Bulletin of Environment, Pharmacology and Life Sciences
Bull. Env. Pharmacol. Life Sci., Spl Issue [1] January 2023: 327-334.

Online ISSN 2277-1808
Journal's URL:http://www.bepls.com
CODEN: BEPLAD

ORIGINAL ARTICLE **OPEN ACCESS**

Inhibition of Mycelial growth and Aflatoxin biosynthesis in *Aspergillus flavus* by extract of various spices and herbs

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ABSTRACT

The aflatoxins are very toxic and carcinogenic secondary metabolites produced by fungi like Aspergillus flavus, A. parasiticus, and A. nomius. The tropical and sub-tropical climatic conditions naturally support the growth of Aspergillus especially A. flavus, particularly when raw peanuts and peanut-based products are stored under inappropriate conditions. Here, various natural plant extracts that would be potential source of controlling aflatoxinin agricultural products are discussed. Naturally infected peanuts collected from trader's godown were tested for aflatoxins and found that after several months' storage, were contaminated with aflatoxin B1. The effect of aflatoxin on seed germination and root elongation was tested on plant model like wheat. Water soluble extracts of these spices were prepared, agar diffusion performed and zone of inhibition was measured after applying different concentrations of extracts against the strain. Our study showed that these extracts are effective to control aflatoxigenic strains of Aspergillus and aflatoxin contamination and may be considered as an alternative to control post-harvest fungal diseases.

Keywords: *Aflatoxins, A .flavus, secondary metabolites, food contamination, toxic effect, Natural extracts; Antifungal*

Received 12.11.2022 Revised 25.11.2022 Accepted 26.12.2022

INTRODUCTION

Aflatoxins are poisonous, carcinogenic, mutagenic, immunosuppressive, and teratogenic secondary metabolites formed by *Aspergillus flavus, A. parasiticus, and A. nomius*. These fungi grow ubiquitously and contaminate agricultural products such as rice, wheat, maize, barley, sorghum, black pepper, chili, ginger, coriander, turmeric, pistachios, almonds, walnuts, Brazil nuts, peanuts, oil seeds (cotton, sunflower, sesame, and soybean), milk, cheese, and animal feed [1-4].

The Food and Agriculture Organization (FAO) estimated that around 25% of the world's cereals are contaminated by mycotoxins, including aflatoxins. Aflatoxins are stable and resistant to degradation. Among the 18 different groups, aflatoxins B1, B2, G1, G2, M1, and M2 are the major classes and derivative of bifuranocoumarins. The aflatoxins B1 and B2 give blue color, while G1 andG2 give a yellowish green color under UV light. Aflatoxins M are hydroxylated derivatives of aflatoxins B and first isolated from milk. *A. flavus* produces only AFB1 and AFB2,but it is also able to synthesize cyclopiazonic acid. However, *A. parasiticus* produces AFBI, AFB2, AFG1 and AFG2 [5, 6].

According to the Food and Drug Administration (FDA), the safe limit of aflatoxins is20ppb [7].

Consumption of aflatoxin contaminated products causes aflatoxicosis in humans and animals which may be acute and chronic. Acute condition caused death,while chronic condition results in immune suppression and cancer. In human, it is characterized by vomiting, abdominal pain, pulmonary edema, convulsions, coma, and death with cerebral edema and fatty involvement of the liver, kidneys and heart. In animal, aflatoxicosisis characterized by gastrointestinal dysfunction, reduced feed utilization, jaundice, liver damage, decreased milk and egg production and immunity suppression[8].In plants, AFs retarded seed germination, seedling growth and root elongation.The members of genus *Aspergillus* mostly contaminate agriculture commodities in tropic and subtropic region. Contamination may occur at different stages such as in preharvesting stage, harvesting stage, post harvesting stage, or in storage and transportation stage. In preharvesting, the field fungi attack on growing crop. It may be the environmental stress like hot and dry condition and soil moisture, mechanical damage by arthropods, birds, rodents, and nematodes, or delayed harvesting. While in postharvesting, contamination occurred due to improper drying, storage in polythene bags, damage during shelling, or storage in poorly ventilated warm environment.

