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Full Length Research Article

MYCOLOGY AND EFFECTS OF DEHAULING LOCAL *ORYZA SATIVA* FOR HUMAN CONSUMPTION ON TOTAL AFLATOXINS WITHIN SOME PARTS OF KADUNA METROPOLIS

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ABSTRACT
Aflatoxins are the most toxic food contaminant and their presence in Oryza Sativa could have a great toxic effect on humans and animals health. The aim of this study was to isolate aflatoxigenic moulds from dehauled Oryza sativa and to assess the effects of small scale mechanized and traditional wooden pestle and mortar processing methods on aflatoxins. Standard techniques was employ to screen for moulds using potato dextrose agar and aflatoxigenic strains using Yeast extract sucrose agar (YESA) modified with 0.3% cyclodextrin and 0.6% sodium desoxycholate (YCSD). The total aflatoxin was determined using enzyme-linked immunosorbent assay (ELISA). Proximate values were also determined using standard techniques. The mould counts for the processed samples were lower than that of undehauled Oriza sativa. The percentage moisture was relatively low (9.69 and 9.79 %) for both small scale mechanized and pestle and mortar processing techniques respectively. Ash content of Oryza sativa obtained through pestle and mortar processing method presented higher level (0.74 %dm) compared to small scale mechanized technique (0.48 %dm). Aspergillus flavus had the highest frequency of occurrence. While thirty nine (39.0 %) of the total samples collected was contaminated with aflatoxigenic moulds, seventeen (17.0 %) represent prevalence of aflatoxin contamination. It was understood from this work that wooden pestle and mortar processing retains higher total aflatoxin level than small scale mechanized technique. The small scale mechanized technique is better but can further be standardized to solve the issue of drying after boiling raw Oryza sativa for dehauling.

INTRODUCTION
Mycotoxins are generally defined as secondary metabolites of fungi that have great pathological changes in humans and animals (livestock) (Reddy, 2010; Ghali *et al*., 2010; Sun *et al*., 2011; Nejati *et al*., 2014). The word mycotoxin has been derived from a Greek word *"myke"* which means fungus and also "*toxicum*" a Latin word which mean poison. Aflatoxins are mycotoxin, most well-known because of its carcinogenicity, toxic health challenging issue (Almeida *et al*., 2012). Besides aflatoxins, ochratoxin A, fumincins, trichothecene, and many other mycotoxin have been discovered. Aflatoxins are produced by two founder mental species of moulds; *Aspergillus flavus* and *Aspergillus parasiticus*. They are natural moulds found in human food supply in areas with wet and warm climates (Sugita-Konishi *et al*., 2006; Andrade *et al*., 2012; Surez-Bonnet *et al*., 2013). The aflatoxigenic mould could be found in grains grown under droughts (Bansal *et al*., 2011) where they secret aflatoxins B_1, B_2, G_2 and G_2 (Hussain and Anwar, 2008). Aflatoxins are the most toxic food contaminant that has a great toxic effect on humans and animals health. International Agency for Research on Cancer has classified aflatoxin B_1 as a "Group I carcinogen" (International Agency for Research on Cancer, 2014). Large populations of humans and livestock have died due to contamination of food with mycotoxins (Sales and Yoshizawa, 2005). The main cause of this contamination is basically due to exposure in the farm before harvest, post-harvest practices. Aflatoxins are not entirely killed off even when contaminated foods such as wheat and pea nuts (Hyun *et al*., 2007) are processed or roasted. Chemical processes are used to remove aflatoxins in foods (Fallah *et al*, 2011). Likewise brewing and germination of barley (Awasthi *et al*., 2012) has also been reported to affect aflatoxin development. The control of aflatoxin contamination on rice is of great important in other to prevent the spread of these mycotoxins. Traditional processing could control the spread of these toxins in food stuffs. The aim of this work was to assess aflatoxigenic moulds and effect of dehauling *Oryza sativa* on total aflatoxins.

MATERIALS AND METHODS

Sampling Site
Oryza sativa samples were obtained from some small scale mechanized processors in station market located in Kaduna metropolis. Samples were also collected after *Oryza sativa* was dehauled using traditional wooden pestle and mortar technique.

