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ORIGINAL ARTICLE

Molecular Characterization of Aflatoxigenic *Aspergillus species* in dried Traditional foods in Zimbabwe

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ABSTRACT
The presence of aflatoxin producing Aspergillusspp in sixty samples of six selected traditional foods sold on the Bulawayo open market in Zimbabwe was investigated. Ten samples of each of the following commodities were bought from the market; dried groundnuts, dried cowpeas, dried maize, dried cowpea leaves, dried mopane worms and dried Cleome gynandra leaves and analysed for the presence of aflatoxigenic aspergillus. Moisture content of the samples was determined and another portion of the samples was plated on petri dishes containing Sabouraud Dextrose Agar (SDA) and incubated at 28°C. A total of 35 isolates was obtained and these were characterised according to their morphology as well as the type of aflatoxins they produced as determined by thin layer chromatography. Four distinct morphological groups were found and they were classified as Aspergillu sparasiticus (57%); Aspergillusniger (17%); Aspergillus tamarii (17%) and Aspergillusflavus (8%). These results were validated by using DNA primers of the structural genes, aflD(nor-1), aflM(ver-1) and aflP(omt-1) and the regulatory gene aflR to discriminate between aflatoxigenic and non-aflatoxigenic strains after amplifying DNA of the fungal strains. None of the isolates produced all the four genes involved in the aflatoxin biosynthetic pathway although they had shown positive results on the biochemical tests. Out of the 35 isolates obtained, 18 of them were aflatoxigenic and these isolates were from mopane worms (9), cowpeas (4), groundnuts (3) and Cleome gynandra (1). This investigation showed that dried traditional foods in Zimbabwe were contaminated by the aflatoxigenic fungus, Aspergillus, probably due to improper drying of the commodities, coupled by prevailing environmental conditions from packaging to the selling points.
***Key words**: Aflatoxins, Aspergillus, traditional foods, DNA primers.*

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INTRODUCTION

Economic and technological growth such as commercial scale plantations and cash-cropping has led to a reduction in the use of traditional food resources in Zimbabwe [1].However, of late traditional food reserves utilization in urban areas has gradually increased as awareness of the health protecting properties of non-nutrient bio-active compounds found in these foods is now recognised [2]. This is evidenced by open markets that are found in urban areas where these traditional foods are sold either in the fresh form or the dried form.

Edible wild leafy vegetables play an important role in the African agricultural and nutritional systems [3]. The cat's whiskers (*Cleome gynandra*L./*Gynandropsisgynandra*(L.) Briq) is one such vegetable. It grows as a weed in most tropical countries, but is a semi-cultivated popular tropical leafy vegetable in many parts of sub-Saharan Africa, especially in countries in eastern and southern Africa [3]. In Zimbabwe traditional leafy vegetables grow naturally, while others are cultivated in traditional subsistence farming [4]. Growing under a wide range of environmental conditions examples of the traditional leafy vegetables include cowpea leaves (*Vignaunguiculata*), Cat whiskers, (*Cleome gynandra* and *C. monophylla*), cucurbits and vegetable amaranth (*Amaranthus*hybridus, *A. thunbergii*). Rural communities supplement grain-based staple diets consisting mainly of maize or sorghum soft porridge with legumes, mufushwa and

tuber-type of traditional vegetables [4]. Grain-based foods are reported to be poor sources of protein, calcium, iron, zinc, riboflavin,, vitamin C, and carotene (pro-vitamin A), but are good sources of magnesium, potassium, and thiamine. When the dried vegetables are cooked and eaten with white maize porridge a more balanced diet results [4]. The vegetables are dried during the rainy season to ensure continual supply, use and consumption of the vegetables throughout the year [5]. During the drying process and the storage period there is a possibility of these vegetables coming into contact with fungi. The vegetables can be contaminated with spores both in the soil and air as they are usually dried on rocks in the open. Infestation with pests during storage can result in elevated temperatures and increases in water activity as a result of respiration processes of these pests. These conditions will then favour the germination of the spores into fungi. These fungi cause food spoilage, biodeterioration and are capable of producing different mycotoxins. *Aspergillus* species (*Aspergillus flavus* and *Aspergillus parasiticus*) are the most common toxigenic species in various stored food commodities [6]. These species produce aflatoxins, the most carcinogenic mycotoxins. Consumption of aflatoxin-tainted foods can lead to chronic aflatoxicosis in animals [7]. Aflatoxins have been shown to be immunosuppressive, mutagenic, teratogenic and hepatocarcinogenic in experimental animals [8]. The significant economic and health hazards caused by fungi and mycotoxins, especially in developing countries that have poor food storages, is of great concern [9], therefore there is need to monitor fungal and mycotoxin contamination periodically so as to meet international and national mycotoxin regulatory standards.

