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THE BIOLOAD AND AFLATOXIN CONTENT OF HERBAL MEDICINES FROM SELECTED STATES IN NIGERIA

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Abstract

Background: There is increased reliance on traditional herbal medicines by several millions of people worldwide, especially in West Africa and Nigeria in particular. This is due to escalating cost of good quality drugs and consequent proliferation of faked cheaper drugs. However, non standardization of production and handling methods have resulted in herbal medicines with varying quality and safety indices, thus resulting in possible public health concerns. This work investigated the microbial load and aflatoxin levels in herbal medicines from selected states in Nigeria.
Materials and Methods: A total of 210 samples obtained from various renowned herbal medicine practitioners from some selected states in Nigeria, based on their medicinal uses, were analyzed to determine the microbial load by the plate count method and aflatoxin contamination levels using thin layer chromatography with aflatoxin standards.
Results: At least six bacterial genera (*Bacillus*, *Pseudomonas*, *Salmonella*, *EPEC*, *EHEC*, *Streptococcus* and other coliforms) and 6 fungal genera (*Aspergillus*, *Penicillium*, *Rhizopus*, *Cladosporium*, *Geotricum* and *Candida*) were isolated. Aflatoxin B1, B2 and G1 were detected in varying concentrations in the samples analyzed, with an average occurrence of 18.6%. Some of these herbal concoctions were found to contain unacceptably high bioload, according to WHO standards.
Conclusion: Microbial contamination and the presence of aflatoxins in herbal medicines appear to be an endemic problem in Nigeria, as observed in this work, probably due to poor observation of basic hygiene during preparations and poor storage conditions. The findings in this work may serve in developing and instituting public health standards for the production and safety of herbal remedies in Nigeria.

Key words: herbal medicines, microbial, bioload, aflatoxin, public health.

Introduction

The history of using herbs is inextricably intertwined with that of modern medicine. Many synthetic drugs listed as conventional medication were originally derived from plants (Abba et al, 2009). Traditional herbalists use various herbal preparations to treat various types of ailments which include diarrhea, urinary tract infections, typhoid fever, skin eruptions, fevers, female infertility and menopausal symptoms etc (Sofowora, 1993). Most of them are boiled with water to make a decoction just before oral administration, or fine powders of the herbal drugs are mixed with other types of solid medicines without heating. Since they are natural products, the drugs are quite often deteriorated by microorganisms before harvesting and during handling and storage (Hitokoto et al, 1978). Quality control to prevent growth of fungi and bacteria is therefore essential.
Most of the herbal preparations are used in different forms and may carry large numbers of various kinds of microorganisms originating from soil, usually adhering to the leaves, stems, flowers, seeds and roots of herbs (Adeleye et al, 2008).

Previous studies have confirmed the presence of potential contaminants in herbal preparations (De Smart, 1999). The contaminants that present serious health hazards are pathogenic bacteria such as *Salmonella*, *Escherichia coli*, *Staphylococcus aureus*, *Shigella* species and other Gram positive and Gram negative strains of bacteria (Arias et al, 1999; Okunola et al, 2007; Adeleye et al, 2008).

The occurrence of toxigenic mycoflora and mycotoxins in medicinal plants and herbal products has also been reported in many studies (Alwakeel, 2009). *Aspergillus* and *Penicillium* are the two major genera reported to produce mycotoxins (Rodriguez-Amaya and Sabino, 2002). Several environmental factors are reported to influence mycotoxin production, but temperature and humidity are considered to be the most critical (Simsek et al, 2002). Mycotoxins are secondary metabolites of some moulds which include highly toxic, mutagenic or teratogenic compounds. These substances are not formed by all mould species but are characteristic of their producers.

Although mycotoxin contamination of foods and feed has been a worldwide problem as demonstrated by an estimate from the Food and Agriculture Organization (FAO) of the United Nations, recent focus has however been on contamination of medicinal herbal products.
According to World Health Organization (WHO, 1993), about 70-80% of the world's population, particularly in developing countries, Nigeria, being no exception, rely on non-conventional medicines for their primary health care (Akerele, 1993). This is because herbal medicines are accessible and cheap (Sofowora, 1993) and fake drugs abound; therefore the quality and safety of herbal preparations are also of great concern.
Contamination of bacterial toxins and mycotoxins pose a wide variety of health hazards. In addition to the individual adverse effects of bacterial toxins, the effects mycotoxins can vary also depending on the type in question. More than 300 mycotoxins have been identified, the most important being aflatoxins, ochratoxins, patulin, zearalenone, fumonisins and trichothecenes (Rodriguez-Amaya and Sabino, 2002).
This study was designed to investigate the presence and extent of bacterial and mycofloral contamination and aflatoxin load of herbal medicines used for specific ailments from five selected states across Nigeria.