Sample collection and Dehauling

Small scale mechanized dehauler processing
Samples of *Oryza satva* was collected before and after dehauling from each sack that customers bring for dehauling as follows: Roughly 200g each *Oryza satva* were collected before and after dehauling aseptically using sterile low density cellophane from four small scale processors at station market, Kaduna. Firstly, the cellophane was swapped with 70 percent ethanol and was allowed to air dry in a safety cabinet fitted with ultraviolet light. It was then left under ultraviolet light for one hour and wrapped in disinfected aluminium foil before use.

Manual traditional wooden pestle and mortar processing
Some few samples (400g) each with visible signs of moulds growth was dehauled using traditional wooden pestle and mortar processing method. The pounded samples were traditionally wind manually to separate the wholesome *Oriza satva.*

Isolation and characterization of the fungi
Moulds were isolated following standard techniques. Fivefold

serial dilution was made. Then a Trial test was carried out by plating 0.1mL each of the dilutions separately on freshly prepared sterile potato dextrose agar and incubated at room temperature (27± 2^{o}C) for seven days. The dilution with colony count between 30 – 300 CFU/g was adopted for subsequent analysis. Briefly, twenty five gram (25g) of each sample was aseptically weighed into a sterile conical flask containing 225mL of sterile peptone water. The mixture was shaken vigorously using vortex mixer to dislodge all fungal contaminants. Dilution two with count 30 – 300 CFU/g was taken for subsequent analysis. One milliliter of each sample was dispensed on duplicate sterile Petri dishes containing sterile potator dextrose agar mixed with (0.5g/l) Chloramphenicol added to the sterile cold to 45^{o}C molten potato dextrose agar. It was gently rocked before it was poured into sterile Petri dishes. The plates were incubated at 28°C for 7 days. The cultures were subcultured and were subsequently identified using morphological characteristics, spore formation, the production of fruiting bodies and appropriate keys by mycology on line by Ellis (2002). The characterization was also done by microscopy using a drop of lacto phenol cotton blue stain placed on a clean grease-free slide. Then a small fragment of (vegetative) colony, woolly or powdery colony was picked using a sterile needle and teased in the stain until a homogenous blue mixture of stain and culture was obtained. A clean cover slip was applied. Excess stain was removed with blotting paper and the slide examined using X10 and X40 objectives.

Aflatoxigenic Moulds Assay and Frequency of Occurrence
Aflatoxigenic moulds was cultured using specific detection media, Yeast extract sucrose agar (YESA) modified with 0.3% cyclodextrin and 0.6% sodium desoxycholate (YCSD) according to the method described by Ordaz *et al*. (2003) and adopted by Wartu *et al*. (2017). The frequency of occurrence of isolated aflatoxigenic strains was determined using the formulae:

Frequency of occurrence =

$$\frac{\text{Number of times fungus encountered}}{\text{Total number of times all fungi were encountered}} \times \frac{100}{1}$$

Proximate Analysis
Proximate composition on *Oryza satva* was carried out according to the methods described by APHA (1999).

Aflatoxin Analysis using Enzyme-Linked Immunosorbent Assay (ELISA)

Aflatoxin Extraction
Aflatoxin extraction was done using extraction kits obtained from the manufacturers (Helica Biosystems Inc. U.S.A.) guide. Briefly, a representative sample was obtained and grinded in a blender so that 75% passes through a 20 mesh screen. Then 5g of the sample was measured and 25mL of 70/30 (v/v) methanol/water extraction solution was added to each sample. Extraction was done in the ratio of 1:5 (w/w) of sample extraction solution respectively. The sample was then vigorously shaked using orbital shaker at 250 rpm for 3mins. The sample was then allowed to settle and filtered.

Aflatoxin Detection
The detection of aflatoxins from the sample was carried out according to manufacturer's instructions: Two hundred (200) µl of conjugate was pipetted using a micro liter pipette and dispensed into the mixing wells**.** Then**,** 100µl of standard and sample was pipetted and added to mixing wells. Each well was mixed carefully by pipetting up and down 3 times and immediately 100µl of contents was transferred from each dilution well into a corresponding antibody coated micro well**.** It was then incubated at 37^{0}C for 15mins**.** The content of the antibody coated micro well strips was then emptied into waste container and washed by filling with distilled water and then dumping the water from the micro well strips using a tissue. This step was repeated 4 times. Then, 100µl of the substrate was pipetted into each micro well strip and Incubated at room temperature for 5mins after which a blue colour developed**.** Then, 100µl of stop solution was measured and dispensed into each micro well strip. On adding stop solution colour changes from blue to yellow, micro well strip was then read using ELISA reader at the wavelength of maximum absorption of 450nm.

