

Inhibitory effects of natural spices extracts on *Aspergillus* growth and aflatoxin synthesis

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Abstract

Aflatoxins are naturally occurring toxic metabolites produced by many *Aspergillus* particularly *Aspergillus flavus* and *Aspergillus parasiticus* in food and feed products. These toxins are hepatotoxic and mutagenic causing severe health disorders including hepatitis and hepatocellular carcinoma (HCC). Various physical and chemical strategies have been introduced to control the growth and aflatoxin production. However, due to safety concerns and potent toxicity of chemicals, researchers are seeking for cost-effective and non-toxic alternates of plant origins to prevent deterioration of grain crops. The aim of this study was to check the effect of natural spices products such as aniseed, black cummin, black pepper, cardamom and turmeric extracts on the growth of pathogenic strains and their ability to produce aflatoxin contamination. Antifungal activity of spices including black cummin, turmeric, black pepper, cardamom and aniseed were performed against aflatoxigenic strain of *Aspergillus parasiticus*. Water soluble extracts of these spices were prepared, agar diffusion assay was performed and zone of inhibition was measured after applying different concentrations of extracts against the above strain. Subsequently, aflatoxin inhibition assay was performed by inoculating sub-inhibitory concentrations of these extracts with aflatoxigenic strain in Czapek Dox liquid medium. After incubation, thin layer chromatography (TLC) was performed to detect and quantify aflatoxins. All extracts have shown concentration dependent antifungal activity and best results were obtained with cardamom which was active even at 0.005% concentration. In addition, it was also observed that the synthesis of toxin was inhibited by all extracts and maximum activity at the concentration of 0.00025% was shown by black cummin, black pepper and aniseed. 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; *Aspergillus* species; Natural extracts; Antifungal.

Introduction

Aflatoxins (AFs) are classified as the most important subgroup of mycotoxins. These toxins are carcinogenic and may cause acute and chronic outcomes including liver damage, immunological consequence and may leads to liver cancer (William et al., 2004). AFs were first identified in the early 1960s in England as the causative agent in turkey X disease that destroyed more than 100,000 turkey fowl (Khun and Ghannoum, 2003). Several types of AFs are produced, however aflatoxin B₁ (AFB₁), B₂ (AFB₂), G₁ (AFG₁) and G₂ (AFG₂) are the four most important and naturally occurring AFs. AFB₁ is considered as the most common naturally occurring carcinogenic compound (IARC, 2002).

Aflatoxin contamination is a major problem that gradually turns more important due to their association in crop production, food quality and in animal and human health. The problem of AFs contamination is more serious in developing countries due to suitable climate, agricultural practices and storage conditions for the proliferation of fungus and toxin production (Aziz and Moussa, 2004). Several strategies have been established including various chemical and physical methods to prevent AFs contamination in susceptible foodstuff, plants and crops (Cleveland and Bhatnagar, 1992). Physical methods including the use of UV

light, treatment with heat, and ionizing radiations are not completely useful. The application of chemical compounds has directed to several health and ecological problems due to their residual toxicity, carcinogenicity, hormonal imbalance and spermatotoxicity (Kumar et al., 2007; Pandey, 2003). To inhibit AFs biosynthesis by aflatoxigenic *Aspergillus*, there is a need to design novel and environmentally safe methods of reducing infection (Razzaghi-Abyaneh, 2009). Adequate control measures to prevent spoilage of grains and foodstuffs are essential to avoid contamination and decrease public health hazards. Natural plant extracts could provide an alternative to chemical preservatives. Recently, there is an increased interest to control the fungal growth and toxin production by using naturally occurring compounds.

Spices are used in the food industry to enhance flavors and fragrances. Spices and herbs are usually considered harmless and proved to be efficient against diseases (Hema et al. 2009). Moreover, they also express beneficial antimicrobial properties (Nychas and Skandamis, 2003). Medicinal and antimicrobial properties of natural extracts have been well-studied. These extracts can provide potential alternatives to control post-harvest fungal deterioration of different crops. Several herb extracts may possibly inhibit the biosynthesis of

AFs by controlling the growth of fungi and mycotoxin production (Steinhart et al., 1996). These compounds are safe to humans, because they have been used as a preservative, flavoring the food and for their antioxidant and medicinal properties (Hesseltine, 1965; Nakatini, 1994).

