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Effect of lemon grass (Cymbopogon citratus (DC.) Stapf.) treatments on *Aspergillus flavus* (SGS-421) infestation and aflatoxin B_1 content of maize grains

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Abstract

Studies were conducted to determine the effect of lemon grass (*Cymbopogon citratus* (DC.) Stapf.) treatments on *A. flavus* (SGS-421) infestation of maize grains and aflatoxin B_1 (AFB_1) content of the grains. The experimental design followed a 2×2×5 factorial combination and effect of three factors: form of leaf addition (whole and powdered), form of water suspension (cold and hot), and level of concentration of leaf (0, 8, 10, 12 and 14%, w/w) and water suspension (0, 8, 10, 12 and 14%, w/v); were evaluated by analysis of the maize grains at 5 day interval for 30 days. The growth of the aflatoxigenic fungus and the aflatoxin content of the maize grains were evaluated with palm kernel agar and by thin layer chromatography method respectively. Powdered leaves of lemon grass at 14% (w/w) concentration prevented the growth of the fungus and AFB_1 formation in the grains by the 25th day of incubation. This was followed by the whole leaves which reduced the fungal growth to 12.17% and aflatoxin content to 42.62%. Both the cold (8.90%) and hot suspensions (4.15%) had no significant ($p > 0.05$) effect on the fungal growth after 10 days of incubation. On the contrary, there were significant ($p < 0.05$) reductions in the aflatoxin content of grains treated with cold (10.24%) and hot suspensions (10.25%) at lowest concentrations of 8%. The findings showed that *A. flavus* infestation and AFB_1 formation in maize grains can be controlled by co-storing powdered leaves of lemon grass with aflatoxin infected maize.

Introduction

Maize (*Zea mays*, L., *Poaceae* family), also known as corn, is a very versatile crop, growing in all sorts of edaphic, altitudinal and fertility conditions, which explains its global adaptability and its many types of varieties. Maize is currently the third most traded cereal, after wheat and rice, with a total production of 822 million tonnes in over 160 million hectares in 2008 (*http://faostat.fao.org*). The five main producers in the world are: the United States of America (USA), China, Brazil, India and Mexico. Maize production in Nigeria increased from 5,840,000 metric tonnes in 1992 to 7,305,530 metric tonnes in 2012 (FAO, 2012). The crop is used as a staple food source especially in Latin America and Africa and because of its low price and worldwide distribution; it has become the most important raw material for animal feed and for several industrial processes (*http://faostat.fao.org*). Maize is used for three main purposes: animal feed, food, and in the industry. Animal feed represents 65% of the total world maize production, while 15% is used for food and the remainining 20% has different industrial uses (UNDP, 2010). The trend for global cereal demand in the next decade is expected to increase, and in the case of maize it is expected to surpass the demand of wheat and rice (UNDP, 2010).

West Africa has a tropical climate with an all year round high ambient temperature and relative humidity that provides optimal conditions for growth of toxigenic moulds (Bankole and Adebanjo, 2003). Maize is an excellent substrate for the growth of aflatoxigenic fungi and aflatoxin contamination (Egal *et al*., 2005; Mutungi *et al*., 2008). Aflatoxigenic strains of *Aspergillus flavus* are capable of contaminating maize in the field and can result in further deterioration of the crop during storage (Bandyopadhyay *et al*., 2005; Atenhkeng *et al.*, 2008). Infection of host plants in the field is unavoidable since factors that promote fungal infection and aflatoxin production such as inoculum availability, weather conditions, and pest infestation during crop growth, maturation, harvesting and storage are difficult to control (Lopez-Garcia and

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Park, 1998).

Aflatoxins have been implicated with acute and chronic aflatoxicosis, genetoxicity and chronic infection of hepatitis B virus (HBV). Similarly, dietary exposure to aflatoxins is one of the two major etiological risk factors responsible for the development of hepatocellular carcinoma (HCC), suppression of immune system, aggravation of kwashiorkor and impaired childhood (Farombi, 2006; Mutungi *et al*., 2008). Furthermore, Nigeria has experienced high recorded aflatoxin exposure levels in humans and has also reported the highest estimated number of cases of hepatocellular carcinoma (HCC-liver cancer) attributable to aflatoxins in the whole world (Liu and Wu, 2010).