Identification of *Aspergillus* species is not an easy task due to the similarities amongst some of the species. Generally identification of the *Aspergillus* species is based on the morphological characteristics of the colony and microscopic examinations [13]. Although molecular methods continue to improve and become more rapidly available, microscopy and culture remain commonly used methods for identification of *Aspergillus spp* [13]. Molecular methods have been widely applied in the identification of a large number of *Aspergillus* species. The most widely used DNA target regions for discriminating *Aspergillus* species are the ones in the rDNA complex, mainly the internal transcribed spacer regions 1 and 2 (ITS1 and ITS2) and the variable regions at the 5' end of the 28S rRNA gene (D1-D2 region) [14]. Also detection of genes or their transcripts involved in the aflatoxin biosynthetic pathway can be used for the identification of Aspergillus [15]. Several genes involved in aflatoxin biosynthesis have been identified, cloned and studied. They include a regulatory gene locus *aflR* from *A. flavus*and *A. parasiticus*, and several structural genes, *e.g. pks*A, *nor*-1, *ver*-1, *uvm*8 and *omt*A [14]. By combining different methods (polyphasic approach), it is possible to achieve are liable identification and a discrimination of putative aflatoxin producers.

Work done in Zimbabwe has shown that the population is exposed to aflatoxins. In 1987, Nyathi and colleagues measured urinary aflatoxin levels in samples collected across the country and found that 4.3% of the samples analysed were contaminated [10]. In the same year 11% of human breast milk analysed was reported to be positive for AFM_1 [11]. In 1996 Siwela, [12], analysed 277 samples of groundnuts, cowpeas, maize, sorghum and millet. He found that 16% of these commodities had a total aflatoxin level of greater than the 20 µg/kg limit. The problem of aflatoxin contamination on commodities is not new in Zimbabwe but this study aims at screening for the presence and identifying aflatoxigenic*Aspergillus* in traditional foods commonly sold at the Bulawayo open market. Early detection of these fungi can serve as a warning signal for the potential health hazard.

MATERIALS AND METHODS

Food samples

A total of sixty samples of selected dried traditional foods (sample size =10) of each of the following; dried groundnuts, dried cowpeas, dried maize, dried cowpea leaves, dried mopane worms and dried *Cleome gynandra* leaves) were purchased from the Shashe Market in Bulawayo during the period of April-May 2011. Moisture content of each sample was determined by heating 10 g of each sample in the oven at 100˚C to a constant weight [16].

Mycoflora isolation and identification

The samples were directly plated onto petri dishes containing sabouraud dextrose agar (SDA). After 5 days incubation at 25˚C the colonies were identified morphologically to genus level using colony and spore colour. Also taken into account was head seriation and conidia morphology through microscopy [17]. These isolates were subcultured onto fresh media until pure cultures were obtained.

Screening for aflatoxin production using ammonium hydroxide

The isolates were grown on yeast extract sucrose agar (YES), comprising of 2% yeast extract, 5% sucrose and 1.5% agar. These were seeded as single colonies in the centre of plate and incubated in the dark at 28°C in duplicates [18]. The isolates were then tested for aflatoxin production using ammonia on days 3

and 7 after incubation. This was done by adding 2 ml of ammonia solution on the lid of the petri dish and inverting the plate. Aflatoxin producing isolates would turn pink on the reverse side whilst there was no colour change for non –aflatoxin producers.

