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MICROBIOLOGICAL ASSESSMENT OF SOME LOCALLY PREPARED HERBAL MEDICINES SOLD IN ILARO

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Abstract
Herbal medicine is an important aspect of African culture in solving health related issues, although the methods of preparation remain as they were handed over by the fore-fathers. Microbial quality of locally prepared herbal medicines sold in Ilaro was assessed in order to determine their safety. Ten commonly consumed herbal medicines in Ilaro, Ogun State were sampled for microbial quality using standard plate technique. The isolates were identified using conventional method. The total viable count ranged from $1.8 \times 10^5 - 4.6 \times 10^5$cfu/ml or cfu/g. The total coliform count ranged from $1.0 \times 10^3 - 9.0 \times 10^3$cfu/ml or cfu/g; some samples showed no coliforms. Total fungal count ranged from $7.0 \times 10^1 - 9.0 \times 10^3$cfu/ml or cfu/g.*Bacillus subtilis, Enterococcus faecalis, Staphylococcus aureus, Escherichia coli, Klebsiella* sp.*, Staphylococcus epidermidis, Corynebacterium* sp.*, Proteus* sp., *Enterobacter* sp., *Citrobacter* sp., *Aspergillus niger, Aspergillus flavus, Saccharomyces cerevisiae, Saccharomyces ellipsoideus* and *Penicillium* sp.were isolated from the samples. Antibiotics susceptibility tests performed on some of the bacterial isolates showedvaried degrees of susceptibility to some common standard antibiotics. The zones of inhibition ranged from 8-20 mm. The microorganisms associated with the herbal samples are of concern, they could pose a great threat to health speakers.
Key: Herbal samples, sanitary measure, microbial contaminants, antibiotics

Introduction

The use of herbs is solving heath challenges is an important part of African culture. Herbal medicine is the oldest form of healthcare known to mankind. Herbs had been used by all cultures throughout history. It was an integral part of the development of modern civilization. The use of herb is an ancient practice usually employed in solving health related issues, it is sometimes use as complementary or alternative medicine (Archibong, Igboeli, Okoro & Obika, 2017). The medicinal use of plants seems to have been developed through observations of wild animals, and by trial and error. As time went on, each tribe added the medicinal power of herbs in their area to its knowledge base (Falodun&Imieje, 2013).

The global use of herbs in solving primary healthcare related issues has been estimated by World Health Organisation (WHO) to be about 80% of the world population (Ampofo, Tetteh & Bello, 2012). The use of herbal medicines has also been recognised by WHO as the surest means of achieving total healthcare coverage of the world population most especially in developing counties. More so, in order to provide accessible and culturally acceptable healthcare across the globe, WHO advocated the integration of herbal medicine into primary healthcare programme in developing countries (Yesuf, Wondimench, Gebrecherkos&Moges, 2016), althoughmost times, the method of preparation, dose and preservation/storage techniques are always issues of public health concern. It is becoming more widespread as improvement in analysis and quality control along with advances in clinical research show the worth of herbal medicine in the treatment and prevention of diseases in developed countries.

Herbal medicines are plants and plant parts such as seed, berries, roots, leaves, and bark that have been transformed into phytochemicals by means of simple processes involving harvesting, drying and storage (Abel & Busia, 2005).Herbs have been recognisedin the treatment of typhoid fever, malaria, infertility, waist pain, pile etc. (Coon, Ernest &Parax, 2002). Most times the plants or plant parts are not without presence of microorganisms. Even herbal preparation assumed to be safe could be contaminated with microorganisms and other foreign materials such as heavy metalsand pesticide residues. During microbial contamination, the herbal materials sometimes serve asnutrition for the growth and proliferation of the microorganisms,which evenly brings about deterioration in quality or with no therapeutic efficacy (Rajapandiyan, Shanthi & Vidya, 2013). The presence of contaminants in herbal preparation or medicine could pose a great threat to health seekers. Presence of potential pathogen and other organisms have been reported in herbal medicines by various authors across the globe (Noor, Huda, Rahman, Bashar & Munshi, 2013; Odedare&Memuletiwon, 2014; Yesuf*et al*., 2016).