The hazardous nature of aflatoxins to human, animals and livestock's necessitate the need for the establishment of control measures and concerted efforts are now being directed at finding very cheap and reliable methods of minimizing aflatoxin formation in stored food commodities.

Lemon grass (*C. citratus* (DC.) Stapf.) is a herbal plant commonly called "*Ewe tea*" in the South Western part of Nigeria. It has potential health benefits as it has been used locally to combat anxiety, insomnia, stomach problems, hypertension, lowering of body temperature and fever (Mdidea, 2007). Lemon grass is known to contain a variety of essential oils including citral, limonene, myrecene, geraniol, decanal and furfurol (Fasihuddin and Ismail, 2003). Although the essential oils of lemon grass had been used to reduce aflatoxigenic fungi and aflatoxin production in food commodities (Paranagama *et al*., 2003; Bankole and Joda, 2004), the extraction process is labour intensive, requires technological skill, and the oil is expensive. The local farmers are however familiar with the simple method of preparing the cold and hot suspensions from lemon grass as they often use it for medicinal purposes.

The present study was conducted to evaluate the effect of whole and powdered leaves, cold and hot suspension of lemon grass on the growth of *Aspergillus flavus* (SGS-421) and AFB_1 content of maize grains.

Materials and Methods

Materials

Fresh *C. citratus* leaves were collected from a local herbarium in Abeokuta. The collected leaves were identified at the Forestry Research Institute of Nigeria (FRIN), Ibadan. Maize grains (white variety) were purchased from a local farmer in Ibadan, Nigeria, shortly after harvesting and sundrying. Aflatoxin B_1 standard was obtained from Sigma-Aldrich Chemical Private Limited (Banglore, India). Fresh palm fruits were purchased from the palm plantation of Federal University of Agriculture, Abeokuta while *Aspergillus flavus* (SGS-421) was obtained from the culture collection of the microbiology laboratory of Food Science and Technology Department, Federal University of Agriculture, Abeokuta. All reagents and chemicals used were analar grades.

Treatment of C. citratus *leaves and maize grains*

They leaves were air-dried under the shade (25-29^{o}C) until the leaves became crispy dry after 5 days. Discoloured and visibly mouldy grains were removed and the grains surface sterilized in 1% NaCl for one min, followed by three successive rinses in sterile distilled water.

Test for aflatoxigenic potential of Aspergillus flavus *(SGS-421)*

The aflatoxigenic potential of *A. flavus* (SGS-421) was determined by incubating pure cultures of the organism in yeast extract sucrose (YES) medium at 30^{o}C for 10 days. The isolates were viewed under ultraviolet light (365 nm) qualitatively for bright blue fluorescence (AFB_1). Pure cultures of the fungus were maintained on potato dextrose agar supplemented with 0.01% chloramphenicol, to suppress bacterial growth, and renewed bimonthly.

Preparation of conidia suspension

Conidia suspension was prepared as reported by Atanda *et al.* (2006). The concentration of the suspension was adjusted with sterile distilled water to approximately 10^6 spore/ml by an improved Neubauer haemocytometer.

Moisture content of maize grains

The moisture content of the freshly purchased maize grains, cold and hot lemon grass suspensions were determined by the AOAC (2000) method. The moisture content of the grains were equilibrated to 13% by the addition of sterile distilled water.

Bioassay with leaves and suspensions of lemon grass

The bioassay of the leaves was as described by Bankole and Joda (2004). Dry leaves corresponding to concentrations of 8.0, 10.0, 12.0 and 14.0% (w/w) were introduced in triplicates into 250ml conical flasks containing maize grains and 1 ml conidia suspension of *A. flavus* (SGS-421). The dried leaves were further powdered in a coffee grinder, and sieved with a 0.5 micron mesh. Powered leaves

corresponding to concentrations of 8.0, 10.0, 12.0 and 14.0% (w/w) were also introduced in triplicates into 250 ml conical flasks containing maize grains and 1 ml conidia suspension of *A. flavus* (SGS-421). The control flasks consisted of maize and conidia suspensions without the addition of the leaves. For cold and hot suspensions of lemon grass, the freshly harvested leaves were blended with appropriate volumes of distilled water in a Waring blender. The slurry was sieved through four layers of muslin cloth into 250 ml conical flasks and 50 g of maize grains soaked in triplicates in the filtrate for 1 h. The soaked grains were inoculated with 1ml conidia suspension of *A. flavus* (SGS-421). The flasks were incubated at room temperature (28 ± 2°C) for 30 days and hand inverted twice daily. They were further assayed for *A. flavus* and aflatoxin concentration at five days interval.