So, The aim of this study was to evaluate the antifungal activity of some natural spices extracts i.e. black cumin, turmeric, black pepper, cardamom and aniseed on the growth of *Aspergillus* species and AF production. The findings achieved during the research have been contributed to the safety assessment of food and agricultural commodities being exposed to AFs contamination.

Results

Effect of spices extracts on fungal growth

Antifungal activity of aqueous extracts of different spices was tested against aflatoxigenic *A. parasiticus* species, by using agar well diffusion method. A total of five (05) 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. The results of all spices extracts are summarized in Table 1. The graphical data representing the zone of inhibition (mm) of individual extracts at different concentrations are illustrated in Fig. 1 (a-e).

All extracts have shown inhibition against *A. parasiticus* at highest concentrations. The activity of each extract was concentration dependent and inhibitory effect of all extracts increased as the concentration increases. Highest percent inhibition was observed when 1% cardamom and 1% aniseed extracts were applied in wells of their respective plates (Fig. 1d, 1e). Furthermore, cardamom was showing its inhibitory effect at lowest concentration of 0.005%. As far as aniseed activity was concerned, it was showing good antifungal activity at higher concentrations, but the activity was diminished at lower concentrations. Black cumin, black pepper and turmeric were consistent with their antifungal activity and their activity became zero at 0.005% concentration of extract (Fig. 1a, 1b, 1c).

Effect of Spices extracts on Aflatoxin (AFs) production

A. parasiticus was also tested to evaluate anti-aflatoxigenic activity of these spices extracts. It was grown on Czapek Dox Medium (Oxoid, UK) supplemented with different concentrations of extracts not inhibitory to fungal growth. AFs were extracted and analysed on TLC plates, and AFB₁ and AFB₂ standards were used as positive controls. AFB₁ and AFB₂ were completely inhibited by black cumin, black pepper and aniseed extracts at all concentrations except the lowest i.e. 0.00005%. On the other hand, turmeric and cardamom had shown inhibition only at higher concentrations i.e. 0.005% and 0.0025% respectively, and its AFs inhibition activity was diminished at lower concentrations. The minimum inhibitory concentrations of all extracts against AFs synthesis are presented in Table 2.

Discussion

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 five different aqueous spices extracts i.e. black cumin, turmeric, black pepper, cardamom and aniseed against FH44 *A. parasiticus* strain. In this study, all of the extracts have shown antifungal activity at different levels in which the best antifungal activity was observed in cardamom even at the minimum concentration of 0.005%. It contains high level of phenolic contents that's why it has shown significant activity (Kapoor et al., 2008). 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 radical scavengers as well as metal chelators (Shahidi and Wanasundara, 1992; Sanchez-Moreno et al., 1998). Madsen and Bertelsen have revealed that terpenoids also plays significant role in antioxidant activity of spices (1995). Furthermore, it is expected that the antifungal activity of cardamom could be due to the antagonistic or synergistic action of its components. It has also demonstrated that majority of the natural antioxidative compounds frequently work synergistically with each other to produce a broad spectrum of antioxidative actions that produces an efficient defence system against free radical attack (Vardar et al., 2003). Similar results with cardamom were observed against *A. niger* by Hema et al. (2009). The rest of the extracts have also shown an effective activity against fungal growth which diminished at lower concentration of extracts. Several studies have reported that the extracts of fruit and herbs possess antifungal activities against *A. parasiticus* (Soliman and Badaea, 2002; Rasooli and Owlia, 2005). The antifungal activity of *Nigella sativa* seeds (black cumin) extract have been reported in several studies against *A. parasiticus*, *Candida albicans* and *Saccharomyces cerevisiae* (De et al., 1999; Khan et al., 2003). Research has also been done to explore the antifungal activity of turmeric against *A. niger* and *A. flavus* which showed predominant activity against these species (Arora, 1999). Furthermore, ethanolic extract of turmeric also showed inhibition against *Rhizopus stolonifer* and *Mucor* spp (Pundir and Jain, 2010). In contrast to the present study, another study has reported that the ethanolic extract of turmeric was highly effective as compared to hexane and water (Gur et al., 2006). 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 commodities is the most important problem and may vary with environmental conditions, production and storage practices. The production of AFs is mainly influenced by physical and biological factors (Bankole and Adebajo, 2003). The anti-aflatoxigenic activity of five (05) 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 black cumin, black pepper and aniseed have shown complete inhibition of AFB₁ and AFB₂ except the lowest, i.e. 0.00005%. However, black cumin and black pepper extracts

