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(RESEARCH ARTICLE)

Microbial quality of herbal preparations sold in some parts of Nigeria

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Abstract

The practice of using herbal drugs has become widespread in recent times without prior knowledge of their microbiological safety. In this context, this research was focused on assessing the microbial quality of different popular herbal preparations registered by the National Agency for Food and Drug Administration Control (NAFDAC) sold within South-West and South-South regions of Nigeria. The microbial diversity of some herbal drugs were examined by aerobic plate count method and microbial isolates were identified based on their morphological, cultural and biochemical characteristics. A total of sixty (60) herbal samples from six (6) herbal products (Kj cleanser, Ok Cleanser, Sk cleanser, Gk cleanser, Y-Bitters and Sw-Bitters) were analyzed and all were found to be free from pathogenic bacteria and indicator organisms of fecal contamination. However, *Bacillus* spp. was isolated from twenty six (26) of the herbal samples. Altogether four different *Bacillus* spp. were identified and the most predominant was *Bacillus subtilis.* The total heterotrophic bacterial count (THBC) of the herbal syrups ranged from 3.10 $x10^2$ cfu/ml to 2.65$x10^3$ cfu/ml whereas in the herbal powder the THBC ranged from 9.0$x10^1$ cfu/g to 1.5$x10^2$ cfu/g. The fungal count of herbal syrups ranged from 2.0$x10^1$cfu/ml to 1.9$x10^2$ cfu/ml while that of the herbal powder ranged from 1.0$x10^1$ cfu/ml to 1.0$x10^2$ cfu/ml. However, the microbial load and organisms present in the herbal samples were within WHO standard, therefore, it is recommended that plants to be used in herbal preparations should be properly washed and heated as to reduce or eliminate the presence of *Bacillus* species.

Keywords: Herbal preparations; Microbial quality; Pathogenic bacteria; *Bacillus* spp.

1. Introduction

Since ancient times, herbal medicines have been used by many different counties throughout the world to treat illnesses and to assist bodily functions. The use of herbal medicines in human health care has developed substantially in both developed and developing countries and it is continually expanding. About 60 to 80 % of the population of every country of the developing world relies on herbal or indigenous forms of medicine. The reasons for the patronage of herbal medicine are the high cost of very effective antibiotics and the problem of antibiotic resistance which is very common in developing countries [1, 2]. The World Health Organization [3] survey indicated that about 70–80 % of the world population particularly in developing countries rely on non-conventional medicines mainly of herbal origins for their primary health care.

The use of herbal medicine has always been part of human culture, as some plants possess important therapeutic properties, which can be used to cure human and other animal diseases [4]. The use of these herbal remedies have increased significantly in the last two to three decades in Nigeria. This has led to the production of herbal products with bogus claims that it can cure all forms of ailments. With the ever increasing use of herbal medicines and the global expansion of the herbal medicines market, safety has become a concern for both health authorities and the public at large. This is because it is difficult to attest to the hygienic nature of the sanitary conditions from the point of collection of the herbs to processing and finally to the point of preparation [5].

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Therefore, the quality and safety of herbal preparations are of great concern because quality is the basis of reproducible efficacy and safety of herbal drugs, and to ensure the standard of research on herbal medicines, the quality of the plant materials or preparations is of utmost importance and must therefore be investigated. [6]

In developing countries, herbal extracts are recognized as pharmaceutical medicine and are used as complementary medicine and without adequate supervision they are released directly into the market. Hence, high microbial contamination may occur in these products [7, 8].

In Nigeria, there appears to be an overwhelming increase in public awareness and usage of herbal medicinal products in the treatment and prevention of diseases. This may not be unconnected to the active mass media advertisements embarked upon by the producers and marketers of the herbal medicinal products who have taken the advantage of the relatively high cost of the conventional pharmaceutical dosage forms, inaccessibility of the orthodox medical services to a vast majority of the people particularly in the rural areas and the reservations by the public due to prevalence of fake, substandard or counterfeit drugs in the market. These have placed the herbal medicinal products as a ready alternative to conventional dosage forms in the treatment of infections and diseases. With this increased usage, the safety, efficacy and quality of these medicines have been an important concern for health authorities and health professionals [9].

