EFFECT OF SHIKONIN ON GROWTH OF *Aspergillus flavus*

A

PROJECT REPORT

Submitted in partial fulfilment of the requirements for the award of the degree of

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UNDER THE GUIDANCE OF

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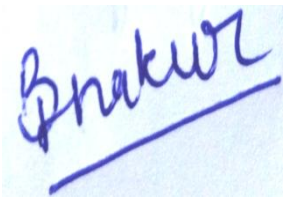
WAKNAGHAT

JUNE-2020

DECLARATION

We hereby declare that the work reported in the B. Tech Project Report entitled **"Effect of Shikonin on growth of *Aspergillus flavus*" s**ubmitted to Department of Biotech Engineering, Jaypee University of Information Technology, Waknaghat is an authentic record of work carried out under the supervision of **Dr. Jata Shankar.**

The matter embodied in the report has not been submitted for the award of any other degree or diploma.



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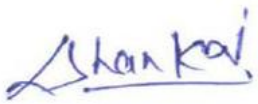
This is to certify that the above statement made by the candidates is correct to best of my knowledge.



Dr. Jata Shankar
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LIST OFFIGURES

ABSTRACT

Aspergillus flavus, a extensively found fungus in our surroundings. They are known to have asexual bodies which are small in size called as conidia which swells in order to form the germ tube which leads to the development of the hyphae finally forming network of white filaments called as mycelia. Aspergillus species produces aflatoxin, a poisonous carcinogen and mutagen which is responsible for causing aflatoxicosis in humans and animals. Nevertheless, an enormous research space for distinct morphological conditions at molecular degree to grow novel anti-aflatoxigenic as well as a compound called anti-*Aspergillus* is still witnessed. There are a number of studies which are being supervised at the mycelial stage of *A. flavus* species and during germination process as it is considered as a major step in the life cycle of *A. flavus*. Also, some transcriptomic and proteomic approaches showed the results that suggested that at mycelia phase *A. flavus* is considered as a powerful mycotoxin producer. Hence, this lead to additional research to develop efficacious anti-aflatoxigenic aggregates and to recognize crucial anti-*Aspergillus* targets [1, 2] Development of resistance against the available antifungal drug is also matter of concern for clinician [3]. Among various genus of fungi the genus *Aspergillus* has high ability to produce a wide mixture of secondary metabolites, including mycotoxins which plays a highly important role in causing food and feed contamination. One of the most prime group of fungi from *Aspergillus* segment *Flavi* is known to manufacture the most strongest carcinogenic compound of the mycotoxins, aflatoxin. Shikonin is considered as an important component of zicao (purple gromwell, the dried root of *Lithospermum erythrorhizon*), which is described as a herbal medicine in the Chinese culture having a variety of biological activities, also known for hampering of the human immunodeficiency virus (HIV) type 1 (HIV-1). To enter the host cells, HIV-1 uses the G protein-coupled chemokine receptors as co receptors. It was found to induce apoptotic features such as chromatin condensation, DNA fragmentation. Our objective is to infer about the effects of shikonin (a phytochemical) on *Aspergillus flavus's* growth.

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CHAPTER 1

INTRODUCTION

The numerous biological functions and host- pathogen interaction makes it a complex process to study biological activities of pathogenic as well as non pathogenic microorganisms in order of the biosynthesis of macromolecules needed for these various functions.Thespecies of *Aspergillus* is famously studied manufacturer of mycotoxin namely carcinogenic compound, a chief cause of diseases in animals and plants as well as responsible for their contamination [4]. Aspergillosis, a fungal disease is primary in variety of occurence of HIV, patients suffering from cancer , leadind to an increase in the death rate in the human beings that are infected. *A. fumigatus, A. flavus, A. terrus* are the prime species held responsible for aspergillosis [5, 6]. The species of *Aspergillus* survives in dissimilar stages of morphology during the lifecycle namely mycelia, conidia, and hyphae [7]. Recently, various approaches to reveal and study it's molecular mechanisms of events that occur in morphological stages have been identified such as trasncriptomic approach but transcriptomic approaches has certain limitations. Therefore proteomic study is quite important for the contribution in the revolution in biological studies of microorganisms. But still a restricted set of studies related to the proteomics on species of *Aspergillus* during contrasting stages of its morphology or throughout the interactivity with phytochemicals or antifungal. The attainability of proteome(which is annotated), of a number of non-pathogenic and pathogenic species of *Aspergillus* (*A. parasiticus, A. fumigatus, A. terrus, A. flavus* etc.) and dataset of proteins that are bounded experimentally, attempts are feasible now to understand the and molecular and biological mechanism of *Aspergillus*. Anyhow, it is important to identify proteins and pathways involved in germination process which will permit to find strategies which are novel for a development of drug which has the ability to battle the diseases related *Aspergillus* [9, 10].

