



## Antifungal activity of Popular Indian herbs against *Aspergillus flavus*

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**Abstract :** *Aspergillus* belongs to the kingdom Fungi are filamentous fungi which have a wide species diversity. Among the pathogenic species of *Aspergillus* are *Aspergillus fumigatus*, *Aspergillus niger* and *Aspergillus flavus*. *Aspergillus flavus* is cosmopolitan in distribution and causes pre-harvest and post-harvest infections. It produces a toxic compound known as mycotoxin which when consumed by humans and animals is toxic. The current research was focused on isolation and identification of *Aspergillus flavus* from the stored peanut and other food materials and its control using some medicinally important plant extracts such as

*Syzygium aromaticum*, *Ocimum basilicum*, *Azadirachta indica*, and *Glycyrrhiza glabra*. All extracts were effective against *Aspergillus flavus* and were able to reduce the mycelial growth at 1% concentration and also they were able to reduce the production of secondary metabolite, aflatoxin as observed by thin layer chromatography (TLC). The most effective extract was clove ethanolic extract which completely diminished mycelia growth at 1% concentration.

**Keywords:** *Aspergillus flavus*, Mycotoxin, aflatoxin, Thin layer chromatography (TLC)

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### Introduction:

*Aspergillus flavus* belongs to the kingdom Fungi and it is a saprophytic and pathogenic (Machida et al., 2010) fungus which has a cosmopolitan distribution (Ramirez-Camejo et al., 2012). It is widely known for its colonization on cereal grains, legumes and tree nuts. *Aspergillus flavus* is a thermotolerant fungus, therefore, it can survive at temperatures that other fungi cannot (Hedayati et al., 2007). The optimum temperature for the growth of *Aspergillus flavus* is 37°C. The minimum and the maximum growth temperatures are 12°C and 48°C, respectively (Agrios et al., 2005). *Aspergillus flavus* in addition to causing pre-harvest and post-harvest infections, also produces significant quantities of toxic compound known as mycotoxin which when consumed by humans and animals is toxic (Agrios et al., 2005).

Aflatoxins are ubiquitous and they have been isolated from different food and feed used for human and animal consumption (Sinha et al., 1989). They are

poisonous, carcinogenic secondary metabolites produced by certain toxigenic strains of *Aspergillus flavus* and *Aspergillus parasiticus*, while colonizing on different agricultural products, like fruits and vegetables, animal and human eatables of animal origin, like meat, eggs, milk produced by livestock and poultry, when fed with aflatoxin contaminated food (Bennett et al., 2003; Herzallah et al., 2013). Aflatoxins produced by *Aspergillus* species have been recognized as one of the most potent teratogens (Wagnikar et al., 2005), carcinogens (Harris et al., 1984), mutagens (Ong et al., 1975) and immunosuppressant (Norbury et al., 1985) and also cause some serious metabolic disorders which result into the damage of heart, lungs and kidney which finally causes death in humans as well as in animals (Herzallah et al., 2013). Thus, aflatoxins constitute an international health hazard problem as well as a fascinating scientific challenge for the scientists. The food products that are contaminated with the toxigenic fungi and the presence of aflatoxin in the food products is a major concern. This has received worldwide attention due to its deleterious effects on the health of both humans as well as animals (Mishra et al., 2003).

In the present time there is a vogue for herbal medicines and drugs as about 80% of the Asian and African communities rely on the traditional herbal medicines for the primary health care. There are evidence of the efficiency of many crude herbal plant products which are being used by tribal and traditional societies in preventing and curing different kinds of disease. The control of aflatoxin has become a global problem in spite of a variety of physical and chemical practices to counteract the mycotoxin (Samarajeeva et al., 1990).

Natural plants are a reservoir of various antimicrobial components and most of them are unexplored. Hence the role of natural plant extracts and their antifungal properties against *Aspergillus* species becomes an important area of research (Hosseini et al., 2013). Our research focuses on the antifungal activity of popular Indian herbs against *Aspergillus flavus* which is responsible for the huge loss of agricultural crops and stored food products thus affecting human health. The aim of this research is to study the antifungal effects of *Syzygium aromaticum*, *Ocimum basilicum*, *Azadirachta indica*, and *Glycyrrhiza glabra* extracts against *Aspergillus flavus* and aflatoxin production.