Optimum condition for fungal growth in cereal is moisture content about 18% which is equal to 85% relative humidity and temperature about 12–42°C with an optimum at 27–30°C in tropical and subtropical areas [9]. The time of incubation also affects the production of toxin by Aspergillus species [10]. Optimum duration for the production of aflatoxins was 14 days of incubation at 30°C. When the incubation time is increased, there will be reduction in aflatoxins level because of re-adsorption or degradation by fungus [11]. The fungal growth is affected by 20%CO2 and 10% O2 level. The metals such as manganese and zinc are crucial for aflatoxin production. But the mixture of cadmium and iron mixture reduces them old growth and aflatoxin synthesis.

In this study we selected *P. betle* which has been used as traditional medicines in Malaysia mainly for treatment of infectious diseases and also cough treatment. Anti-infectious effect of P. beetle against these disease led to the hypothesis that plant-derived phenolic compounds would have an antifungal effect on *Aspergillus spp*. This hypothesis was supported by studies showing that *P. betle* phenolic compounds exhibit antifungal activities (12).

MATERIAL AND METHOD

Sample collection: Samples of infected Peanut (Arches hypogeal) were collected from trader's godown in Pune.

1. Fungal isolation: Collected Peanuts were disinfected using 2.5% sodium hypochlorite for three minutes, rinsed three times in sterile distilled water and dried between layers of sterile filter paper (Whatmann, No. 1). Seeds were plated out on 15 ml of the PDA medium (5 seeds /plate) and incubated at 25°C for 5 days. Sub culturing was repeated several times for mycelia tips to obtain pure cultures which were preserved on potato dextrose agar slant till identified.
2. Fungal strain: The toxigenic isolate Aspergillus flavus was isolated from Peanut and identified onthe basis of colony and morphological characteristics (Lactophenol blue stain). The culture of toxigenic Aspergillus flavus strain was maintained on potato dextrose agar plate in the lab.

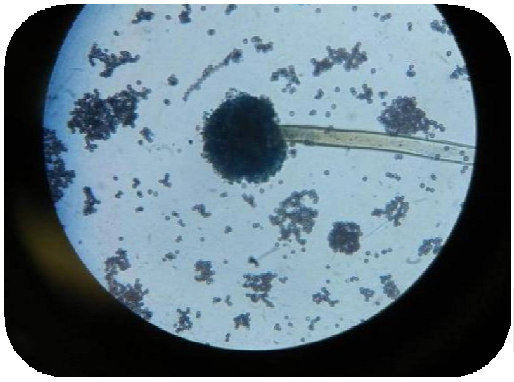

Fig1 Microscopic identification of A.flavus

Materials and Methods for checking toxicity of toxin produced on wheat germination.

1. Inocula: The inoculum was prepared by suspending the spores of A. flavus in 0.1% Tween-80solution. The spore suspension was inoculated in to the broth using a micropipette. 1 ml of the spore suspension was inoculatedper20g of substrate.
2. Media: Potato dextrose broth(PDB)
3. Extraction of Aflatoxin: The filtrate was defatted with equal amount of chloroform in flask was vigorously hand shaken for 30min. The liquid was divided into two separate layers the second layer is removed in crucible and allowed to evaporate. Then 1ml of methanol was added to the extract in the crucible .The purified aflatoxin was weighed.
4. Aflatoxin detection: Chromatographic assay. The culture was filtered and then filtrate was mixed with half the volume of chloroform. The flask were vigorously hand shaken for 30 min. The organic phase was separated and was dried on a heating block at 65°C. The extract was analyzed by TLC using80% Benzene: 15% Methanol: 5% Acetic Acid as the solvent system. The visualization of bands was carried out under UV light at 365 nm.
5. Effect of Aflatoxin on seed germination in wheat: The wheat seeds were soaked overnight in water and these soaked seeds were put into petri plates with tissue paper/cotton. In one set of petri plates water was added and in second set aflatoxin solution in water was added. The effect was seen over a period of 5 days.
6. Piperbetle was collected from agro-farm, Plant leaves were washed and rinsed in tap water, dried

At 40°C using forced air convention oven drier and powdered by grinder machine. 125 g of sample was macerated at room temperature in 1L of 95% methanol for 24 hours and repeated for three times. The extract was filtered through Whatman No. 1 filter paper and evaporated to dryness to provide a sticky crude extract. The crude extract suspended in 90% methanol and subsequently partitioned with an equal volume of chloroform to afford the fraction of chloroform and water. The fractions were concentrated in a rotary evaporator. Concentrated extract was weighed and resuspended in 2% (W/V) dimethyl sulfoxide (DMSO)in distilled water to prepare appropriate concentrations. Crude methanol extract from P. betel powdered leaves was partitioned between chloroform and water. The fractions were tested against *A. flavus* , a strong aflatoxin producing strain. Inhibition of mycelia growth and aflatoxins biosynthesis were tested by well diffusion and macro dilution techniques, respectively. The presence of aflatoxin was determined by thin-layer chromatography (TLC) and fluorescence spectroscopy techniques using AFB1standard.

