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Microbial load and aflatoxin contamination in locally formulated herbal mixtures obtained from Itoku market, Nigeria

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ABSTRACT

Contamination of locally formulated herbs with aflatoxins and pathogenic organisms poses major health concerns to humans and animals, especially in recent times when herbal mixtures are on the rise. This study aimed to determine the herbs' microbial profile and aflatoxin level. Two different herbal medicines (malaria and typhoid; each prepared with water and alcohol) were obtained at Itoku market, Ogun-state, Nigeria. The samples were isolated using the serial dilution technique and isolates were identified morphologically. Aflatoxin quantification was done on the herbal samples using High-Performance Liquid Chromatography (HPLC). The viable bacteria count ranged from 1.0×10^5 cfu/g to 20.0×10^5 cfu/mL with the typhoid herbs prepared with water recording the highest count. The microorganisms obtained in the herbs were confirmed as, *Escherichia coli, Staphylococcus aureus, Micrococcus luteus, Salmonella* sp, *Proteus* sp, *Aspergillus flavus, Penicillium chrysogenum, Saccharomyces cerevisae* and *Fusarium* sp. Typhoid herbs prepared with water showed high aflatoxin detection limits of 7.60 µg/mL. The result showed that the locally formulated herbs were highly contaminated with microorganisms and that consumption of the locally formulated herbs with aflatoxin could cause aflatoxicosis.

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1. Introduction

Herbal medicine production is increasing in Nigeria as a result of the majority of people using it on a regular basis (1). Conventional pharmaceuticals remain out of reach for the poor, and many people mistakenly believe that "natural" is synonymous with "safe." The safety, efficacy, and quality of herbal medicines have become

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a major concern for health authorities as the use of locally compounded herbs has grown (2).

Herbal medicine is the most ancient method of healthcare recognized by humanity and it stands as the sole accessible and pocket-friendly treatment option in many developing nations (3). Because medicinal plants and herbal goods are natural products, all portions of the plants can be damaged by bacteria and fungi, particularly molds, and the presence of bacterium load and aflatoxins contamination has been discovered (4). Herbal mixtures are primarily formulated with a combination of alcohol and a licorice aqueous extract, a liquid derived from the root of Glycyrrhiza glabra, known for its inherent sweet flavor. This aqueous extract is prepared using water as the solvent, and licorice root has a rich history of use in traditional medicine and herbal remedies for various purposes (5). Postharvest diseases in crops and natural remedies pose a significant challenge when it comes to preserving their freshness as agricultural products (6). Aflatoxins are mycotoxins that are produced by *Aspergillus* spp. as secondary metabolites and are among the most significant mycotoxins, having been categorized as Group I carcinogenic substances by the International Agency for Research on Cancer (7). Aflatoxins are mostly found in cereals, oilseeds, and trees in tropical areas where the fungus genus thrives (8), these fungi also thrive well in conditions resembling drought-induced stress, as such stress can elevate the presence of aflatoxin contamination (9). Aflatoxin contamination has increasingly acquired attention in recent years, being recognized as a significant threat to food security (10). In underdeveloped areas, aflatoxins are reported to cause aflatoxicosis in domestic animals and people at a higher rate than in industrialized countries (11). Fungal species such as Aspergillus species have also been shown to create aflatoxins in herbs and herbal products (12). The existence of possible pollutants in herbal remedies has been proven in previous investigations (13). Harmful pollutants can also come from the growing circumstances of medicinal plants, post-harvest treatment (such as fumigants), and the manufacturing stages of finished products (13). For example, substantial effects on growth have been observed in animals given aflatoxin-contaminated agricultural products (14). In reality, with an understanding of the link between aflatoxin consumption, lower food efficiency, and reduced feed intake, detrimental impacts on poultry, swine, and other species are a main concern. Aflatoxin and pathogenic bacteria have been demonstrated in previous studies to be a substantial health hazard for humans and animals in underdeveloped countries. The global popularity of herbal medicines is rapidly on the rise, driven by the expanding market presence of dietary supplements, resulting in year-on-year sales growth (15). However, there remains a dearth of information regarding aflatoxin contamination in locally prepared herbal mixtures. Consequently, this study seeks to offer additional insights into the safety of consuming herbal concoctions.

