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ASSESSMENT OF BACTERIAL AND FUNGAL CONTAMINATION OF HERBAL MIXTURE VENDED WITHIN KADUNA METROPOLIS, NIGERIA

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ABSTRACT

The consumption of herbal mixtures among Nigerians have been on the increase in recent years. Many herbal mixtures sold to the general public are likely to be contaminated by a diverse range of potentially pathogenic microorganisms due to lack of good manufacturing practices (GMPs). Therefore, this study is aimed to evaluate the bacterial and fungal profile of herbal mixtures sold within the Kaduna metropolis. A total of twenty samples of herbal mixtures were randomly purchased fromfour distinct locations within the Kaduna metropolis. The samples were subjected to microbiological analysis using standard methods. Bacterial genera isolated from the herbal mixture samples include Bacillus sp., Staphylococcus sp., Streptococcus sp. while the fungal genera were Aspergillus sp., Saccharomyces sp., Fusarium sp. and Penicillium sp. Herbal mixture obtained from Mandao and Malali district had the highest ($9.68\pm1.70\times10^5$CFU/ml) and lowest ($6.60\pm0.43\times10^5$CFU/ml) bacterial load while the corresponding fungal load involved samples obtained from Central market ($1.48\pm11.25\times10^4$ CFU/ml) and Tudun Wada ($1.48\pm 3.11\times10^3$ CFU/ml), respectively. Bacillus sp. (60%) and Aspergillus sp. (45%) were the dominant bacterial and fungal genera, respectively. Given the substantial microbial contamination of herbal mixtures and the potential pathogenicity of certain isolates, it is imperative to ensure that herbalists are well-informed about the principles and implementation of GMPs. It is recommended that regular microbiological monitoring of herbal mixtures should be conducted.

Keywords: Herbal mixtures, pathogenic microorganisms, *Aspergillus* sp. *Bacillus* sp.

INTRODUCTION

A herb refers to botanical organism or specific plant component that is utilized for its aromatic qualities, taste attributes, or potential therapeutic effects. Herbaceous plants encompass various plant parts, such as leaves, stems, flowers, fruits, seeds, roots, rhizomes, and barks. The utilization of herbs for the treatment of diverse ailments predates recorded human history and serves as the foundation for a significant portion of contemporary medical practices. Prior to the emergence of contemporary medicine, herbal remedies were widely utilized as the primary means of treating various ailments (Barakat et al., 2013).

The term "herbal mixtures" pertains to the utilization of plants and plant components to

create beneficial phyto-pharmaceuticals through uncomplicated procedures that do not necessitate advanced equipment. These processes encompass activities such as harvesting, drying, and storage (Abel and Busia, 2005).

Herbal medicine commonly consists of polyherbal formulations, which are derived from combinations of diverse plant parts sourced from various plant species and families. These formulations may contain multiple bioactive constituents, posing challenges in terms of their characterization (Ogbonnia et al., 2010).

In Nigeria, traditional herbalists employ diverse herbal preparations for the treatment of a wide range of ailments, which include diarrhea, cough, 'neonatal fistula', convulsions, and skin diseases, among others. These preparations are commonly utilized in the form of concoctions, which refer to soups or drinks made by boiling ingredients, or infusions, which involve soaking plant material and allowing it to stand for different durations (Adeleye & Opiah, 2003).

According to a survey conducted by the World Health Organization (WHO) in developing countries such as Nigeria, it is estimated that four billion individuals, accounting for approximately 70-80% of the global population, rely on non-conventional medicines primarily derived from herbal sources for their primary healthcare needs (Ampofo et al., 2012).

The utilization of herbal preparations or medicines by traditional practitioners and patients suffering from various illnesses is increasing globally. This can be attributed to the easy availability, accessibility, and cost-effectiveness of raw materials in comparison to synthetic drugs (Ekor, 2014). Consequently, it is imperative for pharmacists and physicians acquire in-depth and knowledge pertaining to the safety of these preparations (Mosihuzzaman and Choudhury, 2008). The precise identification of bioactive compounds in many herbal preparations is uncertain, and it is plausible that these compounds may interact with one another when combined in a solution. The establishment of quality and safety standards for herbal drugs necessitates a precise scientific delineation of the constituent raw materials employed in their formulation (Ogbonnia et al., 2010).