Screening for aflatoxin production using coconut agar medium (CAM)

In CAM preparation, desiccated coconut was obtained locally. Desiccated coconut powders (100 g) were mixed with 600 ml hot water in a separation funnel. A volume of 350 ml of the clear extract was then measured. Agar-agar (15 g/l) was added and the mixture heated to boiling and autoclaved. The molten agar was poured into sterile petri dishes and left to set. The plate centre was inoculated with spores of the isolates and incubated in the dark at 25˚C for four days and viewed under UV light (365 nm) to check for fluorescence which would reveal aflatoxin production by the isolate either by formation of a blue or green ring depending on the aflatoxin produced by the isolate. The results were scored as positive or negative [19].

Aflatoxin detection by Thin Layer Chromatography

All isolates that showed positive results in the preliminary aflatoxin production tests were then grown on YES agar at 25˚ C for seven days. The agar and the mycelia were homogenised in a warring blender and extracted with 250 ml methanol- water solution (9+1) containing 3 g sodium chloride and allowed to stand for 30 minutes [20]. The slurry was then vigorously shaken in the presence of 100 ml hexane and finally filtered using Whatmann no. 540 filter paper. A 25 ml aliquot of the filtrate was extracted with 25 ml chloroform using a separating funnel. The filtrate was dried using anhydrous sodium sulphate and then evaporated on a steam bath to near dryness. The residue was redissolved in 200 µl of chloroform and 5 µl spotted on TLC plates alongside authentic aflatoxin standards. The plates were developed in chloroform-acetone (9+1) solvent system. Aflatoxin identification was achieved by visual comparison of fluorescence of standards when viewed under UV light. Confirmation of aflatoxin identity was then achieved by spraying plate with 25% sulphuric acid [21].

DNA extraction

A loopful of spores of culture was grown in YES broth for 5 days at 25˚C in 250 ml conical flask on a rotary shaker (100 rpm). Mycelia were then harvested by filtration through filter paper, rinsed with 0.85% NaCl solution. The mycelia was then stored in 1 g aliquots at -70˚C. DNA was then extracted using GeneJET™ Plant Genomic DNA purification Kit #K0791.

PCR amplification

PCR amplifications were done in 25 µl reaction mixtures containing 0.4mM of each primer pair (primer sequences are given in table 1) of the aflatoxin pathway genes *aflR*, *ver-1*, *omt-1* and *nor-1*, 10ngof the DNA sample, 12.5 µl of Maxima Hot start mastermix and the remainder topped up with nuclease-free water. PCR was carried out as follows 94˚C for 4 minutes followed by 5 cycles of 1 minute at 94˚C, 1 minute at 60˚C and 1 minute at 72˚C then 30 cycles of 1 minute at 94˚C,1 minute at 55˚C, 1 minute at 72˚C and a final extension of 6 minutes at 72˚C. A multiplex PCR was developed using the same set of primers employed in single gene PCR protocol so as to simplify PCR approach for detection of aflatoxigenic*Aspergillus*strains. The PCR products separated by electrophoresis on 1.2% agarose gel with 0.5% ethidium bromide in1×TBE (Tris-borate EDTA, pH 8.0) buffer and visualised under UV light.

RESULTS

Dried cowpea leaves had the highest moisture content with an average of 9.5% whereas the rest of the isolates had moisture content of less than 7.5%. A total of 35 isolates was obtained of which 17 were aflatoxin producers. Fig 1 shows typical colonies of the isolates. Four distinct morphological groups were found and were classified as *Aspergillusparasiticus* (57%) showing green colonies, *Aspergillusniger* (17%) showing black colonies, *Aspergillustamarii* (17%) showing brown colonies and *Aspergillusflavus* (8%) showing yellowish-green colonies.

The distribution of the fungi in different commodities is shown in Fig 2. *A. parasiticus* was the most prevalent as it was present in all the food commodities but cowpeas, followed by *A. flavus*, *A. tamarii* and *A. niger*. The highest number of isolates were from the mopane worms whilst only one species was isolated from catwhiskers.

