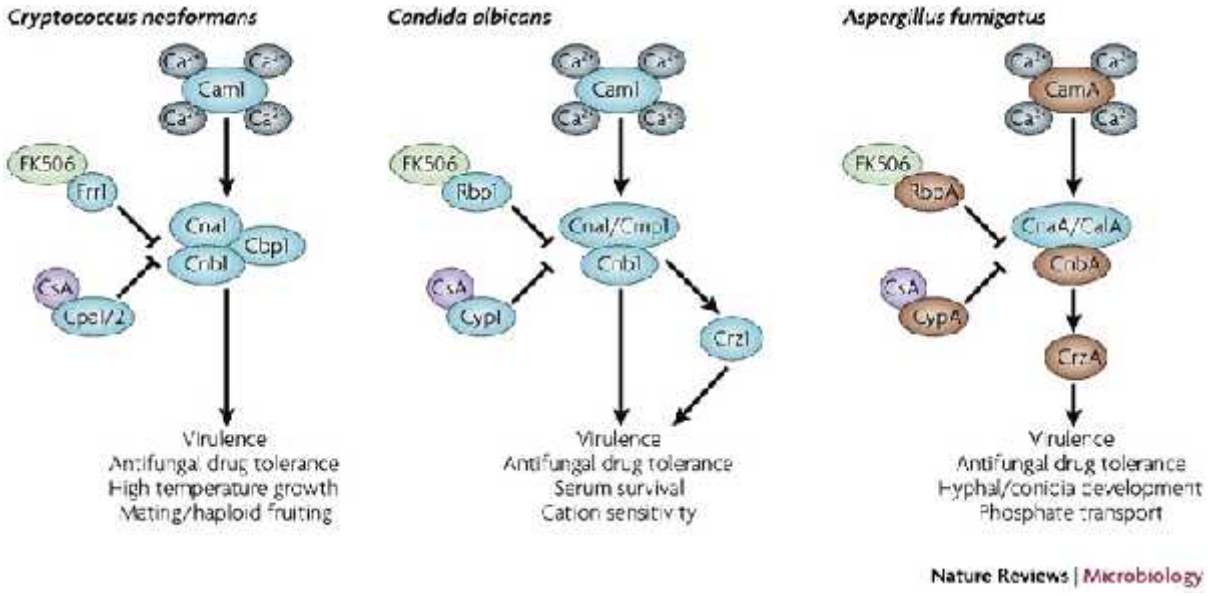

FIGURE 1: The relevant phenotypes attributed to calcineurin signalling in each species are shown as the output of each pathway. Pathway components shown in blue have been

disrupted and studied; the *A. fumigatus* components shown in orange have not yet been studied and have been given a gene designation consistent with expected *A. fumigatus* gene designations. In *C. albicans*, some activities such as azole and ion resistance are partially mediated through Crz1, whereas others such as virulence and serum sensitivity are not mediated through Crz1.

A genome-wide approach comparing *cna*Δ/Δ and *crz1*Δ/Δ mutant strains shows that both genes regulate similar subset of calcium-activated genes, thus suggesting that Crz1p belongs to the calcineurin activation pathway. On the other hand, it was demonstrated that *CRZ1* has no impact on antifungal tolerance in the presence of different drugs and slightly affects virulence in contrast to calcineurin, therefore indicating that calcineurin controls additional components in this signalling pathway (Steinbach et al., 2006).

Apart from *Aspergillus flavus,* the fungus *Aspergillus fumigatus* is a leading cause IA. In an experiment by Steinbach et al., it was demonstrated that a mutant of *A. fumigatus* lacking the calcineurin A (cnaA) catalytic subunit displayed drastic decrease in filamentation due to defective hyphal morphology related to apical extension and polarized growth. The extensive lattice of invading hyphae otherwise well observed in with the wild-type and complemented strains lacked in the ΔcnaA mutant. Effect on sporulation along with morphological conidial defects with the absence of surface rodlets and the added presence of disjunctors creating long conidial chains was also observed in the ΔcnaA mutant. Significant attenuation of pathogenicity of ΔcnaA mutant compared to that with the wild-type and complemented strains was observed when several different animal models with different types of immunosuppression and inoculum delivery were infected. Lung tissue from animals infected with the ΔcnaA mutant displayed a complete absence of hyphae. These findings were confirmed by quantitative fungal burden and pulmonary infarct scoring. The clinical observations substantially supported their claim that decreasing fungal growth can prevent disease establishment and decrease mortality.

In another study by Steinbach et al., it was showed that calcineurin inhibition via pharmacologic inhibitors or the Δ*cnaA* mutant, led to morphological hyphal defects similar or superior to those found with echinocandin treatment of *A. fumigatus*. The radial growth assay, demonstrated that 50-fold lower concentration of FK506 than caspofungin was required to limit filamentation and growth. This hyphal dysmorphic effect is even more significant with the Δ*cnaA* mutant than FK506 treatment suggesting that current calcineurin

inhibitors may not be completely effective in blocking the calcineurin pathway's impact on hyphal growth and therefore allows possibilities that improved drugs could be designed for hyphal growth inhibition by targeting and blocking calcineurin. They also observed that 1, 3-D-glucan measurements which were utilized for quantifying caspofungin antifungal activity led to decreases after calcineurin inhibition, supporting the hypothesis calcineurin inhibition operates through cell wall biosynthesis.

FUNGITOXICITY OF PHYTOCHEMICALS

Antifungal compounds have been often been overshadowed by antibacterials in research interest and application due to the greater impact bacterial infections have had on human health. Resistance to antibacterial drugs and the resultant clinical impact is of widespread concern regarding public health. However, resistance by pathogenic fungal infections to drug treatment has become more common in the last 20 years as well. Some mechanisms of fungal resistance are similar to those of drug resistance in bacteria, and knowledge of those bacterial mechanisms is being applied to understanding fungal drug resistance.

Several key antibiotic compounds function by targeting the integrity of the cell. Many compounds increase the porosity of the cell wall or membrane, or interfere with key steps in the synthesis of cell walls. While prokaryotic bacteria and eukaryotic fungi do not have identical cell wall and membrane components, there are corresponding lipids and key structural molecules (Hussin, et al., 2009). As a result, similar to antibacterials, most antifungal compounds work because they directly or indirectly damage the cell wall or cell membrane.

The fungal cell wall is composed of multiple layers, with mannoproteins being predominantly expressed at the external surface (Figure 2). An underlayer of β-glucan creates a supporting matrix for the mannoproteins providing structural rigidity to the cell wall. The glucan structure is strengthened by frequent $\beta(1\rightarrow3)$ and additional $\beta(1\rightarrow6)$ linkages and by chitin interspersed with the β-glucan. Mannoproteins and glucan make up more than 80% of the cell wall composition, while chitin represents less than 2%. The plasma membranes of fungi are primarily composed of ergosterol, analogous to cholesterol in animal cells. Since ergosterol and cholesterol have sufficient structural differences, the majority of chemicals found to act as fungicides target ergosterol biosynthesis or cell membrane porosity and do not cross react with host cells (Ghannoum et al., 1999).

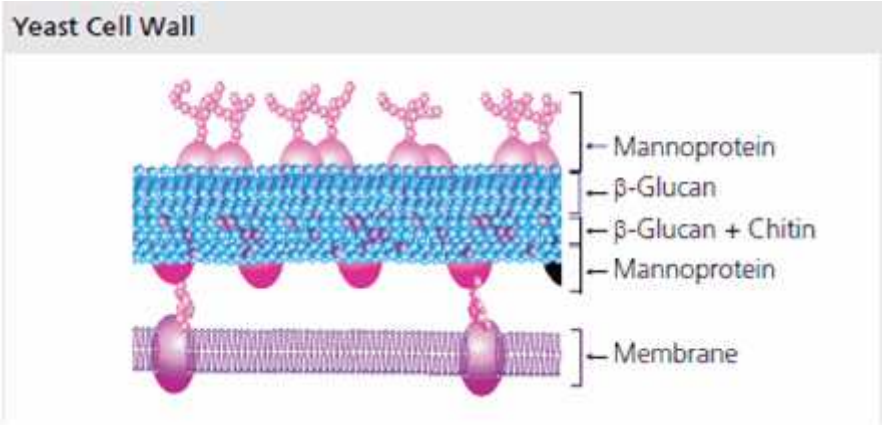

FIGURE 2- Structure of the yeast cell wall. The wall is primarily composed of mannoproteins and β-glucan that is linked (1→3) and (1→6). Ergosterol is the major lipid component of the underlying plasma membrane.

Plant sources of antifungal compounds, though studied extensively, still haven't been able to be of any commercial use- be it as a drug or as a potential antimicrobial compound to control *A. flavus* disease incidence in commercially important crops. Medicinal plants have been associated with the prevention of degenerative diseases such as cancer and cardiovascular diseases. Chemo preventive and cardio protective effects and protecting human body against oxidative damage by free radicals have been shown by a wide range of phytochemicals such as phenolics, thiols, carotenoids, anthocyanins and tocopherol (Naz et al., 2013). Natural phytochemicals derived from fruits, vegetables and herbs have been reported to possess a wide range of biological effects, including antioxidant, antimicrobial and anti-inflammatory actions.

In this study, seven phytochemicals were tested for anti-fungal activity initially. However, due to lack of resources, the final attempt was made with only four.

- Caffeine
- Gallic Acid
- Ascorbic Acid
- Quercetin

Caffeine is a water-soluble alkaloid. Pure caffeine is a white odourless crystalline powder with a very bitter taste. It is closely related to other alkaloids such as theophylline (mainly

found in tea) and theobromine (mainly found in cacao beans). The difference between these three molecules is the position of the methyl groups. It is a methylxanthine naturally occurring in some beverages and also used as a pharmacological agent. Caffeine's most notable pharmacological effect is as a central nervous system stimulant, increasing alertness and producing agitation. It also relaxes smooth muscle, stimulates cardiac muscle, stimulates diuresis, and appears to be useful in the treatment of some types of headache. Several cellular actions of caffeine have been observed, but it is not entirely clear how each contributes to its pharmacological profile. Among the most important are inhibition of cyclic nucleotide phosphodiesterases, antagonism of adenosine receptors, and modulation of intracellular calcium handling. Caffeine has been shown to inhibit growth and polyketide mycotoxin production in a number of Aspergillus and Penicillium species, but little is known about its mode of action. It is said to inhibit aflatoxins G1 and G2 in a study conducted by Maraqa et al. (2007).