Medicines derived from plants and herbs are commonly referred to as "Herbal medicine" and are generally considered to be safe, as they have been used for an extended period of time in diverse cultures (Mosihuzzaman and Iqbal, 2008).

Various forms of herbal preparations are utilized, which may harbour a significant quantity of microorganisms derived from soil, typically found on different components of herbs. The herbal materials that are frequently utilized include herbal powders, herbal mixtures, and suspensions. The majority of these botanical substances are processed and marketed in unsanitary environments, posing a potential risk of microbial contamination of the product and subsequent manifestation of diseases.Consequently, the assessment of the quality of herbal mixtures holds significant significance in determining their suitability within the context of modern technological advancements. Therefore, the objective of this study is to evaluate the bacterial and fungal composition of herbal mixtures sold in the Kaduna metropolis.

MATERIALS AND METHODS

Description of the Study Area

The study was carried out within Kaduna metropolis. Precisely in Malali, Tudun Wada, Mando and Central Markets.Kaduna is a popular state in Northern part of Nigeria.

Collection of Samples

A total of twenty (20) samples of locally prepared herbal mixtureswere purchased randomly from four different locations namely: Malali, Tudun wada, Mando, Central market. Five (5) samples were obtained from each location. The samples were placed in sterile polythene bags labeled accordingly and

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transported to the Kaduna Polytechnic Microbiology Laboratory for further analysis.

Preparation and Sterilization of Media

Nutrient agar (NA) was utilized for the enumeration of bacteria, while Plate Count Agar (PCA) and Sabouraud Dextrose agar (SDA) were employed for the enumeration of fungi. The media for isolation and enumeration of bacteria and fungi were prepared in accordance with the guidelines provided by the manufacturer.

Microbiological Analyses

Serial Dilution and Bacteria Enumeration

The pour plate method, as outlined in the study conducted by Abba et al. (2009), was employed. In this method, 1ml from the serially diluted sample was aseptically transferred into a properly labeled and sterilized Petri dish. Subsequently, 20ml of molten agar media was poured into the plates and were gently swirled in order to facilitate the even distribution of colonies, which entailed performing a serial dilution of the samples in a five-fold manner. One milliliter aliquots of the sample were transferred using a pipette from the dilution tubes with dilutions of 10^{-3} and 10^{-4} into petri dishes that were appropriately labeled.

The inoculated pates for isolation and enumeration of bacterial load were incubated for 24 h at 37 ºC. Thereafter, the colonies that appeared on the culture plates were counted using a colony counter. The bacterial load in each sample was calculated using the formula stated below.

$$\text{CFU/ml} = \frac{\text{Number of colonies x dilution factor}}{\text{Volume plate}}$$

Determination of Pure Isolates

Duplicate samples from each hawker were subjected to microbiological analyses, and the resulting averages were duly recorded. Subsequent to their development, the colonies were sub-cultivated in order to acquire pure isolates. The pure isolates were cultured on Nutrient agar slants in order to facilitate their subsequent identification.

Identification and Biochemical Characterization of Bacterial Isolate

The process of characterizing a bacterial isolate involved examining its cell morphology and performing gram staining and biochemical tests. Using the methods outlined by Oyeleke and Manga (2008). Identification of the isolates was conducted by employing the scheme of Cowan and Steel (1973) and the result was compared with established taxa.

Oxidase

A strip of filter paper was saturated with a small quantity of a freshly prepared 1% solution of Kovac's oxidase reagent.

An aliquot culture was applied onto it using a wire loop. A phenomenon of chromatic alteration was observed.

A positive response is denoted by the emergence of a vivid deep-purple hue within a timeframe of 5 to 10 seconds. A negative response characterized by either the absence of coloration or delayed coloration occurring after a period of 60 seconds.

Catalase

A loop was employed to transfer a minute quantity of bacterial colony onto the surface of a pristine glass slide. A 3% solution of hydrogen peroxide was applied tothe bacterial isolate on the slide and subsequently examined for the presence of bubbles.