Figure 3. shows the ability of the isolates from the different food commodities to produce aflatoxins. All the isolates from maize samples were non-aflatoxigenic. There were equal numbers of aflatoxigenic and non aflatoxigenic isolates obtained from mopane worms and cowpeas. The most prevalent aflatoxin that was produced by the aflatoxigenic species was AFB_1 (Table 1).

Table 1. Target regions and PCR primers

Target region	Primers	Expected amplicon
nor	**F** 5'-ACC-GCTACGCCGGCACTCTCGGCAC-3' **R** 5'-TTGGCCGCCAGCTTCGACACTCCG-3'	400bp
ver	**F** 5'-GCCGCAGGCCGCGGAGAAAGTGGT-3' **R** 5'-GGGGATATACTCCCGCGACACAGCC-3'	600bp
omt	**F** 5'-GTGGACGGACCTAGTCCGACATCAC-3' **R**5'-GTC-GGCGCCACGCACTGGGTTGGGG-3	800bp
AflR	**F** 5'-TATCTCCCCCCGGGCATCTCCCGG-3' **R** 5'-CCGTCAGACAGCCACTGGACACGG-3'	1000bp

Molecular identification

Using gel electrophoresis of the *ver-1* primer pair, a band of 600 bp was produced (Fig 4). Out of the 8 isolates from mopame worms, the gene was amplified in only three isolates (lanes 11, 13 and 19). The same band was produced in all isolates from groundnuts (lanes 8, 9 and 10). Lanes 4, 5, 6 and 7 represents isolates from cowpeas and 3 out of the 4 isolates showed the presence of the *ver-1* gene. The isolate from *Cleome gynandra*did not have the gene. Out of the 17 aflatoxigenic isolates obtained from the study, 9 of them showed the presence of the *ver-1* gene.

Gel electrophoresis of the PCR products of the *omt-1* gene is shown in Fig 5. The *omt-1* gene was found to be present in 9 of the 17 isolates i.e. isolates from CG (lane 3), two isolates from CP (lane4 and 5), and 6 isolates from mopane worms (lanes 12, 13, 14, 15, 18 and 19). For the *nor-1* gene a product of 400 bp was obtained from 15 out of the 17 isolates (Fig 6) whereas none of the isolates showed the presence of the *AflR*gene.

Fig 7 shows multiplex PCR of the genes involved in the aflatoxin biosynthetic pathway. Only one out of the 17 isolates produced 3 bands (lane 16). This isolate was from mopane worms. There was only one isolate with one band and this was from mopane worms as well (lane 10) . The *nor-1* gene was the only gene found in this isolate. The remaining 14 isolates produced two bands. The combination of ver-*1* and *omt-1* was produced by 14% of the isolates whereas 36% had a combination of *omt-1* and *nor-1* the remaining 50% had the *ver-1 and nor-1* gene.

