Potential of *Cinnamon cassia* Oil for Safety of Grains Contaminated with Aflatoxin Induced by *Aspergillus flavus*

Shirurkar D. D. *[1]and Ranadive K. R.[2]

[1]Annasaheb Magar Mahavidyalaya, Hadapsar, Pune, 411028, M.S, India,
[2]Annasaheb Magar Mahavidyalaya, Hadapsar, Pune, 411028, M.S, India.

ABSTRACT

Fungal infestation is one of the common and serious problems faced during gain storage. *Aspergillus flavus* is one of the major storage fungi producing aflatoxins, which is highly toxic, carcinogenic and shows ill-effects on human and animal health. Inhibitory effect of *Cinnamon cassia* oil against the mycelial growth and aflatoxins production by *A. flavus* has been studied in this investigation. *A. flavus* was found dominant fungi during maize grain storage. *Cinnamon cassia* oil showed significant antifungal activity against *A. flavus* when evaluated by agar well diffusion method. Monitoring of aflatoxin levels in grains is a management methodology and it can be applied during storage. Aflatoxins were assessed qualitatively and quantitatively by adopting TLC and HPLC methods in *Cinnamon cassia* oil treated and non-treated grains. Cinnamon oil is a natural essential oil that does not show ill effects to human and animal health. Aflatoxin production was considerably declined by application of *Cinnamon cassia* oil during storage. *Cinnamon cassia* oil has fungi toxic potential against *Aspergillus flavus.* It may be used as fungicidal agrochemical during seed storage.

Keywords: Aflatoxin, *Aspergillus flavus, Cinnamon cassia* oil, fungi toxic potential.

INTRODUCTION:

Stored grains deterioration is a prolonged problem in India. Fungi are prominent destroyer during storage and by producing mycotoxins nutritive value of grains becomes decreased. Dominance of *Aspergillus* species in maize seeds was also reported by Reddy and Reddy (1989), Nishant and Mall (2008), Saleem M. K. *et al*. (2012) and Saleem M. J. *et al*. (2012); Shirurkar and Wahegaonkar (2013). *A. flavus* have been the most prevalent fungal species in samples of maize grains reported by Fandohan *et al*. (2003), Bhutta *et al*. (2004) and Aksun (2006); Shirurkar and Wahegaonkar (2013).

About 300 fungal metabolites are reported to be toxic to man and animals (Galvano *et al*., 2001). Among them aflatoxins were very common mycotoxin produced by *Aspergillus flavus,* which is one of the common storage fungi. Reported toxic effects are carcinogenicity, genotoxicity, teratogenicity, nephrotoxicity, hepatotoxicity, reproductive disorders and immuno-suppression (Diener *et al*. 1987, Lacey, 1988; Desjardins *et al*. 2000). Fungal invasion in grains results into decline of the power of germination, moldering visible

discoloration, odor, chemical, and nutritional changes, with consequent loss of quality, mycotoxin production and making them unfit for consumption (Paster and Bullerman, 1988).

Effective and efficient control of seed borne fungi can be achieved by the use of synthetic fungicides but synthetic fungicides may show pesticide toxicity. Hence there is a necessity to explore different approaches to store cereal grains for human consumption, which are eco-friendly and easily available. Some essential oils can be applied as mold inhibitors to prevent growth of toxigenic fungi in stored food. Plant derived oils can be used as effective and promising fungal inhibitors to control fungal infestation can be used as effective and promising fungal inhibitors to control fungal infestation (Shirurkar and Wahegaonkar, 2012). Some researchers reported inhibitory activity of cinnamon oil against *Aspergillus* species (Chatterjee, 1990; Tiwari and Tiwari, 1997; Dusanee *et al*., 2007). Fungi produce a mixture of secondary metabolites or mycotoxins (Negedu *et al*., 2010). Many researchers found that biological control has played significant role to reduce aflatoxin contamination in various crops. Sinha *et al*. (1993) recorded inhibition of mycelial growth of *A. flavus* and aflatoxin production by Cinnamon oil. Mahmoud (1994) recorded suppressed aflatoxin synthesis due to cinnamaldehyde treatment. Roquia El-Habib (2012) carried out *in vitro* studies on antifungal activity of essential oils against *A. flavus* and aflatoxin B1 production and reported that dill oil was the most effective against aflatoxin production, both thyme and basil oils delayed the growth of *A. flavus* and inhibited aflatoxin production. Namazi *et al.* (2002) revealed that lyses of the mycelia and spores of the toxigenic fungi are characteristics of aflatoxin deactivation process. Fungal growth and aflatoxin production were inhibited by essential oils and in corn samples, the oregano essential oil was more effective than that of mentrasto oil (Renata et al., 2014)

