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Effect of selected spices against *Aspergillus flavus* of pistachio (*Pistachia vera* L.) *in vitro* condition

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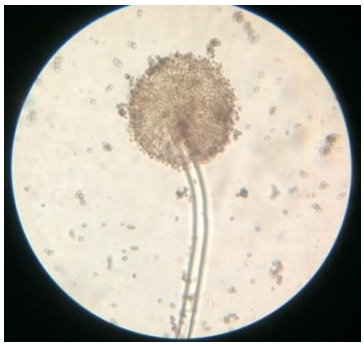
Abstract
Aspergillus flavus is one of the most common and worldwide pathogen which is infected pistachio nuts. The antifungal activity of three selected spices powder namely *Cinnamomum tamal* (Bay leaf), *Cinnamomum rerum* (Cinnamon) and *Cuminum cyminum* (Cumin) at 2 and 5 % concentration was tested for management of *Aspergillus flavus in vitro*. In the present investigation the highest inhibition of mycelial growh of *Aspergillus flavus* was achieved by Bay leaf (94.00%) followed by Cinnamon (72.00%) and Cumin (53.00%). Among all selected spices evaluated, Bay leaf caused the highest reduction of mycelial growth of *Aspergillus flavus* while *Cumin* caused the lowest inhibition of mycelial growth of the pathogen.
Thus the present study revealed that selected spices have shown significant inhibition and proved to be cost effective and eco-friendly for the management of *Aspergillus flavus.*

Keywords: *Aspergillus flavus*, selected spices

Introduction
Pistachio nut (*Pistachia vera* L.) is a favorite tree nut worldwide. Pistachio trees are widely cultivated in saline, hot and dry areas of Mediterranean countries, the Middle East and the USA. Iran has a significant share of the worldwide pistachio production, followed by the USA, Turkey, Syria, China, Greece and Afghanistan (FAOSTAT, 2008; Zheng *et al*., 2012) [6, 12].
Pistachio kernels are a rich source of oil and essential fatty acids for humans depending on the variety, the oleic acid content of pistachio kernels varies between 51.60-67.86%, with linoleic acid contents between 11.56-27.03% in pistachio kernels (Tsantili *et al*., 2010) [10].
Foods of plant origin, such as tree nuts and fruits, are known to foster the growth of various microorganisms including toxigenic and pathogenic fungal species. Tree nuts in particular have been often reported to contain potentially toxigenic molds such as *Aspergillus flavus, A. parasiticus, A. niger,* and other species capable of flourishing in this type of substrates.
Among all mycotoxins, aflatoxins (AFs) are the most important groups and attract a significant amount of attention due to their mutagenic, carcinogenic and teratogenic effects (Bhat *et al*., 2010) [3]. In warm and hot climates, AFs are predominantly produced by *Aspergillus* spp., such as *Aspergillus parasiticus* and *Aspergillus flavus* (AfsahHejri *et al*., 2013a, 2013b) [2]. Aflatoxin B1 (AFB1) is the most toxic aflatoxin with the highest carcinogenic effects (Afsah-Hejri *et al*., 2011) [1].
As a result of early splitting, damaging or cracking of the pistachio nut shell, Aspergillus spores can enter and infect the kernel (Sommer *et al*., 1986) [9]. *Aspergillus* spores enter through the split hull and colonize between the kernel and coat, where the relative humidity is high enough to support spore germination (Mahoney and Rodriguez, 1996) [7]. Infected kernels can rapidly accumulate AFs under high relative humidity and temperature (Diener *et al*., 1987) [4]. More than 80% of pistachio nuts split before harvest (in some varieties up to 94%) (Tsantili *et al*., 2010) [10], making the kernel more susceptible to *Aspergillus* invasion and aflatoxin contamination. Even small numbers of spores can result in high levels of AFs (some times more than the permitted level) under favorable growth conditions. Aflatoxin contamination of pistachio nuts significantly reduces the quality and value of pistachios and directly affects farmers and consumers. Product recall and bans from international export are problems associated with high levels of aflatoxin contamination in pistachios (Doster and Michailides, 1994; Ellis *et al*., 1991) [5]. According to the latest European Commission regulation, the maximum permitted level for nut products has been set at 2 ng/g for AFB1 and 4 ng/g for total AFs (Pearson *et al*., 1999; EC 1998; Sobolev, 2007) [8].

Materials and Methods
The experiment was carried out during 2015-2016 at the Lab of Department of Plant Pathology, Sam Higginbottom Institute of Agriculture, Technology & Sciences (deemed- to- be university) Allahabad, Uttar Pradesh, India.