Table 1. Antifungal activity of spices extracts on *Aspergillus parasiticus* isolated from food and feed commodities.

S. No.	Extracts	Concentration of extract (%)						
		0 (Control)	1	0.5	0.1	0.05	0.01	0.005
1.	Aniseed							
	Zone of Inhibition mm. (mean ±SD)	0.0	72.6±2.05 ^a	62.6±3.29 ^b	29.3±4.92 ^c	0.0	0.0	0.0
	Percentage Inhibition %	0.0	80.7	69.6	32.6	0.0	0.0	0.0
2.	Black Cumin							
	Zone of Inhibition mm. (mean ±SD)	0.0	46.3±5.50 ^a	31.0±6.55 ^b	29.0±3.60 ^b	27.0±3.60 ^b	19.3±4.04 ^c	0.0
	Percentage Inhibition %	0.0	51.5	34.5	32.3	30.0	21.5	0.0
3.	Black Pepper							
	Zone of Inhibition mm. (mean ±SD)	0.0	47.3±2.05 ^a	40.0±4.08 ^b	31.0±2.94 ^c	30.3±3.68 ^c	24.3±4.18 ^d	0.0
	Percentage Inhibition %	0.0	52.6	44.5	34.5	33.7	27.0	0.0
4.	Cardamom							
	Zone of Inhibition mm. (mean ±SD)	0.0	74.3±7.58 ^a	64.6±1.69 ^b	60.3±3.68 ^b	49.6±0.45 ^c	25.7±1.53 ^d	17.0±1.73 ^e
	Percentage Inhibition %	0.0	82.6	71.8	67.0	55.2	28.5	18.8
5.	Turmeric							
	Zone of Inhibition mm. (mean ±SD)	0.0	64.0±2.97 ^a	51.6±4.98 ^b	47.3±2.05 ^b	34.0±4.32 ^c	25.7±4.04 ^d	0.0
	Percentage Inhibition %	0.0	71.2	57.4	52.6	37.8	28.5	0.0

Any two means not sharing a letter in a row differ significantly at P<0.05 (LSD) (n=3)