Detection of A. flavus *(SGS-421) in maize grains*

The treated maize grains were milled with a hammer mill (Glean, USA) and 0.1 ml portion spread plated on PKA agar supplemented with 0.01% chloramphenicol as described by Atanda *et al*. (2005). Plates were incubated at 30°C for 72 h and aflatoxigenic fungus detected visually by yellow pigmentation and fluorescence of palm kernel agar under ultraviolet light (365 nm). Maize samples that were not treated served as control.

Aflatoxin content of maize grains

The level of aflatoxins in the treated maize grains was assayed with the AOAC (2000) official method and AFB_1 quantified by thin layer chromatography. AFB_1 was identified on the basis of co-migration with AFB_1 standard and their characteristic fluorescent colour under long wave UV light. The identity of aflatoxin was confirmed by derivatization with trifluoroacetic acid (Stack and Pohland, 1975). The detection limit was 5 ng/g.

The spot that gave the minimum fluorescence was noted and the quantity of aflatoxin calculated from the formula:

$$\mu g/l\ AFB_1 = \frac{S \times Y \times V}{X \times W}$$

Where S = µl aflatoxin B_1 reference standard
Y = Mass concentration of aflatoxin B_1 reference standard (µl/ml)
V = Final dilution of sample extract (µl)
X = µl of sample extract spotted giving fluorescence intensity equal to S (aflatoxin B_1 standard).
W = Effective weight of sample in g.

Rate of reduction of growth of A. flavus *and aflatoxin B_1 content of maze grains*

The rate of reduction (%) of the growth of *A. flavus* and AFB_1 content of maize grains was calculated as:

Reduction rate (%) = 1- Treatment/Control

Statistical analysis

Data obtained were analyzed using SPSS 12.0 version. Means were calculated and tested for significant differences at 95% confidence level using analysis of variance while mean separation was by the Duncan's multiple range test.

Results

Tables 1-4 show the effect of different lemon grass treatments on *A. flavus* and AFB_1 content of the maize grains. There was no growth of *A. flavus* (SGS-421) and formation of AFB_1 on the maize grains at the initial day of incubation up to the 10th day. Aflatoxin formation did not occur till the 15th day. The rate of reduction of aflatoxigenic colonies and AFB_1 content of the grains increased with incubation time and was dose dependant. In particular, powdered leaves of lemon grass at 14% (w/w) concentration prevented the growth of the fungus except for the 20th day (Figure 1) and AFB_1 formation in the grains by the 25th day of incubation (Figure 2). This was followed by the whole leaves which reduced the fungal growth to 12.17% and aflatoxin content to 42.62%. Tables 5-8 show the effect of cold and hot suspensions of lemon grass on *A. flavus* and AFB_1 content of maize grains. Both the cold (8.90%) and hot suspensions (4.15%) did not have significant effect ($p > 0.05$) on the fungal growth of the fungus after the 10th day of incubation but there were significant ($p < 0.05$) reductions in the aflatoxin contents of grains treated with cold (10.24%) and hot suspensions (10.25%) at lowest concentrations of 8%.

Discussion

Although *Aspergillus* species are able to metabolize a range of food commodities after seven days (Passone *et al*., 2003), the delayed appearance of *A. flavus* at the 10th day and formation of AFB_1 at the 15th day of incubation may be due to the fact that essential oils of lemon grass have been reported to be capable of delaying fungal growth (Guynot *et al*., 2003).