Although, herbal remedies are often perceived as being natural and therefore safe microbial contamination of medicinal herbal preparations could be influenced by environmental factors such as temperature, humidity, rainfall during pre-harvesting and post-harvesting periods, handling practices, the production of this herbal medicines by unlicensed vendors with no or poor educational levels and also poor knowledge of food hygiene [10].

The storage conditions of crude and processed medicinal-plant materials are also not left out in the contamination of this herbal medicine. The presence of microbial contaminant in non-sterile pharmaceutical products can reduce or even inactivate the therapeutic activity of the products and has the potential to adversely affect patients taking the medicines. It is desirable that the microbiological status of medicinal plant which is susceptible to microbial attack be ascertained before their usage. The widespread disregard for possible association of microorganisms to the production of some herbal drugs has tarnished the reputation of many herbal drugs. Without proper microbial analysis, there is no assurance that the herb contained in the bottle is the same as what is stated on the outside label and that it is free from contaminations.

Microbial analysis is a term that refers to the detection of microorganism in a given sample or product to ascertain its hygienic state. Regardless of the form of herbal preparation, some microbial analysis is vital. Hence, it is doubtful to say that herbal preparations with codes Sk cleanser, Gk cleanser, Ok cleanser, Y-bitters, Sw bitters and Kj cleanser products commonly sold in some parts of Nigeria is free from pathogenic microorganism. The aim of this study was to establish the microbial quality of herbal preparations obtained from vendors in South-West and South-South regions of Nigeria.

2. Material and methods

2.1. Sample collection

A total of sixty (60) samples representing different types of herbal preparations were collected from random sources in Nigeria. Out of the 60 samples, 50 were in liquid form and 10 were in powdered form. All commercial herbal preparations samples (Sk cleanser, Gk cleanser, Ok cleanser, Y-bitters, Sw bitters and Kj cleansers) were examined for their bacteriological profile.

2.2. Sample preparation and bacteriological analysis

1 ml/g each of the samples was dissolved in 9 ml of normal saline solution. Serial dilutions were made and viability assessed using spread plate method. The plates were incubated at 37 °C for 24 hrs for bacteriological analyses and 5 days for fungal analysis. After incubation visible colonies that developed were enumerated and recorded as colony forming units/ml (cfu/ml). Total colony counts were recorded in cfu/ml using equation:

$$\text{Total colony counts (cfu/ml7877)} = \frac{\text{No. of colony formed}}{\text{Volume plated}} \text{x dilution factor}$$

The media utilized were Nutrient agar, Violet Red Bile agar, Lauryl sulphate broth, Salmonella Shigella agar, Centrimide agar, TCBS agar, Chromcot agar, Sabouraud dextrose agar. All isolates were identified using standard biochemical tests [11].

2.2.1. Preparation of samples

The liquid sample was prepared by dispensing 1 ml of herbal sample in 9 ml of physiological saline under aseptic condition while the powdered sample was prepared by weighing 1 g of herbal product and dissolving same in 9 ml of physiological saline. The herbal suspension was steamed thoroughly while stirring using a sterile rod thereafter, a 10-fold serial dilution was performed following aseptic technique protocol.

2.2.2. Preparation of culture media

All media were prepared according to manufacturer's instruction and standard preparation methods.

Nutrient agar

This is an all-purpose medium, very rich for the support of bacterial growth, 28 g of Nutrient agar was weighted and dissolved in 1000 ml of de-ionized water and autoclaved, it was allowed to cool before it was aseptically poured in sterile Petri dishes.