The aim of this study was to develop ecofriendly management technique against *Aspergillus flavus* with respective mycelium growth and aflatoxins production during grain storage with the help of *Cinnamon cassia* oil.

MATERIALS AND METHODS

- **Collection of Maize samples:** Three different grain samples of Maize varieties, African Tall and Amber, were collected from the College of Agriculture, Pune and Local variety from farmers.

- **Isolation of *A. flavus*:** Toxigenic fungus *A. flavus* was isolated from the selected maize samples and identified. Conformation of fungal species was carried out by Agarkar Research Institute, Pune. Pure cultures of isolated fungi grown on PDA media by Hi Media Laboratories Pvt. Ltd., Mumbai and maintained throughout the investigations by sub-culturing. These pure cultures served as the test fungi for toxin production and antifungal activity assay (Pundir and Jain, 2010).

- **Preparation of spore suspension:** The spore suspension was prepared according to Florl *et al*. (2003) in saline water, spores taken from the 7-day-old pure culture of the recovered fungal species grown on PDA medium. The stock suspensions of fungal isolates were standardized at 0.20 OD at 530 nm. This stock solution was used further for study of antifungal activities of fungicides and botanicals against test fungi and toxigenicity study of *Aspergillus flavus*.

- **Antifungal activity assay:** Antifungal activity study was carried out by agar well diffusion method (Perez, 1990). *Aspergillus flavus* spore suspension (0.1 ml) was spread on sterile agar plates. With sterile cork borer of size 5mm well were made on agar plate and 50 µl of commercial *Cinnamon cassia* oil and commercial fungicide (Mancozeb and Bavistin) of different concentrations (0.1%, 0.2%, upto 1.0%) were loaded. Plates were kept for pre-incubation for 30 min in refrigerator after sealing of plates with cling film and then were incubated at room temperature (28^0 C) for 4 days.

- **Extraction of aflatoxin from *Aspergillus flavus*:**
  Presences of aflatoxins were confirmed by preparing culture filtrate and TLC for identification of aflatoxins. Culture filtrate was prepared with spore suspension of *A. flavus.* 500 µl of spore suspension of *A. flavus* were added to 50 ml Diener and Davis (1966) liquid medium (0.5 g/ lit $MgSO_4.7H_2O$; 3.0 g/ lit KNO3; 200 g/ lit Sucrose; 7.0 g/ lit Yeast extract) in 250 ml Erlenmeyer flasks. It was incubated at 28 ± 1^oC on a mechanical shaker at 120 rpm for 20 days. After incubation the filtrates were collected by filtering through Whatman's filter paper No. 1. In separating funnel 40 ml of culture filtrate and 20 ml of chloroform was added and shaken for 5 minutes. Collected chloroform layer was washed with 10 ml of hexane, chloroform was evaporated to dryness on a rotary vacuum evaporator and the residue was dissolved in 100 ml of chloroform and used for analysis of aflatoxins by TLC **(**Krishna Kishore, 2002). TLC was done in Chloroform**:** Methanol (9:1) solvent system.

- **Qualitative analysis of aflatoxins by TLC:** Ten µl of sample extracts were spotted on Silica Gel plates (Merk F254). Based on the results of earlier experiments solvent system chloroform: methanol (9:1) was considered for the detection of aflatoxins. After the development, the plates were viewed under long wavelengths UV light (365 nm) (Banu and Muthumary, 2008). Aflatoxins B1, B2 fluoresce as blue, G1, G2 fluoresce as green under long UV light.