2. Materials and Methods

2.1. Plant source

Two different locally formulated herbs which are malaria and typhoid herbs prepared with water and dry gin typically contain an alcohol (ethanol) content

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ranging from (40% to 45%) which was obtained from Itoku market, Abeokuta, Ogun State. Vendors of these herbs usually prepare each of the herbs separately with water and alcohol and sell them locally to consumers based on their preferences. The choice of alcohol for producing herbal mixtures was predicated on its multifaceted attributes. Ethanol preservative qualities, which curtail the proliferation of microorganisms like bacteria and fungi, enhance the longevity of herbal blends. Additionally, its versatility enables the efficient extraction of a diverse array of bioactive compounds from herbs, including phytochemicals. Its solubility, which extends to both hydrophilic and lipophilic compounds, renders it well-suited for the comprehensive extraction of herbal constituents. Moreover, its established safety profile, generally regarded as safe for moderate human consumption, further supported its choice. The locally formulated herbal samples were collected in a sterile container, labeled, sealed and stored until use.

2.2. Preparation of herbal samples

2.2.1. Preparation of Malaria herbs

Bark of *Lawsonia inermis*, *Morinda lucida*, *Terminalia glaucescens*, *Mangifera indica, Sarcocephalus latifolium, Azadirachta indica* and *Enantia chlorantha* were soaked in water and alcohol respectively.

2.2.2. Preparation of typhoid herbs

Bark of *Enantia chlorantha, Sarcocephalus latifolium, Garcinia kola* and *Cocos nucifera* were soaked in water and alcohol respectively.

2.3. Bacteriological analysis of locally formulated herbs

One mL of each sample was aseptically introduced into 9 mL of sterile distilled water and properly mixed and a 10-fold serial dilution was done. One mL from the 10^3 and 10^5 dilutions was pipetted into the sterile petri dish and 10 mL of freshly prepared cooled nutrient agar each was dispensed into sterile petri dishes, mixed well and allowed to solidify. Plates for bacteria were incubated at 37°C for 24 h. Developed colonies were counted to obtain the total viable count. Discrete colonies were subcultured onto fresh agar plates for proper identification.

Microscopic examination of bacteria isolated from herbs was carried out using the Gram staining procedure and biochemical tests were carried out for the identification of the bacteria isolates and the results were interpreted according to (16).

2.4. Mycological analysis of locally formulated herbs

One mL of each sample was aseptically introduced into 9 mL of sterile distilled water and properly mixed for a 10-fold serial dilution. One ml from the 10^3 and 10^5 dilutions was pipetted into the sterile petri dish and 10 mL of freshly prepared cooled potato dextrose agar supplemented with a drop of acetic acid was dispensed into sterile petri dishes, mixed well and was then allowed to solidify.

The potato dextrose agar plates (for fungi) Plates for fungi were kept at room temperature for 72 h. They were examined macroscopically and microscopically. The fungi isolates were examined macroscopically for growth, color and texture.