RESULTS AND DISCUSSION
The isolation of moulds and aflatoxigenic strains from *Oryza sativa* indicated the presence of the moulds strains and aflatoxin contamination of the samples collected from the small scale processors. Proximate analysis was carried out for the chemical evaluation of the *Oryza sativa*, where the samples were subjected to series of standard analysis, so as to determine the percentage ash, protein, moisture content and free fatty acid as shown in Table 1. The percentage moisture was relatively low (9.69 and 9.79 %) for both small scale mechanized and wooden pestle and mortar processing techniques samples respectively. High moisture in food and homes encourage the growth of moulds (Pitt, 1995; Bankole and Joda, 2004; Niemeier *et al*., 2006). However, the moulds spores could be the principal avenue for contamination of *Oryza sativa* and other similar food crops. Ash content of *Oryza sativa* obtained through pestle and mortar processing method presented higher level (0.74 %dm) compared to small scale mechanized technique (0.48 %dm). The high ash in the former could be due to improper separation of bran from the processed *Oriza sativa*. Bran has been reported to host bulk of the aflatoxins produced by moulds (Oh-Kyung Kwon *et al*., 2004). This was explained earlier by Wartu *et al*. (2017) when aflatoxin contaminated wheat was processed into wheat flour at different extraction rates. Generally, there was no significant difference ($p \geq 0.05$) between the proximate values of the two processing techniques except for their ash contents.
The samples were cultured in other to check for the presence of the two fundamental aflatoxigenic strains and other moulds contaminants that were present in the samples collected. The results indicated that *Aspergillus flavus*, *Aspergillus niger, Fusarium* sp, *Penicillium* sp and *Rhizopus* sp were present in some of the samples, but *Fusarium* sp was dominant followed by *Aspergillus flavu*s. Aflatoxigenic strain assay showed that *Aspergillus flavus* had higher frequency of occurrence than *Aspergillus parasiticus* (Table 4).
The mean total moulds count and aflatoxins detected from *Oryza sativa* processed using small scale mechanized rice dehauler is presented in Table 2.The mould counts for the processed samples were lower than that of undehauled *Oriza sativa.* This correlates well with the earlier work of Wartu *et al*. (2017) who reported that milled grain products with higher extraction or bran harbor more aflatoxigenic strains and aflatoxins. However,

efficient separation of the bran from the products greatly counts in the reduction of aflatoxins. The mean moulds count of the processed samples was within the limits (10 µg/Kg) recommended by National Agency for Food and Drug Administration and Control and Standard organization of Nigeria. However, 15.0% of the total samples screened contravene these regulatory bodies' limits. The aflatoxin values for undehauled samples were all above the 10 µg/Kg limit enacted by NAFDAC. However, all the samples from both small scale mechanized and manual pestle and mortar processing were within the NAFDAC limit but could be higher when the product stays longer before consumption due to reinfection by the aflatoxigenic strains. Certain times the *Oryza sativa* samples are cooked before drying. This opens the grains for contamination by the aflatoxigenic strains and subsequent production of aflatoxin before being dried. But generally manual pounding samples had higher aflatoxin values than the former. The high aflatoxin level in the undehauled samples could be due to poor drying techniques since all the samples were from non-mechanized farmers and the usual drying method has been by sun drying on mat or bare floors. This technique takes long time to archive and the samples become vulnerable to material contacts and ubiquitous aflatoxigenic strain contamination. Poor storage condition before processing and unstandardized processing conditions could also promote proliferation of aflatoxigenic moulds and consequently aflatoxin production when conditions are adequate. The work of other authors (Chaelae *et al*., 2002; Bankole *et al*., 2005) illustrated contamination of crops when exposed in an open field for drying. The higher total aflatoxins in manual pestle and mortar pounded samples (Table 3) could be as a result of dusty contaminated bran arising from improper separation of dehauled *Sativa oriza* from bran or probably from damaged grains (Paterson and Lima 2010) contaminated right from the rice farm. Other authors (Ordaze *et al*., 2003; Wartu *et al*., 2017) reported moulds and aflatoxigenic strain contamination of other crops such as *Triticum* sp used for wheat flour processing, soybean (Negedu *et al*., 2010), fresh and harvested groundnut (Wartu *et al*., 2005) and groundnuts oil (Wartu *et al*., 2011).