1.1 *Aspergillus flavus*:

Aspergillus species enormously present is known for producing mycotoxins like carcinogenic compound, which is a major cause of diseases in animals and plants as well as in contamination, also it is well known for causing a disease, aspergillosis which is of fungal nature and origin and is also chiefly responsible in various occurrence of patients suffering from cancer and HIV, which higher the death rate in humans suffering with the same and also within mice model [6,11,12]. *A. fumigatus, A. flavus, A. terreus* are the species which are accountable for aspergillosis.

Aspergillusflavus is also one of the prime producer of aflatoxin (AF) within food crops. It was recorded that aflatoxin is produced during mycelia phase in the species of *A. flavus* however the collection of data on proteins on conversion from conidia to mycelia of *A. flavus* is restricted. So, it becomes more important to identify the distinctive active biochemical and proteins as well as activities related to the metabolic pathway at various stages of germination for identifying the agent having antimicrobial effect against the species of *Aspergillus* [7].

.*Aspergillus flavus* is established as a saprophyte growing in soils and as a disease causing agent on a vast number of agriculture crops. Cereal grains, legumes, and tree nuts are found to be common hosts for pathogen causing disease. Specifically, *A. flavus* infection is responsible for causing yellow mold in peanutsand ear rot in corn before or after harvest, also it has the possibilty to contaminate seedlings by sporulation in wounded seeds. In the field infection can be present , postharvest, preharvest, as well as during the storage, and during transit. It is customary for the microbe to arise while the crops that are the host are still present in the field; however, symptoms of the pathogen are frequently hidden.

Taxonomy:

Aspergillusflavus

Kingdom--Fungi,

Phyllum--Ascomycota,

Order--Eurotiales,

Class--Eurotiomycetes,

Family--Trichocomaceae,

Genus--*Aspergillus*,

Species-- *flavus.*

1.1.1 Ecology and geographical distribution :

Apergillusflavus produces conidia which disperses with the help of air and insects that makes *A. flavus* being dispersed within the whole world considering the both i.e. ecology amd geography .

How so ever, *A. flavus* population elevation is to be achieved at a variety of temperature ranges (12 to 48ºC) this shows the ability of fungi to survive high temperatures. Hence, atmosphere and humidity plays crucial role for its growth. In crops which are post harvested, *A. flavus* generates rots, this proposes that for *A. flavus* to grow it requires high humidity and hot or piquant conditions [13].

Temperature:

The minimum growth temperature and maximum growth temperature for *A. flavus* are 12 °C (54 °F) and 48 °C (118 °F) respectively. The temperature for maximum growth is found to b 48 °C (118 °F), the temperature required for growth that is optimum is said to be is 37 °C . Quick growth of *A. flavus* is seen at 30–55 °C, and a development much slower at 12–15 °C is seen, and at 5–8 °C the developmental mostly halts.

Moisture:

A. flavus has the capability to carry on to subsist and grow in difficult scenarios arbitrates cut-throat obstruction of additional organisms for different substrates within plants.

A. *flavus* growth is noted to occur at distinct moisture levels for various crops.*A. flavus* growth is widespread in tropical countries.

Growth occurs at 13.0–13.2% for cereals that are starchy. Growth occurs at 11.5–11.8% for soybean and at 14% for other crops.

Occurance of *A. flavus* in drinking water was shown in a number of studies.The phrase 'aflatoxicosis' was common in India in 1960's in the public knowledge realm when an incident took place in Mysorepoultry farms in Karnataka which led to the death of 2219 chicks .For India, because of the climate variations (50 °C in Rajasthan, summery and stifling afternoon in Punjab and Uttar Pradesh and −40°C in Cargill) causes imprudent warming and floods and making favourable environment for colonization of *A. flavus*. The encampment of *A. flavus* on satisfactory substrate aggregates in AF contamination[13].

1.1.2 Morphology:

Aspergillus genus is characterized by identifying the features of it'sconidiophore, anyhow recognition and variance of this species is not easy. Macro-morphological characters such as colour of conidia and mycelia, diameter of the colony,reverse color of the colonies present in the germinated *Aspergillus* species, analysis of the pigment and occurrence of sclerotia. Micro-morphology characters such as size of thevesicles, shape of the vesicle , seriation, morphology of conidia,seriation and ascospores morphology should be taken into account while identification and characterization..

Distinguishing features of Aspergillus genus conidiophores is as follows:

Colour: un-coloured,

Size: up to 800 µm long x 15 – 20 µm wide,

Vesicle shape : globose to subglobose; Vesicle size: 20 – 45 µm,

Seriation: metulae (8 – 10 x 5 – 7 µm) covering nearly the entire vesicle in biseriate species [14].