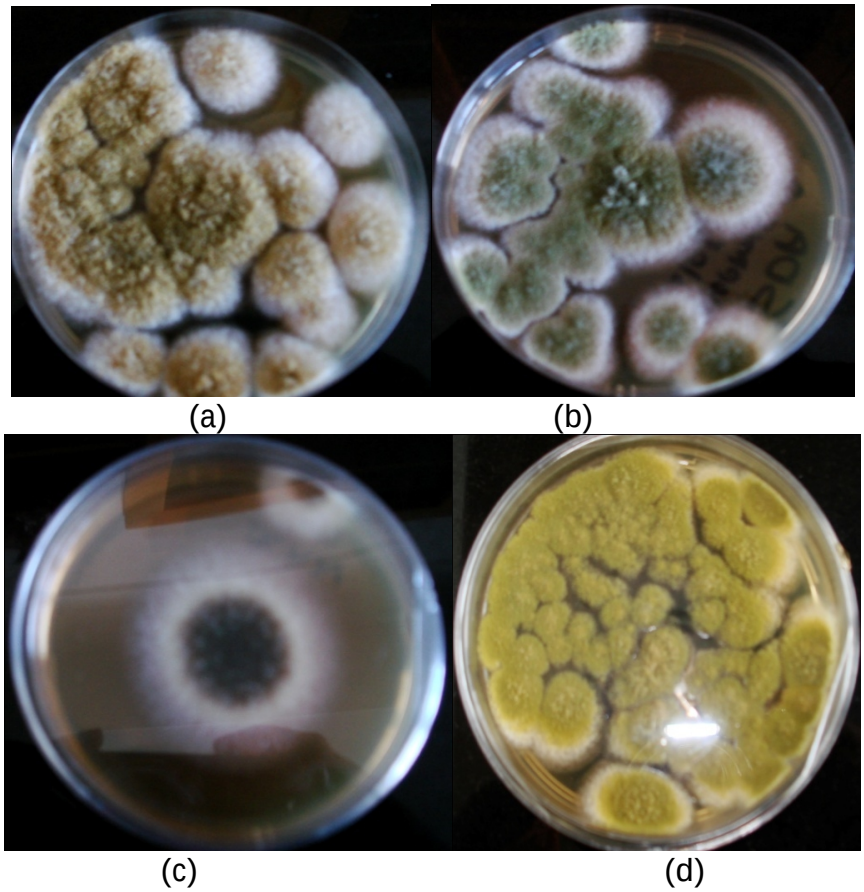

Fig 1. Identification of the isolates based on colony morphology. Plate (a) brown colony identified as *A. tamarii*, (b) dark green colony identified as *A. parasiticus*, (c) black colony identified as *A. niger* and (d) light green colony identified as *A. flavus*.

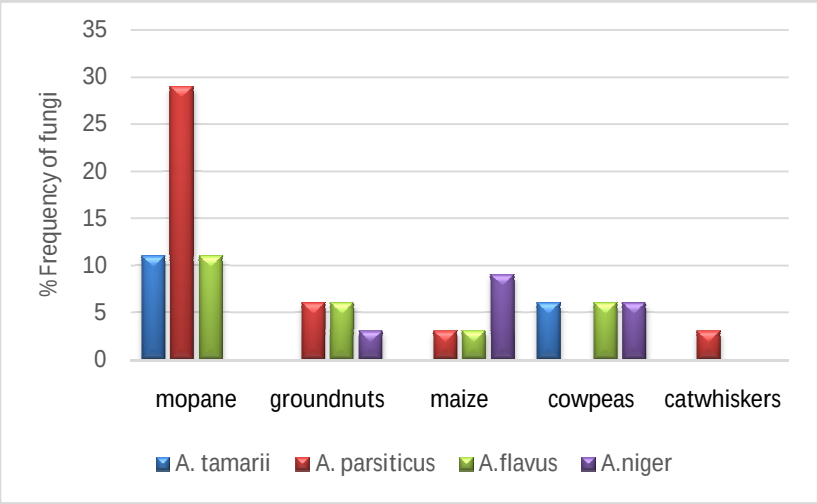

Fig. 2 Distribution of *Aspergillus spp* isolated from dried foods.

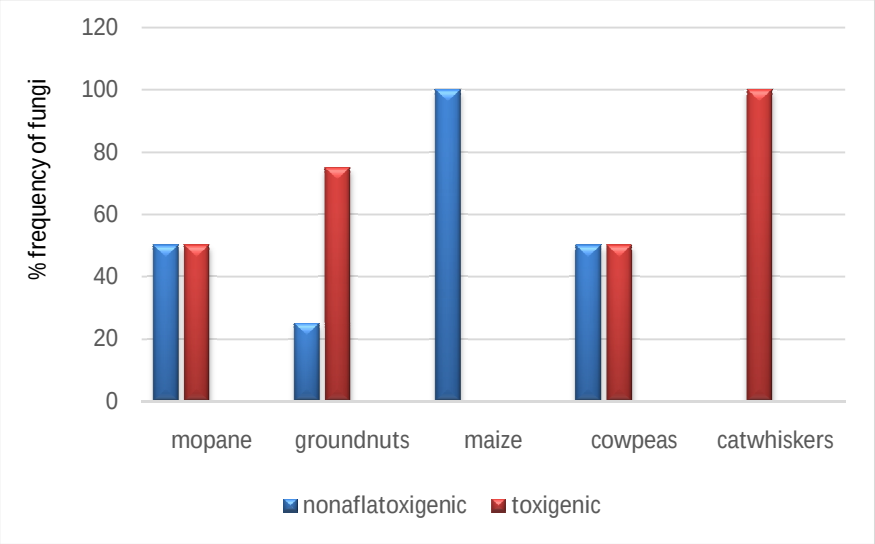

Fig .3 Comparison of aflatoxigenic and non-aflatoxigenic *Aspergillus spp* isolated from different dried food commodities

Table 2. Morphological and chemical characterization of isolates of aflatoxigenic*Aspergillus*isolates.