- **Application of Cinnamon oil on maize grains:** *Cinnmon cassia* oil was obtained from the market. Each variety was divided into two lots. One lot of untreated seeds was kept as control. Other lot treated with cinnamon oil (0.2 ml/100g v/w). Treated and untreated seeds were stored for 2 months in plastic containers.

- **Extraction of mycotoxin from stored seeds:** It was carried out according to Sundaram *et al.,* 2001 and Banu and Muthumary, 2008**.** 36 ml of acetonitrile was added in 10 gram of non-treated grains powder sample. 4 ml of 4% KCl and 0.8 ml of 5N HCl added in it and macerated using mortar and pestle for 3 minutes. The sample was filtered through Whatman filter paper No.1. In 250 ml separating funnel, 20 ml of this filtrate was transferred and 20 ml of distilled water and 20 ml of hexane were added to it. This flask was shaken well for few minutes. The upper hexane layer was rejected and in the lower layer 20 ml of hexane was added and shaken well for 3 minutes. From this lower layer was again collected. The acetonitrile phase was extracted with 20 ml of chloroform. The chloroform was evaporated to dryness. The final residue was dissolved in 0.2 ml of chloroform. Same procedure was used for extraction of toxins from *Cinnamon cassia* oil treated grains and qualitative and quantitative analysis for aflatoxins was

carried out.

- **Quantitative analysis of aflatoxin after storage period by HPLC:**

  Treated and non-treated grains grind in to powder. HPLC analysis was carried out from National Agriculture and Food Analysis and Research Institute (NAFARI), Tilak Road, Pune, Maharashtra, India, according to AOAC Method 990.33, 1995 by following HPLC Conditions (AOAC 968.22, AOAC 990.33). Column: Zorbas ODS (250 X 4.6 mm, 5 µm); Mobile phase: Deionized water: Acetonitrile: Methanol (60:20:20); Flow rate: 1 mL / min.; Temperature: 30^0 C; Detector: Fluorescence at 360nm excitation and 440nm emission; Injection volume**:** 20 µl; Run time: 12 minute; Approximate Run Time: Aflatoxin B1 = 5.100 minute, Aflatoxin B2 = 7.491 minute, Aflatoxin G1 = 4.625 minute, Aflatoxin G2 = 6.336 minute.

- **Statistical analysis:** Statistical analysis was done by using Graph Pad In Stat version 3.06 (Graph Pad Software, San Diego, California, USA; Trial Version). One-way ANOVA with Dunnett multiple comparison test was performed to test if the difference between the mean of control and other treatments is significant. ns: Non-significant; * P≤0.05 (significant); ** P≤0.01 (highly significant).

RESULT AND DISCUSSION:

- **Occurrence of *A. flavus***

  In the present investigation there was dominance of *Aspergillus* species in all selected maize varieties, among that *A. flavus* was occurred in large frequency. Identification of *Aspergillus* species was conformed from Agarkar research Institute, Pune, (MS).

- **Antifungal activity of Cinnamon cassia oil and commercial synthetic fungicides against *Aspergillus flavus*:** (Table – 1, Figure – 1, Plate -1).

  Commercial synthetic fungicides Bavistin and Mancozeb inhibits mycelium growth of *Aspergillus flavus* with all concentrations (0.1 to 1.0 %) in a range 13 mm to 27 mm. and 10 mm to 18 mm. respectively. Among these two synthetic fungicides Bavistin is more effective than Mancozeb as an antifungal agent. *Cinnamon cassia* oil is a plant derived oil which have ultimate antifungal activity against mycelium growth of *Aspergillus flavus*. Efficacy of the *Cinnamon cassia* oil was much more when compared with the synthetic fungicides such as Mancozeb and Bavistin.