Mycelium of *Aspergillus* :

Mycelia of *Aspergillus* is similar to that of most of other fungi. It well developed and loosely interwoven mass of hyaline, pale or bright coloured, highly branched, septate hyphae. Several of the hyphae, ostensibly break up upon the substratum however some others infiltrate deeply into the substratum which in turn helps the entire mycelium for absorbing the food. The freely branched hyphae form thick mats on the substratum. The slender, delicate hyphae have thin and even walls.

1.1.3 MODE OF REPRODUCTION:

The species of *A.flavus* generally shows asexual mode of reproduction i.e. by conidia(reproductive bodies).The conidia of the species are 2-5 µm in size which is quite a small number. These conidia are less active when seen metabolically and they are generally comatose both in water and air which needs triggering 6 mechanism for germination, this necessitates utilizing of sugars,amino acids and organic salts. However, recently it is reported that A. flavus can reproduce through sexual mode of reproduction i.e. (Development of ascospores inside sclerotia) but very rarely.

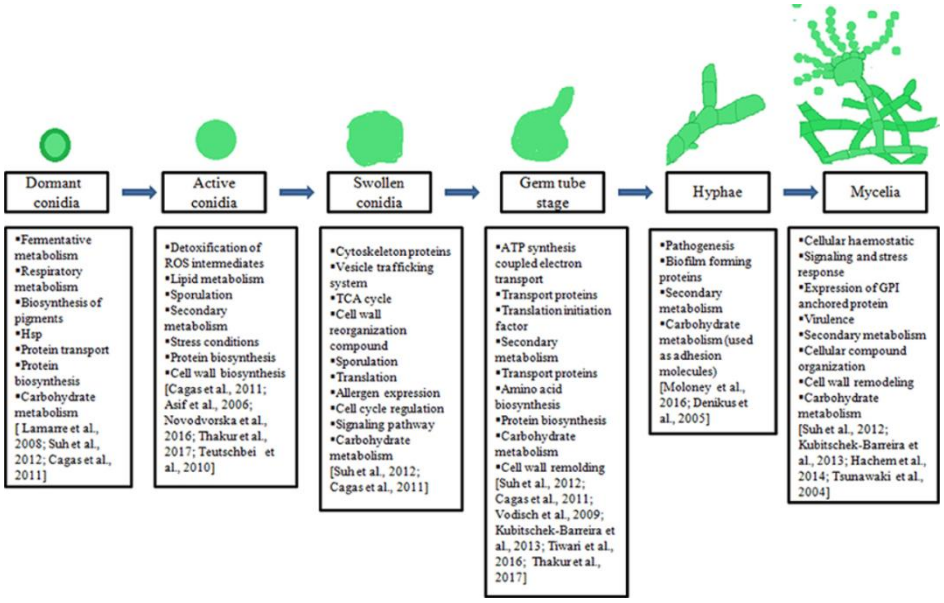

Fig.1.1: Development/germination of conida in *Aspergillus* (Shankar et 2018)

Asexual Reproduction :

Asexual reproduction is fulfilled by the help of spores which are asexual known as conidia theseare manufactured extrinsicly in chains present at the spikes of perpendicularly growing aerial hyphae known as the conidiophores.

(a) Conidiophores :

Certain cells in the older parts of the young, vigorously growing prostrate mycelium become thick-walled. These thick-walled, T-shaped cells are characteristic of the genus *Aspergillus* and are known as the foot cells.

Each foot cell produces a special erect branch as an outgrowth. These upright hyphal branches are the young conidiophores. A length of about 2.5 mm is obtained by the conidiophores and swells at its apexto result into an elliptical bulbous head.

This swollen end of the conidiophore is called the vesicle. The vesicle may, in some species, be hemispherical or clavate. The lumen of the vesicle is continuous with the upper part of the conidiophore and is multinucleate.

At maturity,the conidiophores, are stout, long, generally unseptate and unbranched, hardly ever septate structures. They appear one by one from the mycelial foot cells and are not arranged into any kind of asexual fruit bodies.

A number of spirallyarranged, tubular outgrowths arise from the multinucleate surface of the vesicles. Sterigmata (Sing, sterigma) or philades are these specialized conidiogenous outgrowths or cells .

The sterigmata are arranged compactly side by side and thus completely cover the entire surface of the vesicle. The species with conidia bearing phialides arising directly from the vesicle are termed uniseriate species

In some other species, the phialides are borne on intermediate cells, the metullae (primary sterigmata) which are attached to the vesicle. These are termed biseriate species. In biseriate species such as A. fonsecaceus the conidia bearing sterigmata are called secondary sterigmata.

The entire structure comprising of the foot cell, the vesicle,the upright hypha, the phialides andthe metullae comprises the conidiophore .

Sexual reproduction:

The sexual or perfect stage is rare which is probably the consequence of the evolution. This view is supported by the fact that even in species that form asci there is evidence of sexual degeneration.

Different species of this genus show variation in their sexual behaviour. The antheridium is absent in some species or if present it is non-functional. The asci, however, develop from the female sex organ (ascogonium).

