Original article

Fungal contamination of crude herbal remedies as a possible source of mycotoxin exposure in man

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Received April 24, 2009; *Accepted June* 14, 2009

Abstract

Objective: The documented evidence of toxigenic fungi and their toxic metabolites on medicinal plants, coupled with the ability of these toxins to resist decomposition and temperature treatments necessitated this study, with a view of surveying for a possible carry over into the final medicinal products. As such popular indigenous crude herbal preparations widely consumed for various ailments in south-western Nigeria, were screened for fungal contamination, mycoflora enumeration, flora mycotoxin productibility, detection and quantification of a potent human carcinogen (aflatoxin). **Methods**: Fungal contamination was assessed on acidified potato dextrose agar using the plate count method, while mycotoxin detection, extraction and quantification were achieved by the thin – layer chromatography and chemical confirmation techniques. Mycoflora were characterized by standard procedures. **Results**: The total plate count ranged from 1.80×10^4 CFU/ML to 1.10×10^5 CFU/ML and 2.00×10^3 CFU/ML to 1.38×10^5 CFU/ML for water and dry gin extracted preparations respectively. The mycoflora consisted of six genera (*Aspergillus*, *Penicillium*, *Fusarium*, *Mucor*, *Alternaria* and *Rhizopus*). Thirty-four percent (34 %) of the potential toxigenic species (*Aspergillus*, *Penicillium* and *Fusarium*) produced mycotoxins in culture, while further characterization indicated production of aflatoxin B1 (42 %), ochratoxin A (50 %) and penicillic acid (8 %) by the mycotoxigenic strains respectively. The aflatoxin content of the herbal medicines ranged between 0.004 μg/kg and 0.345 μg/kg. **Conclusion**: The study confirmed the carry over of the fungal contaminants and their toxic metabolites into the final herbal medicines in quantities that exceeded some of the available limits. The implication of this is that the chronic exposure to mycotoxins particularly aflatoxins as a result of long term consumption of these preparations, could lead to impaired growth, nutritional interference, immunologic suppression and hepatocellular carcinoma in the consumers.

Keywords: Contamination; Drug safety; Mycoflora; Mycotoxins; Herbal medicines

INTRODUCTION

Herbal medicines are gaining popularity, with a high level of acceptance by patients and the majority of the population[1-3]. Even, World Health Organization estimated that more than 80 % of the World's populations rely solely or largely on traditional remedies, from home gardens, forests, alpine pastures and other multiple-use habitats[4]. On September 30, 1992, the government of Nigeria promulgated the Medical, and Dental Practitioners (Amendment) Decree No. 78, which placed natural medicine (traditional and alternative medicine) side by side with the orthodox system. Since then, advertising in various forms by the herbal practitioners is unparalleled in Nigeria[5].

Pre – harvest practices, storage, prevailing climate, the genotype of the crop, soil type, minimum and max-

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imum daily temperatures, daily net evaporation, insect activity, poor timing of harvest, heavy rains at harvest and post harvest, inadequate drying of the crop before storage, humidity, temperature, aeration during drying and storage have all been documented to increase the susceptibility of medicinal plants to contamination[6-9]. The greatest risk for health impact of toxigenic fungi and their mycotoxins lies in tropical developing countries where the agricultural practices, the various storage methods and trade practices have encouraged the contamination of harvested products with soil fungi. The warm and humid tropical conditions have further helped to compound the problem[10]. Similarly, contamination of traditional drugs by toxigenic fungi and their mycotoxins has become a concern for safety issue, which is largely because of the current unregulated nature of indigenous herbal medicines in Nigeria[11].

Extensive studies have shown that medicinal plants are associated with a broad variety of fungal contaminants[12-16]. According to Roy et al, [12] the traditional, unscientific methods of collecting, storing, and marketing medicinal plants promoted their association with several fungi. The prolonged storage of stuck - pilled medicinal plants under warm and humid ambient tropical conditions often created favourable microenvironments which further enhanced microbial growth and activities[13]. The eventual use of contaminated plant materials for medicinal preparation implies the contamination of the final herbal medicines. The effects of mycotoxins especially aflatoxins on human health have been documented and consequently over 5 billion people in developing countries worldwide are at risk of chronic exposure to aflatoxins through contaminated foods[17]. Unfortunately, mycotoxins have been reported to be carcinogenic, teratogenic, tremorogenic, haemorrhagic and dermatitic to a wide range of organisms, and may cause hepatic carcinoma in man[18].