Materials and Methods

A total of 210 samples were randomly bought from traditional medical practitioners from 5 selected states in Nigeria i.e. Edo State (South South), Enugu State (South East), Anambra State (South East), Oyo State (South West) and Plateau State (Central North). These states represent subtle geoclimatic differences, ranging from hot and humid (Oyo, Edo, Anambra and Enugu) to sometimes extremely cold and humid (Plateau). The

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herbal preparations were grouped according to their specific medicinal use, namely: arthritis, fertility, menopause and typhoid. All samples were analyzed in the laboratories of the Department of Medical Laboratory Sciences, University of Nigeria, Enugu Campus.

Laboratory analyses
Bacterial
Different bacterial species and genera were cultured and isolated from the different batches of ethnomedical preparations using general purpose media, enrichment media and other appropriate selective media (Mackonkey agar, blood agar, eosin-methylene blue agar), all obtained from Oxoid, UK, according to the method of Prescott et al (1999). Biochemical tests were also carried out with Gram reaction and motility tests. Total aerobic bacteria plate count was performed according to a modified method of Colle and Miles (1989).

Differentiation of species of *Escherichia coli*
Entero haemorrhagic *E. coli* (EHEC)

Some *E. coli* colonies were streaked onto sorbitol Mackonkey agar (Oxoid, UK). EHEC colonies were identified as those which were pale coloured and non-sorbitol fermenting. Other *E. coli* colonies ferment sorbitol, producing pinkish colonies. Serological tests were also carried out using the latex agglutination kit for 'O' 157:H7 (Oxoid). EHEC strains agglutinated with the antiserum within 60 seconds.

Enteropathogenic *E. coli* (EPEC)

The *E. coli* strains were tested serologically using pooled antisera produced by Wellcome, UK, and also with monovalent antisera. Agglutination revealed the presence of EPEC strain 0126.

Fungal

The herbal preparations were cultured onto petri dishes of potato dextrose agar (PDA) supplemented with chloramphenicol (50mg/L), according to Mahon et al (2011). The plates were sealed with tapes to minimize dehydration and to prevent the spread of fungal spores. Triplicate plates of each sample were incubated at room temperature for 7 days. The mean number of colony forming units (CFU) was also recorded. Plates were examined macroscopically and microscopically. The fungal agents were identified based on examination of morphological characteristics (conidial heads, phialides, conidiophores and the presence or absence of foot cells of rhizoids). Tease mounts were prepared using two teasing needles to remove a portion of the mycelium from the growth, placed on a drop of lactophenol cotton blue on a slide and gently teased apart with the two needles. A cover slip was placed on top and the slide examined microscopically using low power (x 10) and high power (x 40) and confirmed using a mycology atlas (Gilman, 1975).

Mycotoxin standard preparation

The standard solutions were prepared by dissolving the pure aflatoxins (AfB1, AfB2, AfG1 and AfG2) in acetonitrile:water (1:1, v/v) to give concentrations of 1 mg/ml each. The solutions were stored at 4^0C. Aflatoxin standards were obtained from the Sigma Chemical Co. (St Louis, MO, USA).

Aflatoxin Extraction and Detection

The aflatoxin contents of the various samples were extracted and detected according to standard methods (Singh, 1988). 10ml of sample was extracted three times with 25 ml of chloroform in a separating funnel. The pooled chloroform extract was passed through an anhydrous sodium sulfate funnel, and then evaporated to dryness using a rotary evaporator. Each residue was re-dissolved in 1.0ml of chloroform and spotted (10μL), using a micropipette, on pre-coated silica gel G TLC plates (0.5mm thickness, 5 x 10cm, 0.5mm thickness Merck, Germany). Aflatoxin standards were also spotted alongside the extracts and all the TLC plates were developed in the solvent system: chloroform:methanol:water; 88.5:11:0.5 v/v/v). Subsequently the spots were viewed under UV light (365nm) and the presence of aflatoxins was confirmed by spraying the plates with 28% H_2SO_4 in water according to Nabney and Nesbitt (1965). For identification, the fluorescence and Rf value of the samples spot on TLC plates were matched with the fluorescent intensity and Rf value of the aflatoxin standards.