In others it is well developed and functional. *Aspergillus* is homothallic. A. heterothallicus is the only species that has been reported to be truly heterothallic [15].

CHAPTER 2

REVIEW OF LITERATURE

Aspergillus flavus

Aspergillus genera constitutes fungi which are saprophytic in nature that enlarges on different types of organic matter and actively involves in recycling of nitrogen and carbon by decomposing dead organic debris. This genus has almost more than 500 species. Aspergillosis caused due to *Aspergillus* species is alarming. Several studies on host response how Aspergilli induces immune responses or cytokine/ chemokines or signaling pathways are important in elucidating the mechanism of pathogenesis [17,7]. Among these *Aspergillus* species, *A. flavus* represents unique scenario. *A. flavus* is responsible for causing yellow mold in cottonseeds and peanuts, eat rot in maize and other various crops. Damage in plant parts and seeds, drought stress, osmotic stress, oxidative stress etc are the stress factors which plays vital role in the capability of *Aspergillus flavus* to elicit infections. It's carcinogenic secondary metabolite is another distinguishing factor of of *A. flavus* [4].

Aspergillus flavus produces aflatoxin that has the ability to cause aflatoxicosis and aspergillosis following from ingesting or inhaling *Aspergillus flavus* particulates in higher quantities which is dangerous in humans having a compromised immune system. Also, phytochemicals such as quercetin, gallic acid etc have shown to inhibit aflatoxin biosynthesis [18, 19].

Hence, *A. flavus* becomes a crucial fungus for studies as it has been proven a potential threat in agriculture and medical fields. *A. flavus* has various properties as a potent pathogen such as cultural and morphological characteristics, damage to host-tissues, significant stress- tolerance chemistry, antifungal drugs resistance development, ability to infiltrate host immune system and colonization are the aspects of *A. flavus's* eco-physiology that makes it a thriving pathogen. Genome wide

studies proteins including heat shock protein are differentially regulated in response to *Aspergillus* [20] and specifically Hsp90 confer resistance against the antifungal drugs [21].

Quantification of A. flavus DNA done by the technique of quantitative PCR (qPCR) as a substitute for fitness of aflatoxigenic and non - aflatoxigenic field isolates grown in soil microcosms. Aflatoxigenic isolates had noteworthy lower fitness than non- aflatoxigenic isolates have in natural soils across three temperatures (25, 37, and 42°C). The addition of aflatoxin to soils (500 ng/g) had no effect on the growth of *A. flavus*. Amplicon sequencing showed that neither the aflatoxin-producing ability of the fungus nor the addition of aflatoxin had a significant effect on the composition of fungal or bacterial communities in soil.

Aspergillus flavus contaminates and infect various agricultural product around the world with carcinogenic aflatoxins. The high genetic diversity of *A. flavus* is contributed by its feature of sexual reproduction which is done with the help of formation ascocarps which bears ascospores embedded within sclerotia. During the time of harvest the sclerotia which is formed in the crops gets dispersed onto the surface of the soil and these are majorly formed by the mating of the solitary strains of the similar mating type.The ascospores on the soil which are produced from the single strain sclerotia and the offspring showcased an inheritance pattern which is biparental that involved alleles derived from fertilization by native soil strains. An evidence of recombination in progeny is seen when laboratory fertilized sclerotia is applied to soil and ascospores production is seen before formation of ascocarp, however detection of known parental alleles were witnessed. Sclerotia as well as conidia from both the strains worked as male and female in case of a reciprocal cross, giving the hint that *A. flavus* is hermaphroditic however the extent of fertility depended on the sources which were parental of both the sclerotia and conidia. Maternal inheritance of mitochondria from the sclerotia was seen in the progengy. Fertilization of sclerotia may even happen during co-infection of crops with strains that are sexually compatible. Genetic recombination can be seen in progenies which are produced with in the laboratories where crosses

of opposite mating type strain are carried out thus showing that *A.flavus* is heterothallic in nature.In this study, to examine sexual reproduction in fertilized sclerotia and in single strains,laboratory and field experiments were performed following exposure of sclerotia to natural fungal populations in soil. Through reciprocal crosses between sclerotia and conidia the male and female roles and mitochondrial inheritance in *A. flavus* were also examined. Compared to *A. flavus* populations in crops, soil populations would provide a higher likelihood of exposure of sclerotia to sexually compatible strains and a more diverse source of genetic material for out-crossing. Phytochemical or their derivative has emerged as promising molecules against *A. fumigatus* effecting wall organization or pigmentation, modulating heat shock protein or cell wall morphology *A. flavus* [25] and *A. terreus* [25].

Shikonin

Molecular Formula:--$C_{16}H_{16}O_5$

IUPAC Name:-- 5, 8-dihydroxy-2-[(1R)-1- hydroxy-4-methyl-3-pentenyl]-1,4-naphthoquinone .