Isolate code	Colony colour	Seriation	Conidia	Reaction with ammonia vapour	Reaction on CAM under UV	AFB1	AFB2	AFG1	AFG2	Classification
MW3	G	u/b	R	+	+	+	-	+	-	*A parasiticus*
MW4	G	u/b	R	+	-	+		+	+	*A parasiticus*
MW5	G	u/b	R	+	+	+	+	+	+	*A parasiticus*
MW6	b	n.d	R	+	+	+	-	-	-	*A tamarii*
MW12	G	u/b	R	+	+	+	+	+	+	*A parasiticus*
MW14	G	u/b	R	+	+	+	-	+	+	*A parasiticus*
MW16	G	u/b	R	+	+	+	-	+	-	*A parasiticus*
MW17	g	b/u	S	+	+	+	-	-	-	*A flavus*
MW18	g	b/u	S	+	+	+	-	-	-	*A flavus*
GN3	g	b/u	S	+	+	+	-	-	-	*A flavus*
GN4	G	u/b	R	+	+	+	+	-	+	*A parasiticus*
GN5	g	b/u	S	+	+	+	-	-	-	*A flavus*
CP1	B	n.d	R	+	+	+	-	+	-	*A tamarii*
CP2	B	B	R	+	+	+	+	+	+	*A niger*
CP3	g	b/u	S	+	+	+	+	-	-	*A flavus*
CP5	B	n.d	R	+	-	+	-	+	+	*A tamarii*
CG1	G	u/b	R	+	+	+	-	+	-	*A parasiticus*

KEY: B- Black, b-Brown, G- Green dominating (dark green or green-yellow), g- Yellow dominating (yellow-green), +(present), - (absent), R- rough, S-smooth, b- biserieate, u- uniseriate, u/b-predominantly uniseriate, b/u-predominantly biseriate, MW- mopane worms, CP-cowpeas, GN- groundnuts, CG- *Cleome gynandra.*

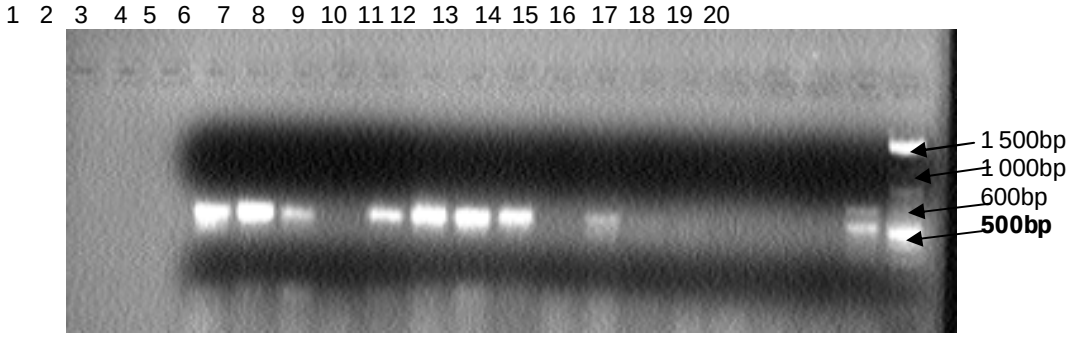

Fig 4. Gel electrophoretic analysis of PCR products using *ver-1* primers andDNA obtained from the aflatoxigenic isolates.

Lane 20 is the 1500bp marker, lane 3-*Cleome gynandra* isolates, lanes 4, 5, 6, and 7 are cowpeas isolates, lanes8, 9 and 10 are groundnuts isolates, lanes 11 to 19 are isolates from mopane worms.