2.5. Aflatoxin detection and extraction of locally formulated herbs

High-performance liquid chromatography (HPLC) technique was employed in the extraction and quantification of aflatoxin from the herbal and rat feed sample at the National Agency for Food and Drug

Administration and Control (NAFDAC) according to (17). The locally formulated herbs and rat feed sample was mixed in a 500 mL conical flask with 50 mL methanol: water (8:2) to extract aflatoxin from samples according to the standard method (18). The flask was securely stopped with masking tape and shaken on a wrist action shaker for 30 min to extract the toxin. Then it was filtered through fluted filter paper. The mixture was transferred to a Buchner funnel pre-coated with about 0.45 µm micro-syringe filter membrane if the filtration was slow and it was filtered using a light vacuum. The filtrate was collected. Clean-up was done using immune-affinity columns. The Aflatoxin extracted samples were added to 100 µL of tri-fluoroacetic acid (TFA) and were mixed well for 30 s, and the mixture was allowed to rest for 15 min. Extracted samples were analyzed by an HPLC system consisting of a Waters 6000 A solvent delivery system and a WISP 71OB sample processor for sample injections (Water Associates India). Samples were eluted isocratically on a radically compressed 10 µm octadeclsilane cartridge (Water Associates India) with a mobile phase of acetronile: methanol: water (15:15:70) at a flow rate of 0.8 mL/min. A pre-filter was placed between the injector and the cartridge. The aflatoxin was detected fluorometrically (excitation wavelength, 365 nm; emission wavelength, 425 nm) with a fluorescence detector (model 420C, Water Associates). The HPLC chromatograms were recorded on a Water Data Module (Water Associates) at a chart speed of 1.0 cm/min. The concentration of aflatoxin in the herbal mixtures was determined by peak area and comparison with samples containing known concentrations of aflatoxin. Table 1 shows LOD and LOQ values.

Table 1. Limit of Detection (LOD) and Limit of Quantification (LOQ) (µg/mL)

LOD and LOQ for aflatoxins	AFB1	AFB2	AFG1	AFG2
LOD	0.30	0.30	0.12	0.12
LOQ	1.00	0.10	0.40	0.40

2.6. Aflatoxin recovery

Five hundred grams of blank samples were used and the recoveries were done in duplicate. Each test matrix underwent single-laboratory validation, adhering to the stipulations of regulation EC401/2006 (EC401/2006). The recovery and precision studies were carried out at a concentration of 0.5 mg/kg. Five feeding stuff samples were spiked with 0.3, 0.4, 0.5, 0.8 and 1.0 mg/kg and analyzed by the same operator on the same day with the same HPLC system.

2.7. Statistical analysis

Data obtained were subjected to statistical analysis using the Statistical Package for Social Sciences (SPSS) version 20.0 (19). Mean values were compared using Analysis of Variance (ANOVA). Results were presented as Mean±Standard deviation. A post hoc test was done using the Student-Newman-Keuls (SNK) to compare mean values between the treatment groups. A probability value (p-value) less than 0.05 was considered to be statistically significant.

Results

3.1. Bacterial colony count of locally formulated herbs

Fig. 1 shows the viable bacteria counts of locally formulated herbs. The typhoid and malaria herbs prepared with water have bacterial counts of 20.0×10^5 cfu/mL and 17.0×10^5 cfu/mL respectively. Typhoid and malaria herbs prepared with alcohol had bacterial

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counts of 19.0×10^5 cfu/mL and 15.0-19.0×10^5 cfu/mL respectively.

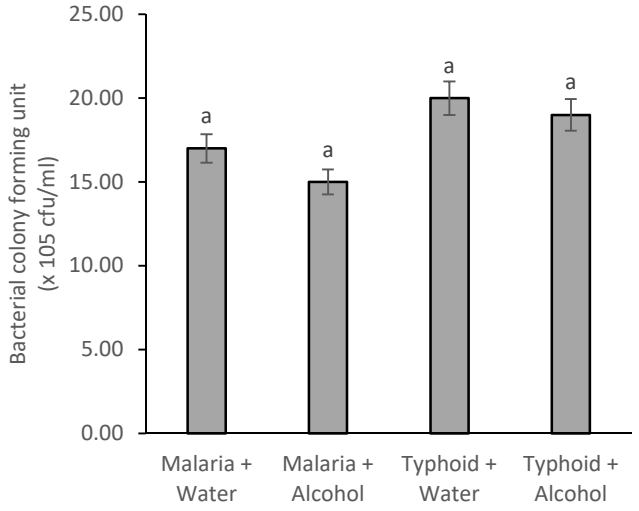

Figure 1. Bacterial colony count of locally formulated herbs (x10^5 cfu/mL)

3.2. Morphological characteristics of bacteria isolated from herbs

Table 2 shows the morphological characteristics of bacteria isolated from the locally formulated herbs. A total number of 25 bacteria were isolated from the herbs with each bacterium characterized on the basis of the color of the colony, its surface, edges, shape and opacity of the colony.