Coagulase

Two separate slides were used to place a drop of normal saline. Two thick suspensions were created by emulsifying a portion of the isolated colony in each drop using a loop. One suspension was gently mixed with a drop of human plasma, resulting in the observation of clumping of the organisms. A negative result is recorded in the absence of clumping of the plasma.

Citrate

A sample of Simmons citrate agar was prepared following manufacturer's instruction. The slant was gently streaked in a back-and-forth motion using a small portion of inoculum obtained from the centre of a colony that was well-isolated. The tube was subjected to incubation for a minimum48 hours at a temperature of 37^{o}C. The slant was observed after the incubation period to for a change in colour.

Starch Hydrolysis

The bacterial culture was inoculated onto a plate containing starch agar under sterile conditions. The plate that had been inoculated was placed in an inverted position and incubated at a temperature of 37 ^{o}C for 18 to 24 hours. After the incubation period, the media's surface was inundated with a solution containing 10% iodine.

Hemolysis

The test organisms were streaked on to a blood agar plate using a sterile inoculating loop and the inoculum was stabbed with the loop along the streak line. The inoculated plate was incubated at 37 ^{o}C for 24 hours and the results was recorded.

Methyl Red (MR)

Methyl Red Voges-Proskauer (MRVP) broth was prepared in test tubes. The broth was inoculated aseptically with loopful of bacterial culture. The test tubes were incubated at 37 ^{o}C for 48 hours, few drops of methyl red indicator was added in the incubated tubes and was observed for colour changes.

Voges-Proskauer (VP)

MRVP broth prepared in test tubes was inoculated with the test organism taken from 18-24 hours' pure culture. The test tubes were incubated at 37 ^{o}C for 24 hours. Following 24 hours of incubation, 6 drops of 5% naphthol was added and properly mixed to aerate. Two drops of 40% potassium hydroxide was added and mixed. It was observed for a pink-red colour at the surface within 30 minutes.

Fungal Enumeration and Isolation

The fungal count was determined by pipetting 1ml of the serially diluted herbal mixture on Sabouraud Dextrose Agar (SDA) containing 0.01% chloramphenicol. The plates were incubated for 3 to 7 days at ambient temperature.

Identification of Fungal Isolates

The identification of fungal isolates was conducted by assessing various characteristics, including the colour of aerial and substrate hyphae, the type and shape of hyphae, the presence of foot cells, sporangiophores, and conidiophores, as well as the characteristics of the spore head.

A minute fraction of the mycelial expansion was meticulously extracted using a sterile inoculating needle and deposited onto a microscopic slide containing a droplet of lactophenol cotton blue. Subsequently, a cover slip was positioned over the sample. The slide was subsequently subjected to microscopic examination, initially employing a (x10) objective lens, followed by a (x40) objective lens, in order to identify the distinctive structural characteristics of the fungal spores. The identification of the isolates was conducted by comparing their characteristics with established taxa, using the guide put forward by Domsch and Gams (1970).

Data Analysis

Data generated was subjected to statistical analysis using Analyses of Variance (ANOVA) and tables where necessary.

RESULTS

In this study, all the herbal mixtures samples analyzed were contaminated. However, the bacterial and fungal load of the herbal mixture varied among the samples obtained from different vendors and locations.

Table 1 shows the total bacterial and fungal counts of herbal mixtures from different locations in Kaduna metropolis. The total bacteria and fungi count in the herbal mixture samples varied.

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Samples from Mando had the highest bacterial load of 9.68±1.70 x 10^5CFU/ml while samples from Malali had the least (6.60±0.43 x 10^5CFU/ml). The highest fungal counts of 1.48±11.25 x 10^4 CFU/ml were recorded in the sample obtained from central market and the least count 4.80±3.11 x 10^3 CFU/ml) involved the sample from Tudun Wada.