Statistical Analyses

Statistical analyses were done using Graph Pad Prism software version 4.

Results and Discussion

In general, several bacterial species were detected, including *EPEC*, *EHEC*, *Bacillus species*, *Salmonella*, *Streptococcus faecalis*, *Pseudomonas* and other coliforms such as *Klebsiella*, *Aeromonas*, etc (Table 1). *Bacillus* species was the most prevalent in all batches of herbal medicines except batch 4, used to treat typhoid fever, with the highest levels detected in batch 3, used to treat menopausal symptoms from all the states. This may be due to the fact that *Bacillus* species produce spores which are resistant to harsh conditions such as elevated heat and drying that might have been employed during processing and preparation of the herbal medicines. Other species present were *EPEC* and *EHEC* at varying levels in different batches and locations. *Salmonella* was only significant in batch 2 samples from Enugu State in Eastern Nigeria at $p < 0.05$. Batch for herbal medicines used for the treatment of typhoid fever showed the least contamination, with only *Streptococcus faecalis* being significant ($p < 0.05$) only in Edo State, South West, Nigeria. Bacterial contaminants that present serious health hazards are pathogenic bacteria such as *Salmonella*, *E. coli*, *Staphylococcus*, *Shigella* and other gram positive and gram negative strains (Abba *et al*, 2009). Of special interest is the *E. coli* which is an indication

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of urine and fecal contamination thus pointing to very poor hygienic conditions (Ogunshe *et al*, 2006). The limits of bacterial contamination given in European pharmacopoeia as reported by Okunola *et al*, 2007 and EHIA, 2011, are total aerobic bacteria 10^5 cfu/g, *Enterobacteria* and other Gram negative 10^3 cfu/g; while *E. coli* and *Salmonella* should be absent. Some of the herbal products under study did not meet these specifications and so are unacceptable.

Table 2 shows the results of the fungal isolates comprising *Aspergillus*, *Mucor*, *Penicillium*, *Curvullaria*, *Rhizopus*, *Cladosporium*, *Geotricum* and *Candida*. *Aspergillus* was the most predominant and widely distributed, followed by *Candida* and *Penicillium*. *Curvullaria* occurred mainly in batch 2 herbal medicines used for fertility, while *Geotricum* was predominant only in Enugu State in most of the batches.

The occurrence of toxigenic bacterial and mycotoxins in herbal medicines have been reported in many studies. (Hitokoto et al, 1978; Kneifel et al, 2002; Sewaram et al, 2006; Pavlovic et al, 2006; Alwakeel, 2008). This poses great concerns over their safety. Although not all fungi produce mycotoxins, *Aspergillus* and *Penicillium* are the two major genera reported to produce toxigenic mycotoxins (Riba et al, 2008). Several environmental factors are reported to influence mycotoxin production, but temperature and humidity are considered to be the most critical (Simsek et al, 2002). The prevalent weather conditions of the selected states are a predisposing factor for bacterial and fungal growth. Other contributory factors include high pH and unsanitary conditions.

The effects of mycotoxins especially aflatoxins and ochratoxins on various organs have been reported in both experimental animals and man (Alwakeel, 2009). Impaired liver, kidney and brain function are well known consequences of ingesting even minute quantities of mycotoxin. Since they are generally regarded as indestructible in all contaminated consumable items, they cannot be removed or destroyed, so prevention is the only real way forward. Aflatoxins are both hepatotoxic and hepatocarcinogenic while ochratoxins are both nephrotoxic and nephrocarcinogenic (Orsi *et al*, 2007). Aflatoxin B1 was the most frequent mycotoxin recorded in some of the samples beyond the tolerance level fixed by the World Health Organization with an average occurrence rate of 18.6% in the samples contaminated with *Aspergillus flavus* (highest in Edo state). Aflatoxins B2 and G1 were also isolated in varying amounts in the different locations (Table 3). Aflatoxin G2 was not detected in any of the samples.

Conclusion

Microbial contamination and the presence of mycotoxins in herbal medicines appear to be an endemic problem in Nigeria as observed in this study and so deserve a closer research emphasis with a view to curtailing the resultant adverse health effects. As a result of microbial contamination, risk assessment of the bioload of medicinal plants has therefore become an important subject in the establishment of modern Hazard Analysis and Critical Control Point (HACCP) schemes. However, it is difficult to establish comprehensive quality criteria for herbal drugs due to "professional secrecy" of herbalists, but in order to improve the purity and safety of the products, observations of basic hygiene during preparations, standardization of some physical characteristics such as moisture content, pH and microbial contamination levels are desirable (Baeur, 1998). Of vital importance also are the length of period of storage prior to preparation and thereafter. The findings obtained in this work may be relevant in developing and instituting public health standards for the production and safety of herbal medicines sold to the public.