Although medicinal plant materials usually harbor microorganisms which are probably originated from soil, additional contaminants might also be added as a result of methods of handling and preparation. The use of herbal medicines in developing countries keeps on increasing but the methods of preparation still remain as they were handled over by our fore-fathers. Only little efforts have been put in place by the traditional health givers

as means of quality control, has been itemized by WHO and National Agency for Food and Drug Administration and Control (NAFDAC) in order for herbal medicines to play vital role in primary healthcare delivery. Herbal medicines prepared under unhygienic condition could pose serious health problems. Therefore there is need to assess the quality of local herbal medicine sold in our community.

Materials and Methods

Samples Collection
A total of ten (10) herbal samples were purchased from different herb sellers in Ilaro, Yewa-South Local Government area of Ogun State. The samples were collected in sterile polythene bags and were transported to Microbiology Laboratory section of Department of Science Laboratory Technology, The Federal Polytechnic, Ilaro. The microbial analysis of the samples was carried out within 6 h of collection.

Isolation of microorganisms
Pour plate technique as described by Rajapandiyan*et al*. (2013) was adopted for the isolation of the organisms. A ten-fold serial dilution was carried out and required dilution levels were plated on Nutrient agar and McConkey agar for total viable count and total coliform count respectively.Potato dextrose agar incorporated with chloramphenicol was used determine the total fungal count. The inoculum (1 ml) was introduced and the medium (15-20 ml) was dispensed into the plate (Petri-dish) under aseptic condition. The plates were rocked for the inoculums to be evenly distributed, and they were allowed to solidify before inversion. Nutrient agar and McConkey agar plates were incubated at 37 ˚C for 18-24 h. Potato dextrose agar plates were incubated at 25 ˚C for 2-3 days. All the plates count were expressed as colony forming unit (cfu/ml or cfu/g). The isolates were sub-cultured on a freshly prepared medium by streaking and incubated appropriately. The pure cultures were maintained on agar slants and kept in the refrigerator until further analyses.

Identification of Bacterial Isolates
The bacterial isolates were identified using conventional method on the basis of colonial morphology, cellular characteristics and biochemical tests. The pure isolates were identified by macroscopic examination of the colonies. The colony size, colour, surface, elevation and consistency of the pure isolates were examined. Gram-stain was carried out in order to determine the cellular characteristics of the bacteria.Likewise biochemical tests such as catalase, coagulase, indole and sugar fermentation were carried out as described by Cheesbrough (2006).Bergey's Manual of Determinative Bacteriology (Holt, Krieg, Sneath, Staley & Williams, 1994) was used as reference for the identification.

Identification of Mould
The mould Isolates were identified on the basis of their cultural and cellular characteristics using fungal atlas and diagnostic keys as guides. The pure isolates were examined for their mycelia or sporescolour, surface texture and reverse pigment formation as means of determine the cultural characteristics. They were also stained with lactophenol cotton blue dye to determine the cellular/microscopic characteristics of the isolates (Archibong*et al*., 2017). Microscopic examination was carried out afterthe mycelia of the organism were stained with 1 or 2 drops of lactophenol cotton blue on a clean glass slide as described by Archibong*et al*. (2017). The glass slide was observed under 10x and 40x objectives of a compound microscope. Cultural and cellular characteristics of the organisms were compared with fungal diagnostic keys and atlas.

Antibiotic Susceptibility Test
The antibiotic susceptibility test of the bacterial isolates was carried out using disc diffusion method on Mueller Hinton Agar,according to National Committee for Clinical Laboratory Standards (NCCLS) (2007). The organisms were inoculated into sterile peptone water and incubated for 18-24 h. The broth was further diluted until the turbidity of the suspension matches 0.5 McFarland standard (Cheesbrough, 2006). Each plate was seeded with 0.1 ml of the cell suspension by spread on the agar plate using sterile bent glass rod. Standard antibiotic discs were aseptically placed on the inoculated plates, and allowed stand on the work bench for 5 min before incubation at 35 ^{o}C for 24 h. The diameter of zones of inhibition was measured using transparent ruler and recorded accordingly,this was used in determiningthe sensitivity of the bacterial isolates to the antibiotics.