Table 1: Bacterial and Fungal Load of Herbal Mixtures Sold within Kaduna Metropolis

Location	Bacterial Load (CFU/ml)±SD	Fungal load (CFU/ml) ± SD
Tudun wada	$9.66\times10^5 \pm 3.50^a$	$4.80\times10^3 \pm 3.11^a$
Central Market	$7.30\times10^5 \pm 2.59^b$	$1.48\times10^4 \pm 11.25^b$
Malali	$6.60\times10^5 \pm 0.43^b$	$9.60\times10^3 \pm 3.05^c$
Mando	$9.68\times10^5 \pm 1.70^c$	$6.40\times10^3 \pm 1.14^a$

Values with the same superscript across the column were not statistically significant ($p>0.05$) While values with different superscript across the column were statistically significant ($p\leq0.05$)

Table 2 describes the cultural and biochemical characteristics of bacterial isolates. They include *Staphylococcus* sp., *Streptococcus* sp. and *Bacillus* sp.

Table 2: Cultural and Biochemical Characteristics of Bacterial Isolates from Herbal Mixture

Cultural Characteristics	Shape	Cell Arrangement	Gram's reaction	Biochemical Tests								Probable organism
				CAT	COA	CIT	VP	MR	HEM	S/H	OXI	
Rough,opaque, raised, large, irregular white colonies	Rods	Single and in pairs	+	+	-	+	+	-	-	+	+	*Bacillus* sp.
Smooth, circular, medium size, golden to milky colonies	Cocci	Clusters	+	+	+	+	+	+	+	+	-	*Staphylococcus* sp.
Smooth, circular, flat, small, light yellow to milky colonies	Cocci	Chain	+	-	-	-	+	-	+	-	+	*Streptococcus* sp.

Key: + = Positive, - = Negative, CAT = Catalase, COA = Coagulase, CIT = Citrate, VP = Voges/Proskauer, MR = Methyl red, HEM = Hemolysis, S/H = Starch hydrolysis, OXI = Oxidase

Table 3 depicts the morphological and microscopic characteristics of fungal isolates. They were identified as *Aspergillus* sp., *Saccharomyces* sp., *Fusarium* sp. and *Penicillium* sp.

Table 3: Morphological and microscopic characteristics of fungal isolates from herbal mixture

Isolates	Morphological characteristics	Microscopic characteristics
Aspergillus sp.	Initially white turning to brown to black colour with white periphery. Cottony to granular texture.	Presence of conidiophores, septate- hyphae and branched foot cell
Saccharomyces sp.	Off-white to cream, dry smooth colonies, discrete and scanty growth on plate.	Oval to convex, budding yeast cell, purple coloured colonies cocci shape
Fusarium sp.	Woolly to cottony, flat spreading colonies appear brown or pink in centre with white edges	Macro conidia formed with chlamydospores arising in the mycelium and conidia
Penicillium sp.	Green-greyish colonies, flat and powdery growth.	Non-septate hyphae, presence of brush-like conidiophore

Table 4 shows the distribution of bacterial and fungal isolates from the herbal mixture from Kaduna metropolis. The result shows that herbal mixture samples obtained from the four locations were contaminated with *Bacillus* sp. and *Staphylococcus* sp. while one sample from Malali was contaminated with *Streptococcus* sp.

Aspergillus sp. was encountered in the samples obtained from all the locations while *Fusarium* sp. and *Penicillium* sp. were encountered in samples obtained from 2 out of 4 locations.

Table 4: Distribution of Bacterial and Fungal Isolates in Herbal Mixtures from Kaduna Metropolis

Location	Samples	Bacterial isolates	Fungal isolates
Tudun wada	A	*Bacillus* sp.	*Aspergillus* sp.
	B	*Staphylococcus* sp.	*Fusarium* sp.
	C	*Staphylococcus* sp.	*Saccharomyces* sp.
	D	*Bacillus* sp.	*Aspergillus* sp.
	E	*Bacillus* sp.	*Aspergillus* sp.
Central market	A	*Bacillus* sp.	*Saccharomyces* sp.
	B	*Bacillus* sp.	*Saccharomyces* sp.
	C	*Bacillus* sp.	*Penicillium* sp.

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	D	*Bacillus* sp.	*Aspergillus* sp.
	E	*Staphylococcus* sp.	*Saccharomyces* sp.
Malali	A	*Staphylococcus* sp.	*Aspergillus* sp.
	B	*Streptococcus* sp.	*Saccharomyces* sp.
	C	*Bacillus* sp.	*Aspergillus* sp.
	D	*Streptococcus* sp.	*Saccharomyces* sp.
	E	*Bacillus* sp.	*Penicillium* sp.
Mando	A	*Bacillus* sp.	*Fusarium* sp.
	B	*Staphylococcus* sp.	*Aspergillus* sp.
	C	*Bacillus* sp.	*Aspergillus* sp.
	D	*Bacillus* sp.	*Fusarium* sp.
	E	*Staphylococcus* sp.	*Aspergillus* sp.