Saboroud dextrose agar

65 g of saboraud dextrose agar was dissolved in 1000 ml of de-ionized water, the powder was allowed to dissolve and the tube was plugged with cotton wool, covered with aluminium foil and autoclaved at 121 °C for 15 minutes. It was allowed to cool aseptically and poured into a sterile plate. The surface was flamed to remove air bubbles. The plates were allowed to solidify then dried in the hot air dryer 10% lactic acid (0.1 ml) was incorporated to prevent the growth of bacteria and other organisms apart from fungi.

Violet red bile agar (VRB)

39.5 g of virulent red bile agar (MERCK) powder was weighed and dissolved in 1000ml of distilled water, heated in a heating mantle with frequent swirling until it boils.

Lauryl sulphate broth (LSB)

35.6 grams of lauryl sulphate (MERCK) powder was dissolved in 1000ml of distilled water, autoclaved at 121 °C for 15 minutes at 15 psi.

Salmonella Shigella agar (SSA)

60 g of the agar (MERCK) was dissolved in 1000 ml of distilled water and heated to boil in the heating mantle. This is selective medium for the isolation of *Salmonella* and *Shigella.*

Centrimide agar (CA)

44.5 g of Centrimide agar (Merck) powder was dissolved in 1000 ml of distilled water, 10 ml of glycerol was added (as a source of carbon, it aids the microorganism to grow by increasing the nutrient value and also help the agar to gelatinize very well), autoclaved at 121 °C for 15 minutes at 15 psi, after which it was put in the water bath that has been set at 45 % with rotation of about 2000 revolution per minute so it does not gel.

TCBS agar

88 g of TCBS agar (Merck) powder was dissolved in 1000 ml of distilled water and heat to boil in the heating mantle.

Chromcot agar

26.5 g of Chromocot agar (Merck) powder was dissolved in 1000 ml of distilled water and Autoclaved at 121 °C for 15 minutes at 15 psi.

2.3. Microbiological evaluation of sample

2.3.1. Culture of samples

Each of the herbal samples was shaken vigorously and inoculated into the seven different media: Nutrient agar for the cultivation of microbes, SDA for isolation and cultivation of pathogenic fungi and yeast, VRB for enumeration of *E. coli*, LSB for enumeration of coliform, Chromcot agar for isolation and identification of *Pseudomonas aeruginosa*, SSA for isolation and identification of *Salmonella* and *Shigella,* TCBS for the isolation and identification of *Vibrio cholera*, Chromocot agar for isolation and identification of *E. coli* and coliform. The powdered herbal product was diluted by dissolving 1g of the sample in 9 ml of normal saline, the aliquots (0.1ml) of the herbal preparations was inoculated into plates containing different culture agar and incubated at 37°C for 24-48 hrs for visible colonies. The plates containing sabouraud dextrose agar were incubated at room temperature for 5 days.

After incubation, visible colonies that developed were enumerated and recorded as colony forming units/ml (cfu/ml) of cfu/g.

2.3.2. Identification of colonies on the different media

The following parameters were used to identify colonies on Nutrient Agar and Saboraud dextrose Agar. The different parameters used include; Elevation, opacity, size, and surface.

Subculture

This was done in order to obtain a pure culture for the identification of organism using biochemical test. A discrete colony of the organism to be identified was collected from cultured plates, using sterile wire loop and subculture on nutrient agar plates using streaking method and incubated for 24 hrs at 37 °C. After incubation, the isolates were further transferred to nutrient agar slants for further test.

2.3.3. General biochemical and identification of isolates

Gram staining

This is a differential staining which classifies bacteria into gram positive or negative. Thin smears of fresh, pure bacteria cultures were made on a clean grease free glass slides. The slides were allowed to air dry and heat fixed by passing it over a Bunsen burner flame.

The slides were placed on a rack over a sink and flooded with crystal violet for 30 seconds, the slides were rinsed with clean running tap water and flooded with iodine for 60 seconds, the slides were rinsed with water and decolorized with 95% alcohol for 30 seconds, the slides were counter stain with safranin for 30 second's, they were rinsed with clean running water and allowed to air dry after which they are viewed under the microscope using oil immersion lens (100x). Gram positive bacteria stained purple while gram negative bacteria stained red.