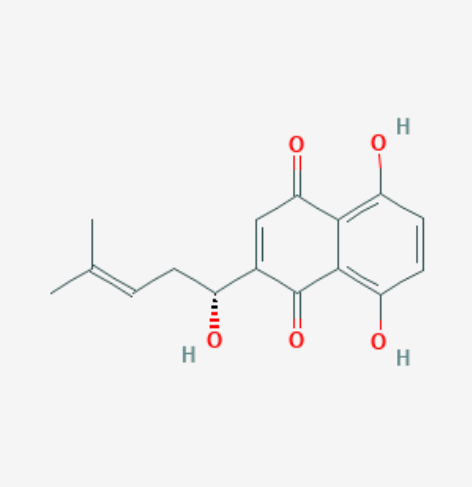

Fig.2.1 : Structure of Shikoin

(Prenosil JE et al; Ullmanns encyclopedia of industrial chemistry 7th edition 1999-2012)

Shikonin, a napthoquinone compound, it's chemical structure discovered by Brockmann in 1936.Shikonin is an enantiomer of alkannin, and shikalkin is the racemic mixture of these two. Shikonin was first isolated as acetylshikonin from the roots of *Lithospermum erythrorhizon.* The

naphtoquinones shikonin and its derivatives are the main active molecules present in traditional Chinese herbal medicine Zicao. It is widely planted in Korea, Japan, and China, and it is used as a dye for food colorants and fabrics staining. This plant has been classified as edible by the KFDA (Korea Food & Drug Administration)[30].

Shikoninis the major constituent of the red pigment extracts from the roots of the plant *Lithospermum erythrorhizon*. Shikonin, derived components are lipophilic red pigments, with naphthazalin skeleton are present as low molecular weight fatty acid ester in the roots.

Various pharmacological effects of shikonin have been recorded such as , inhibition of platelet activation and antimicrobial activity, anti- HIV1, antigonadotropic,anti-inflammatory, modulating enzyme estrogen by down- regulating the steroid sulfatase activities, healing of wounds, suppression of local acute inflammatory reactions, inhibition of angiogenesis, enhanced granuloma formation, inhibition of select chemokine ligands, inhibition of DNA topoisomerase activity.[27]. Shikonin was also reported to exhibit certain functions which were widely used to prepare an ointment to treat wounds, burns and hemorrhoids in Japan. Also it was used to induce necrosis or apoptosis through generating reactive oxygen species to treat gastric cancers; and to serve therapeutic roles in brain disorders involving uncontrolled inflammatory responses.

Shikonin was found to induce apoptotic features such as chromatin condensation, DNA fragmentation. Recently it was found that shikonin have an antifungal effect on almost all *C. albicans* isolates tested. [28].

The structural integrity of cell wall is crucial for the survival and growth of fungal cells, as it provides immunity from osmotic pressure and other stresses . Damages to the cell wall due to increased synthesis or decreased degradation of chitin, may increase the tolerance to antifungal drugs . Shikonin could induce the endogenous reactive oxygen species production, reduce the

mitochondrial membrane potential, and alter mitochondrial aerobic aspiration as induction of reactive oxygen species can lead to cell arrest and hence, the apoptosis. [29]

Biosynthesis of Shikonin

The biosynthetic pathway of shikonin was investigated by Inouye who administered labelled precursors, *para*-[3-^{3}H] hydroxybenzoic acid and [2-^{14}C] mevalonic acid, to *Lithospermum erythrorhizon* callus cultures (strains M18 and M231a) grown on LS agar medium containing IAA and kinetin, in the dark. The results of experiments indicated that para-hydroxybenzoic acid (**20**) was incorporated into the hydroquinone portion of shikonin, whereas two molecules of [2-^{14}C]MVA were incorporated into shikonin, labeling C-1' and C-5' positions of the side chain. Furthermore, the chemically synthesized labeled substances *meta*-geranyl-*para*-[8′-^{3}H] hydroxybenzoic acid and [8′-^{3}H] geranylhydroquinone were almost specifically incorporated into shikonin. Tracer experiments showed that both meta-geranyl-para-hydroxybenzoic acid (**21**) and geranylhydroquinone (**22**) are the biosynthetic intermediates of shikonin (Scheme 1). The biosynthetic pathway of shikonin proposed by Inouye's group has been supported by the isolation of these intermediates from shikonin-producing cells of the strain M18 [30].