Table 5 shows the percentage of occurrence of bacterial and fungal isolates from the herbal mixtures. Among the bacterial genera isolated from the herbal mixtures, *Bacillus* sp. (60%) and *Streptococcus* sp. (10%) had the highest and least percentage occurrence, respectively. Meanwhile, the fungal genera which had the highest and least percentage occurrence was *Aspergillus* sp. (45%) and *Penicillium* sp. (10%)

Table 5: Percentage occurrence of bacterial and fungal isolates from herbal mixture

Bacterial isolates	**% occurrence**	**Fungal isolate**	**% occurrence**
Bacillus sp.	60	*Aspergillus* sp.	45
Staphylococcus sp.	30	*Saccharomyces* sp.	30
Streptococcus sp.	10	*Fusarium* sp.	15
		Penicillium sp.	10
Total	**100**		**100**

DISCUSSION

The findings of this study shows that samples of herbal mixtures obtained from Kaduna metropolis were contaminated with three (3) bacterial and four (4) fungal genera. The herbal mixture samples collected from Mando

had the highest bacteria counts (9.68±1.70 $\times 10^5$ CFU/ml), whereas the samples from Malali had the lowest counts ($6.60\times10^5 \pm 0.43$ CFU/ml). Conversely, the herbal mixture samples obtained from central market had the highest fungal counts ($48\pm11.25\times10^4$CFU/ml), whereas the samples from Tudun wada had the least count (4.80± 3.11×103 CFU/ml). The microbial count of herbal mixture samples evaluated in this study exceeded 1 x 10^5 CFU/ml which is above the standard recommended by the United States Pharmacopeia (USP 30). Consequently, the herbal mixture samples are unacceptable based on the limit of microbial count recommended by the World Health Organization (WHO, 2007).On the contrary, Oluyege (2010) reported that microbial load of herbal products hawked in Ado-Ekiti is between 4.0 x 104 to 1.7 x 106 CFU/ml.

High microbial count observed in the herbal mixture analyzed in this study can be attributed to factors such as inadequate hygiene practices, utilization of contaminated equipment, use of contaminated water, contamination from personnel, and unsterilized packaging materials

The herbal mixtures examined in this study were contaminated with *Bacillus* sp., S*taphylococcusaureus*, *Streptococcus* sp., *Aspergillus* sp., *Saccharomyces* sp., *Fusarium* sp., and *Penicillium* sp. The occurrence of *Bacillus*sp. and *Aspergillus* sp. were dominant in the samples of herbal mixture, whereas *Streptococcus* sp. and *Penicillium* sp. were the least among the bacterial and fungal genera, respectively.

The findings from this study is in agreement with the report by Idu et al. (2015) which evaluated the microbial load of polyherbal products from Lagos state, Nigeria. researchers reported that polyherbal samples were contaminated with Bacillus sp. (100%), *Staphylococcus* aureus (33.3%), *Pseudomonas* sp. (50%), *Aspergillus flavus* (43.3%), *Fusarium* sp. (10%), *Penicillium* sp. (13.3%), and *Mucor* sp. (3.3%).

The occurrence of *Bacillus* sp. in the herbal mixtures could be attributed to insufficient heat treatment, improper product handling, and contamination of processing equipment.

CONCLUSION

The investigation revealed that all the herbal mixtures analyzed in this study were contaminated with bacterial and fungal loads that exceeded the acceptable limit. *Bacillus* sp., sp., *Streptococcus* sp., A*spergillus* sp., *Penicillium* sp., *Fusarium* sp. were identified in the samples analyzed. As a result of increasing global consumption of herbal mixtures, it is pertinent that the activities of the manufacturers or producers of herbal mixtures be regulated by regulatory agencies. The agencies ought to educate the producers and the consumers the dangers of not producing under aseptic condition and the need to adhere to good manufacturers practices.

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