Spore staining

This is done to detect the presence of endospores smears of the isolates were made on a clean grease free glass slides; air dried and heat fixed. The slides were flooded with Malachite green and steamed over a beaker containing boiling water for 15 minutes. They were washed off with clean water and counter stained with safranin for 30 seconds. The slides were rinsed with water, dried and viewed under the microscope. Spores stained green while vegetative ells stained red.

2.3.4. Biochemical test

Citrate utilization test

This is a test used to identify the organisms that utilize citrate thus using carbon as its sole carbon source. Simmon's citrate agar slant was inoculated with the test organism using a sterile wire loop and incubated at 37 °C for 48 hrs. Positive result shows a blue colour change while a negative result shows a green colour, meaning that the organism did not utilize citrate.

Indole test

This test is carried out to test the ability of an organism to break down tryptophan, an amino acid to pyruvate and indole. The test organism is inoculated into 5 ml of sterile peptone water and incubated aerobically at 37 °C for 24 hrs. The production of indole was tested by adding 0.5 ml of kovac's reagent to the broth culture and shaken gently. A positive result shows a red colour in the alcohol layer which indicates that indole was produced while a negative result shows absence of red colour.

Motility test

This test detects if an organism is motile or not. The test was carried out by inoculating the test organism into a SIM medium by stabbing the butt using a sterile stabbing needle. A positive motility test is indicated when the organism spreads throughout the medium while a negative motility test is indicated when the organism grows along the line of the stab.

Catalase test

This test differentiates between Staphylococci that produce catalase from non-catalase producing bacteria such as Streptococci. Catalase breaks down H_2O_2 to hydrogen and water. This test was carried out by adding a drop of distilled water on a grease free glass slide and emulsifying the test organism in it, after which 3% hydrogen peroxide was added. The presence of gas bubbles shows a negative result.

Sugar fermentation

Utilization of sugar (glucose, sucrose, lactose) as sole source of carbon with the production of either acid, gas or both simultaneously was investigated. The production of the acid or gas indicates the ability of the organism to use the sugar as source of carbon. 1 gram of peptone was dissolved in 220 ml of de-ionized water, an indicator (Bromocresol, 0.3 ml) which indicates the reactivity by colour change was also added to the peptone water. Equal quantity of the peptone water was distributed into three (3) sterile conical flasks. The available sugar (2.2 g) was respectively dissolved into the peptone water and 10 ml of peptone water with sugar were dispensed into test tubes containing inverted clean Durham tube and sterilized in an autoclave, respective isolates were inoculated aseptically into the sterilized sugar solution and incubated for 48 hours at 37 °C. Positive results had colour change from purple to yellow, indicating acid production while gas was noted by the presence of air space in the Durham tubes within the test tube.

Oxidase test

This test detect the organism that produces the enzyme cytochrome oxidase and is used to distinguish Pseudomonas which is oxidase positive from all enteric bacteria. It was done by moistening a filter paper with few drops of oxidase reagent (tetra-methyl-p- phenylene diamine dihydrochloride) and a smear of the test organism was made on the filter paper. A deep purple colour is an indication of a positive oxidase test while absence of this colour is a negative test.

2.4. Fungi identification

The fungal cultures were checked for their morphological characteristics such as colour, texture, edge and form.

2.4.1. Microscopy

It detects the presence of spores, conidia, whether it is septate or aseptate. A drop of lactophenol blue was added on a clean grease free glass slide and a sterile needle was used to pick a little of the fungal culture from the edge and teased into the lactophenol blue. The smear was covered with cover slip and viewed under the microscope using 10x and 40x objective.