Detection and quantification of aflatoxins:

The detection and quantification of aflatoxin was done by thin-layer chromatography (TLC) and fluorescence spectroscopy techniques respectively. TLC was carried out on a silica gel, using toluene: chloroform: acetone (15:75:10 v/v) as mobile phases. 20 µl of test samples and aflatoxin standards B1and B2with 1 µg/ml concentration were spotted on TLC plates in a TLC tank at room temperature. The developed plates were viewed under UV light at 254 and365 nm.

Extraction of spices

Several raw spices products were used to check their effects on aflatoxin. These include: black pepper (Piper nigrum), clove, cinnamon and bay leaves. These spices were purchased from local market. Spices were washed with deionized water and dried in oven at 50°C for24 hours. Dried spices were kept in a plastic bag until further use. Hot water extraction was performed to separate water soluble extracts of spices. Spices products were grinded separately and 10g of each was added in 100ml of distilled water. Boiling was done for 1 hour and supernatant was separated after filtration/centrifugation. The extracts were concentrated through rotary evaporator and dried in oven at 75–80°C. One gram of each extract was weighed and dissolved in 10ml of sterile distilled water and it was further serially diluted in sterile distilled water and several dilutions, i.e. 1%, 0.5, 0.1were prepared to determine the minimum inhibitory concentration.

Antifungal Activity

Agar well diffusion method:

Agar well diffusion method was performed (2010). Potato dextrose agar was autoclaved and spore suspension (105/ml)was added into the molten medium. Culture Medium was poured into sterile petri plates and allowed to solidify, followed by the formation of 10mm size diameter wells with sterile borer. One hundred microlitre of the different concentration of spices extract were added in their respective wells and incubated at 25 ± 2°C for 5–7 days. Sterilized distilled water was used as a negative control and acetic acid (Sigma-Aldrich, USA) was used as a positive control which was introduced into the well instead of spices extract. After incubation, plates were observed for any zone of inhibition surrounding the wells containing the spices extract.(13)

Aflatoxin inhibition assay:

The efficiency of spices extracts to reduce AFs production were evaluated by the method stated by Zohra and Fawzia (2013)(14). CzapekDox liquid medium was prepared, supplemented with different concentrations of extracts separately, followed by the inoculation of fungal spore suspension. Positive control was also prepared, contained only spore suspension, the flasks were incubated at 25 ± 2°C for10 days .After incubation, thin layer chromatography (TLC) was performed.

Samples were spotted as 10µland standards were separately applied on the TLC plates on imagining line about 4cm from bottom edge. The plates were developed in unlined tank containing 20ml of chloroform: xylene : acetone(6:3:1;v/v)and was observed under long wavelength UV light(λ=254and366nm)in UV visualizer.

RESULTS

1. TLC After the extract was spotted on to the Silica gel TLC plate, the plate was developed using the developing solvent and was visualized under UV light. Blue colored bands were observed, Confirming the presence of aflatoxins.

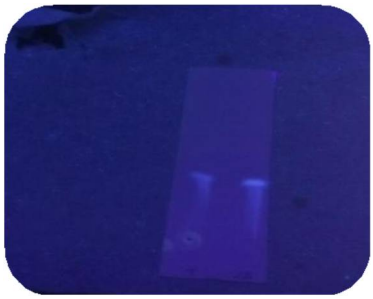

Fig2: TLC of aflatoxin and standard aflatoxinB1

2. Effect of Aflatoxin on seed germination in wheat:
The wheat seeds in petri plates with water germinated after 24 hours of incubation whereas there was no germination in plates in which aflatoxins solution was added even after 5 days. It was observed that in wheat roots growing in absence of the Aflatoxins showed root elongation of 53mm but the roots allowed to grow in presence of aflatoxins grew only 4mm.

Effect of aflatoxin on seed germination in wheat

Sample

Processed /test

Figure 3

The results, evaluated by measuring the mycelial growth and quantification of aflatoxin B1 (AFLB1)production in broth medium revealed that chloroform soluble compounds extract from P.betle dried leaves was able to block the aflatoxins biosynthesis pathway at concentration of 800 ul without a significant effect on mycelial growth.