Based on the fact that mycotoxins are not destroyed by heat[19,20] during herbal preparation and also considering the dearth of information on the toxigenic moulds in herbal medicines, which are widely consumed in the south-western part of Nigeria, it is imperative for survey of fungal contaminants and their toxic secondary metabolites in such alternative remedies. The present study therefore, tries to evaluate the predominant mycoflora, the extent of fungal contamination, the toxigenic potential of the fungal isolate and mycotoxins especially, the most potent and potentially lethal human carcinogen - aflatoxin in crude indigenous herbal preparations.

MATERIALS AND METHODS

Sampling

Thirty samples of crude medicinal plant decoctions and extracts were evaluated to assess the extent of fungal contamination, the predominant mycoflora, the toxigenic potential of the isolate and the quantification of aflatoxins. The preparations were chosen on the basis of their commercial availability and popularity of use, in treating fever, fistula, cough, sore throat, skin infections, pains and aches. Some were also used as male invigorants for alleviating erectile dysfunction and few for general health maintenance.

Evaluation of fungal contamination and identification of isolates

Ten fold serial dilutions of preparations were performed in buffered peptone water. Enumeration of fungi was by the pour plating method[21] using modified potato dextrose agar (PDA). Culture plates were incubated at 25℃ for 3-5days. The fungal contaminants were calculated as a mean of triplicates and compared with the Indonesian[22], United States[23], Malaysia[24], German[25] and WHO[21] limits for herbal medicines. Mould colonies representative of all the morphologically different colonial types were subcultured onto sterile PDA plates to obtain pure cultures. Isolates were identified by micromorphological criteria (shape, colour, size of the phialides, vesicles, conidia and conidiophores) according to the method of Domsch et al 1981[26].

Evaluation of toxigenic potential of isolates

Seven day old cultures of *Aspergillus* spp on Czapek agar (CZA), *Fusarium* spp on potato sucrose agar (PSA) and *Penicillium* spp on Czapek yeast autolysate agar (CYA) were investigated for mycotoxin production, using the agar plug method of Frisvad et al. 1989[27].

Thin layer chromatography (TLC) determination of mycotoxins

TLC was performed by touching the agar plugs on the pre-coated 20 cm x 20 cm TLC silica gel plate. After drying, plates were developed in toluene/ethylacetate/90 % formic acid (5: 4: 1, V/V/V). Plates dried at ambient temperature were observed in daylight and un-

der long wave (366 nm) and short wave (254 nm) UV light[28].

Chemical confirmation of mycotoxins

This was performed directly on the developed TLC plate. Plates were exposed to a general spray reagent: 20 % sulphuric acid in ethanol (W/V) and heat treatment at 110 ℃ for 10 minutes. This aided visualization and increases the fluorescence intensity of the mycotoxins. TLC plate was finally observed under UV light at 366nm. Fluorescence was compared with published data on colours of mycotoxin[29].

Extraction, detection and quantification of aflatoxins in crude herbal medicines

Extraction

To 5 g of herbal sample weighed into a 100 mL conical flask was added 2.5 mL of distilled water and 25 mL of chloroform. Mixture was shaken for 30 minutes and then filtered. Ten milliliter (10 mL) of the filtrate was evaporated to dryness on a water bath.

Detection

A mixture of 1 mL of chloroform and 0.2 mL of the reconstituted extract was spotted on a pre-coated 20 cm x 20 cm TLC plate, along with aflatoxin standard of known concentration. TLC plate was developed in an equilibrated tank containing chloroform: acetone (9: 1 V/V). Aflatoxins were detected under UV light at 360 nm.

Quantification

Preparative TLC plate (0.5 μm) was used and 0.8mL of aflatoxin extract was applied to the plate as a band, to chromatograph the maximum amount of sample. Development of plate was as in detection. Plate was removed from tank when solvent reached 3/4 of the plate length. Following examination under the UV light, the area containing the toxin was scrapped, and extracted with chloroform. Three milliliter (3 mL) of the reconstituted solution and aflatoxin standard of 20 μg/mL concentration was used to determine absorbance or optical density on a UV spectrophotometer at 360 nm. Aflatoxin concentration was calculated thus:

$$\frac{\text{Absorbance of sample} \times \text{Concentration of standard} \times \text{Dilution factor}}{\text{Absorbance of standard}}$$

RESULTS

The fungal enumeration of the herbal samples yielded between 1.80×10^4 CFU/mL-1.10×10^5 CFU/mL and 2.00×10^3 CFU/mL - 1.38×10^5 CFU/mL in the water and dry gin extracted preparations respectively. The microbiological suitability of preparations for human consumption was assessed, by comparing fungal load to available international standards (Indonesian, United States, Malaysian, German and the World Health Organization). Only one dry gin extracted herbal medicine (3 %) was found to be safe, by the German standard which permitted a fungal load of $< 10^4$ CFU/g/mL. However the remaining 97 % crude herbal medicines failed by the reason of very high load which was unacceptable by all the standards used.