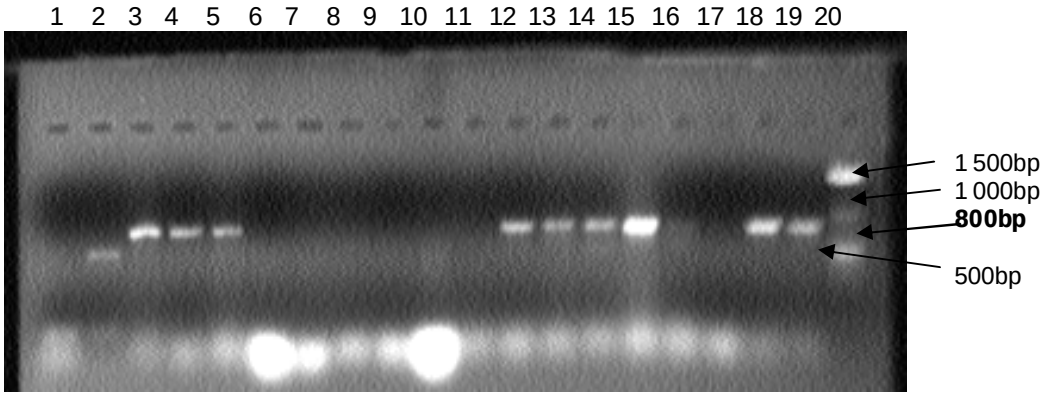

Fig 5. Gel electrophoresis of PCR products using *omt-1* primers and DNA obtained from the aflatoxigenic isolates.

Lane 20 is the 1500bp marker, lane 3-*Cleome gynandra* isolates, lanes 4, 5, 6, and 7 are cowpeas isolate lanes8, 9 and 10 are groundnuts isolates, lanes 11 to 19 are isolates from mopane worms.

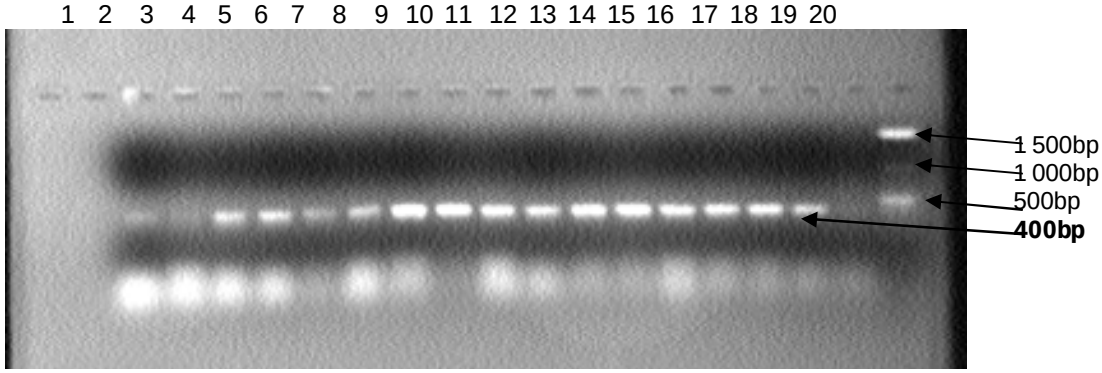

Fig 6. Gel electrophoresis of PCR products using *nor-1* primers and DNA obtained from the aflatoxigenic

Lane 20 is the 1500bp marker, lane 3-*Cleome gynandra* isolates, lanes 4, 5, 6, and 7 are cowpeas isolates, lanes8, 9 and 10 are groundnuts isolates, lanes 11 to 19 are isolates from mopane worms.