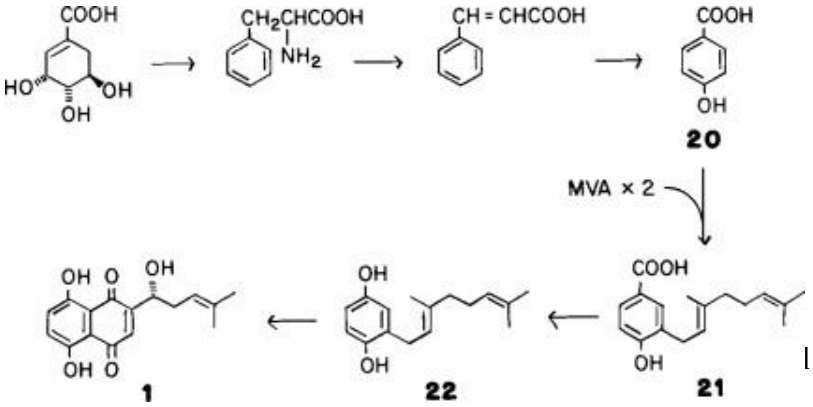

Fig. 2.2 : Biosynthesis of Shikonin [31]

l or production of shikonin and its derivatives. Hairy root cultures of *Lithospermum erythrorhizon* have been used to produce and secrete shikonin. Derivative of shikonin usually accumulates in root tissues of *L. erythrorhizon*. The core structure of shikonin is biosynthesized from *p*-hydroxybenzoic acid and geranyl diphosphate, which are coupled by a membrane-bound geranyltransferase

localized at endoplasmic reticulum. From this key biosynthetic step, intermediates are to be hydrophobic leading to shikonin formation. Shikonin derivatives are water–insoluble so when they are secreted by the cells they accumulate in apoplastic cells. After various experimentations it was found that hairy roots in liquid cultures of *L. erythrhorizon* have a reddish appearance in M9 medium, due to the excretion of shikonin derivatives into the apoplast [30]. Thus, *A. flavus* being important for both agricultural crops e.g., Rice [32] and as human pathogen [33[, studies are needed to control the contamination in crops and to look for the phytochemical that can inhibit the colonization of *A. flavus*.

CHAPTER 3

MATERIALS REQUIRED ANDMETHODOLOGIES

3.1 MATERIALS REQUIRED:

3.1.1 DEVICES AND INSTRUMENTATIONS:

Aspergillus flavus strain , Bunsen burner, Pipettes,Tips, Marker, Petri plates, Matchsticks, Eppendorf tubes, Haemocytometer, Refrigerated centrifuge, Autoclave, Incubator, Laminar air flow, Flasks, Weighing balance, Measuring cylinders.

3.1.2 CHEMICALS REQUIRED:PBST (Phosphate buffered saline Tween-20), PBS(Phosphate bufferd saline), PDA (Potato Dextrose Agar), 70% Ethanol, Lactophenol cotton blue, Shikonin.

3.2 METHODOLOGIES:

3.2.1 PREPARATION OF MEDIA AND SUBCULTURING OF FUNGUS:

- 19.5g of PDA (potato dextrose agar) was measured.
- 500ml of distilled water was added to the weighed PDA.
- PDA and distilled water were then dissolved by mixing and boiling.
- Prepared media was autoclaved.
- Pouring of the autoclaved media in to the petri dishes was done.
- Petri dishes were then kept undisturbed for solidification in LAF .
- *A. flavus* fungal strain was streaked over PDA plates for culturing and plates were marked accordingly.

3.2.2 HARVESTING OF CONIDIA FROM CULTURE PLATES:

- *A. flavus* strain culture plates were taken.
- 4ml-6ml of PBST was spreaded all over the fungal plate using spreader.
- Around 2 ml of PBST and conidia solution was pipette out in the Eppendorf.
- It was then centrifuged at 10000rpm for 5min. at 4^0 C.
- Supernatant was discarded.
- 2ml PBS was added to pellet obtained in the eppendorf.
- Centrifugation step repeated again.
- Above 2 steps were repeated 2 times.

3.2.3 COUNTING OF SPORES:

- 10microlitre of *A. flavus* culture was taken and 15 microlitre of lactophenol cotton blue dye was taken and mixed in eppendorf tube.
- The mixture of culture and dye was pipetted out on haemocytometer .
- Cover slip was put very carefully on the haemocytometer.
- The culture was observed under microscope.
- Spores were counted [33].

Conidia count= average cell count from each set of 16 corner squares * 16 * 10^4Conidia per microlitre

3.2.4 Experimentation with the shikonin:

A. Trial#1

1. Harvested spores of *A. flavus* were used for making culture plates.
2. 50 microlitre of conidia suspension was taken and spread on the media plate.
3. 1 microlitre of shikonin was put in the middle of plate on which the culture was spread.
4. Then plates were observed .
5. A control culture plates were made which does not contain the shikonin to study the effect of shikonin on growth of *A. flavus*.

B. Trial#2

1. Diluted the shikonin using DMSO in the ratio 4:1.

2. 4 microlitre of DMSO and 1 microlitre of shikonin was taken in an eppendorf to make the diluted solution of shikonin in the ratio 4:1.