Table 2. Morphological characteristics of bacteria isolated from locally formulated herbs

Isolate code	shape	color	surface	edges	opacity
LFH A	Circular	Creamy yellow	Flat	Smooth	Opaque
LFH B	Circular	Yellow	Flat	Smooth	Opaque
LFH C	Circular	Greyish white	Flat	Smooth	Opaque
LFH D	Circular	Greyish white	Flat	Smooth	Opaque

LFH A= Locally formulated herb A LFH C= Locally formulated herb C
LFH B= Locally formulated herb B LFH D= Locally formulated herb D

3.3. Biochemical characteristics of bacteria isolated from herbs

Table 3 shows the biochemical characterization of bacteria isolated from herbs including Gram Reaction, Catalase, Citrate, Hydrogen sulphide production, Indole and Motility.

Table 3. Biochemical characteristics of bacteria isolated from locally formulated herbs

Isolate	GR	CT	CI	H_2S	IN	MT	ORG
LFH A	+	+	+	-	-	-	*Micrococcus luteus*
LFH B	+	+	-	+	-	-	*Staphylococcus aureus*
LFH C	-	+	-	-	-	+	*Salmonella* sp
LFH D	-	+	-	-	+	+	*Escherichia coli*

GR= Gram Reaction, CT= Catalase, CI= Citrate, H_2S= Hydrogen sulphide production, IN= Indole, MT= Motility, ORG= Organism, += Positive, -= Negative. LFH= Locally formulated herb

3.4. Morphological characteristics of fungi isolated from herbs

Table 4 shows the morphological characteristics of fungi isolated from the locally formulated herbs. A total of 8 fungi were isolated from the locally formulated herb with fungi characterized carried out on the basis of the color of the colony, the color of the reverse side, type of spore and septation.

Table 4. Morphological characteristics of fungi isolated from locally formulated herbs

Isolate code	CMG	CRS	TS	SEP	ORG
M	Green	White	Conidia	Septate	*Aspergillus flavus*
N	White but with violet centre	White	Conidia	Septate	*Fusarium sp*
P	Bluish-green	White	Conidia	Septate	*Penicillium chrysogenum*
Q	Cream, slimming	Cream	Ascoconidia	Septate	*Saccharomyces cerevisae*

CMG= Colony morphology, CRS= Color of reverse side, TS= Type of spore, SEP= Septation, ORG= Organism

3.5. Microbial load in herbal samples (colony forming units)

Table 5 shows the microbial load of the locally formulated herbs ranging from 15.0×10^5 to 530.0×10^5 cfu/mL.

Table 5. Microbial load of herbal samples (colony forming units)

Samples	Dilution plated (10^5) cfu/mL	Dilution plated (10^5) cfu/mL
Malaria + Water	17.0×10^5	530.0×10^5
Malaria + Alcohol	15.0×10^5	500.0×10^5
Typhoid + Water	20.0×10^5	520.0×10^5
Typhoid + Alcohol	19.0×10^5	300.0×10^5

3.6. Aflatoxin quantification and extraction of locally formulated herbs

Table 6 shows the aflatoxin quantification and extraction of the locally formulated herbs. The maximum contents detected for AFB1, AFB2, AFG1 and total aflatoxin were 1.20±0.14, 0.60±0.14, 0.001±0.00 and 7.60±0.28 µg/mL respectively. AFG2 was not detected in any samples.