Table 1: Antifungal activity of *Cinnamon cassia* oils and synthetic fungicides

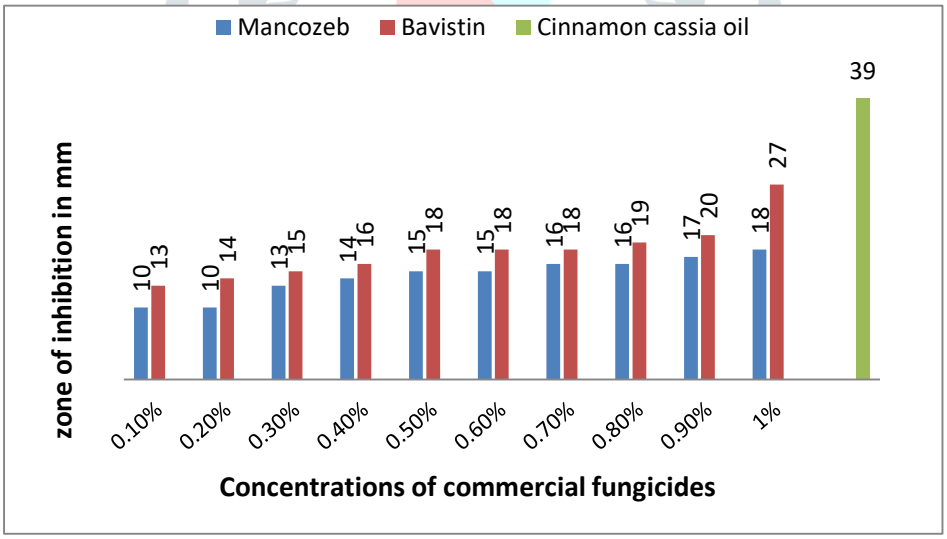
Concentration	Bavistin	Mancozeb	*Cinnamon cassia* oil
0.1%	13±0.32**	10±0.32**	39±0.24**
0.2%	14±0.32**	10±0.32**	
0.3%	15±0.32**	13±0.24**	
0.4%	16±0.32**	14±0.32**	
0.5%	18±0.32**	15±0.4**	
0.6%	18±0.32**	15.4±0.4**	
0.7%	18±0.32**	16±0.32**	
0.8%	19±0.32**	16±0.00**	

0.9%	20±0.32**	17±0.04**	
1%	27±0.32**	18±0.00**	

Plate 1: Antifungal activity of *Cinnamon cassia* oils and 1% synthetic fungicides

***Cinnamon cassia* oil** **1% Bavistin** **1% Mancozeb**

Figure 1: Comparison of antifungal activity of *Cinnamon cassia* oils and synthetic fungicides



- **Toxicity of *Aspergillus flavus*:**

Fluorescing spots of aflatoxin B1, B2 G1 and G2 were observed at different Rf values when crude culture filtrate of *A. flavus* was spotted on TLC plates and developed in chloroform: methanol (9:1) solvent systems (Table - 2). Some unknown spots were also observed at different Rf values. *A. flavus* may produce mixture of mycotoxins.

Table 2a: Standard Rf values of aflatoxins

Toxin	Colour of spot	Standard Rf value
Aflatoxin B1	Blue	0.77
Aflatoxin B2	Blue	0.54
Aflatoxin G1	Greenish Yellow	0.37
Aflatoxin G2	Greenish Yellow	0.25

Table 2b: Occurrence of aflatoxins in pure culture of *Aspergillus flavus*

Colour of spot	Toxin	Rf value in pure culture
Blue	Aflatoxin B1	0.77
Blue	Aflatoxin B2	0.54
Greenish Yellow	Aflatoxin G1	0.37
Greenish Yellow	Aflatoxin G2	0.24
Blue	Unknown	0.96
Greenish	Unknown	0.4
Blue	Unknown	0.33
Blue	Unknown	0.18

- **Detection of aflatoxins from non-treated and treated seed extracts by TLC:**

Individual maize variety was investigated for mycotoxin contamination. Seed extracts prepared for mycotoxin analysis were spotted on TLC plates and developed in chloroform: methanol (9:1) solvent system and results were recorded (Table 3). Under non-treated storage condition, presence of aflatoxin B1, aflatoxin B2 and aflatoxin G1 was appeared in Local and Amber variety of maize. African tall variety only contaminated with aflatoxin B1. Aflatoxin G2 was not found in all varieties of maize. The results suggested that all selected maize varieties were contaminated with hazardous aflatoxins under non-treated storage condition. In the present study thus, compounds with florescent colour intensities and retention factors (Rf) values different from the available mycotoxin standards were observed in the sample extracts. During storage fungi may produce mixture of mycotoxins.