Antifungal activity of Indian herbs against *Aspergillus flavus* can be used to prevent the contamination of various agricultural products and

stored food thus preventing humans and animals from the hazardous effects of the food contaminated with toxins produced by *Aspergillus flavus*. The conventional technique used to detect aflatoxins is High Performance Liquid Chromatography (HPLC) (Morshdy et al., 2015) but thin layer chromatography can also be used to detect the aflatoxin secreted in the medium while growing the culture of *A. flavus*.

#### Methodology :

**Isolation and identification of *Aspergillus flavus*:** *Aspergillus flavus* was isolated from contaminated peanuts according to Rajarajan et al., 2013). The collected peanuts were disinfected using 2.5% sodium hypochlorite for three minutes, rinsed three times with sterile distilled water and dried between layers of sterile filter paper. Surface sterilized peanuts were plated out on Potato dextrose agar (PDA) under sterile condition and incubated at 25 °C for 5 days. Sub-culturing was done several times to get a pure culture of *A. flavus*. The species was confirmed based on the colony and morphological characteristics.

**Plant material and Plant extract preparation :** In the present study, five different commonly used herbs have been selected (Table 1) to analyze their effectiveness on the growth of *Aspergillus flavus*. The ethanolic extracts of *Syzygium aromaticum*, *Ocimum basilicum*, *Azadirachta indica*, glabridin and *Glycyrrhiza glabra* were prepared by following method : These were washed with distilled water to separate their waste materials, and then shade dried. After drying, they were grounded using mechanical grinder (with low heating), and then coarse particles were removed by passing through a sieve and the obtained powder was used for extract preparation. For herbal extract preparation, 2.5 gm of ground plant material was mixed with 25 ml of 70% ethanol for 24 hours with shaking condition. The extracts were then filtered using Whatmann filter paper No.1 and sterilized (Shanmugam et al., 2010) and used for the antimicrobial assays. Glabridin was available in pure (62%) form so it was just dissolved in ethanol as per concentration 20mg/ml.

**Antifungal susceptibility testing of the plant extracts using Well Diffusion Assay:** Antifungal activity of the herbal plant extracts was determined by Agar well diffusion method according to National Committee for Clinical Laboratory Standards (NCCLS). Inoculum containing the fungal culture of *Aspergillus flavus* was spread on the PDA plates with a sterile swab moistened with the fungal suspension. Subsequently, three wells were punched into the agar medium to which plant extract, solvent (negative control) and antifungal

Fluconazole were added separately in wells. The plates were then incubated at 28°C for 48 hours. After incubation, the diameters of inhibition zones were measured in mm. The experiments were performed in triplicates.

#### **Effect of plant extracts on mycelia biomass:**

The isolated fungus was propagated as a pure culture in 100 ml Potato dextrose broth which served as control for mycelia biomass estimation. Fungus was inoculated with 1% of each herbal extract in separate flask namely, clove extract, neem leaves extracts, basil leaves extract, Glycyrrhiza glabra root extract and glabridin to estimate the inhibition of mycelia biomass by these plant extracts. Then the flask was incubated on shaker incubator for 10 days at 28°C and examined for the presence of mycelia and spores. After 10 days of incubation the content of the each flask was filtered with Whatman filter paper No. 1. Biomass was weighed and correlated with the control.

**Production and Extraction of Aflatoxin from the culture medium :** The pure culture of *Aspergillus flavus* was inoculated in PDA broth for aflatoxin production (Munimbazi et al, 1998) and allowed to grow with and without the presence of herbal extract. The fungal broth culture were filtered and aflatoxin were extracted from the culture filtrate with the help of chloroform (Kumar et al, 2010). 25 ml of the filtrate was added to 10 ml of chloroform and was shaken for half-an-hour. The chloroform containing aflatoxin was separated using a separating funnel which was left for sometimes until the two layers appeared. The upper aqueous layer was re-extracted several times with chloroform for complete separation.