Table 6. Aflatoxins quantification of locally formulated herbs (µg/mL)

	AFB1	AFB2	AFG1	AFG2	Total Aflatoxin
Malaria + Water	1.20 ± 0.14^b	ND^b	ND^b	ND^a	1.20 ± 0.14^b
Malaria + Alcohol	0.10 ± 0.01^c	ND^b	ND^b	ND^a	0.10 ± 0.01^c
Typhoid + Water	7.00 ± 0.71^a	0.60 ± 0.14^a	ND^b	ND^a	7.60 ± 0.28^a
Typhoid + Alcohol	ND^d	ND^b	0.001 ± 0.00^a	ND^a	ND^d

^{abcd}Means (±Standard deviation) in the same column having similar superscripts are not significantly different at $p < 0.05$, ND: Not Detected

3.7. Frequency of occurrence of bacteria isolated from herbs

Fig. 2 shows the frequency of occurrence of bacteria isolated from the locally formulated herbs. *E.coli* revealed the most frequency (32.00%) while *Salmonella* sp. showed the least (11.50%).

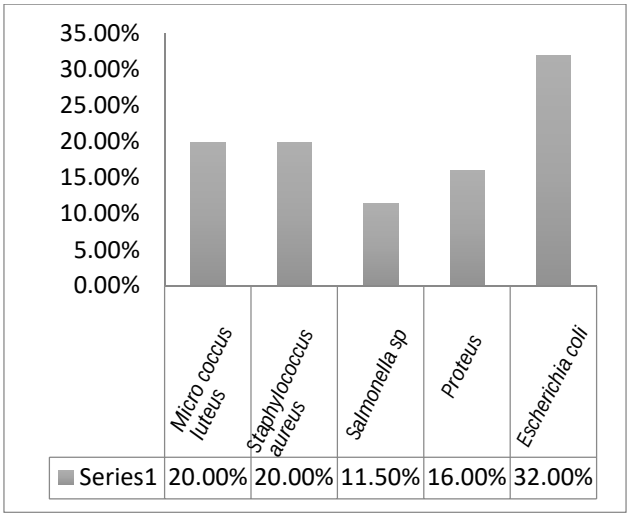

Figure 2. Frequency of occurrence (%) of bacteria isolated from locally formulated herbs

3.8. Frequency of occurrence of fungi isolated from herbs

Fig 3. shows the frequency of occurrence of bacteria isolated from the locally formulated herbs. *Aspergillus flavus* had the highest frequency (37.50 %) while *Saccharomyces cerevisae* showed the lowest (20.00%).

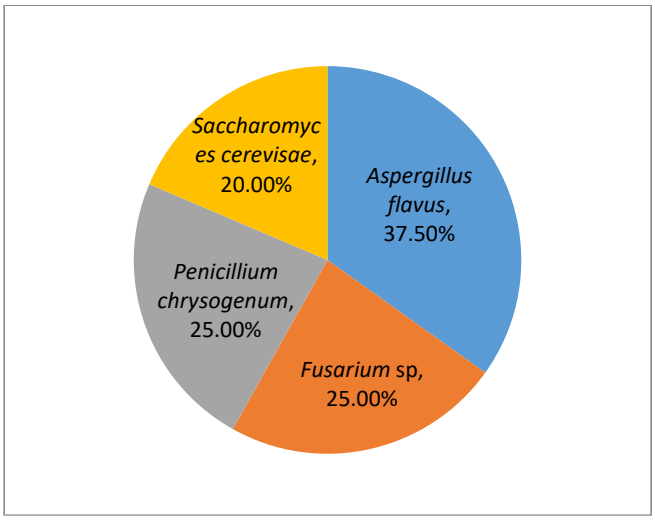

Figure 3. Frequency of occurrence of fungi isolated from locally formulated herbs

4. Discussion

The viable bacteria counts of the locally formulated herbal samples ranged from 1.0×10^5 cfu/g to 20.0×10^5 cfu/mL, with Typhoid herbs prepared with water having the highest bacteria count of 20.0×10^5 cfu/mL and it was similarly reported by EzekwesilI-Ofili et al. (12).