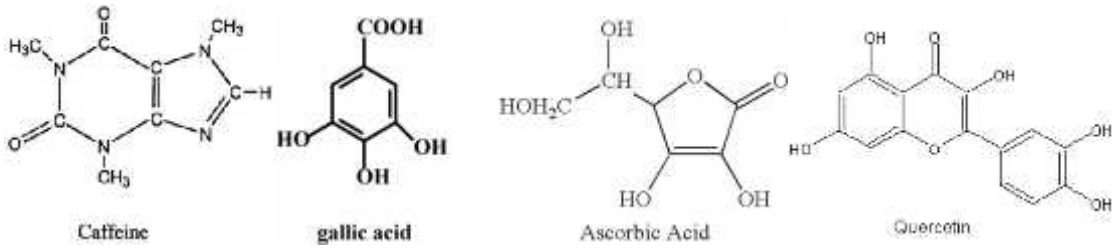

FIGURE 3: Structures of various Phytochemicals used.

Gallic acid exists in plant material in the form of free acids, esters, catechin derivatives and hydrolysable tannins. This ubiquitous chemical is one of the most biologically-active phenolic compounds of plant origin. The antioxidant activity of gallic acid and its derivatives has been reported in several studies. Gallic acid has been shown to possess antimicrobial activity against human pathogens (Staphylococcus aureus, Corynobacterium accolans), a plant pathogen (Erwinia carotovora) and human pathogenic yeast (Candida albicans). Gallic acid is known to exhibit good antifungal activity against *M. grisea* and *Erysiphe graminis*. Moderate and significant inhibition of conidial germination (64%) and appressorium formation (5%) of M. grisea, respectively, were observed with gallic acid. Gallic acid was said to act on a cAMP-related signaling pathway regulating appressorium formation in M. grisea (Naz et al., 2013).

Ascorbic Acid is a six carbon compound related to glucose. It is found naturally in citrus fruits and many vegetables. Ascorbic acid is an essential nutrient in human diets, and

necessary to maintain connective tissue and bone. Its biologically active form, vitamin C, functions as a reducing agent and coenzyme in several metabolic pathways. Vitamin C is considered an antioxidant. It is a well-known anti-microbial compound. The antimicrobial action of ascorbic acid may probably be due to its ability to promote the cyclic reduction of cupric ions which then combine with ion- containing sites within the cell and/or the oxidative products. This has also been attributed to some undefined processes involving the cell components. It is known to show 14.3% inhibition against *A. flavus* at 1% concentration according to Obaleye et al. in 1994 in a study of fungitoxic effects of ascorbic acid and its acid-metal complexes on four standard strains of bacterial species - *Staphylococcus aureus, Escherichia coli, Bacillus subtilis* and *Klebsiella pneumonia* and four fungal species - *Trichophyton* sp., *Penicillium* sp., *Aspergillus flavus* and *Aspergillus niger.*

Quercetin belongs to a group of plant pigments called flavonoids that give many fruits, flowers, and vegetables their color. Flavonoids are ubiquitous in photosynthesising cells and are commonly found in fruit, vegetables, nuts, seeds, stems, flowers, tea, wine, propolis and honey. The antimicrobial activity of quercetin has been at least partially attributed to inhibition of DNA gyrase. Its antifungal activity is not very extensively studied.

3. MATERIALS AND METHODS

Aspergillus flavus strain MTCC-AF9367, which is an active producer of aflatoxins, was used for the whole project work.

1. Identification of *Aspergillus flavus* strain MTCC-AF9367

For the identification of *Aspergillus flavus* strain MTCC-AF9367 in laboratory, Lactophenol Cotton Blue Staining was used. The Lactophenol Cotton Blue (LPCB) wet mount preparation is the most widely used method of staining and observing fungi and is simple to prepare. The preparation has three components:

- Phenol: kills any live organisms;
- Lactic acid : It preserves fungal structures, and
- Cotton blue: It stains the chitin in the fungal cell walls.

The following protocol was applied:

- Took a slide and wiped it with ethanol.
- Poured a drop of Lactophenol Cotton blue.
- Took the fungal culture from the agar slants with the help of a loop.
- Mixed it well and teased it with the help of a needle.
- Observed it under the microscope at 100X.

2. Counting of conidia through Hemocytometer

Hemocytometer is a device used for determining the number of cells per unit volume of a suspension is called a counting chamber. It is the most widely used type of chamber, since it was mainly designed for performing blood cell counts. It is now used to count other types of cells and other microscopic particles as well.

The ruled area of the hemocytometer consists of several large 1 x 1 mm ($1mm^2$) squares, which are subdivided in three ways; 0.25 x 0.25 mm ($0.0625\ mm^2$), 0.25 x 0.20 mm ($0.05\ mm^2$) and 0.20 x 0.20 mm ($0.04\ mm^2$). The central, 0.20 x 0.20 mm marked, 1 x 1 mm square is further subdivided into 0.05 x 0.05 mm ($0.0025\ mm^2$) squares. Hold the cover slip (0.1 mm) at the raised edges of hemocytometer, which gives each square a defined volume.

TABLE 1: Dimensions of Hemocytometer

Dimensions	Area	Volume at 0.1mm depth
1 x 1 mm	1 mm^2	100 nl
0.25 x 0.25 mm (1/16)	0.0625 mm^2	6.25 nl
0.25 x 0.20 mm (1/20)	0.05 mm^2	5 nl
0.20 x 0.20 mm (1/25)	0.04 mm^2	4 nl
0.05 x 0.05 mm (1/400)	0.0025 mm^2	0.25 nl

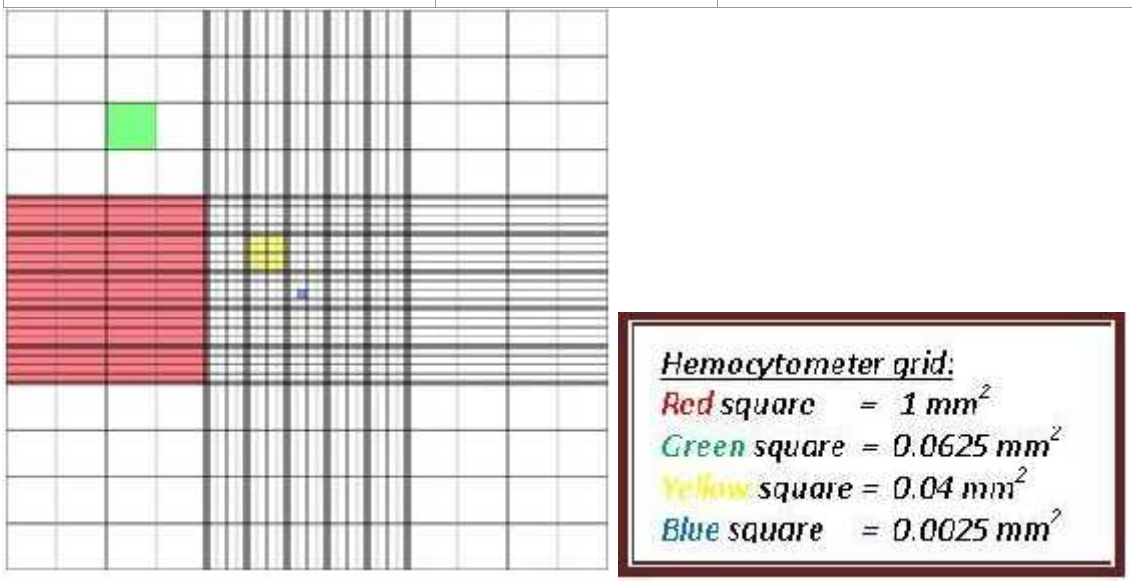

FIGURE 4: Divisions on a Hemocytometer and their dimensions.

The following protocol was used:

- Prepared a spore suspension in 1mL Phosphate Buffer Saline with Tween-20.
- Cleaned all surfaces of the hemocytometer and cover-slip.
- Pipetted approximately 10 µL of the cell suspension into one of the two counting chambers.
- Counted the conidia.

Formula used:

$$\textbf{Cells/mL} = (n) \times 10^4 \times \textbf{dilution factor}$$

where n = the average cell count per square of the four corner squares counted.

Phosphate Buffer Saline with Tween-20 (PBST):

PBST was used for the maintenance of cell viability Saline maintains osmotic balance between internal and external environment of cell. Phosphate buffer maintain physiological pH. It was made using the following composition:

Ingredients	Grams / Litre	Grams/100mL
Sodium Chloride	8.000	0.800
Potassium chloride	0.02	0.02
Disodium Hydrogen Phosphate	1.440	0.144
Potassium Dihydrogen phosphate	0.240	0.024
Tween-20	2mL	0.2mL

The above components were dissolved in 80 mL of water and the pH was adjusted to 7.2. The volume of the solution was then raised to 100 mL and autoclaved.

3. Culturing of *Aspergillus flavus* strains MTCC-AF9367 at different Calcium concentrations of 10mM, 20mM, 30mM, 40mM and Control (No Calcium)

Media Used: 50 mL of Czapek Dox Broth (CDB)

CDB is a semisynthetic medium was used for the cultivation of A. flavus. Czapek Dox contains sodium nitrate as the sole source of nitrogen. This medium is prepared according to the formula developed by Thom and Church, which has a defined chemical composition. Czapek Dox Broth is the modification of the original medium of Czapek Dox as per Thomas and Raper. Sucrose serves as the sole source of carbon while sodium nitrate serves as the sole source of nitrogen. Dipotassium phosphate buffers the medium. Magnesium sulphate, potassium chloride, ferrous sulphate serves as sources of essential ions.

Composition:

Ingredients	Grams / Litre
Sucrose	30.000
Sodium nitrate	3.000

Dipotassium phosphate	1.000
Magnesium sulphate	0.500
Potassium chloride	0.500
Ferrous sulphate	0.010
Final pH (at 25°C)	7.3±0.