**Thin layer chromatography (TLC) analysis for aflatoxin :** Thin layer chromatography was performed to detect aflatoxin (Calvo et al, 2004). The fungal culture was grown in broth medium along with 1% herbal extract in different flasks for 10 days at 28°C. fungal culture without plant extract served as control. After 10 days it was filtered and the filtrate was used for aflatoxin separation by thin layer chromatography technique. The spot of different culture filtrate was spotted on TLC and was sprayed with Ninhydrin. Aflatoxin was detected as purple spots.

#### **Results and Discussion :**

Herbs have been a reliable source for the drug and plant extracts can also be used for the inhibition of growth of various microorganisms. The *Aspergillus flavus* was isolated on PDA media from infected peanuts. Fig.1 shows the characteristic growth of the *A. flavus* with light green spores whereas Fig. 2 shows the microscopic view of it. The ethanolic extracts of all the

plant extracts used for the study had the detrimental effect on the growth of the *Aspergillus flavus* as indicated by the inhibition zones in well diffusion assay. Representative Plate which contains inhibition zone by *Syzygium aromaticum*, fluconazole and solvent has been shown as Fig. 3. Zone of inhibitions are presented in the Table-2 and statistically correlated. The zone of inhibition determines the effectiveness of the herbal plant extracts used to determine the antifungal activity. Higher the zone diameter more potent is the plant extract against that microbe. The ethanolic extract of clove (*Syzygium aromaticum*) was found to be highly effective in inhibition of *Aspergillus flavus* growth and showed the maximum zone of inhibition than any other plant extract used in this study. The weight of the mycelial mats obtained after 3<sup>rd</sup> day, 6<sup>th</sup> day and 10<sup>th</sup> day were calculated and tabulated in table 3. The mean of mycelia mat shows that the least growth was observed in the broth supplemented with 1% ethanolic extract of clove followed by neem, glabridin, *G. glabra* and tulsi, respectively increasing the mycelia weight. Fig. 4 shows the growth of the *Aspergillus flavus* in PDA broth without plant extract. These extracts were able to inhibit the mycelia growth as well as they were able to inhibit the aflatoxin production which was showed by thin layer chromatography (Fig.5).

#### **Conclusion :**

Most of the time, peanut gets spoiled during storage and is responsible for the bitter taste and low quality. Similarly, many other food grains, meat and poultry products also get spoiled during storage. This leads to greater loss of various food products as well as agricultural crops due to the production of wide variety of mycotoxin, especially aflatoxin produced by *Aspergillus flavus*. The source of contamination is not only during storage but it may come from field, during harvesting and processing. An effective way to control the problem caused by aflatoxin is by preventing the growth of fungi on different food products. The present study proposes the effectiveness of different plant extracts against *Aspergillus flavus*. Among them clove was the most potent inhibitor and can be further explored to control the *Aspergillus flavus* infection and aflatoxin production in food crops by using as natural chemical spray or during any step of processing of these foods.

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**Table 1: List of plant species tested for antifungal activity**

SL. No.	Plant Name		Plant part used	Family
	Common Name	Scientific Name		
1.	Liquorice	<i>Glycyrrhiza glabra</i>	Roots	<i>Fabaceae</i>
2.	Liquorice roots	<i>Glabridin</i>	Roots	<i>Fabaceae</i>
3.	Clove	<i>Syzygium aromaticum</i>	Fruit	<i>Myrtaceae</i>
4.	Basil	<i>Ocimum basilicum</i>	Leaves	<i>Lamiaceae</i>
5.	Neem	<i>Azadirachta indica</i>	Leaves	<i>Meliceae</i>