The probable reason why powdered leaves were more effective than the whole leaves in controlling the aflatoxigenic fungus and preventing formation

Table 1. Effect of whole leaves of *C. citratus* on *A. flavus* (SGS-421) infestation of maize grains

Treatment Conc.(%)	Storage period (Day)										
	5	10	*RR(%)	15	RR(%)	20	RR(%)	25	RR(%)	30	RR(%)
0	0.35**	4.57^{a}	-	5.17^{a}	-	5.43^{a}	-	4.67^{a}	-	3.37^{a}	-
8	-	3.53^{b}	27.79	5.17^{a}	0.00	5.23^{a}	3.68	4.43^{b}	5.14	3.20^{a,b}	5.04
10	-	3.43^{b,c}	24.95	5.13^{a}	0.77	5.23^{a}	3.68	4.43^{b}	5.14	3.07^{a,b}	8.90
12	-	3.43^{b,c}	24.95	4.17^{b}	19.34	5.20^{a}	4.26	4.37^{b}	6.42	3.13^{a,b}	7.12
14	-	3.30^{c}	22.75	4.10^{b}	20.70	5.20^{a}	4.26	4.30^{b}	7.92	2.96^{b}	12.17

Values with different superscripts within a column are significantly different at $p \leq 0.05$.
*RR = rate of reduction, ** = 10^{1} cfu/ml

Table 2. Effect of whole leaves of *C. citratus* on AFB_1 (µg/kg) content of maize grains

Treatment Conc. (%)	Storage period (Day)							
	15	*RR(%)	20	RR(%)	25	RR(%)	30	RR(%)
0	2.44^{a**}	-	2.94^{a}	-	2.56^{a}	-	2.44^{a}	-
8	2.00^{a,b}	18.03	2.80^{a}	4.76	2.44^{a}	4.69	2.27^{a,b}	6.96
10	2.00^{a,b}	18.03	2.27^{b}	22.79	2.22^{a,b}	13.28	2.17^{a,b}	11.06
12	2.00^{a,b}	18.03	2.15^{b}	26.87	2.22^{a,b}	13.28	2.16^{a,b}	11.06
14	1.40^{b}	42.62	2.09^{b}	28.91	2.00^{b}	21.88	1.40^{b}	42.62

Values with different superscripts within a column are significantly different at $p \leq 0.05$.
*RR = rate of reduction, ** = 10^{1} cfu/ml

Table 3. Effect of powdered leaves of *C. citratus* on *A. flavus* (SGS-421) infestation of maize grains

Treatment Conc. (%)	Storage period (Day)										
	5	10	*RR(%)	15	RR(%)	20	RR(%)	25	RR(%)	30	RR(%)
0	0.35**	4.57^{a}	-	5.17^{a}	-	5.43^{a}	-	4.67^{a}	-	3.37^{a}	-
8	-	1.50^{b}	67.17	1.27^{b}	75.43	1.17^{b}	78.45	0.93^{b}	80.09	0.86^{b}	74.48
10	-	1.50^{b}	67.17	1.07^{c}	79.30	0.93^{c}	82.87	0.70^{c}	85.01	0.53^{c}	84.27
12	-	1.43^{b}	68.71	1.03^{c}	80.07	0.90^{c}	83.42	0.60^{c}	87.15	0.40^{c}	88.13
14	-	0.77^{c}	83.15	0.50^{d}	90.33	0.17^{d}	96.87	0.00	100.0	0.00	100.0

Values with different superscripts within a column are significantly different at $p \leq 0.05$.
*RR = rate of reduction, ** = 10^{1} cfu/ml

Table 4. Effect of powdered leaves of *C. citratus* on AFB_1 (µg/kg) content of maize grains

Treatment Conc. (%)	Storage period (Day)										
	5	10	*RR(%)	15	RR(%)	20	RR(%)	25	RR(%)	30	RR(%)
0	0.35**	4.57^{a}	-	5.17^{a}	-	5.43^{a}	-	4.67^{a}	-	3.37^{a}	-
8	-	1.50^{b}	67.17	1.27^{b}	75.43	1.17^{b}	78.45	0.93^{b}	80.09	0.86^{b}	74.48
10	-	1.50^{b}	67.17	1.07^{c}	79.30	0.93^{c}	82.87	0.70^{c}	85.01	0.53^{c}	84.27
12	-	1.43^{b}	68.71	1.03^{c}	80.07	0.90^{c}	83.42	0.60^{c}	87.15	0.40^{c}	88.13
14	-	0.77$_{c}$	83.15	0.50^{d}	90.33	0.17^{d}	96.87	0.00	100.0	0.00	100.0

Values with different superscripts within a column are significantly different at $p \leq 0.05$.
*RR = rate of reduction, ** = 10^{1} cfu/ml

of AFB_1 on the maize grains may be due to the fact that they had a lower surface area of contact. This is in contrast to the work of Atanda (2005) who found 10% whole basil (*Ocimum basilicum*) leaves more effective but is in agreement with the work of Bankole and Joda (2004) who found out that 10% (w/w) powdered leaves of lemon grass was able to reduce the rate of deterioration of melon seeds inoculated with toxigenic *A. flavus*.