3. Serial dilutions were prepared of the stock solution using PBS.

4. 6 eppendorf tubes were taken and 900 microlitre of PBS was added to each tube .

5. Eppendorf tubes were marked as 10^{-1} , 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} respectively.

6. 100 microlitre of stock culture was added to eppendorf tube marked as 10^{-1} and mix well.

7. 100 microlitre of diluted solution was taken from eppendorf marked 10^{-1} and added to the eppendorf marked as 10^{-2} and mix well.

8. The previous step was repeated for the other dilutions.

9. 50 microlitre of diluted suspension culture marked 10^{-5} were taken and spread uniformly over the agar medium plate using a spreader .

10. A small disc paper was put carefully over the agar plate containing streaked culture and 1 microlitre of diluted shikonin was added to the disc.

11. Plates were then observed after 0hr, 24hr, 48 hr and 72hr of incubation.

Precautions:

1. All the work should be done under LAF.
2. Hands and other equipments used must be properly sterilized
3. Disposing of the plates should be done properly.
4. Make sure to clean the working bench properly with ethanol after work is done.
5. Pipetting and calculations should be done properly.
6. Media and tips used should be autoclaved properly.

CHAPTER 4

RESULTS AND DISSCUSION

Trial#1

- **Conidia count:**

Average cell count= $5 + 5 + 9 + \frac{7}{4} = \frac{8}{4} = 2$

Conidia count= $2 * 16 * 10^4 = 104 * 10^4 = 1 * 10^6$ per microlitre

Result: *1*$*10^6$*conidia per microlitre*

- The shikonin affected the growth of the *Aspergillus flavus* by inhibiting its growth.
- Normal growth was observed in control cultured plates of *Aspergillus flavus*.