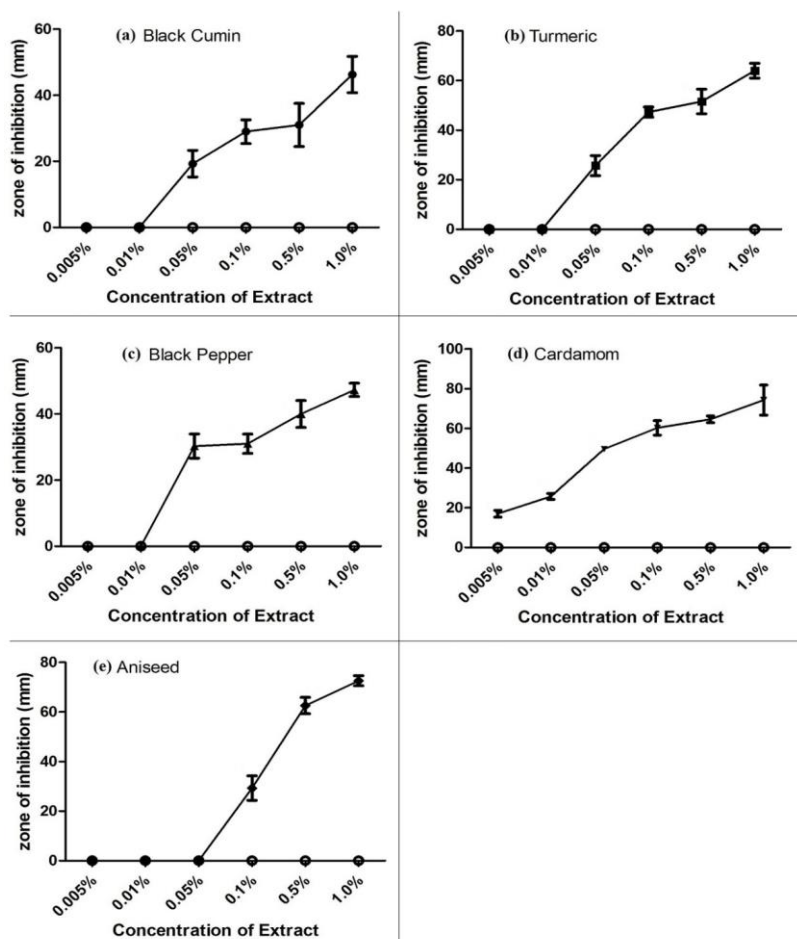


Fig 1. Antifungal activity of a) Black cumin, b) Turmeric, c) Black pepper, d) Cardamom, and e) Aniseed against Aflatoxigenic *Aspergillus parasiticus* on PDA after 07 days of incubation. Different concentrations of extracts were applied and zone of inhibition was measured after incubation.

Table 2. Anti-aflatoxigenic activity of spices extracts on *Aspergillus parasiticus* isolated from food and feed commodities.

S.No.	Extracts	Concentration of extract (%)					
		0 (Control)	0.005	0.0025	0.0005	0.00025	0.00005
1.	Aniseed						
	Aflatoxin B ₁	+	ND	ND	ND	ND	+
2.	Aflatoxin B ₂	+	ND	ND	ND	ND	+
	Black Cumin						
3.	Aflatoxin B ₁	+	ND	ND	ND	ND	+
	Aflatoxin B ₂	+	ND	ND	ND	ND	+
4.	Black Pepper						
	Aflatoxin B ₁	+	ND	ND	ND	ND	+
5.	Aflatoxin B ₂	+	ND	ND	ND	ND	+
	Cardamom						
6.	Aflatoxin B ₁	+	NT	ND	+	+	+
	Aflatoxin B ₂	+	NT	ND	+	+	+
7.	Turmeric						
	Aflatoxin B ₁	+	ND	+	+	+	+
8.	Aflatoxin B ₂	+	ND	+	+	+	+

(ND = Toxin Not Detected, + = Toxin Detected, NT = Not Tested)

have shown moderate antifungal activity, but they have potential to inhibit the production of AFs. As far as the aniseed activity was concerned it has shown good antifungal activity only at higher concentrations but they have inhibited the AFs production at all concentrations. On the other hand, turmeric and cardamom had shown inhibition only at higher concentrations i.e. 0.005% and 0.0025% respectively, and its AFs inhibition activity was diminished at lower concentrations. Although, turmeric and cardamom have shown good antifungal activity, they are not able to inhibit the AFs production in lower concentrations. 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. Similarly, turmeric leaf oil showed toxin inhibition at 1.0% and 1.5% (Sindhu et al., 2011). The level of AFs inhibition and fungal growth was dependent on the essential oil concentration. It has been shown that the essential oils mainly target the cell membrane and cell wall (Nazzaro et al., 2013). In case of thyme essential oil at 250 ppm, the plasma membrane of *A. parasiticus*, was seen to be irregular and invaginated. Such variations might be related to the interference of essential oil components with enzymatic reactions of cell wall synthesis, which influences fungal morphogenesis and growth (Abdolamir et al., 2011).