**Table 2: Zone of inhibition (In mm) by different plant extracts (Mean±SD)**

Inhibitory Agents	Zone of inhibition (in mm) (mean±SD)					
	Fluconazole	<i>Glycyrrhiza glabra</i>	<i>Glabridin</i>	<i>Syzygium aromaticum</i>	<i>Ocimum basilicum</i>	<i>Azadirachta indica</i>
<i>A. flavus</i>	29.83±0.2887	10.90±0.1000	22.10±2.455	21.52±5.001	19.40±0.100	21.90±0.1000

**Table 3: Weight of the mycelium at different time interval (in days)**

Plant extracts	Weight of the mycelium			
	3 <sup>rd</sup> Day	6 <sup>th</sup> Day	10 <sup>th</sup> Day	Mean
Control	1.2 g	2.7 g	3.45	2.45 g
<i>Glycyrrhiza glabra</i>	0.5 g	0.82 g	1.42 g	0.91 g
<i>Glabridin</i>	No growth	0.14 g	0.44 g	0.19 g
<i>Syzygium aromaticum</i>	No growth	No growth	0.2 g	0.06 g
<i>Ocimum basilicum</i>	0.4 g	1.6 g	2.2 g	1.4 g
<i>Azadirachta indica</i>	No growth	0.1 g	0.31 g	0.13 g

## LIST OF FIGURES



Fig. 1. *Aspergillus flavus* on PDA media

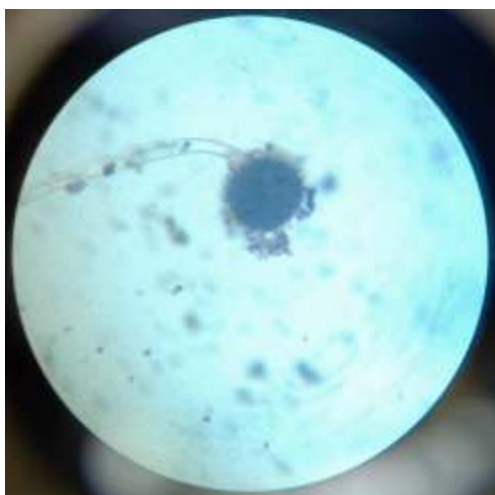


Fig. 2. Microscopic view of *Aspergillus flavus*

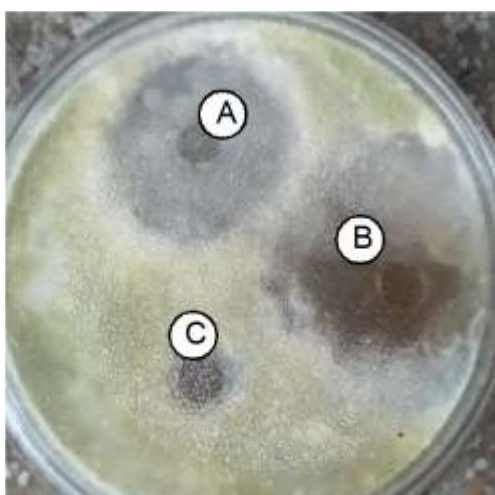


Fig. 3. Inhibition zone of the antifungal (fluconazole) -A, *Syzygium aromaticum* extract- B and solvent (ethanol)-C



Fig. 4. *Aspergillus flavus* after 10 days incubation in PDA broth

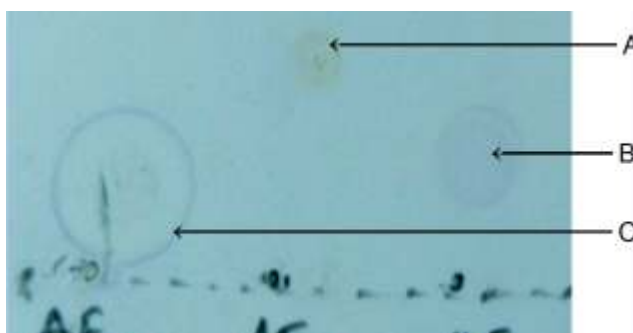


Fig. 5. Ninhydrin reacted Spots of aflatoxin in purple colour – A) Aflatoxin produced with 1% neem extract B) Aflatoxin produced with 1% *G. glabra* C) Aflatoxin produced without plant extract.

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