The predominant mycoflora obtained from the herbal medicines was distributed into 6 genera, namely, *Aspergillus*, *Penicillium*, *Fusarium*, *Mucor*, *Rhizopus* and *Alternaria*. The genus *Aspergillus* was the dominant mycoflora consisting of seven species; *Aspergilus flavus*, *Aspergillus parasititcus*, *Aspergillus niger*, *Aspergillus wentii*, *Aspergillus erythrocephalus*, *Aspergillus terreus* and *Aspergillus* tamarii. However, *Aspergillus niger* was found to have the highest recovery rate (Table 1).

Table 1 Distribution of the fungi detected in samples of herbal medicine (*N*,%).

S/n	solated fungi	Recovery rates of isolates
1	*Aspergillus niger*	13 (24.53)
2	*Aspergillus wentii*	2 (4.08)
3	*Aspergillus flavus*	4 (8.16)
4	*Aspergillus parasiticus*	2 (4.08)
5	*Aspergillus erythrocephalus*	6 (12.24)
6	*Aspergillus terreus*	1 (2.04)
7	*Aspergillus tamari*	1 (2.04)
8	*Fusarium poae*	3 (6.12)
9	*Rhizopus oryzae*	2 (4.08)
10	*Rhizopus stolonifer*	1 (2.04)
11	*Mucor hiemalis*	6 (12.24)
12	*Penicillium oxalicum*	2 (4.08)
13	*Penicillium chrysogenum*	1 (2.04)
14	*Alternaria citri*	5 (10.20)
15	*Alternaria tenius*	3 (6.12)

Two species of Rhizopus, *Penicillium* and *Alternaria* were identified, while the genera *Mucor* and *Fusarium* had one specie each (Table 1). The 36 potential toxigenic isolates of *Aspergillus*, *Penicillium* and *Fusarium* were evaluated for their ability to produce mycotoxins *in vitro* and three species of *Aspergillus* namely: *Aspergillus flavus*, *Aspergillus niger* and *Aspergillus parasiticus* produced mycotoxins in culture (Figure 1). These were aflatoxin B1 and ochratoxin A. The highest number of mycotoxin producers was found in the specie *Asp. flavus* (4 of 4 isolates). Only one Penicillium specie was mycotoxigenic producing penicillic acid. None of the Fusarium isolates produced mycotoxin. All the crude herbal medicines however, contained aflatoxins in varying concentrations, ranging from 0.004 μg/kg to 0.345 μg/kg (Figure 2).

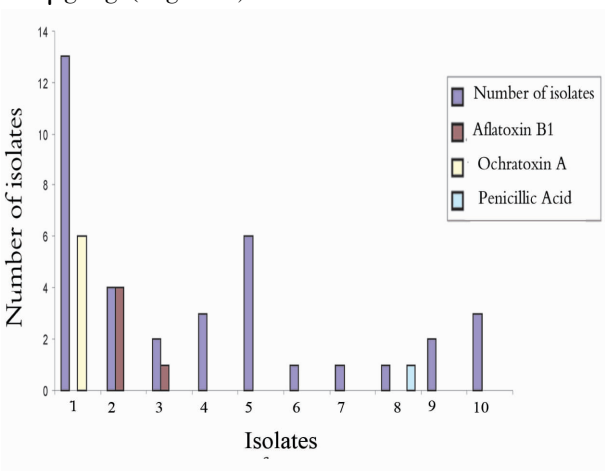

Figure 1 Distribution of toxigence isolates detected in herbal medicines according to the type of mycotoxins produced.
1 Aspergillus niger; 2 Aspergillus flavus;3 Aspergillus parasiticus;4 Aspergillus wentii; 5 Aspergillus erythrocephalus; 6 Aspergillus terreus;7 Aspergillus tamaril; 8 Penicillium chrysogenum; 9 Penicillium qxalicum;Fusarium poae.