Escherichia coli and *Staphylococcus aureus* had the highest percentage of occurrence of all the bacteria isolated from the locally formulated herbal samples whereas *Aspergillus flavus* had the highest fungal occurrence, which agrees with the findings of Dersjant-Li et al. (14). The identification of *Escherichia coli, Staphylococcus aureus,* along with various other gram-positive and gram-negative bacterial strains aligns with the findings reported by Abba et al. (20). Notably, pathogenic bacteria like Salmonella, *E. coli, Staphylococcus*, and Shigella are known to pose substantial health risks.

The elevated incidence of harmful bacterial infection, as evidenced by the prevalence of *Escherichia coli* and

Staphylococcus aureus in the analyzed herbal samples, may be attributed to the selection of raw materials and the methodologies employed in their preparation. The presence of *E. coli* in the herbal mixture is of great significant concern, as it serves as an indicator of potential urine and fecal contamination. This suggests substandard hygiene conditions, which aligns with the findings of Ogunshe et al. (21). Contamination of raw materials can happen at any point in the food chain, including harvesting, drying, and storage, all of which might impact the final product by raising the microbial load of herbal samples. As a result, good production processes must be followed. The bacterial contamination thresholds and permitted limits specified in the European Pharmacopoeia, as detailed by Okunola et al. (22), are as follows: total aerobic bacteria at 10^5 cfu/g, Enterobacteria and other Gram-negative bacteria at 10^3 cfu/g; and the absence of *E. coli* and Salmonella. It is noteworthy that certain herbal products examined in this study failed to comply with these established criteria, rendering them unsuitable for use. The low incidence of fungal presence and mold decay observed in the locally prepared herbal products may be attributed to the composition of the licorice aqueous extract (5). Licorice aqueous extract is known for its potential health benefits, such as soothing properties for coughs and sore throats, anti-inflammatory effects, and more. However, it's important to note that excessive consumption of licorice, especially for extended periods, can have adverse health effects due to the glycyrrhizin content, which can lead to high blood pressure and other health issues. Therefore, it should be used judiciously and in moderation (5).

The presence of aflatoxins in herbal medicines has been reported in prior investigations (4,23), raising significant apprehensions regarding their safety. Except for typhoid herbs prepared with water, which had an aflatoxin load (AFB1) of 7.60 µg/mL which surpassed the Aflatoxin permissible limit set by European union (AFBI; 2 µg/mL, Total aflatoxin; 4 µg/mL) which corresponds to the findings of EzekwesilI-Ofili et al. (12) and AOAC (18).

The low aflatoxin level measured in this present study is in agreement with the research of Bagheri et al. (24) which opined that aflatoxins in pistachio crops are lower than the maximum tolerance established in the national standard for aflatoxin B1 (5 µg/kg) and the total aflatoxin (15 µg/kg) and concluded that the density of aflatoxin in highly consumed pistachio cultivars supplied in Tehran is within acceptable health and safety standards

5. Conclusion

In light of the discovery of a diverse range of aflatoxins and pathogenic bacteria in locally prepared herbal mixtures, it is evident that this poses a significant health concern in Nigeria. Therefore, it is imperative to prioritize quality assurance measures during both the production processes and distribution of locally formulated herbal products. Regulatory authorities and herbal practitioners should work collaboratively to establish and enforce stringent quality control standards to ensure the safety and well-being of consumers. Furthermore, a heightened awareness of natural remedies labeling, particularly in terms of their composition and recommended dosage, can encourage

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increased attentiveness to the safety and quality of such products.

Conflict of interest

The authors declare that there is no conflict of interest.

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