Suspended individual components (1.755g) in 50 ml distilled water. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

For Calcium Stress: 500mM Calcium Chloride solution was prepared by dissolving 5.55g (MW= 110.99g) in 100 mL distilled water. It was autoclaved separately from the media at 15 lbs pressure (121°C) for 15 minutes.

- Five concentrations of $CaCl_2$-containing media was prepared in Laminar Air Flow:

TABLE 2: Volume of Water and $CaCl_2$ solution added in CDB

Concentration	**Distilled Water (mL)**	**500 mM $CaCl_2$ (mL)**
Control	50	-
10mM	49	1
20mM	48	2
30mM	47	3
40 mM	46	4

- Each flask was inoculated with 10^6 conidia of *Aspergillus flavus* strain MTCC-AF9367 and kept at **28-30ºC at 150 rpm for 24 hours**.

4. Isolation of Genomic DNA from *Aspergillus flavus* MTCC-AF9367

Genomic Isolation was carried out from the mycelium filtered through an autoclaved muslin cloth in Laminar Air Flow after 24 hours. The main aim of isolation of genomic DNA was to standardize primers and ensure easy and efficient extraction of DNA from our organism of interest.

Reagents for Genomic DNA Isolation:

- **Lysis buffer:**
  - 40mmol/l Tris-acetate
  - 20mmol/l Sodium Acetate
  - 1mmol/l EDTA
  - 1% w/v SDS (pH- 7·8)
- **Sodium Chloride (5mol/l) :** To facilitate the precipitation of most polysaccharides, protein and cell debris.
- **Chloroform**
- **TE buffer:**
  - 10mmol/l Tris-HCl
  - 0·1mmol/l EDTA (pH- 7·8)

ISOLATION BY FREEZE/THAW METHOD:

Procedure:

Pre chilled the mortar and pestle at -80°C for 15 minutes prior to the start of the experiment. Transferred the filtered fungal cultures into chilled mortar and pestle and kept at - 80°C for 20 minutes. Fungal culture was then ground to fine powder with pestle and transferred into a microfuge tube and incubated it at 60°C for 15 minutes. The tube was again kept at - 80°C for 15 minutes to freeze. The powdered tissue was finally thawed by pouring 500 µl of lysis buffer, pipetting until the viscosity of the suspension was significantly reduced and the formation of froth indicated the detachment of DNA from polysaccharides. 165 ml of 5mol/l NaCl solution was added and the components mixed by inverting the tube several times. The suspension was centrifuged at 13000 rpm for 20min at 4°C, the supernatant was immediately transferred to a fresh tube and 400 ml of chloroform and 400 ml of phenol were added. The solution was mixed by gently inverting the tube until the solution became milky. After centrifugation for 20 minutes, the aqueous phase was removed and extracted with an equal volume of chloroform. The DNA in the aqueous supernatant was precipitated with two volumes of 95% ethanol. After centrifugation for 10 minutes, when the aqueous phase was clear, DNA was precipitated with 95% ethanol. The precipitated DNA was washed three times with 70% ice-cold ethanol, dried and dissolved in 50 ml TE buffer and stored at −20°C.

ISOLATION BY LIQUID NITROGEN CUTTING METHOD:

Liquid nitrogen breaks down the thick fungal cell wall and exposes the fungal chromosomes containing genetic material outside. Liquid nitrogen freezes the tissue to become fragile to be a fine powder which increase the surface area of extraction, and the very low temperature prevent DNase activation.

Procedure:

30 mg of freeze-dried mycelium was ground to a fine powder in an Eppendorf tube in liquid nitrogen using a pre-cooled. The ground mycelium was resuspended and lysed in 500 ml of lysis buffer by pipetting until the viscosity of the suspension was significantly reduced and the formation of froth indicated the detachment of DNA from polysaccharides. 165 ml of 5mol/l NaCl solution was added and the components mixed by inverting the tube several times. The suspension was centrifuged at 13000 rpm for 20min at 4°C, the supernatant was immediately transferred to a fresh tube and 400 ml of chloroform and 400 ml of phenol were added. The solution was mixed by gently inverting the tube until the solution became milky. After centrifugation for 20 minutes, the aqueous phase was removed and extracted with an equal volume of chloroform. The DNA in the aqueous supernatant was precipitated with two volumes of 95% ethanol. After centrifugation for 10 minutes, when the aqueous phase was clear, DNA was precipitated with 95% ethanol. The precipitated DNA was washed three times with 70% ice-cold ethanol, dried and dissolved in 50 ml TE buffer and stored at −20°C.

DNA quantification: Agarose gel electrophoresis

1. Prepared 0.8% solution of agarose by melting 0.4 g of agarose in 50ml of 1X TAE buffer in a microwave for approximately 2 minutes.
2. Allowed it to cool for a couple of minutes then add 2µl Ethidium Bromide, stirred to mix.
3. Casted a gel using a supplied tray and comb. Allowed the gel to set for a minimum of 20 minutes at room temperature on a flat surface.
4. After proper solidification of gel remove the comb and keep gel in gel running tank.
5. Poured TAE buffer gently.
6. Load samples with DNA loading dye in separate wells.

7. Ran the gel for 45-1hr at 100.
8. Exposed the gel to UV light in Gel Documentation System and took a photograph.
9. Confirmed DNA quality, presence of a highly resolved high molecular weight band indicates good quality DNA, presence of a smeared band indicates DNA degradation.
10. A Nanodrop reading can also be taken for the estimation of nucleic acid content.

5. Total RNA isolation from Aspergillus flavus MTCC-AF9367

RNA isolation and purification is one of the key factors for the RT-PCR assays and other related molecular biology detections. TRIzol Reagent is a ready-to-use reagent for the isolation of total RNA from cells and tissues. The reagent, a mono-phasic solution of phenol and guanidine isothiocyanate, is an improvement to the single-step RNA isolation method developed by Chomczynski and Sacchi.

Reagents for RNA Isolation:

- **DEPC (Diethyl Pyro Carbonate) -** inactivates RNase enzymes by the covalent modification of histidine (most strongly), lysine, cysteine, and tyrosine residues.
- **TRIzol**- maintains the integrity of the RNA, while disrupting cells and dissolving cell components.
- **Chloroform**- separates the solution into an aqueous phase and an organic phase.
- **Isopropanol**- to recover RNA through precipitation.
- **75% ethanol in DEPC water**- Washing

Procedure:

Took 100 mg of fungal culture and ground it in liquid nitrogen into fine powder in a mortar and pestle. Added TRIzol reagent (1ml reagent for 100 mg culture). Incubated for 5 minutes at room temperature to permit complete dissociation of nucleoprotein complex. Added 0.2 ml of chloroform per ml of TRIzol reagent under the hood and closed the eppendorf. Shook eppendorfs vigorously for 15 seconds

and incubated at room temperature for 2-3 minutes. The sample was centrifuged at 12000 rpm for 15 minutes at 4°C and transferred the supernatant or aqueous phase to the fresh eppendorf. RNA was precipitated from aqueous phase by mixing with isopropanol (0.5ml per 1 ml of TRIzol). The sample was incubated at room temperature for 10 minutes and centrifuged at 12000 rpm for 10 minutes at 4°C. The supernatant was discarded. Then RNA pellet was washed with 75% ethanol and mixed well. Centrifugation at 7500 rpm for 5 minutes at 4°C was carried out. This step was repeated twice. Discarded supernatant after RNA pellet was obtained. The pellet was air-dried at room temperature for 20 minutes and dissolved in DEPC treated water.

RNA quantification: Agarose gel electrophoresis

1. Prepared 1.5% solution of agarose by melting 0.75 g of agarose in 50ml of 1X TAE buffer in a microwave for approximately 2 minutes.
2. Allowed it to cool for a couple of minutes then add 2µl Ethidium Bromide, stirred to mix.
3. Casted a gel using a supplied tray and comb. Allowed the gel to set for a minimum of 20 minutes at room temperature on a flat surface.
4. After proper solidification of gel remove the comb and keep gel in gel running tank.
5. Poured running buffer (TAE) gently.
6. Load samples with DNA loading dye in separate wells.
7. Ran the gel till the tracking dye reached the middle of the well.
8. Exposed the gel to UV light in Gel Documentation System and took a photograph.
9. Confirmed RNA quality, presence of two highly resolved high molecular weight band indicates good quality RNA, presence of a smeared band indicates RNA degradation.
10. A Nanodrop reading can also be taken for the estimation of nucleic acid content.

6. cDNA Synthesis and PCR Using Housekeeping Genes

Thermo-scientific Verso cDNA Synthesis Kit was used to synthesize cDNA. It provides robust transcription of RNA to create a complete cDNA pool. The Verso system achieves robust and sensitive reverse transcription through the combination of a high affinity RT enzyme, a unique RNA priming method, and an optimized buffering system. The Verso RT enzyme has high RNA template affinity and reduced RNase H activity, to transcribe even sections with high secondary structure. The included anchored oligo dT priming method further enhances sensitivity by increasing transcription efficiency, and the cDNA synthesis buffer has been optimized to achieve a full and diverse cDNA pool. Verso is RNA dependent DNA polymerase with the significantly attenuated RNase H activity. Verso can synthesize long cDNA strands up to 11kb at temperature range of 42 °C to 57 °C. The recommended amount of total RNA to use is between 1 pg to 1 µg. It should be stored at –20 °C until ready for use and repeated freeze thawing should be avoided.

Following are the components of Verso cDNA Synthesis Kit:

- **Verso Enzyme Mix:** It includes Verso Reverse Transcriptase which is active at high temperatures, is highly sensitive and can generate long cDNA strands. This mix also contains RNase inhibitor to protect RNA templates from degradation.
- **5X cDNA Synthesis Buffer:** a proprietary reaction buffer which has been optimized to improve reverse transcription across a wide range of templates.
- **Anchored oligo dT primers and random hexamers:** provide flexible RNA priming methods for cDNA synthesis.
- **RT enhancer:** It is included to remove contaminating DNA, eliminating the need for DNase I treatment. It degrades double stranded DNA during transcription of RNA and inactivated after 2 minutes at 95 °C.