DISCUSSION

Herbal remedies are perhaps the most common form of alternative medicine. In fact, almost every nation or people have at one time used herbs and preparations of various sorts to treat illnesses and diseases. However, in spite of the use of herbs in medicine throughout the centuries, only a relatively small number of plant species and their extracts have been carefully studied[30]. The indigenous crude herbal medicines consumed in four states of South-Western Nigeria were screened for their compliance with acceptable standards regarding

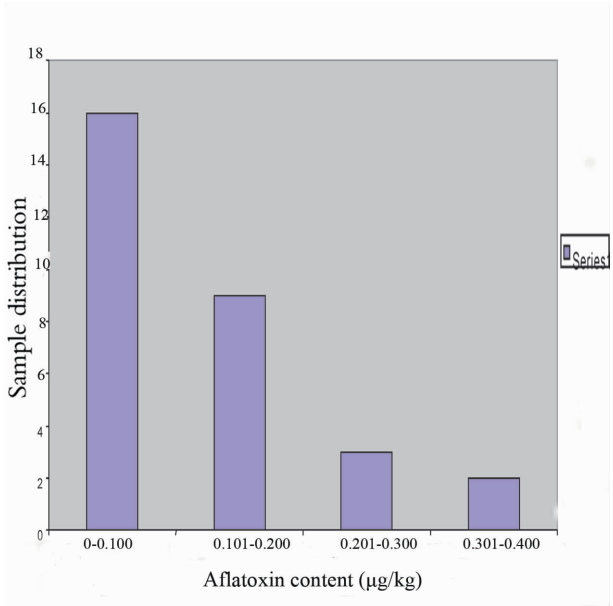

Figure 2 Distribution of Aflatoxin content in samples of crude herbal medicines.

mycoflora and mycotoxins. The results obtained in this study revealed a very high level of fungal contamination that exceeded approved standards, with the level of contamination being satisfactory in only one sample (2.04 %), using the German standards. The fact that the fungal load exceeded all the recommended limits in this study may be due to the natural origin of the herbal components, as well as extraneous contamination by dust during storage in humid conditions[26].

As earlier reported by Ogunshe et al. 2006 [5], although consumer preference for indigenous herbal medications in Nigeria is on the increase, associated microbial hazards have not been fully documented. The study of Ogunshe et al. 2006[5], reported 24 bacterial species from orally-consumed indigenous herbal medications in Nigeria. Similarly, in the present study, the mycoflora of herbal samples included mycotoxin- producing moulds as also earlier reported[12-15,31]. These were ochratoxigenic, aflatoxigenic and penicillic acid- producing strains. Screening of the crude herbal medicines revealed that all had aflatoxins, which ranged from 0.004 μg/kg to 0.345 μg/kg.

The genera *Aspergillus*, *Penicillium* and *Fusarium* have been reported to be toxigenic to human beings and animals[31]. The detection of toxigenic fungal strains and their toxic metabolites indicates potential mycotoxigenesis through the herbal products. This, according to the Argentinian national limit was hazardous, as afla-

toxins should be absent in herbal products for internal and topical use[21]. However the concentration was found satisfactory by the German Standard of 2 μg/kg for Aflatoxin B1 and 4 μg/kg for sum total of aflatoxins. In spite of the low level of aflatoxin in the herbal samples analysed in this study, the safety problem lies in the prolonged intake of these medicines.

In the 1970s, when herbs began their rise in popularity, numerous articles appearing in medical journals and the lay press questioned the safety of herbal products, however, since then, herb usage has increased dramatically, while toxicity reports have not. Perhaps even more important than whether an herbal remedy works, i.e., has the desired therapeutic utility, is the matter of whether it is safe[30,32-34]. Acute exposure to aflatoxins has resulted in aflatoxicosis and its outbreak has been a recurring public health problem throughout the world[35-38]. Acute aflatoxicosis manifest as severe acute hepatotoxicity, which has resulted in approximately 25 % fatality[39]. Chronic exposure to aflatoxin can also result in hepatocellular carcinoma[40], immunologic suppression, impaired growth and nutritional interference[38]. The overwhelming danger associated with the exposure to aflatoxins, makes quality control of traditional medicine a necessity. According to Roy et al. 1988[12] it would be ironic if the treatment of one disease was the unintended cause of another. Above all the various post- harvest methods of storing medicinal plants should be improved upon to exclude conditions favourable for the contamination and growth of fungi. Also simple preparation methods such as sorting and washing may reduce aflatoxin content of the final herbal product.

ACKNOWLEDGEMENTS

The authors are grateful to Mr Oladapo of the Analytical laboratory, Institute of Agriculture Research and Training, Ibadan for his assistance in the analysis of some of the samples and Dr. Adenike Ogunshe for editorial assistance.

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Executive Editor: Yan Lei