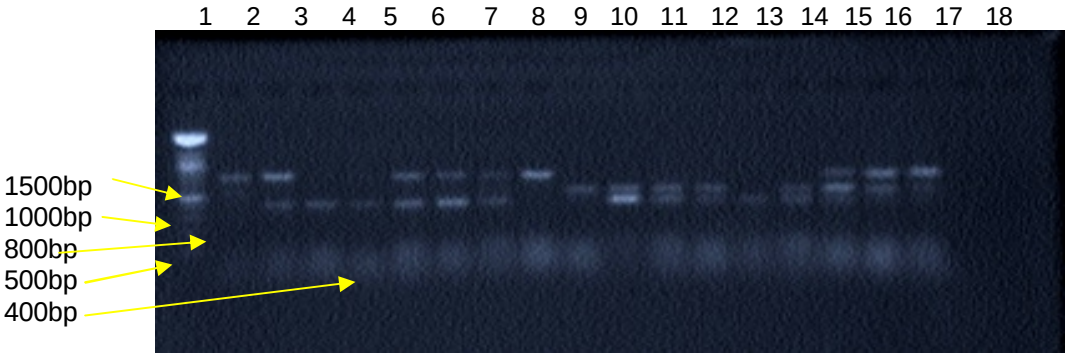

Fig 7. Bands amplified by multiplex PCR of the extracted DNA from the aflatoxigenic isolates and the four primers involved in the aflatoxin biosynthetic pathway. Lane 1 is the molecular marker, lanes 2-10 isolates from mopane worms, lanes 11-13 isolates from groundnuts, lanes 14-17 isolates from cowpeas and lane 18 from *Cleome gynandra*.

DISCUSSION

This study showed that some dried traditional foods are contaminated with *Aspergillus*and the predominant species was *Aparasiticus*. The most contaminated commodity was the dried mopane worms. The results based on biochemical tests for aflatoxin production were in agreement except for a few cases where the same isolates gave positive results for a particular test and negative results on a different test, e.g. isolates MW4 and CP5 had positive results for ammonia reaction and TLC but negative for CAM (Table .1). Studies have indicated that coconut has constituents that have a negative effect on the fluorescent pigment production and as such coconut based media do not always give reliable results [22].

In this study, most of the *Aspergillus spp* isolated from the dried foods did not generate any PCR products of the *AlfR* gene within the aflatoxin gene cluster. However when biochemical tests on aflatoxin production were done, these isolates gave positive results. This suggests that aflatoxin production is not regulated only by the *AflR* gene. In agreement with our study, Obrian, [23] showed that*aflS* is another gene that has a regulatory role in aflatoxin biosynthesis. Wang, [24] also indicated that it is uncertain to judge the aflatoxigenicity of an *Aspergillus* strain using the *aflR*gene as an indicator. Magan, [25] had earlier suggested that the *AflR*gene may not have an important role in the regulation of *nor-1* expression in food matrices. It is believed that the nor-1 (*AflD*) gene encodes an enzyme that catalyses the ketoreduction of norsolorinic acid to averantin in the aflatoxin biosynthetic pathway. Disruption of the nor-1 gene in *A. Parasiticus*was shown to result in accumulation of norsolorinicacid [26], confirming the important function of the nor-1 (*aflD*) in aflatoxin synthesis. In our study most of the isolates showed the presence of *nor-1 gene* meaning that these isolates were able to produce averantin from norsolorinic acid. The presence of this gene is the first indicator that an isolate is capable of producing aflatoxins [27].

Our study showed that the most prevalent aflatoxin that was produced by most of the isolates was AFB_1. This is in agreement with Ehrlich [28] who stated that all aflatoxin positive strains produce AFB_1. AFB_1 is known to be the most potent toxin of all the aflatoxins so its presence in food is of great concern. However, this study also showed that not all *Aspergillus spp* found on dried foods are aflatoxigenic. The non-aflatoxigenic species may have a symbiotic relationship with the commodity where the fungi get food whilst the food commodity is protected from being attacked by toxigenic strains. It has been shown that increasing soil densities with nontoxigenic strains results in seeds being colonised with nontoxigenic strains and a decrease in aflatoxin concentration in seeds [29]. All in all this study revealed that some of the *Aspergillus* spp isolated were aflatoxigenic, and as such, there is a need to prevent contamination of the foods by these fungi. The most effective way of preventing the fungi from getting to the food is by drying the commodities in a dust free environment with as little contact with the soil as possible as *Aspergillus* are found in the soil.

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