TABLE 3: Reaction mixture for PCR reaction

Reaction Mix:	**Reaction Volume:**

5X cDNA Synthesis Buffer	4 µl
dNTP Mix	2 µl
RNA Primer	1 µl
RT Enhancer	1 µl
Verso Enzyme Mix	1 µl
Total RNA	According to nano-drop reading of the RNA samples. Ideally, 1-1.5 µl
Water	Made up to 20 µl
Total Volume	20 µl

Polymerase Chain Reaction for cDNA Synthesis

As the name implies, PCR is a chain reaction, a small fragment of the DNA section of interest needs to be identified which serves as the template for producing the primers that initiate the reaction. One DNA molecule is used to produce two copies, then four, then eight and so forth. This continuous doubling is accomplished by specific proteins known as polymerases, enzymes that are able to string together individual DNA building blocks to form long molecular strands. To do their job polymerases require a supply of DNA building blocks, i.e., the nucleotides consisting of the four bases adenine (A), thymine (T), cytosine (C) and guanine (G). They also need a small fragment of DNA, known as the primer, to which they attach the building blocks as well as a longer DNA molecule to serve as a template for constructing the new strand. If these three ingredients are supplied, the enzymes will construct exact copies of the templates.

Number of copies of DNA obtained after 'n' cycles = 2(n+1)

It is then possible to clone DNA whose sequence is unknown. This is one of the method's major advantages. Genes are commonly flanked by similar stretches of nucleic acid. Once identified, these patterns can be used to clone unknown genes - a method that has supplanted the technique of molecular cloning in which DNA fragments are tediously copied in bacteria or other host organisms. With the PCR method this goal can be achieved faster, more easily and above all in vitro, i.e., in the test-tube. Moreover, known sections of long DNA molecules, e.g. of chromosomes, can be used in PCR to scout further into unknown areas.

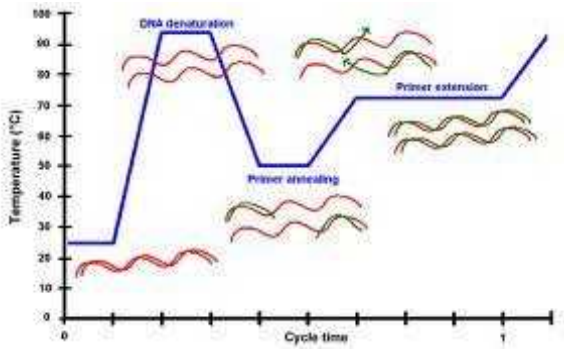

FIGURE 5: General Strategy for Polymerase Chain Reaction

TABLE 4: PCR conditions for cDNA synthesis (33 minutes)

Conditions	Temperature	Time	No. of cycles
cDNA synthesis	42°C	30 minutes	1
	72°C	2 minutes	1

PCR Using Housekeeping Genes

Tubulin (size: 122 bp) was used to perform PCR to check cDNA synthesis: Housekeeping genes are involved in basic cell maintenance and, therefore, are expected to maintain constant expression levels in all cells and conditions. Other housekeeping genes include HSP6 (size 121bp), HSP70 (size 141bp), HSP 989 (size 102bp) and GAPDH (size 119bp).

Thermo Scientific Luminaris HiGreen Master Mixes are universal ready-to-use solutions optimized for SYBR Green chemistry. The master mixes include:

- Thermo Scientific Hot Start Taq DNA polymerase which ensures PCR specificity and sensitivity.
- dUTP and uracil DNA glycosylase (UDG) are included in the mix for carry-over contamination control.
- Inert blue dye and a separate Yellow Sample Buffer that contains a yellow dye. The reaction mix containing both components is green allowing easy monitoring of pipetting steps.

TABLE 5: PCR Reaction Mix for Amplification of Housekeeping Genes

Reaction mixture	Volume
Forward primer	.5 µl
Reverse primer	.5 µl
DNA	1 µl
Luminaris HiGreen PCR Master mix	6 µl
dH2O	4 µl
Total	12 µl

TABLE 6: PCR Reaction for the Amplification using Housekeeping Genes.

Temperature	Time
95°C	5 min
95°C	45 sec
53.7°C	30 sec

72°C	45 sec
72°C	7 min

7. **Semi Quantitative PCR for checking the expression of Calcineurin Gene**

The Reverse Transcription –Polymerase Chain Reaction method can be used not only to detect specific mRNAs but also to semi-quantitate their levels. Thus, one can compare levels of transcripts in different samples. This can be done in two different ways. One is to quantitate against levels of transcripts from a control, house-keeping gene (such as tubulin and GAPDH). Transcription of house-keeping genes is believed to be unaffected by almost all experimental conditions. The second method is to add an exogenous, primer-specific PCR template during PCR. Quantitating against house-keeping gene transcript involves reverse transcription using an oligo-dT or random hexamer primer. The resulting cDNA thus represents both house-keeping gene transcripts as well as specific transcripts one is quantitating. The RT reaction is then amplified in a pair of PCR series - one series is to amplify the house-keeping gene cDNA (using GAPDH-, etc., specific primers), and the other is for the specific cDNA of interest (in separate PCR, using gene-specific primers). After PCR, same volume of reaction products are electrophoresed on an agarose gel (preferably on the same gel).

The following pre-standardized primer sequences were used for calcineurin gene using the *Aspergillus flavus* strain NRRL 3357 Calcineurin gene, complete cds (Accession number- gi|62956525|gb|AY974342.1):

Forward: TACTTCTTCTCGTACCCCG (19)

Reverse: CATCACGCTAGGGAAACC (18)

Product Length: 138

The primers were standardized using Primer-BLAST tool on the NCBI website.

TABLE 7: PCR Reaction Mix for Calcineurin Gene Amplification

Reaction mixture	volume
Calcineurin forward primer	0.30µl
Calcineurin reverse primer	0.30 µl

Luminaris HiGreen Master Mix	6.25 µl
cDNA	1.00 µl
Water	4.63 µl
Total	12.5 µl

TABLE 8: PCR Reaction for the Amplification of Calcineurin Gene.

Temperature	Time
95°C	5 min
95°C	45 sec
53.7°C	30 sec
72°C	45 sec
72°C	7 min

FUNGITOXIC EFFECTS OF PHYTOCHEMICALS

Media Used:

Fungal culture was grown in Petri Plates containing Potato Dextrose Agar (PDA) and incubated 37ºC. PDA contains the essentials for bacterial and fungal species to grow and therefor widely used. Potato Dextrose Agar is recommended by APHA and F.D.A. for plate counts of yeasts and moulds in the examination of foods and dairy products. Potato Dextrose Agar is also used for stimulating sporulation, for maintaining stock cultures of certain dermatophytes and for differentiation of typical varieties of dermatophytes on the basis of pigment production.

Composition

Ingredients	**Gms / Litre**
Potatoes infusion	200.000
Dextrose	20.000
Agar	15.000
Final pH (at 25°C)	5.6±0.2

Suspended 39 grams in 1000 ml distilled water. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

Preparation of Stock Solutions: Stock solutions of various phytochemicals were made in methanol and water since not every phytochemical dissolves in water or methanol. 1.5g of each phytochemical was dissolved in 3mL of methanol/water making a stock solution of 500 mg/mL. Specific amount was substituted in 20mL PDA media used for making plates. The mixture was then mixed using a sterilized glass rod to achieve even consistency of the phytochemical throughout the media.

TABLE 9: Preparation of Agar Plates of different concentrations of phytochemicals.

Concentration of Phytochemical (mg/mL)	Amount pipetted from Stock solution of 500 mg/mL (µl)
1	40
5	200
10	400
15	600
20	800

First Attempt: Agar Disc Diffusion Method: The antimicrobial activity of the various Phytochemicals was evaluated by using agar disc diffusion method. PDA medium was poured in Petri plates. Paper discs of 5mm size were made and autoclaved. Methanolic extracts of the phytochemicals ranging from 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 µl were used to dip the paper discs in and and placed on agar plates containing the fungal inoculum (10^6 cells/mL in PBST plated). All the work was carried under aseptic conditions in Laminar Air Flow. The petri plates were kept at 37ºC for 72 hours.

Second Attempt: Agar Well Diffusion Method: The antimicrobial activity of the various Phytochemicals was evaluated for the second time using Cork-Borer method. PDA medium was poured in Petri plates. Wells of 7mm size with the help

of sterile tips were bored in Petri plates. 10µl of methanolic extracts of the phytochemicals ranging from 1, 5, 10, 15 and 20 mg/ml were dispensed in the wells on the agar plates containing the fungal inoculum (10^6 cells/mL in PBST plated). All the work was carried under aseptic conditions in Laminar Air Flow. The petriplates were kept at 37ºC for 72 hours.

Third Attempt: Agar Disc Diffusion Method: The antimicrobial activity of the various Phytochemicals was evaluated by using agar disc diffusion method for the third time. PDA medium was poured in Petri plates. Paper discs of 5mm size were made and autoclaved. 10 µl of methanolic and water-mixed extracts of the phytochemicals ranging from 1, 5, 10, 15 and 20 mg/ml was pipetted on the paper discs after placing on agar plates containing the fungal inoculum (10^6 cells/mL in PBST plated). All the work was carried under aseptic conditions in Laminar Air Flow. The petriplates were kept at 37ºC for 16 and 24 hours. A methanol control was also set up to determine the activity antifungal activity of methanol.