Inhibition of mycelia growth:

Chloroform and water fractions were preliminary tested for inhibitory effect against *A. flavus* mycelia growth using well diffusion technique. 100µl of spore suspension was added to the solid CYAmedium in 9 cm plates and distributed uniformly using a sterile glass rod aseptically. The extract was dissolved in 5% dimethyl-sulfoxide (DMSO)in water(v/v) to a final concentration of 180,160,140,120,100,80,60,40,20. ul. Sterile well puncher were impregnated with 20µl of extract and placed on the inoculated plates. The plates were incubated at 28°C and diameter of the inhibition zone(mm) around the well was measured after 48 hours.

Inhibition of aflatoxins biosynthesis:

One milliliter of fungus spore suspension were added to 150ml conical flasks containing 25 ml of YES broth containing extract at final concentrations of 200,400and800 µg/ml. At the end of the incubation period (5 days at 28°C without agitation) the liquid culture was filtered through filter paper (Whatman No. 1), the fungal mycelium washed with distilled water, dried at 50°C for 72 h and dry weight were recorded (15). Toxin extraction was performed by removing 5ml of culture fluid from each flask, filtered through 0.2 µm syringe filter (Pall Acrodisc, USA) and extracted with 5 ml of chloroform (16).The organic phase was collected in 25 ml glass beaker and evaporated to dryness under 40°C in an air circulated oven dryer. The residue was resuspended in 500µl of methanol and kept in dark glass vial at-20°C before use.

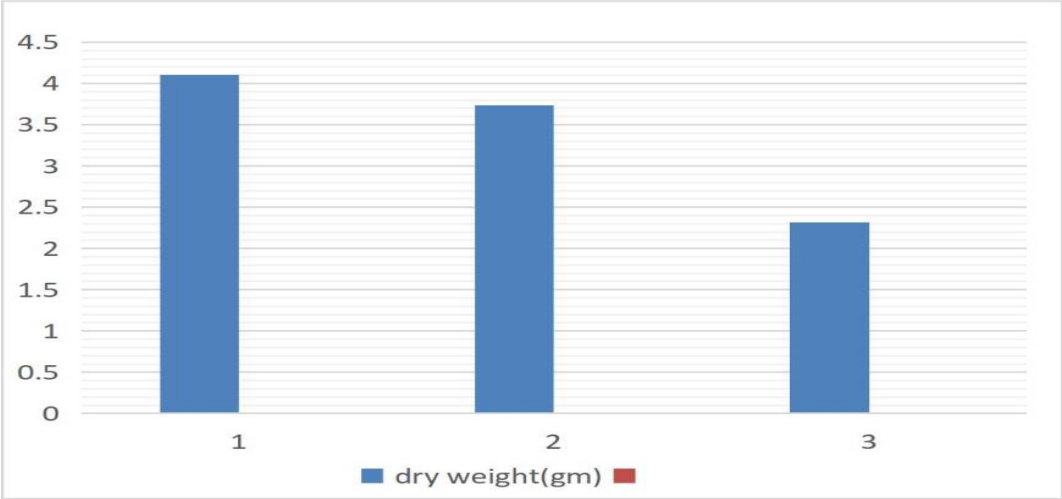

Fig4: AF Effect on dry weight of mycelium

Effect of P.betle on mycelial growth and toxin biosynthesis.

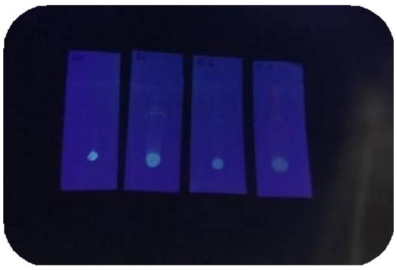

Fig 5: TLC plates of P.betle crude extract against A. flavus mycelial growth

Effect of P. betle crude extract against A. flavus mycelial growth and toxin biosynthesis. The results, evaluated by measuring the mycelial growth and quantification of aflatoxin B1(AFLB1) production in broth medium revealed that chloroform soluble compounds extract from *P. betle* dried leaves was able to block the aflatoxin biosynthesis pathway at concentration of 800ul without a significant effect on mycelium growth. A significant reduction in aflatoxin B1 production was observed for *P. betle* chloroform fraction.

This fraction at 800µg/ml caused a reduction (91%) in mycelial growth and completely inhibited toxin biosynthesis by A. flavus in present experiment.