When cinnamon oil treated maize grains were detected for the aflatoxins during storage, there will be no occurrence of fluorescent blue or greenish yellow spot on TLC plate (Table 3).

Table 3: Occurrence of aflatoxins in stored grains

	Non-treated grains			Treated grains		
Variety	**Rf value**	**Colour of spot**	**Toxin**	**Rf value**	**Colour of spot**	**Toxin**
Local	1	Blue	Unknown	--	--	--
	0.77	Blue	Aflatoxin B1	--	--	--
	0.54	Blue	Aflatoxin B2	--	--	--
	0.37	Greenish Yellow	Aflatoxin G1	--	--	--
	0.11	Greenish	Unknown	--	--	--
African tall	0.77	Blue	Aflatoxin B1	--	--	--
	0.66	Blue	Unknown	--	--	--
	0.63	Blue	Unknown	--	--	--
	0.51	Blue	Unknown	--	--	--
Amber	0.93	Blue	Unknown	--	--	--
	0.77	Blue	Aflatoxin B1	--	--	--
	0.54	Blue	Aflatoxin B2	--	--	--
	0.40	Blue	Unknown	--	--	--

	0.40	Greenish Yellow	Aflatoxin G1	--	--	--

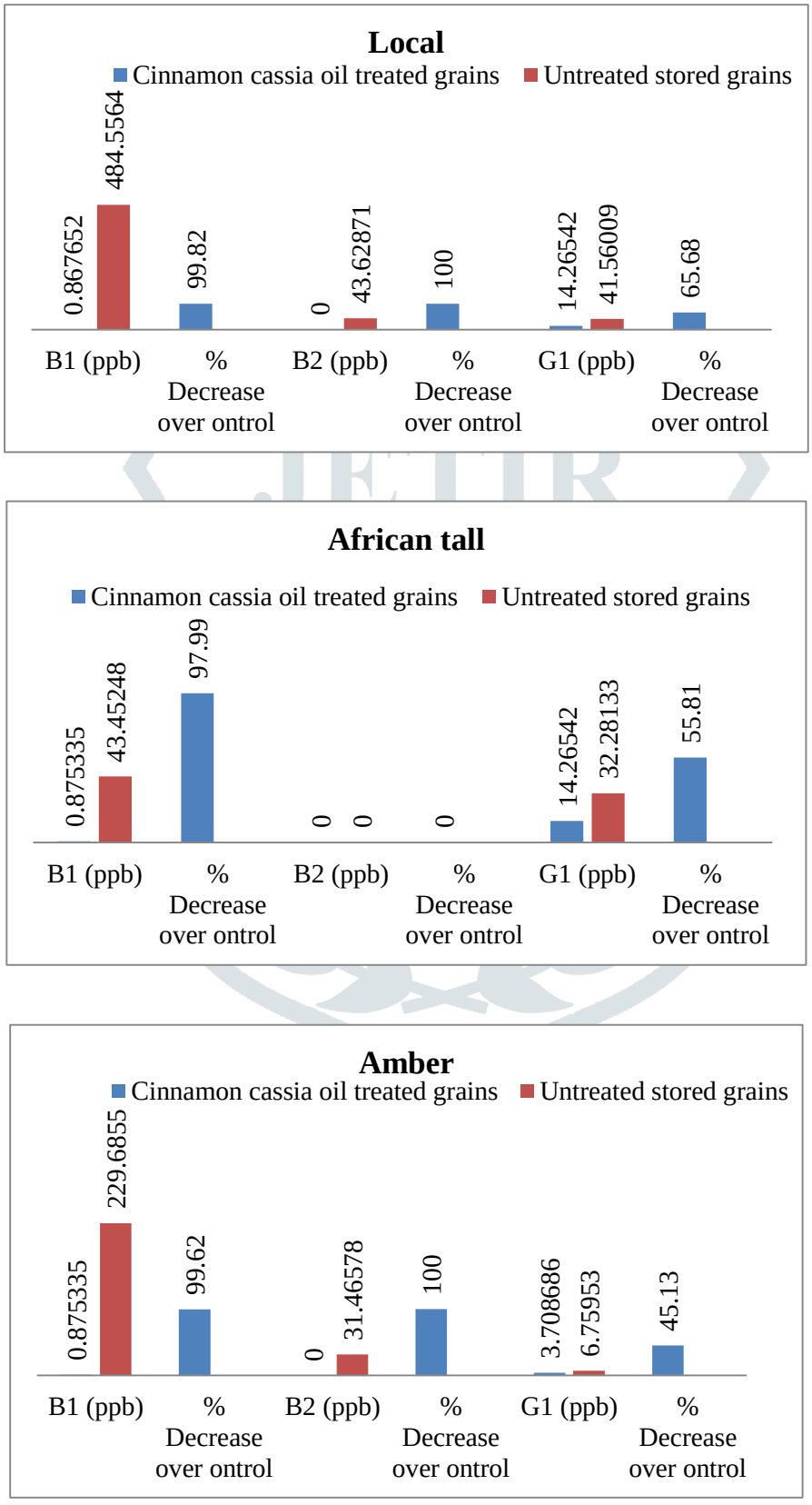
- **Detection of aflatoxins from non-treated and treated seed extracts by HPLC:**