Conidium of *Aspergillus flavu*

2.1. Isolation and identification of the pathogen
The infected pistachio nuts were cut into small pieces of two to three mm dimension in a manner so that pieces had some green portion also. Such nut bits was surface sterilized with

0.1 per cent mercuric chloride ($HgCl_2$) solution for 30 seconds and washed three times with sterile distilled water to remove any traces of mercuric chloride adhered with nut bits. 2-3 nut bits were transferred on PDA medium contained in sterilized Petri plates with the help of forceps. These Petri plates were incubated at 27˚C± 2˚C, after 3 days mycelia growth was observed around nut bits. With the help of cork borer from this colony growth a portion from the periphery having single hyphal tip was separated and transferred to other Petri plates having medium to get pure culture and identification of the pathogen were confirmed by observing the morphological features of colony, spore characteristics and referring the relevant literature.

2.2. Preparation of plant extracts

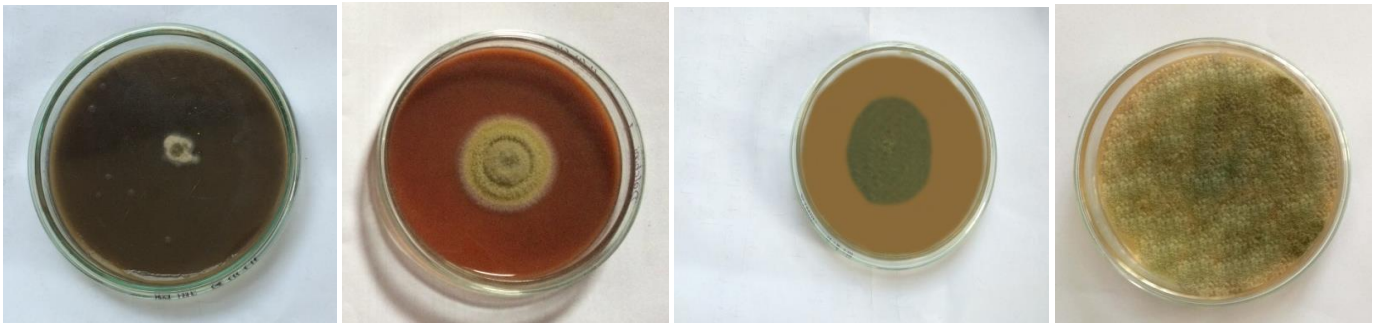
Three selected spices such as Bay leaf (*Cinnamomum tamala*), Cinnamon (*Cinnamomum verum*) and Cumin (*Cuminum cyminum*), were used in this procedure. Each plant seeds leaves and barks were collected then washed with water and surface sterilized with 0.1 per cent mercuric chloride ($HgCl_2$) solution for 30 seconds then washed with distilled water and dried the selected spices grounded in a pestle and mortar and the powder were filtered through double layered muslin cloth and made to the 2 % and 5% concentration and tested for their efficacy in reducing the mycelia growth of *Aspergillus flavus.*

2.3. Determination of effect of plant extracts against the target pathogen by poison food technique

Efficacy of plant extracts against *A. flavus*, *in vitro* was determined by poison food technique. 2 and 5 g of selected spices powder was added to 98 and 95 mL of sterilized PDA medium in a conical flask, mixed thoroughly, sterilized in autoclave and poured in Petri plates to make up 2% and 5% of extract in the plated media. A 5 mm diameter of actively growing mycelium disc of the pathogen of 6–7-day-old culture was placed in the centre of the Petri plates. Plates were incubated at 27°C. Five replicates were maintained for each treatment. Radial growth of mycelium was measured after inoculation. The results were compared with control. The percent inhibition of the fungus in treatments was calculated using following formula given by Vincent (1947) [11].

$$I = \frac{C-T}{C} \times 100$$

Where:
I = Per cent inhibition of mycelia growth
C = Growth of mycelium in control (mm)
T = Growth of intreatment (mm)mycelium



(T_1) Bay leaf **(T_2)** Cinnamon **(T_3)** Cumin **(T_0)** Control

Results and Discussion

The result of three selected spices against Aspergillus flavus at 2 and 5 % concentration in PDA media