Fourth Attempt: Poisoned Food Technique: The antifungal activity of the phytochemicals was evaluated by using Poisoned food technique (Shiva Rani et al., 2013) at three-five concentrations (1, 5, 10, 15 and 20 mg/ml). *Aspergillus* flavus strain MTCC- AF9367 was inoculated on Potato Dextrose Agar supplemented with phytochemicals extract at three-five concentrations via 5mm diameter disc and kept up-side down under aseptic conditions at 37ºC. PDA plate without any extract served as control and PDA plate substituted with 2ml of methanol served as methanol control. All the work was carried under aseptic conditions in Laminar Air Flow. Colony diameter was measured after 24, 48 and 36 hours. Data were statistically analysed and percent inhibition of each phytochemical was counted with respect to the control using the following formula.

$$\text{Inhibition Percentage:} \quad \frac{\text{(Control-Test)}}{\text{Control}} \times 100$$

4. RESULTS AND DISCUSSIONS

1. Identification of *Aspergillus flavus* strain MTCC-AF9367

The following was observed:

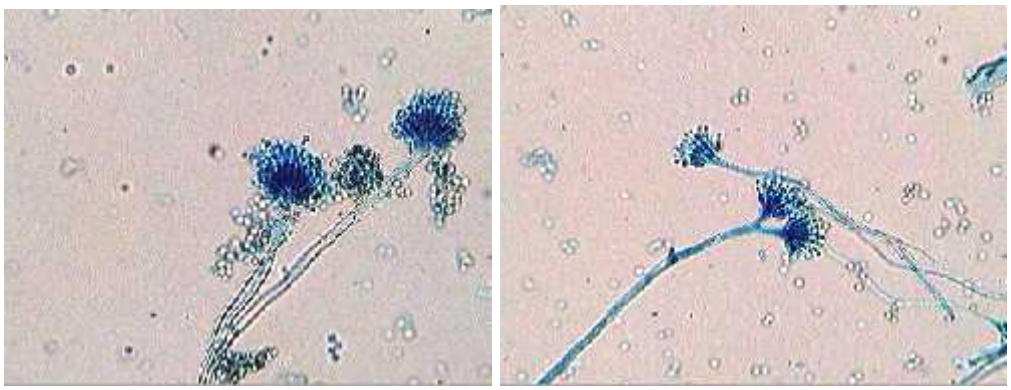

FIGURE 6: *Aspergillus flavus* MTCC AF9367 when viewed under 100 x

The following structures were observed under the microscope:

- Conidia
- Phialides
- Vesicles
- Conidiophore

Identification was done in order to ensure pure culture os *Aspergillus flavus* MTCC-AF9367 for further experimental use. The presence of above structures were a proof that the cultture used was pure *Aspergillus flavus* MTCC-AF9367 strain**.**

2. Counting of conidia through Hemocytometer

Counting of conidia through hemocytometer was done to make working solutions of PBST containing $1x10^6$ cells per ml. The stock solution containing indefinite amount of conidia was serially diluted, mostly four times, and conidia count for each of the dilutions was done. The working solutions were stored at 4ºC for further use.

3. Culturing of *Aspergillus flavus* strains MTCC-AF9367 at different Calcium concentrations of 10mM, 20mM, 30mM, 40mM and Control (No Calcium)

The culture flasks were harvested after 24 hours. Visible growth of white balls-like conidia was observed in each flask with the amount varying with concentration. The observed number of conidia increased with the increase in Calcium concentration.

4. Isolation of Genomic DNA from *Aspergillus flavus* MTCC-AF9367

Several attempts were made in order to extract genomic DNA from the cultures. Due to initial lack of liquid nitrogen, freeze/thaw method was used, where the fungal culture was given heat shock followed by immediate freezing. This method of lysis causes cells to swell and ultimately break as ice crystals form during the freezing process and then contract during thawing. Multiple cycles are necessary for efficient lysis, and the process can be quite lengthy. However, freeze/thaw has been shown to effectively release recombinant proteins located in the cytoplasm of bacteria and is recommended for the lysis of mammalian cells in some protocols. However, in our case the method was inefficient in breaking down the tough chitin fungal cell wall.

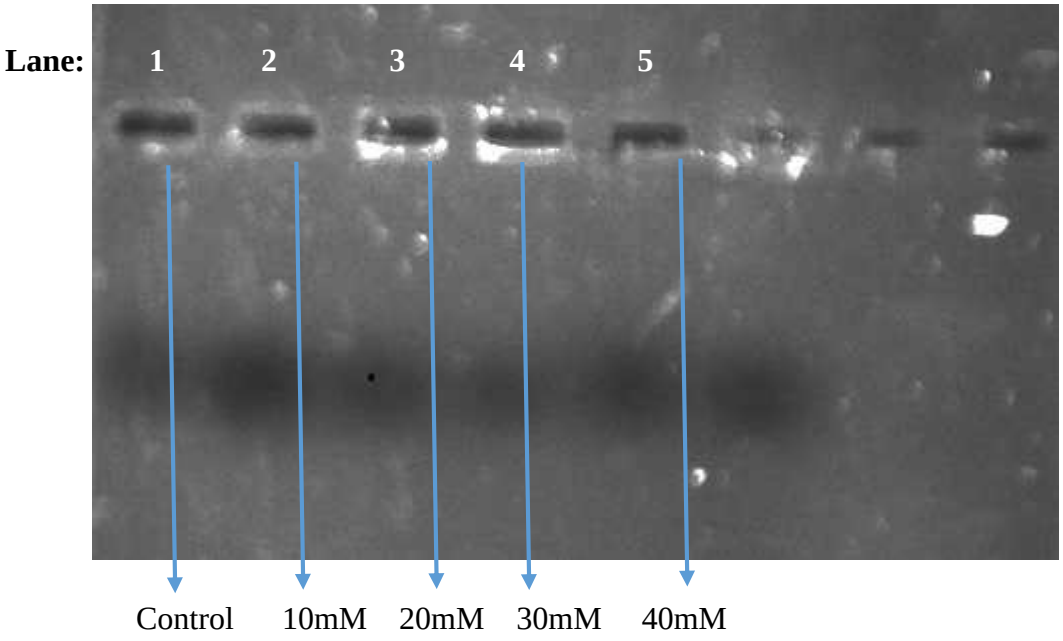

FIGURE 7: Agarose Gel Electrophoresis of Genomic DNA using Freeze/Thaw Method. No visible bands were observed.

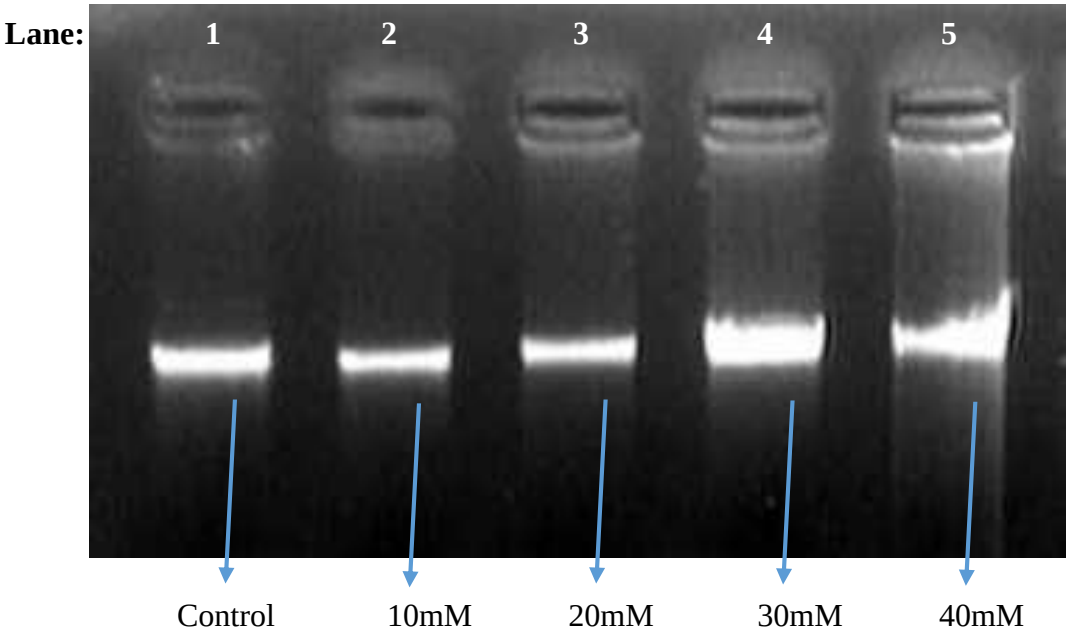

FIGURE 8: Agarose Gel Electrophoresis of Genomic DNA using Liquid Nitrogen Grinding Method. Genomic DNA was observed by this method.

5. Total RNA isolation from Aspergillus flavus MTCC-AF9367

Following are the 1.5% agarose gel pictures obtained after using TRIzol method to extract RNA. Ideally, intact total RNA run on a denaturing gel will have sharp 28S and 18S rRNA bands in eukaryotic samples. The 28S rRNA band should be approximately twice as intense as the 18S rRNA band. This 2:1 ratio (28S:18S) is a good indication that the RNA is intact. Partially degraded RNA will have a smeared appearance, will lack the sharp rRNA bands, or will not exhibit a 2:1 ratio. Completely degraded RNA will appear as a very low molecular weight smear.

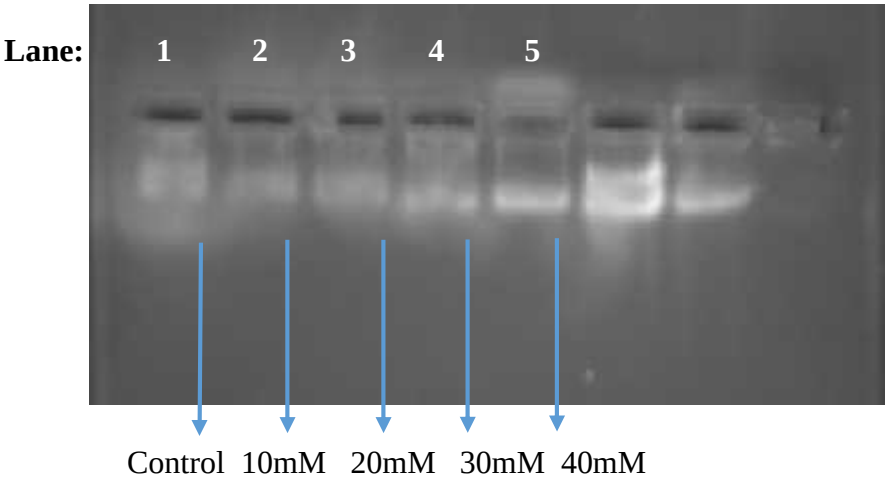

FIGURE 9: Agarose Gel Electrophoresis of Genomic RNA using TRIzol Method.

Partially degraded RNA was observed. The samples were carried forward for cDNA synthesis based on their Nanodrop readings. The required RNA concentration for RNA synthesis is 1000ng/µl.