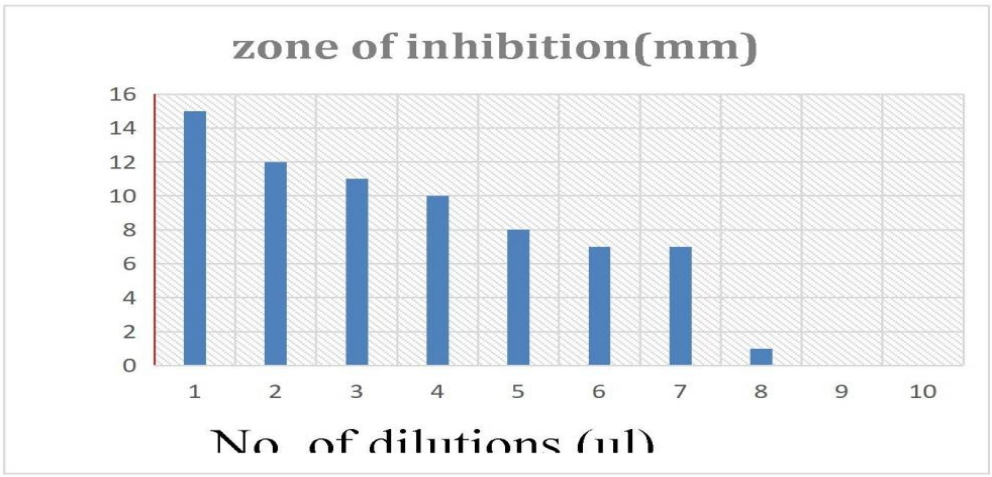

Fig6: Graph of Effect of P. Betle crude extract against *A.flavus*(mm).

Other plant extract we used were aloevera, and pepper mint (Mentha piperita) leaves extract which were found to be inhibiting the growth of *A.flavus* by giving zone of inhibition of 12mm and 2 mm respectively.

Effect of spices extracts on fungal growth

Antifungal activity of aqueous extracts of different spices was tested against aflatoxigenic *A.flavus* by using agar well diffusion method. A total of four different aqueous spices extracts were used to evaluate their antifungal activity. Various concentrations of extracts were tested to find out the inhibitory effect by measuring zone of inhibitions. All extracts have shown inhibition against *A.flavus* at highest concentrations. The activity of each extract was concentration dependent and inhibitory effect of all extracts increased as the concentration increases. black pepper and bay leaves were consistent with their antifungal activity and their activity became zero at low concentration of extract.

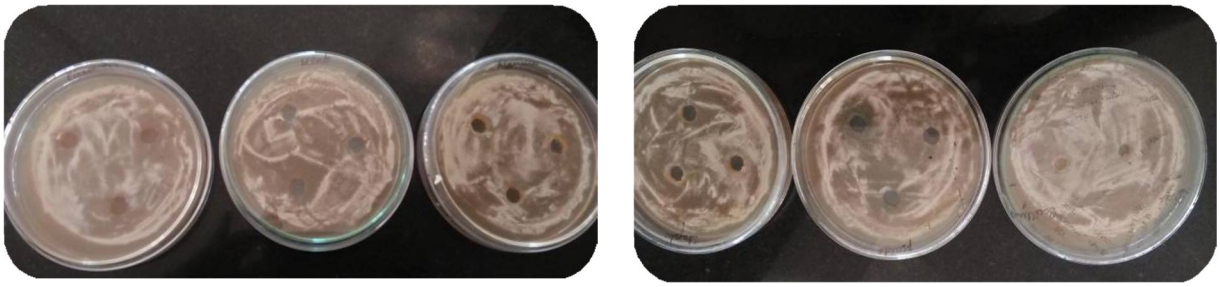

Fig 7: Effect of spices extracts on fungal growth

Effect of Spices extracts on Aflatoxin (AFs) production:

A. flavus was also tested to evaluate anti-aflatoxigenic activity of these spices extracts. It was grown on CzapekDox Medium (Oxoid, UK) supplemented with different concentrations of extracts not inhibitory to fungal growth. AFs were extracted and analysed on TLC plates, and AFB1and AFB2standards were used as positive controls. AFB1and AFB2were not completely inhibited by black pepper, clove, cinnamon, bay leaves extracts at all concentrations.

Fig8 TLC of spice extract grown in Czapek Dox Medium.