Aflatoxins were detected under non-treated condition in all varieties maize during storage (Table 4, Figure 2). The toxins, aflatoxin B1, B2 and G1 were detected in different levels in each of the samples. Aflatoxin B1, B2 and G1 were detected in Local and Amber variety of maize while aflatoxin B1 and aflatoxin G1 detected in African tall variety. Among these three aflatoxins, highest amount of toxin was aflatoxin B1 followed by B2 and G1, was detected from the un-treated grains of Local and Amber variety. In African tall variety amount of aflatoxin B1 is more than aflatoxin G1. This indicates that overall aflatoxin B1 production is dominant one in all varieties. Amounts detected from the all varieties of maize were much higher than the acceptable limits. Aflatoxin B1, B2 and G1 production was tremendously decreased in all varieties of maize when grains were treated with cinnamon oil (Table 4, Figure 2).

Table – 4: Aflatoxin production in cinnamon oil treated and non-treated stored grains with HPLC technique.

Variety	Treatment	B1 (ppb)	% Decrease over ontrol	B2 (ppb)	% Decrease over control	G1 (ppb)	% Decrease over control
Local	Untreated stored grains	484.5564	--	43.62871	--	41.56009	--
	Cinnamon oil treated grains	0.867652	99.82	00	100	14.26542	65.68
African tall	Untreated stored grains	43.45248	--	00	--	32.28133	--
	Cinnamon oil treated grains	0.875335	97.99	00	--	14.26542	55.81
Amber	Untreated stored grains	229.6855	--	31.46578	--	6.75953	--
	Cinnamon oil treated grains	0.875335	99.62	00	100	3.708686	45.13

Figure 2: Aflatoxin production in cinnamon oil treated and non-treated stored grains with HPLC technique



CONCLUSION:

The *Cinnamon cassia* oil not only prevents the mycelial growth of *A. flavus* but also suppressed the ability of aflatoxin production. Inhibition of the mycelia and spores of the *A. flavus* may deactivate aflatoxin synthesis process. *Cinnamon cassia* oil have a great potential as eco-friendly antifungal agent because of their easy methods of preparation and application, safe and effective nature, easy biodegradability and no residual toxicity. *Cinnamon cassia* oil thus could be considered suitable alternatives to synthetic fungicides, attending to the needs for safety and satisfying the demand of consumers for natural components. Such reports will facilitate towards the development of proper seed storage systems and suitable management practices in the agriculture. Improved methods of application like mechanical mixers for uniform and bulk coating of oil on grain, use of gentle release dispensers or sachets which were kept at different depths in storage bins or bags, could be enhancing efficacy of oil during storage.

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