3. Results

3.1. Enumeration of bacterial and fungal counts

The microbiological quality of some popular liquid and powdered herbal preparations which are registered with National Agency for Food and Drug Administration Control (NAFDAC) obtained from the South-West and South-South regions of Nigeria was investigated and the results obtained are presented in Tables 1-5. The total heterotrophic bacterial counts (THBC) of the liquid herbal preparation ranged from $3.1x10^2$ cfu/ml to $2.65x10^3$ cfu/ml whereas, the total fungal counts (TFC) of the liquid herbal preparations ranged from $1x10^1$ cfu/ml to $1.9x10^2$ cfu/ml. The THBC for

powdered herbal preparation, ranged from$1.1x10^2$ cfu/g to $1.5x10^2$ cfu/g while the total fungal counts (TFC) of the powered herbal preparations was $1x10^1$ cfu/g.

Table 1 Enumeration of Total Heterotrophic bacteria on Herbal samples (cfu/ml)

Samples	Kj cleanser (cfu/ml)	Ok Cleanser (cfu/ml)	Sk cleanser (cfu/g)	Gk cleanser (cfu/ml)	Y-Bitters (cfu/ml)	Sw-Bitters
1	-	1.1×10^2	1.1×10^2	7.8×10^2	1.30×10^3	1.0×10^1
2	3×10^2	-	1.3×10^2	8.6×10^2	2.65×10^3	1.2×10^1
3	3×10^2	1.0×10^2	1.5×10^2	5.9×10^2	$9{,}9 \times 10^3$	2.9×10^1
4	-	9×10^2	1.2×10^2	4.9×10^2	1.10×10^3	1.0×10^1
5	2×10^2	1.2×10^2	9×10^2	-	1.90×10^3	1.9×10^1

Table 2 Enumeration of total heterotrophic fungi on herbal samples

Samples	KJ cleanser (cfu/ml)	Ok Cleanser (cfu/ml)	Sk cleanser (cfu/ml)	Gk cleanser (cfu/g)	Y-Bitters cfu/ml)	Sw- Bitters (cfu/ml)
1	-	3×10^1	-	1×10^1	-	-
2	-	-	-	-	-	1.9×10^1
3	-	2×10^1	-	1×10^1	-	-
4	-	-	2	-	-	1.4×10^2
5	-	2×10^1	-	1×10^1	-	-

Table 3 Diversity of bacterial isolates on herbal samples

Samples	*Vibrio*	*E. coli*	*Pseudomonas*	*Shigella and Salmonella*	*Coliform*	*Bacilli*	*Staphylococci*
Sw-bitter	-	-	-	-	-	+	-
Kj cleanser	-	-	-	-	-	+	-
Ok cleanser	-	-	-	-	-	+	-
Sk cleanser	-	-	-	-	-	+	-
Gk cleanser	-	-	-	-	-	+	-
Y- Bitters	-	-	-	-	-	+	-

Key: - = No Bacterial growth; + = Bacterial growth

Table 4 Diversity of fungal cultures on herbal samples

Samples	Aspergillus	Mould	*Penicillium*	Yeast
Sw bitter	+	-	-	-
Kj cleanser	+	-	-	-
Ok Cleanser	+	-	+	-
Sk cleanser	+	-	+	-
Gk Cleanser	+	-	+	-
Y- Bitters	+	-	+	-

Key: - = No Fungal growth; + = Fungal growth

Table 5 Diversity of Bacillus spp. on herbal samples

Samples	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Sw bitter	*B. subtilis, B. polymyxa*	*B. polymyxa*	*B. subtilis*	*B. cereus*	*B. subtilis*
Kj cleanser	-	*B. licheniformis, B. subtilis*	*B. subtilis*	-	*B. cereus, B. subtilis*
Ok cleanser	*B. polymyxa, B. subtilis*	-	*B. subtilis, B. licheniformis*	*B. subtilis*	*B. cereus*
Sk cleanser	*B. cereus, B. subtilis,*	*B. subtilis*	*B. subtilis*	*B. licheniformis*	*B. subtilis, B. licheniformis*
Gk cleanser	*B. cereus, B. subtilis*	*B. cereus*	*B. cereus, B. subtilis*	*B. subtilis*	-
Y-bitters	*B. licheniformis, B. cereus, B. subtilis*	*B. subtilis, B. polymyxa*	*B. cereus, B. licheniformis*	*B. licheniformis, B. cereus, B. subtilis*	*B. subtilis, B. cereus*