Furthermore, the effectiveness of the powder may be due to the fact that the citral content of a 10 gram ground sample of *C. citratus* has been reported to range between 40.7 and 75.4% of the essential oil

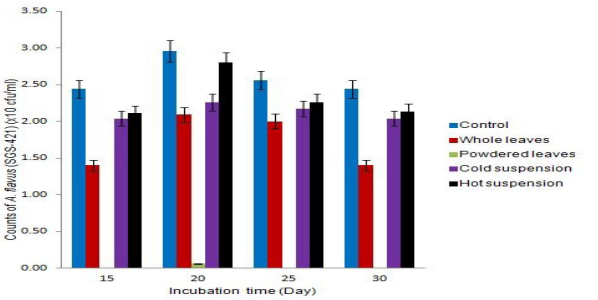

Figure 1. Comparative effect of lemon grass treatments at 14% concentration on *A. flavus* (SGS-421) infestation of maize grains

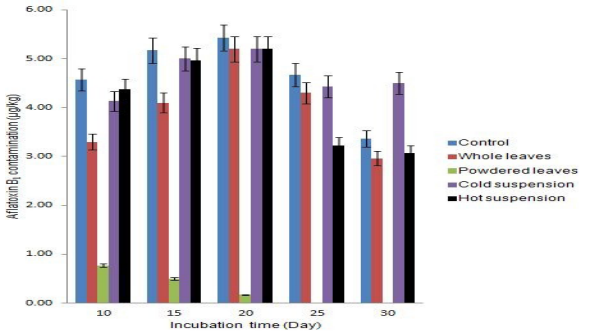

Figure 2. Comparative effect of lemon grass treatments at 14% concentration on aflatoxin B_1 content of maize grains

components (Barbosa *et al.*, 2008). The complete inhibition of aflatoxin and the aflatoxigenic fungus at 14% concentration confirms the fungicidal properties of lemon grass (*C. citratus*). Similar results were obtained by Fiori *et al.* (2002) who found out that *C. citratus* provided 100% inhibition of mycelia growth and germination of spores of *Didymella byroniae* (casual agent of the gummy stem blight of melon crop). Adegoke and Odesola (1996) also reported that the inhibitory activities of lemon grass are connected to the presence of some phytochemical components like tannins, alkaloids and glycosides which are present in the powder.

Cold suspension treatments had significant effect on *A. flavus* at 14% concentration on the 10th day of incubation due to the fact that suspension treatments from fresh *C. citratus* may contain between 0.4% and 0.76% of citral and some other components such as caffeic acid, geraniol, myrecene and citronellol (Barbosa *et al*., 2008). Caffeic acid is known to be a potent anti-aflatoxigenic agent against *A. flavus* and other oil components in the lemon grass (Kim *et al.*, 2008) in addition to citral, geraniol and citronellol that are fungicidal (Paranagama *et al*., 2003). In other studies, chloropylin, a parent compound of chlorophyll and water soluble chlorophyll which is readily available by consumption of green leaves like lemon grass, has been reported to

Table 6. Effect of cold suspension of *C. citratus* on AFB_1 (µg/kg) content of maize grains

Treatment Conc.(%)	Storage period (Day)							
	15	*RR(%)	20	RR(%)	25	RR(%)	30	RR(%)
0	2.44^{a**}	-	2.94^{a}	-	2.56^{a}	-	2.44^{a}	-
8	2.44^{a}	0.00	2.80^{a}	4.76	$2.30^{a,b}$	10.16	$2.19^{a,b}$	10.24
10	2.44^{a}	0.00	2.80^{a}	4.76	$2.30^{a,b}$	10.16	2.11^{b}	13.52
12	2.34^{a}	4.10	2.94^{a}	0.00	2.21^{b}	13.67	2.06^{b}	15.57
14	2.04^{b}	16.39	2.26^{b}	23.13	2.17^{b}	15.23	2.04^{b}	16.39