6. cDNA Synthesis and PCR Using Housekeeping Genes

The synthesis of DNA from an RNA template, via reverse transcription, produces complementary DNA (cDNA). Reverse transcriptases (RTs) use an RNA template and a primer complementary to the 3' end of the RNA to direct the synthesis of the first strand cDNA, which can be used directly as a template for the Polymerase Chain Reaction (PCR). This combination of reverse transcription and PCR (RT-PCR) allows the detection of low abundance RNAs in a sample, and production of the corresponding cDNA, thereby facilitating the cloning of low copy genes.

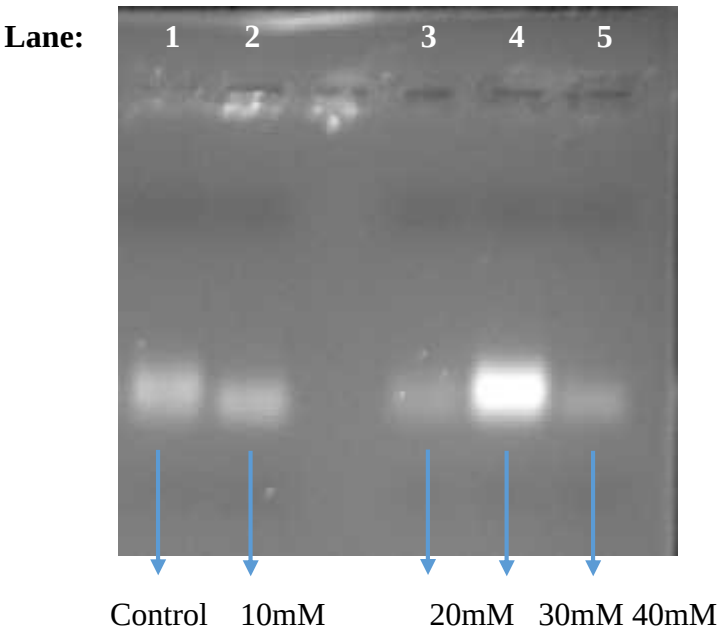

FIGURE 10: Agarose Gel Electrophoresis of cDNA after PCR using Housekeeping Genes -1.

Partially degraded cDNA smears appeared in 1.5% Agarose Gel Electrophoresis. The cDNA synthesis step was repeated to obtain bands with better resolution.

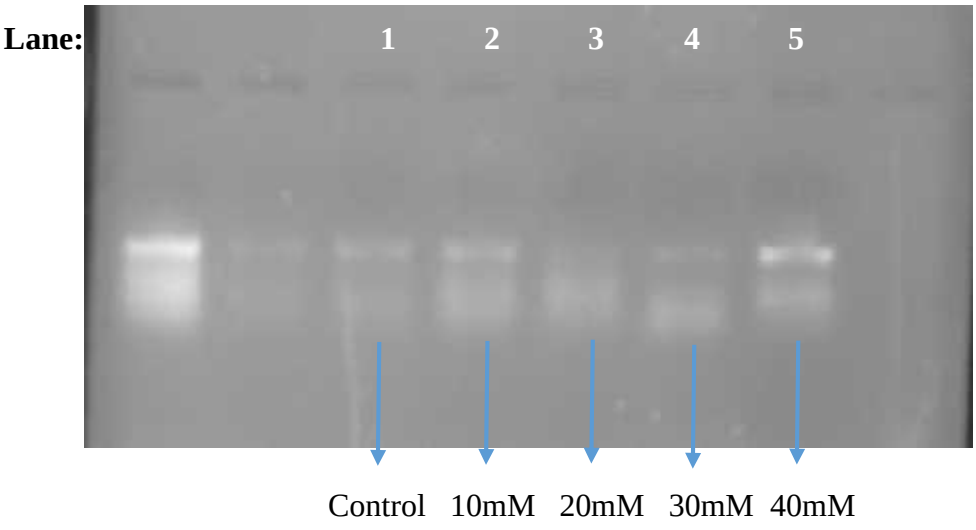

FIGURE 11: Agarose Gel Electrophoresis of cDNA after PCR using Housekeeping Genes -2.

Faint cDNA bands were observed. The samples were carried forward for Quantitative PCR with calcineurin gene.

7. Semi Quantitative PCR for checking the expression of Calcineurin Gene

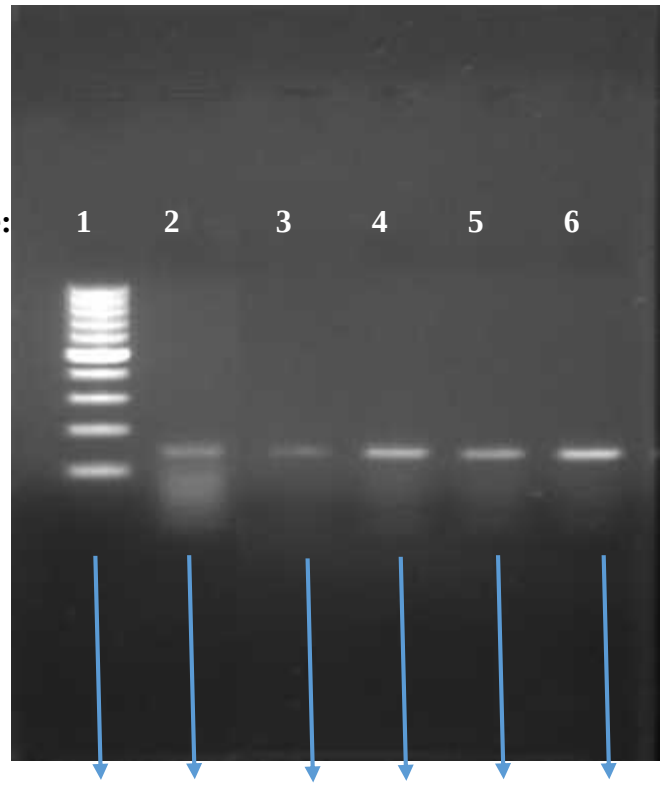

FIGURE 12: Agarose Gel Electrophoresis of cDNA after qPCR using primers for calcineurin gene.

Through Quantitative Polymerase Chain Reaction, the expression of calcineurin gene was checked by amplification of the cDNA with primers specific to the calcineurin gene. The PCR product was then run on an Agarose Gel electrophoresis of 1.5% at 100V along with a ladder of 100bp to detect its size as well. The results obtained after viewing the gel under Gel Documentation System were as follows. The control and the 10mM sample had comparable sharpness of band with 10mM having a slightly less intense band. The possible reason for this however may be loading error. Thereafter, the sharpness of bands increased with the increase in concentration. Therefore, we can conclude that the calcineurin gene is affected positively by increase in Ca^{+2} concentration and has a role in conidiation of *Aspergillus flavus.*

FUNGITOXIC EFFECTS OF PHYTOCHEMICALS

Several attempts to check the MIC or Minimum Inhibitory Concentration of various phytochemicals for *Aspergillus flavus* were made. The MIC is the minimum concentration of the antimicrobial compound that will inhibit this particular isolate. Agar Disc Diffusion Method was used in the first attempt. In this, a paper disk with a defined amount of antibiotic is used to generate a dynamically changing gradient of antibiotic concentrations in the agar in the vicinity of the disk. Inhibition zone edge is formed at the critical time where a particular concentration of the antibiotic is just able to inhibit the organism before it reaches an overwhelming cell mass or critical mass. However, the protocol was improvised for the next attempt, since no zone of inhibitions were observed.

The experiment was performed again using Cork-borer method. Wells were bored instead of placing paper discs. 10 µl of test phytochemical concentration was added and allowed to diffuse through the agar seeded with 1×10^6 conidia. Unfortunately, this test too was unsuccessful in achieving zone of inhibitions probably due to seepage of the phytochemical beneath the agar in the petri plate or heavy inoculum density, the major disadvantage of agar diffusion method. Another reason could have been keeping the plates for too long in the incubator which resulted in over-growth of the culture.

For the third attempt, paper discs were used again, with the phytochemical pipetted over the disc instead of it being dipped in the phytochemical. Also, the incubation period was reduced to 16-24 hours. However, this attempt too didn't yield any results.

Finally, after extensive review of literature, poisoned food technique was implied, which required Agar dilution method. But due to unavailability of phytochemicals in a large amount, the experiment was restricted to four phytochemicals- Ascorbic acid, Caffeine, Gallic acid, and Quercetin. The results obtained through this method were promising and by measuring the radial growth of the fungal inoculum, percentage inhibition could be calculated for different concentrations of different phytochemicals. The diameter was observed for three days and the result was as follows:

Ascorbic Acid

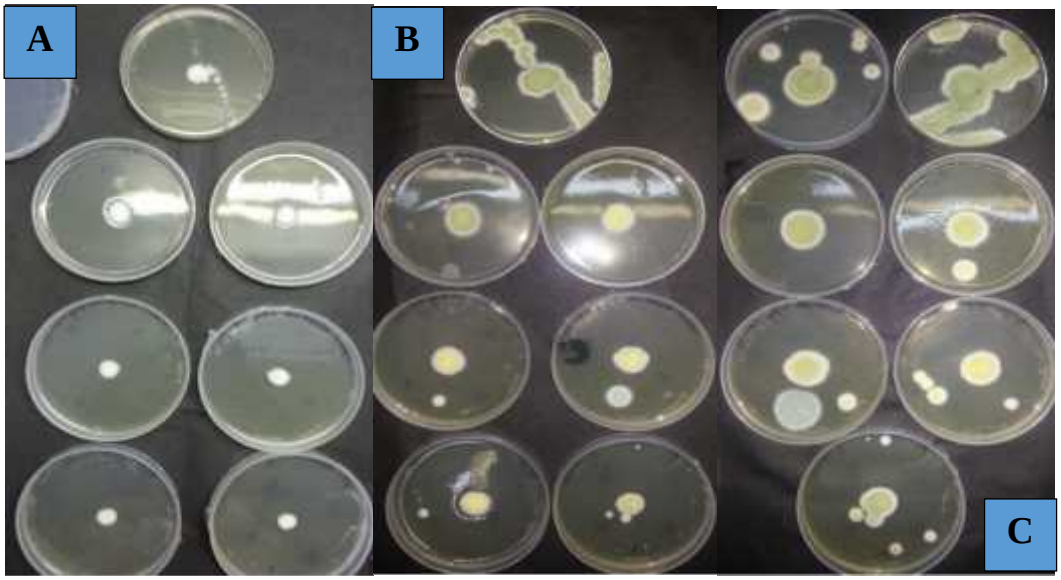

FIGURE 13: Plates containing Ascorbic acid, in increasing order of concentrations. First two plates in every pictures, traversing in left-right fashion, are Control with water and methanol followed by 1, 5, 10, 15 and 20 mg/ml concentrations. A, B and C are the pictures of the plates on Day 1, Day 2 and Day 3 respectively.