Minimum radial mycelial growth inhibition of *A. flavus* was shown in T_1 Bay leaf (56 %) as compared in T_2 (40 %), T_3 Cumin (29 %) with control at 24 hours after incubation where, in 48 hours after incubation radial mycelial growth inhibition of A. *flavus* was shown in T_1 Bay leaf (76 %) as compared in T_2 (61 %), T_3 Cumin (35 %) with control. Minimum mycelial growth inhibition of *A. flavus* was shown in T_1 Bay leaf (77 %) as compared in T_2 (55 %), T_3 Cumin (24 %) with control after 72 hours after incubation. After 96 hours minimum mycelial growth inhibition of *A. flavus* was shown in T_1 Bay leaf (85 %) as compared in T_2 (63 %), T_3 Cumin (37 %) with control after incubation as well as inhibition of radial mycelial growth of *A. flavus* was shown in T_1 Bay leaf (87 %) as compared in T_2 (63 %), T_3 Cumin (49 %) with control at 2 percentage concentration (table 4.1 and figure 4.1) at 120 hours after incubation.

Table 4.1: *In vitro* effect of selected spices extract at 2% concentration on radial growth (mm) and percentage inhibition of *Aspergillus flavus* at different hours of incubation

Treatments		24 hours		48 hours		72 hours		96 hours		120 hours	
		Radial growth (mm)	% Inhibition	Radial growth (mm)	% Inhibition	Radial growth (mm)	% Inhibition	Radial growth (mm)	% Inhibition	Radial growth (mm)	% Inhibition
(T_1)	Bay leaf	5.00	56	6.00	76	6.80	77	6.80	85	8.80	87
(T_2)	Cinnamon	6.80	40	10.00	61	13.20	55	16.80	63	25.60	63
(T_3)	Cumin	8.00	29	16.80	35	22.40	24	28.80	37	35.60	49
(T_0)	Control	11.40	0	26.00	0	29.60	0	46.00	0	70.80	0
F-Test		S		S		S		S		S	
S.Em±		0.500		0.812		0.632		0.529		0.447	
C.D. (0.05%)		1.499		1.436		1.896		1.586		1.341	

Inhibition of Mycelial growth (%) as affected by different selected spices on *Aspergillus flavus, in- vitro* at 5 percent concentration.

Minimum radial mycelial growth inhibition of *A. flavus* was shown in T_1 Bay leaf (72 %) as compared in T_2 (45 %), T_3 Cumin (32 %) with control at 24 hours after incubation but at 48 hour the radial growth of A. *flavus* was shown in T_1 Bay leaf (84 %) as compared in T_2 (58 %), T_3 Cumin (48 %) with control. minimum inhibition radial growth of A. *flavus* was shown in T_1 Bay leaf (82 %) as compared in T_2 (58 %), T_3 Cumin (35 %) with control at 72 hours after incubation where at 96 hour mycelial growth of A. *flavus* was shown in T_1 Bay leaf (92 %) as compared in T_2 (64 %), T_3 Cumin (42 %) with control as well as inhibition of radial mycelial growth of *A. flavus* was shown in T_1 Bay leaf (94 %) as compared in T_2 (72 %), T_3 Cumin (53 %) with control at 120 hours after incubation.

Treatments		24 hours		48 hours		72 hours		96 hours		120 hours	
		Radial growth (mm)	% Inhibition	Radial growth (mm)	% Inhibition	Radial growth (mm)	% Inhibition	Radial growth (mm)	% Inhibition	Radial growth (mm)	% Inhibition
(T_1)	Bay leaf	4.00	72	4.00	84	4.00	82	4.00	92	4.00	94
(T_2)	Cinnamon	8.00	45	11.00	58	15.00	58	18.00	64	20.40	72
(T_3)	Cumin	10.00	32	13.40	48	23.40	35	29.20	42	34.00	53
(T_0)	Control	14.80	0	26.20	0	36.40	0	51.20	0	73.20	0
F-Test		S		S		S		S		S	
S.Em±		0.424		0.548		0.574		0.849		0.548	
C.D. (0.05%)		1.272		1.642		1.722		2.544		1.642	

Conclusion

The present study concluded that effect of selected spices such as Bay leaf, Cinnamon and Cumin powder compared control against *Aspergillus flavus* of Pistachio at the various concentration.

From the critical analysis of the present findings it was concluded that among all treatments, the treatment Bay leaf was significantly reduced mycelial growth of the pathogen followed by Cinnamon and Cumin as compared to control.

From the above study we can conclude that the botanicals can be used as an alternative of fungicides in future and can be employed for sustainable Agriculture, but it still needs more investigation to be conducted in this regard for valid recommendation.

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