Values with different superscripts within a column are significantly different at $p \leq 0.05$.
*RR = rate of reduction, ** = 10^1 cfu/ml

Table 7. Effect of hot suspension of *C. citratus* on *A. flavus* (SGS-421) infestation of maize grains

Treatment Conc. (%)	Storage period (Day)									
	5	10	*RR(%)	15	RR(%)	20	RR(%)	25	RR(%)	30	RR(%)
0	0.35^{a**}	4.57^{a}	-	5.17^{a}	-	5.43^{a}	-	4.67^{a}	-	3.37^{a}	-
8	-	4.43^{a}	3.06	5.13^{a}	0.77	5.33^{a}	1.84	4.67^{a}	0.00	3.37^{a}	0.00
10	-	4.43^{a}	3.06	5.07^{a}	1.93	5.27^{a}	2.95	4.67^{a}	0.00	3.37^{a}	0.00
12	-	4.36^{a}	4.60	5.03^{a}	2.71	5.20^{a}	4.26	4.50^{a}	3.64	3.26^{a}	3.26
14	-	4.37^{a}	4.38	4.97^{a}	3.87	5.20^{a}	4.26	4.50^{a}	3.64	3.23^{a}	4.15

Values with different superscripts within a column are significantly different at $p \leq 0.05$.
*RR = rate of reduction, ** = 10^1 cfu/ml

Table 8. Effect of hot suspension of *C. citratus* on AFB_1 (µg/kg) content of maize grains

Treatment Conc .(%)	Storage period (Day)							
	15	*RR(%)	20	RR(%)	25	RR (%)	30	RR(%)
0	2.44^{a**}	-	2.94^{a}	-	2.56^{a}	-	2.44^{a}	-
8	2.40^{a}	1.64	2.80^{a}	4.76	2.56^{a}	0.00	2.19^{b}	10.25
10	2.39^{a}	2.05	2.80^{a}	4.76	2.36^{a}	7.81	2.15^{b}	11.88
12	2.30^{a}	5.24	2.80^{a}	4.76	2.26^{b}	11.72	2.13^{b}	12.70
14	2.11^{a}	13.52	2.80^{a}	4.76	2.26^{b}	11.72	2.13^{b}	12.70

Values with different superscripts within a column are significantly different at $p \leq 0.05$.
*RR = rate of reduction, ** = 10^1 cfu/ml

exhibit chemopreventive activity against aflatoxin carcinogenesis *in vivo* by forming complexes with carcinogen resulting in reduced bioavailability to target organs (Simonich *et al.*, 2008). On the other hand, the inability of the hot suspension treatments to control the growth of aflatoxigenic fungus may be due to the evaporation of volatile oils when heat was applied. This may have caused a probable denaturing and inactivation of the active and essential oil compounds that could have effect on *A. flavus*.

The order of effectiveness of the treatments was; powdered leaves, whole leaves and cold and hot suspensions. The suspensions were less effective because water may not extract all the essential components of the lemon grass. Guynot *et al.* (2003) had reported that more components of the essential oils present in dried plants are likely to be more effective in influencing fungal growth and reaction time. In addition, there was an increase in the level of formation of AFB_1 by the 20th day of the lemon grass treatments. This is in agreement with the work of Krishnamurthy and Shashikala (2006) who reported that production of AFB1 was higher at 20 day incubation period than 10 and 30 days. Similar results were obtained by Huynh and Lloyd (1984) who had an increase in the level of formation of AFB_1 by the 20th day of incubation and a gradual decline afterwards which he attributed to the degradation of AFB_1 by ageing mycelia.

Many of the individual constituents of the essential oils are themselves used as flavouring substances and pose no toxicological threats (Smith *et al*., 2005). Furthermore, Souza *et al*. (1986) observed that an infusion prepared from leaves of *C. citratus* orally administered to adult rats at dosages up to 20 times than the corresponding human dosages did not induce any toxic effect. Therefore, the different treatments used in this research work are not likely to pose any health risk considering their ethno medical use. In conclusion, the findings showed that *A. flavus* infestation and AFB_1 formation in maize grains can be controlled by co-storing powdered leaves of lemon grass with aflatoxin infected maize. The importance of the study lies in the simplified technique for controlling the aflatoxigenic fungus and aflatoxin contamination and the benefits derivable from the use of local resources

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