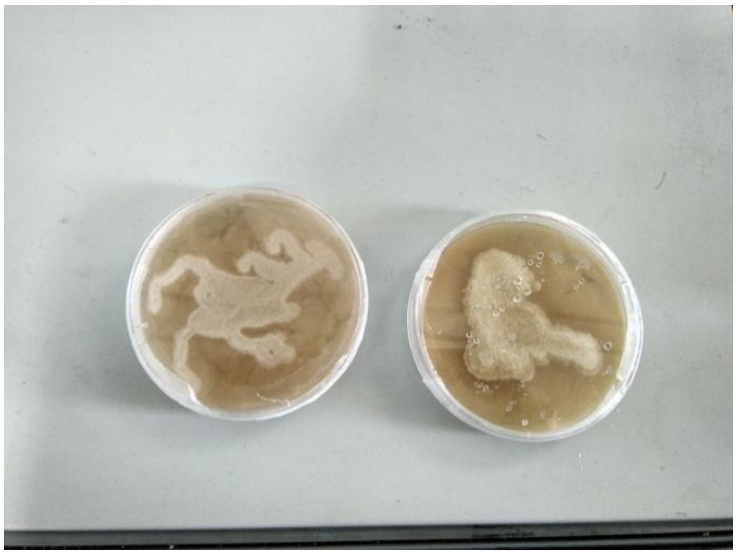

Fig. 4.1 (a): Control cultured plates of *A. flavus*

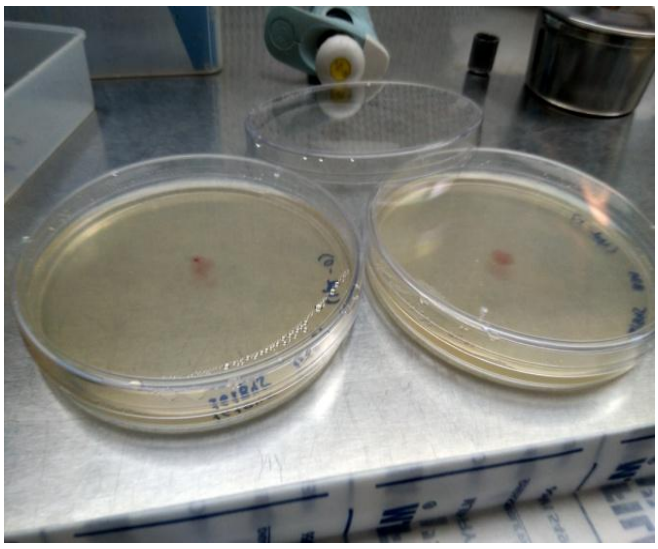

Fig. 4.1 (b) : Cultured plates after 0 hr of adding shikonin

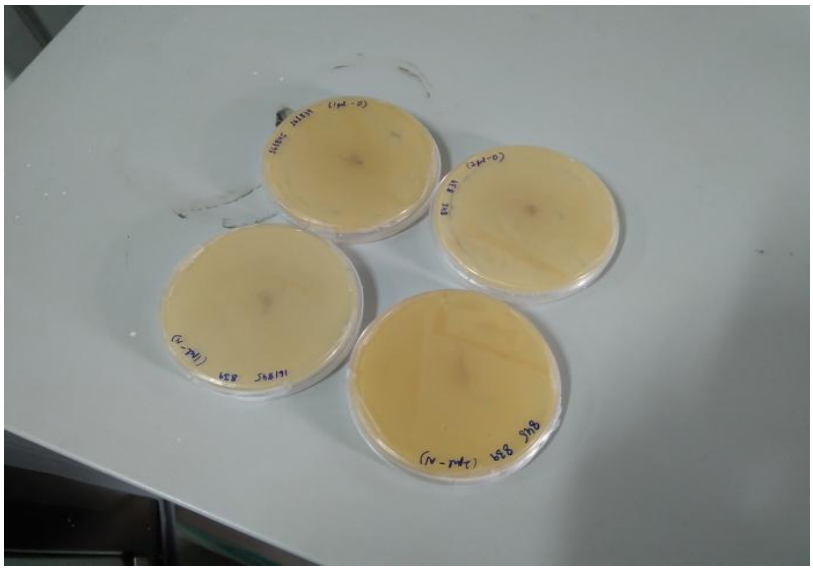

Fig. 4.1 (c) : Cultured plates after 24 hrs adding of shikonin

Trial#2

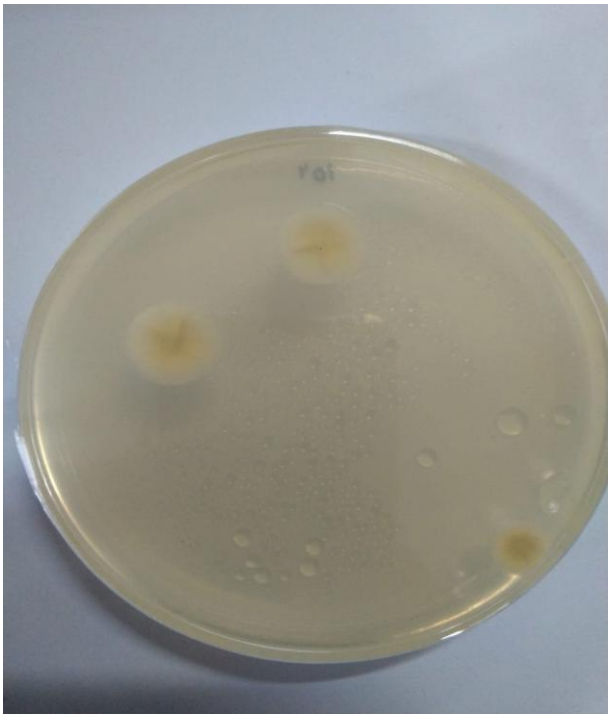

Fig. 4.2 (a): Control plate containing 10^{-5} of *Aspergillus flavus* after48 hrs

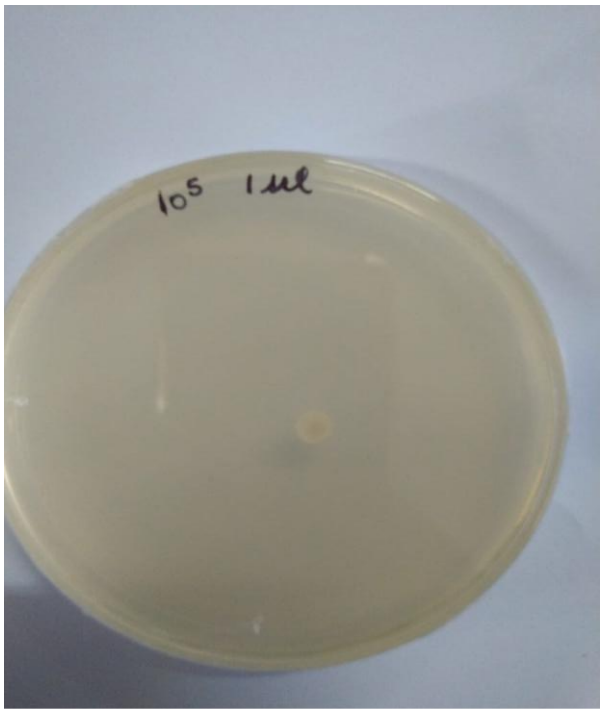

Fig. 4.2 (b) : Cultured plate having 1 micolitre of shikonin added to it after 48 hrs

Result & discussion:

- Fig. 4.2 (a) showed the growth of *Aspergillus flavus* after 48 hrs of incubation.
- Fig. 4.2 (b) showed no growth, as 1microlitre shikonin inhibited the growth of the *A. flavus*.

These experiments have carried out only once, no clear conclusion could be drawn. Experiment needs to be repeated again to assert the effect of shikonin on *A. flavus*.

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