Table 1: Isolation of bacterial agents according to source of sample and medicinal use

TRIPLICATE BATCH POOL	ORGANISMS	EDO STATE	ENUGU STATE	ANAMBRA STATE	OYO STATE	PLATEAU STATE
BATCH 1 ARTHRITIS	*ASPERGILLUS FLAVUS*	++	+	+	++	++
	CLADOSPORIUM	+	+	-	+	-
	RHIZOPUS	+	+	-	+	-
	PENICILLIUM SPP.	+	+	++	-	++
BATCH 2 FERTILITY	*MUCOR SPP.*	+	+	+	+	+
	ASPERGILLUS NIGER	+	-	+	-	+
	CURVULARIA	-	++	-	++	-
	CANDIDA	+	+	+	++	++
BATCH 3 MENOPAUSE	*ASPERGILLUS FUMIGATUS*	+	+	-	+	-
	ASPERGILLUS FLAVUS	+	+	+	+	+
	GEOTRICUM	-	++	-	-	+
BATCH 4 TYPHOID FEVER	*ASPERGILLUS FLAVUS*	++	++	+	++	-
	CANDIDA	+	+	+	-	-
	GEOTRICUM	+	++	-	+	-
	ASPERGILLUS NIGER	+	+	-	+	-
	PENICILLIUM SPP.	+	-	+	++	+

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Table 2: Isolation of fungal agents according to source of sample and medicinal use

TRIPLICATE BATCH POOL	ORGANISMS	EDO STATE	ENUGU STATE	ANAMBRA STATE	OYO STATE	PLATEAU STATE
BATCH 1 (ARTHRITIS)	*EPEC*	+	-	+	++	+
	EHEC	++	+	+	-	+
	BACILLUS SPP.	++	++	++	+	++
	PSEUDOMONAS	-	+	-	+	-
BATCH 2 (FERTILITY)	*BACILLUS SPP*	+	++	+	++	+
	EHEC	+	+	-	++	+
	COLIFORMS	-	-	++	-	++
	SALMONELLA	+	++	-	+	-
BATCH 3 (MENOPAUSE)	*EPEC*	-	+	+	-	+
	STREPT. FAEC.	+	+	+	+	+
	BACILLUS SPP.	++	+++	++	+++	++
	SALMONELLA	+	-	-	+	-
BATCH 4 (TYPHOID FEVER)	*STREPT. FAEC.*	++	-	+	-	-
	COLIFORM	-	-	-	+	-
	SALMONELLA	+	+	+	-	-
% TOTAL COLIFORM = 956 CFU/ML i.e. COLONY FORMING UNITS/ML						

KEY: + - 10 KEY
+ = 10 - 10^2;
++ = $10^3 - 10^4$;
+++ $\geq 10^5$.
EPEC- Enteropathogenic E. coli;
EHEC – Enterohaemorrhagic E. coli
STREPT FAECAL - Streptococcus faecalis;
OTHER COLIFORMS INCLUDE KLEBSIELLA, AEROMONAS- 10^2 ; ++ - $10^3 - 10^4$; +++ $\geq 10^5$

Table 3: Aflatoxin content according to source of sample and medicinal use

TRIPLICATE BATCH POOL	AFLATOXIN (PPM)	EDO STATE	ENUGU STATE	ANAMBRA STATE	OYO STATE	PLATEAU STATE
BATCH 1 ARTHRITIS	B1	32.5*	13.6	ND	23.8*	38.0*
	B2	8.1	ND	ND	15.4	ND
	G1	3.9	ND	ND	ND	ND
BATCH 2 FERTILITY	B1	18.6*	7.7	20.5*	11.2	ND
	B2	ND	ND	ND	8.3	ND
	G1	ND	ND	ND	ND	ND
BATCH 3 MENOPAUSE	B1	14.4	5.8	12.7	ND	2.5
	B2	6.4	ND	ND	ND	ND
	G1	2.9	ND	ND	ND	ND
BATCH 4 TYPHOID FEVER	B1	16.3*	8.4	ND	17.0*	9.8
	B2	ND	6.2	ND	10.6	4.2
	G1	ND	3.3	ND	ND	ND

ND – NOT DETECTED
* Unacceptable according to WHO standards (Patel et al. 2011).

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