Caffeine:

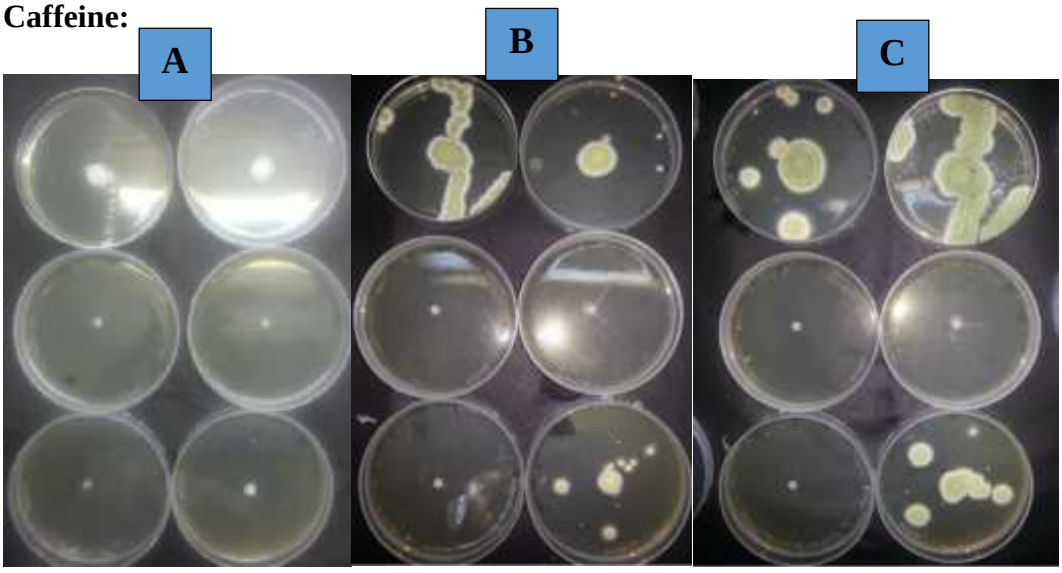

FIGURE 14: Plates containing different concentrations of Caffeine, in increasing order. First two plates in every pictures, traversing in left-right fashion, are Control with water and methanol followed by 1, 5, 10 and 15 mg/ml concentrations. A, B and C are the pictures of the plates on Day 1, Day 2 and Day 3 respectively.

Quercetin

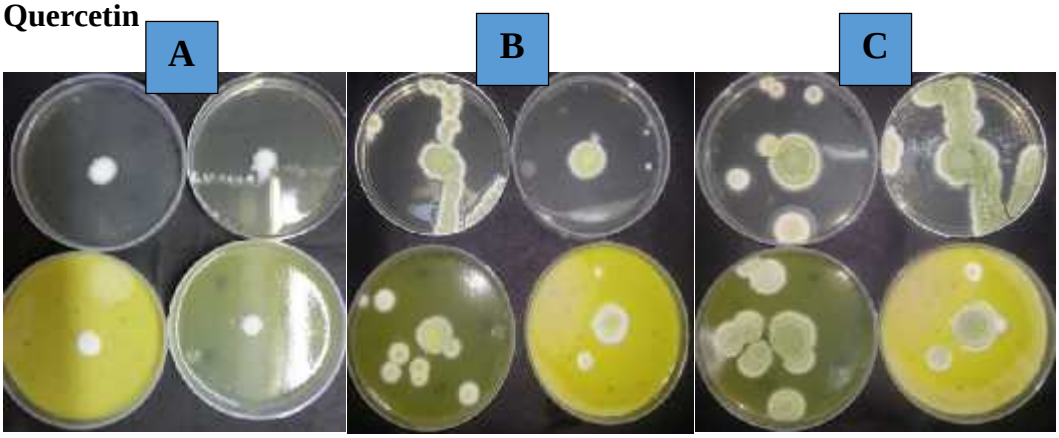

FIGURE 15: Plates containing different concentrations of Quercetin, in increasing order. First two plates in every pictures, traversing in left-right fashion, are Control with water and methanol followed by 1 and 5 mg/ml concentrations. A, B and C are the pictures of the plates on Day 1, Day 2 and Day 3 respectively.

Gallic Acid:

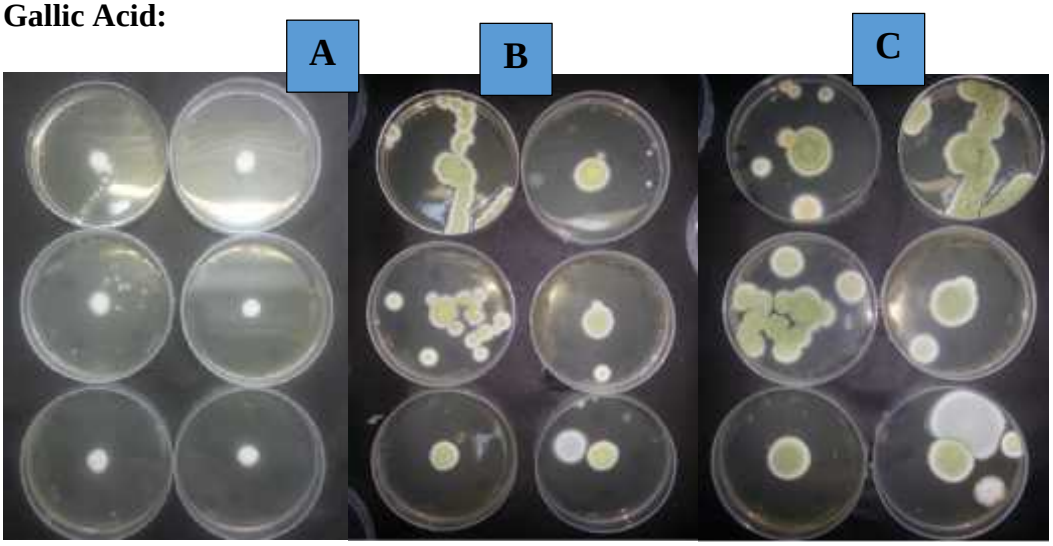

FIGURE 16: Plates containing different concentrations of Gallic Acid, in increasing order. First two plates in every pictures, traversing in left-right fashion, are Control with water and methanol followed by 1 and 5 mg/ml concentrations. A, B and C are the pictures of the plates on Day 1, Day 2 and Day 3 respectively.

TABLE 10: Diameter of *A. flavus* and percentage inhibition for every concentration of different phytochemicals with respect to Control (Average of Methanol and Water Controls). Diameter of each fungal inoculum containing 1x 10^6cells were measured on 1st, 2nd and 3rd day and %age inhibition was calculated.

Phytochemicals	Conc. (mg/ml)	Day 1 (mm)	Day 2 (mm)	Day 3 (mm)	Inhibition % (Day 1)	Inhibition % (Day 2)	Inhibition % (Day 3)	Remarks
Ascorbic acid	1	13	22	28	21.21	12.5	16.41	Maximum % inhibition on in 15 mg/ml
	5	12	21	26	27.27	17.64	22.38	
	10	12	19	25	27.27	25.49	25.37	
	15	13	19	24	21.21	25.49	34.32	
Caffeine	1	0	0	0	100	100	100	Complete inhibition on from 1st day.
	5	0	0	0	100	100	100	
	10	0	0	0	100	100	100	
	15	7	15.5	21	58	39.1	35.21	
Gallic acid	1	12	22.5	32	27.27	11.76	8.4	Least inhibitory phytochemical overall.
	5	15	24	25	9	5.8	11.26	
	10	13	22	29	21.21	13.72	13.43	
	15	14	18	29	15.15	29.41	13.43	
Quercetin	1	12	22	28	27.27	13.72	16.41	Maximum percentage inhibition on in 5mg/ml. Potentia
	5	19	23	23	±21.21	7.8	31.34	

								lly strong phytochemical as comparable % inhibition with 15mg/ml Ascorbic Acid at only 5mg/ml
Control								
Methanol		16	26	34				
Water		17	25	33				

From the observed table, Caffeine proved to be a promising candidate for an environment friendly antimicrobial compound since it shows complete inhibition at concentrations 1, 5 and 10 mg/ml. However, it showed only 35.21% inhibition in its 15 mg/ml concentration plate which is a matter of great concern. The possible reason for this can be attributed to "Feedback Inhibition". However, this cannot be proved for sure since the mechanism of action of caffeine is not well established. While gallic acid shows minimum inhibition- 13.43% at 10 and 15 mg/ml, quercetin seems to be a promising inhibitory phytochemical as it shows 31.34% inhibition at only 5mg/ml- comparable to Ascorbic acid's 34.32% at 15 mg/ml. Higher concentrations of quercetin could not be tested due to constraint of resources.