Aspergillus flavus had the highest frequency (25.0%) at dehauling station 003. There was no *Aspergillus parasiticus* isolated at dehauling station 002. However, station 001 presented highest frequency (15.0%) of occurrence of *Aspergillus parasiticus*. Most of the moulds that were isolated have public health implication in man and animals (Umoh *et al*., 2011). The occurrence of the moulds could be due to the ability of the spores of the aflatoxigenic strains to survive high processing temperature and dehauling sheer of the machinery and the manual pounding. Individuals within the study area take samples for dehauling resulting to different infection of moulds and aflatoxin contamination results.

Table 1: Means of Proximate valves of Dehauled *Oryza sativa*

Serial No	Parameters	Mean Proximate Values Dehauled Rice	
		Mechanized	Wooden Pestle and Mortar
1	Moisture content (%)	9.69[a]	9.79[a]
2	Ash (% dm)	0.48[b]	0.74[d]
3	Protein (%)	5.81[c]	5.69[c]
4	Free fatty acid (%)	0.76[d]	0.82[d]
5	Carbohydrate	83.18[e]	83.02[e]

Results are Means of Duplicate Samples and values with the same superscript along the same row do not vary significantly ($p \leq 0.05$)

Table 2: Mean Total Moulds Count and Aflatoxins of Oryza *sativa* processed using small scale mechanized rice dehauler

Dehauling Station	Undehauled *Oryza sativa*		Dehauled *Oryza sativa*	
	Moulds count (CFU/g)	Aflatoxins (µg/kg)	Moulds count (CFU/g)	Aflatoxins (µg/kg)
001	3.6×10^3	20.5	1.2×10^2	2.3
002	3.3×10^3	27.8	1.4×10^2	2.7
003	2.6×10^3	25.5	1.2×10^2	2.5
004	3.9×10^3	18.5	1.5×10^2	3.3

Table 3: Mean Total Moulds Count and Aflatoxins from *Oryza sativa* dehauled through pounding using traditional wooden pestle and mortar

Dehauling Repeats	Undehauled *Oryza sativa*		Dehauled *Oryza sativa*	
	Moulds count (µg/kg)	Aflatoxins (ppb)	Moulds count (CFU/g)	Aflatoxins (µg/kg)
1	3.4×10^3	23.5	2.1×10^2	4.3
2	3.3×10^3	27.7	2.4×10^2	3.7
3	3.5×10^3	20.8	1.1×10^2	4.3
4	3.1×10^3	19.9	2.1×10^2	4.3

Results are means of duplicate samples

Table 4: Frequency (%) of Occurrence in parenthesis of Aflatoxigenic Moulds Isolated from Dehauled *Oryza Sativa (N =80)*

Aflatoxigenic moulds	Dehauling Stations			
	001	002	003	004
Aspergillus flavus	4(20.0)	2(10.0)	5(25.0)	3(15.0)
Aspergillus parasiticus	3(15.0)	0 (0.0)	1(5.0)	2(10.0)

Conclusion

The percentage moisture was relatively low (9.69 and 9.79 %) for both small scale mechanized and pestle and mortar processing techniques respectively. Pestle and mortar processing technique presented (0.74 %dm) ash content and (0.48 %dm) for small scale mechanized processing.

It is clear from this work that some of the different samples brought to the dehauling station were contaminated with moulds species (*Aspergillus flavus*, *Aspergillus niger, Fusarium* sp, *Penicillium* sp, *Aspergillus parasiticus* and *Rhizopus* sp.). 15.0% of the total samples of dehauled Oriza sativa were above NAFDAC limits. Aflatoxigenic *Aspergillus flavus* had the highest frequency of occurrence than Aspergillus *parasiticus* at all processing stations. Traditional Pestle and mortar processing technique had high level of aflatoxins than the small scale mechanized technique. The use of resistant variety and the use of controlled drying techniques is hereby advocated.

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