4. Discussion

Data obtained showed that the Total heterotrophic bacterial count (THBC) of herbal samples ranged from $3.1x10^2$ cfu/ml to $2.65x10^3$ cfu/ml whereas in the herbal powder the THBC ranged from $9x10^1$ cfu/g to $1.5x10^2$ cfu/g. Fungi count of herbal syrups ranged from $2x10^1$ cfu/ml to $1.9x10^2$ cfu/ml whereas fungi count of herbal powder ranged from $1x10^1$ cfu/ml to $1.0x10^2$ cfu/ml. Microbial load of samples was within WHO limit of microbial contamination of $1x\ 10^5$ for total aerobic bacteria, yeasts and mould 10^3 cfu/g Enterobacteria and other Gram negative organisms 10^3 cfu/g while *E. coli* and *Salmonella* should not be present in herbal preparations. It was observed that liquid herbal preparation had more microbial growth when compared to the powder. Herbal medicines usually contain bacteria and fungi from soil and atmosphere [12-15].

This study revealed that *Bacillus* spp. were the predominant bacteria in the herbal medicines. This result is comparable with the report of [16]. They assessed Nepal herbal medicines and reported all herbal medicines tested were contaminated with *Bacillus* sp which are commonly found in the soil, air, dust etc. Many aerobic species of *Bacillus* produce endospore that helps them not only to resist environmental stress but also to ensure their long term survival under adverse conditions.

Among the *Bacillus* spp, the most predominant was *Bacillus subtilis,* this was followed by *Bacillus cereus*. The result is comparable to the result obtained by [17]. He had also reported *Bacillus subtilis* as the predominant one in herbal medicines. [18], reported *Staphylococcus aureus*, *Salmonella* sp, *E. coli* and *Shigella* sp. in herbal medicines. In this research, such microorganisms were not detected this showed that the herbal drugs used in this research meet the guidelines for medicinal practice, lowest possible level of microorganisms in the raw material, finished dosage forms and the packaging components to maintain appropriate quality, safety and efficacy of the natural products, [18] Presence of *Bacillus subtilis* and *Bacillus cereus* in herbal medicines also matches with similar study carried out by [19]. The presence of *Bacillus* could come from the soil, since the herbal preparations were made from plants grows on soil, which is the natural habitat of Bacillus. Improper drying, inadequate heat processing, improper handling of products and contaminated equipment are other possible sources of *Bacillus* sp in herb. Though, study revealed the presence of *Bacillus*, however it is documented that the isolated *Bacillus* spp are not pathogenic, though *Bacillus cereus* and *Bacillus licheniformis* can cause food poisoning. *Aspergillus* sp and *Penicillium* sp isolated from this work is similar to the report of [20], Fungi causes deterioration which adversely affects the chemical composition of the raw materials and thereby decreases the medicinal potency of herbal drugs.

Microbial contamination results in degradation of the plant constituents, it can also render plant materials toxic either by transforming the chemicals in the plant material or through the production of toxic compounds by the microbes. Microbial quality tests should be applied starting from plants materials, intermediate and finished products where necessary. During the quality analysis, precautions must be taken to ensure that conditions do not adversely affect any

microorganisms that are not to be measured. It is also important that appropriate techniques and standards be followed during packaging and storage to ensure the safety of herbal preparations before consumption.

5. Conclusion

The herbal products used in the research meet WHO Good Manufacturing practice guidelines and techniques, good harvesting practices and the safe handling and storage of herbal products. Microbial load and isolates were within WHO limit and of acceptable pharmaceutical and microbial quality. Organisms isolated indicate product is of natural origin and these herbal products are safe for human consumption. However, plants to be used in herbal preparation should be properly washed and heated to eliminate the presence of *Bacillus* species.

Compliance with ethical standards

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Disclosure of conflict of interest

There is no conflicting interest.

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