DISCUSSION

The effect of Aflatoxin on plant models suggests that it inhibits the growth of roots and seedlings to a considerable extent. The potential of aflatoxin in inhibiting seed germination can also be related to its activity in inactivating DNA. In addition to the embryo, the seed contains a reservoir of preformed, essential molecules to sustain the developing embryo and seedling until it becomes nutritionally self-sufficient. Anti-metabolites, such as aflatoxin, interfere with the synthesis rather than the utilization of metabolites and can only exert their toxicity when biosynthesis becomes essential to growth after the supply of preformed metabolites has been exhausted. When this occurs, continued growth of the seedling ceases or becomes dose dependent if the toxicant functions competitively by binding to an essential molecule such as DNA. Fungi are commonly distributed as environmental contaminants. These fungi produce toxic compounds known as mycotoxins. Mycotoxins contaminate variety of agricultural products such as cereals, grains, spices, etc. Among several types of mycotoxins, aflatoxins (AFs) are considered to be the most important in terms of their toxicity and spectrum of activity. AFs are the secondary metabolites produced by some *Aspergillus species*, such as *A. flavus* and *A. parasiticus*. AFs have been considered as one of the most carcinogenic substances.

Spices and herbs have been used to enhance the flavor and aroma of food for thousands of centuries. Spices are commonly used as natural antimicrobial agents in foods. This study demonstrated the antifungal evaluation of four different aqueous spices extracts i.e. black pepper, clove, cinnamon andbay leaves against *A.flavus* strain. Several studies reported that the phenolic antioxidants compounds play a significant role in antimicrobial activity of spices, it has been found to act as free radicals cavengers as well as metal chelators (19). The rest of the extracts havealso shown an effective activity against fungal growth which diminished at lower concentration of extracts.

Several studies have reported that the extracts of herbs possess antifungal activities against *A.parasiticu*)(20).

Our findings suggest that the aqueous extract are equally effective and have shown good antifungal activity. It was observed in this study that these spices extracts are also very effective against fungal strains. AFs contamination in many food and feed stuffs and agricultural commoditiesis the most important problem and may vary with environmental conditions, production and storage practices. The anti-aflatoxigenic activity of four different aqueous spices extracts were examined for their effects on AFs production by TLC. For AFs inhibition, sub-optimal concentrations of extracts, i.e. the concentration below the inhibitory value of extracts, have been used. The extracts of cinnamon have shown complete inhibition of AFB1and AFB2except the lowest, concentration. It might be possible that these extracts do not target the synthesis machinery of the cell and only damage the structural components of an organism result in growth inhibition.

It is widely accepted that the use of artificial chemicals to control post-harvest diseases has been limited due to their carcinogenicity, teratogenicity, long degradation period, environmental pollution and their adverse effects on food and human health. Inactivation of AFs through physical and chemical means has not yet proved to be effective and economically feasible (17). Instead of chemicals, natural spices extracts may provide an alternative to protect foods or feeds from fungal contamination and AFs production. Our study endorses the idea that these spices extracts will most likely to be the best to use as food additives in different food industries to prevent contamination and deterioration by aflatoxigenic *Aspergillus*strains .Post-harvest treatments to remove aflatoxins such as alkalization, ammonization, and heat or gamma radiation are not generally used by farmers. However, some of the microorganisms naturally present in soil have the ability to degrade and reduce the aflatoxins contamination in different types of agricultural produce. Therefore, methods of using these organisms to reduce aflatoxins are currently being focused on.

CONCLUSION

The results showed that the certain phenolic compounds are able to decline the aflatoxins production in *A.flavus* with no significant effect on the fungus mycelial growth. The result also suggested *P.betle* could be used as potential antitoxin product.

The phytochemicals extracted from *P.betle* inhibited the toxin production via effect on toxin biosynthesis pathway with less effect on fungus mycelial growth. This result was in concordance with certain phenolic compounds when added to *A.flavus* media, the aflatoxins production significantly declines causingno effect on fungal growth. Maggon (18)showed that aflatoxins production was no trelated to fungal mycelium growth.

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CITATION OF THIS ARTICLE

Dr. J. K. Oberoi, S. P.Tambe, A. Shaikh, S. Pathan, M. More and Nisar Tamboli: Inhibition of Mycelial growth and Aflatoxin biosynthesis in *Aspergillus flavus* by extract of various spices and herbs. Bull. Env.Pharmacol. Life Sci., Spl Issue [1]: 2023: 327-334.