Several reviews of antifungal compounds group them into structural classes and have associated certain structures with particular modes of actions. Examples of some of the key structures of fungicides are shown in Figure 15. The binding and synthesis of ergosterol,

the major cell membrane component, are the targets for several antifungal structures. The **azoles and triazoles** interfere with the ergosterol biosynthesis pathway by inhibiting cytochrome P450-dependent 14α-demethylase and blocking the oxidative removal of 14α-methyl from lanosterol (Arif et al., 2009). This incomplete processing of lanosterol results in an increase in ergosterol precursors and a decrease in ergosterol, leading to structural changes in the lipid membrane. Azoles have also been reported to inhibit membrane-surface enzymes and lipid biosynthesis. **Caffeine seems to contain imidazole group, which might be an explanation for its strong antifungal activity.**

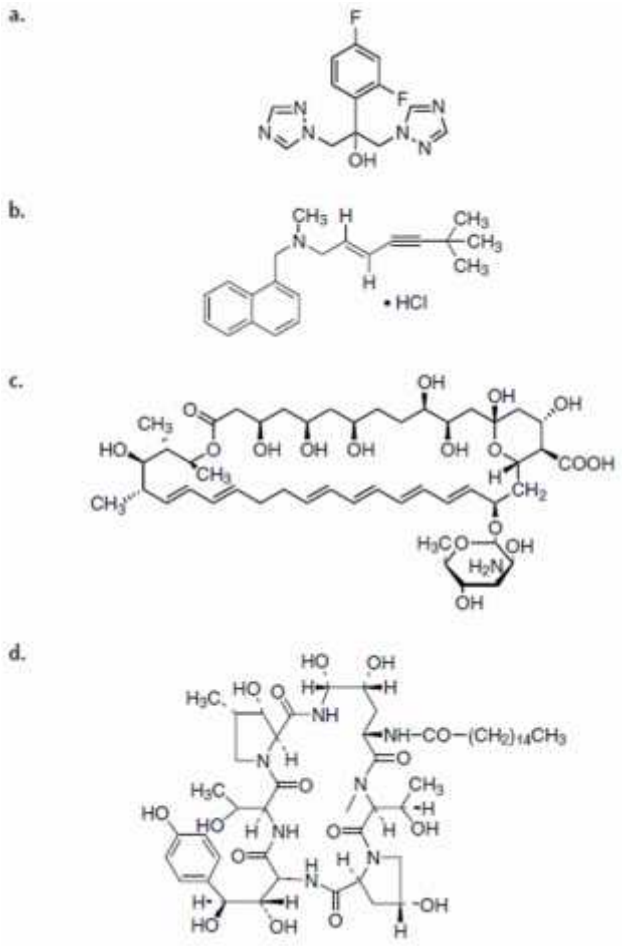

FIGURE 17- Examples of antifungal structure classes. a. Fluconazole (triazole) b. Terbinafine (allylamine) c. Nystatin A1 (polyene) d. Aculeacin A (echinocandin)

Ascorbic acid has 4 alcohol functional groups along with an alkene and an ester.

Gallic acid [3,4,5-trihydroxybenzoic acid**]** has two functional groups in the same molecule, hydroxyl groups and a carboxylic acid group. Benzoic acid derivatives substituted by hydroxyl group or ether containing oxygen atom have bacteriostatic and fragrant

properties. They are typically used in pharmaceutical and perfumery industry. The destructive metabolic property of oxygen containing benzoic acid derivatives such as protocatechuic acid (3,4-dihydroxybenzoic acid) and veratric acid (3,4-dimethoxybenzoic acid) is used in the application for pharmaceuticals which possibly explains its anti-fungal activity too.

Quercetin, being a flavonoid, is a phenolic structure. Flavonoids are hydroxylated phenolic substances synthesized by plants in response to microbial infection. They have been found to be effective antimicrobial substances against a wide array of microorganisms. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with fungal cell walls. More lipophilic nature of flavonoids disrupt fungal membranes. A flavon 3,4',5,7-tetraacetyl quercetin isolated from heartwood of *Adina cordifolia* exihibited moderate antifungal activity against *A. fumigatus* and *Cryptococcus neoformans.*

Allylamines, of which terbinafine is the most common example, also block ergosterol biosynthesis, but at an earlier step. Terbinatine inhibits the enzyme squalene epoxidase, which participates in the conversion of squalene to lanosterol. The resulting build-up of squalene is toxic to the fungal cell. A third structural class, polyenes, increases the permeability of the plasma membrane. Amphotericin B, a polyene with high affinity for sterol binding, is one of the most potent antifungal drugs; its mechanism produces pores in the membrane surface of the yeast, resulting in leakage of the cell contents.

In addition to the plasma membrane and its lipid surface, fungicidal compounds may damage the cell wall of yeast. The major cell wall component $\beta(1\rightarrow 3)$ glucan is the target of echinocandins and aculeacins such as aculeacin A. Echinocandins are semisynthetic lipopeptides that competitively inhibit β-glucan synthetase; the mechanism of action is not well defined but does not involve cytochrome P450 inhibition or P-glycoprotein transport.

Chitin is a trace, critical component of the fungal cell wall, and some inhibitors of chitin synthesis demonstrate antifungal activity. Members of this family of antifungals (i.e., polyoxins and nikkomycins) have structures analogous to UDP-N-acetyl-D-glucosamine (UDP-GlcNAc). This nucleoside phosphate is a glycosyl donor substrate for chitin synthesis, and the antifungals act as competitive substrates to inhibit chitin synthetase.

Not all antifungal compounds have known mechanisms of action, and some of them are relatively unique. While there are several antibacterials that function by preventing DNA or RNA replication, 5-fluorocytosine is a novel antifungal in that its mechanism of action involves blocking DNA synthesis and inhibiting thymidylate synthetase. Sordarin is one of the few compounds that selectively inhibit fungal protein synthesis. Other antifungal antibiotics target sphingolipid biosynthesis and electron transport. The mode of action of griseofulvin is not completely clear, but it has been speculated that griseofulvin inhibits microtubule binding within the mitotic spindle, weakening the cell structure (Chloe McClanahan, 2009).

5. CONCLUSION

From this study, role of calcineurin was established in *Aspergillus flavus*. It was observed that its expression increases with increase in calcium concentration. This can form basis for further investigation on what inactivation or knocking-out of calcineurin gene would result in conidia structure and count in *Aspergillus flavus*. This has been well established in species like *Aspergillus fumigatus*. If the findings prove to be as promising as in the case of *A. fumigatus*, calcineurin gene can be a potential therapeutic drug target for management and control of Invasive Aspergillosis. The only challenge then will be to identify a fungal-calcineurin specific target, targeting which, would not affect the host calcineurin signal transduction machinery in any way, like CRZ1, has been suggested as a fungus-specific target of the calcineurin pathway in *Candida albicans*.

Also, it was observed that caffeine shows maximum inhibition and is one of the most promising component of a natural antimicrobial compound. Never the less, other phytochemicals may also work better in combination with caffeine or other known antifungal compounds. This study, therefore, may form basis for future studies on combinations, derivatives, metal-complexes etc. of these phytochemicals to fight fungal infections better than conventional chemical pesticides. This study may also be utilized in developing novel therapeutic drugs from such phytochemicals in combination with pre-existing therapeutics in order to control, manage and cure deadly diseases like Invasive Aspergillosis. Likewise, identification of medicinal plant species which possess such antimicrobial compounds against plant pathogens may be useful in characterization of inhibitory fractions, their synthesis and development for management of plant diseases.

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CAREER OBJECTIVE

To grow synergistically with a leading organization and enhance my skill set along with contributing significantly to the organization's growth.

EDUCATIONAL BACKGROUND

	Class	Institution	Board/University	Percentage
1.	B.Tech in Biotechnology (Presently pursuing 8th Semester)	Jaypee University of Information Technology	Jaypee University of Information Technology	CGPA 7.1 (76%)
2.	12th (Physics, Biology, Chemistry, Computer Science)	KBDAV Senior Secondary Public School, Chandigarh	CBSE	79.6%
3.	10th	St. Kabir Public School, Chandigarh	ICSE	82.57%

CORE TECHNICAL SKILLS

- Molecular Biology - DNA and RNA isolation, PCR, Ligation, Plasmid Isolation, Primer designing by DNA STAR, Primer 3, Gel Electrophoresis.
- Proteomics - SDS-PAGE, Western Blotting, Protein Estimation Methods
- Diagnostics and vaccine management – ELISA, Immunoassays
- Fermentation Technology and Downstream Processing
- Cell Tissue Culture

INDUSTRIAL TRAINING AND CERTIFICATIONS

- **Summer Trainee at National Institute of Pharmaceutical Education and Research**

  Topic: Enzyme Extraction, Purification and Estimation

  Duration: June 2014 – July 2014

  Description: Training programme under Dr. Ipsita Roy, Department of Biotechnology, NIPER- Mohali. Learnt techniques related to protein:. Enzyme

extraction, purification and determination of activity. Analysis techniques like Protein estimation, activity assay, SDS-PAGE, Western Blot.

- **GBioFin Entrepreneurship and Innovation Certificate**
  Duration: Jan 2013 - Mar 2013
  Description: A program to promote developing of innovative ideas and learning entrepreneurship in Biotechnology in India.

PROJECTS AND PRESENTATIONS UNDERTAKEN

- **Project on Basic Bioinformatics tools used in Genetic Engineering**- October, 2013- November, 2013.
- **Presentation on Bio-ethanol Production in Brazil in Fermentation Technology and Downstream Processing-** April, 2014.
- **Project on Production of "Vermouth" made of Green Grapes and fortified with Rosemary herb, on laboratory scale in Fermentation Technology and Downstream Processing**- March, 2014- May, 2014
- **Final year project on Role of *calcineurin* gene and anti-microbial compounds in conidiation of *Aspergillus flavus***- August, 2014- May, 2015.

ACHIEVEMENTS

- Secured All India Rank 526 in First Nationwide Biotechnology Olympiad organized by
- EduHeal Foundation, New Delhi.
- Secured All India Rank 264 in Second Nationwide Biotechnology Olympiad organized by
- EduHeal Foundation, New Delhi.
- Won First Prize in Floral Quiz, Chrysanthemum Show organized by Municipal Corporation, Chandigarh.
- Won Third Prize in the Slogan Writing Competition organized by the Chandigarh Librarians' Association.
- Won Second Prize in HIV/AIDS Awareness Quiz organized by Municipal Corporation, Chandigarh at school level.

EXTRA CURICULLAR ACTIVITIES

- Active member of a charitable organization- SANKALP for one year.
- Active member of Rotaract Club of Waknaghat for two years.
- Active Member of the Hospitality Committee of the Jaypee Youth Club (JYC) for one year.

ADDITIONAL COMPUTATIONAL SKILLS

Programming Languages	Basics of C/C++, Data Structures in C, Java

Bioinformatics Tools & Software's	Online Bioinformatics databases, BLAST, FASTA, EMBOSS, CLUSTALW, PyMOL, FGENESH, DNASTAR, NebCutter, Restriction Mapper, Primer 3, Restriction Free Cloning etc.
Others	Well versed with Microsoft Word, Excel, e-mail & social media

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Name	:	Nupur Gupta
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