Materials and Methods

Fungal strain

Several food and feed samples were screened, as reported earlier, for the presence of aflatoxigenic *Aspergillus* strains (Ibrahim et al., 2016). *Aspergillus parasiticus* (FH44) was selected for the experiment on the basis of its toxicity. This strain has shown the highest amount of AFs when isolated from a sample of maize (Ibrahim et al., 2016). Inoculum was prepared according to the method described by Ghadeer and Al-Delamiy (2012). Briefly, the growth of *Aspergillus* species in a petri plate was washed with 10ml sterile distilled water, 10-fold serial dilutions were made and spore numbers were adjusted to 10⁵ spores/ml.

Extraction of spices

Several raw spices products were used to check the effects of these products on aflatoxigenic *A. parasiticus* species. These include: black cumin (*Nigella sativa*), turmeric (*Curcuma*

longa), black pepper (*Piper nigrum*), cardamom (*Elettaria cardamomum*) and aniseed (*Pimpinella anisum*). These spices were purchased from local market in Karachi-Pakistan. Spices were washed with deionized water and dried in oven at 50°C for 24 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, 0.1, 0.05, 0.01 and 0.005% were prepared to determine the minimum inhibitory concentration. The left over dried extracts were preserved in a sterile vial for future use.

Antifungal Activity

Agar well diffusion method: Agar well diffusion method was performed as reported earlier by Pundir and Jain with slight modification (2010). Potato dextrose agar (Oxoid-UK) 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 micro litre 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. All combinations were tested on duplicate plates and all experiments were performed in triplicate. After incubation, plates were observed for any zone of inhibition surrounding the wells containing the spices extract. The zone of inhibition was measured; mean and standard deviation of the diameter were calculated. Furthermore, percent inhibition was calculated as described by Singh and Tripathi using the following equation (1999);

$$\text{Inhibition (\%)} = \frac{dc - dt}{Dc} \times 100$$

Where, d_c is the average diameter of mycelia colony of control and d_t is the average diameter of treated plate with extract.

Aflatoxin inhibition assay: The efficiency of spices extracts to reduce AFs production were evaluated by the method stated by Zohra and Fawzia (2013). Czapek Dox liquid medium (Oxoid, UK) was prepared, supplemented with different concentrations of extracts separately, followed by the inoculation of fungal spore suspension (10^5 spores / ml). Sub-inhibitory concentration i.e., concentrations of each spices extracts below inhibitory concentration were used to evaluate their effect on AFs production. Positive control was also prepared, contained only spore suspension, the flasks were incubated at $25 \pm 2^\circ\text{C}$ for 10 days.

After incubation, thin layer chromatography (TLC) was performed as described earlier for detection and quantification of AFs (Asghar et al., 2014). Dried extracts were suspended in 100ml of benzene (Merck, Germany):acetonitrile (Merck, Germany) (98:2; v/v) and shake well. Samples were spotted as 2, 5 and 10 μ l and standards were separately applied on the TLC plates on imaging line about 4cm from bottom edge. The plates were developed in unlined tank containing 20ml of chloroform (Merck, Germany): xylene (Merck, Germany): acetone (Merck, Germany) (6:3:1; v/v) and was observed under long wavelength UV light ($\lambda= 254$ and 366nm) in UV visualizer. The retention factor (Rf) of each AFs (B_1 , B_2 , G_1 or G_2) was calculated according to the following equation.

$$\text{Rf} = \frac{\text{Distance moved by compound}}{\text{Distance moved by solvent}}$$

Furthermore, all positive results of AFs, naturally existing in the tested samples, were confirmed by spraying the TLC plates using 50% (v/v) H_2SO_4 (Sigma-Aldrich, USA). All combinations were tested in duplicate and all experiments were repeated three times.

Statistical analysis

As mentioned earlier, all experiments were performed in triplicate. Mean and standard deviation was calculated and treatment results of antifungal activity were statistically evaluated using one-way ANOVA through Graphpad Prism5 software. Mean separation test was done using least significance difference (LSD) (Steel et al. 1997).

Conclusion

It is widely accepted that the use of artificial chemicals to control postharvest diseases has been limited due to their carcinogenicity, teratogenicity, long degradation period, environmental pollution and their adverse effects on food and human health. Moreover, Inactivation of AFs through physical and chemical means has not yet proved to be effective and economically feasible (Mishra and Das, 2003). 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.

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