Results and Discussion

Results
The microbial loads of some locally prepared herbal medicine samples sold in Ilaro are shown in Table 1. The total viable count ranged from $1.8x10^5$ – $4.6x10^6$cfu/ml. Sample L_1E recorded the least total viable count

($1.8x10^5$ cfu/ml) while the highest count ($4.6x10^6$cfu/ml) was recorded in sample L_5L. The presence of coliform was recorded in most of the herbal samples collected, this ranged from $1.0x10^3$ – $9.0x10^3$cfu/g. There was no presence of coliform in samples L_1E, L_2L, L_3L and L_5L, while sample P_2O had the highest total coliform count ($9.0x10^3$cfu/g). Presence of fungal was recorded in all the herbal samples, the fungal count also ranged from $7.0x10^1$ – $9.0x10^4$cfu/ml. Sample L_1E had the least fungal count ($7.0x10^1$ cfu/ml) while the highest count ($9.0x10^4$cfu/ml) was recorded in sample L_5L.

A total of twenty-one (21) bacteria were isolated from the herbal samples and they belong to nine (9) genera (Table 2). They showed different colonial morphology, microscopic characteristics and biochemical tests (Table 2). These bacteria include *Bacillus subtilis, Enterococcus faecalis, Staphylococcus aureus, Escherichia coli, Klebsiella* sp.*, Staphylococcus epidermidis, Corynebacterium* sp.*, Proteus* sp., *Enterobacter* sp.and *Citrobacter* sp. Fungal associated with the herbal samples were identified on the basis of colonial morphology and microscopic characteristics (Table 3). The fungal isolates include *Aspergillus niger, Aspergillus flavus, Saccharomyces cerevisiae, Saccharomyces ellipsoideus* and *Penicillium* sp.

The microorganisms associated with the herbal samples are shown in Table 4. The presence of *Bacillus subtilis*, *Aspergillus niger* and *Saccharomyces* spp. was recorded in most of the samples. *Escherichia coli* was isolated from samples P_1E and P_4O. Presence of *Staphylococcus aureus* was recorded in sample P_5O. More so, *Penicillium* sp. and *Proteus* sp. were isolated from samples P_4O and P_3O respectively.

The antibiotic susceptibility of some of the bacteria associated with the herbal samples is shown in Table 5. The bacteria showed varied degrees of susceptibility to some standard antibiotics. Only Gentamycin (GEN) and Septrin (STX) were active against all the Gram-positive isolates. Pefloxacin (PEF), Ciprofloxacin (CPX), Amoxicillin (AM) and Tarivid (OFX) were active against all the Gram-negative bacteria. Isolated from the herbal samples. The zones of inhibition ranged from 8-20 mm. The highest zone of inhibition (20 mm) was recorded by OFX against *Enterococcus faecalis* while the least (8 mm) was by GEN against *Klebsiella* sp.

Table 1: Microbial loads of some locally prepared herbal medicines sold in Ilaro

Sample Code	Microbial Load cfu/lml or cfu/g		
	Total Viable Count	Total Coliform Count	Mould Count
L_1E	$1.8x10^5$	Nil	$7.0x10^1$
L_2E	$3.1x10^5$	Nil	$2.6x10^4$
L_3B	$1.0x10^6$	Nil	$9.0x10^4$
L_4B	$2.5x10^6$	$2.1x10^3$	$3.0x10^4$
L_5B	$4.6x10^6$	Nil	$2.3x10^4$
P_1E	$2.5x10^5$	$3.0x10^3$	$3.5x10^4$
P_2E	$4.2x10^5$	$2.7x10^3$	$4.1x10^4$
P_3O	$3.1x10^6$	$2.3x10^3$	$1.5x10^4$
P_4O	$2.6x10^6$	$1.0x10^3$	$1.7x10^4$
P_5O	$1.1x10^6$	$9.0x10^3$	$2.7x10^4$

Key: L= Liquid sample P= Powdered sample E= Express B= Library O= Orita

Table 2: Identification of bacteria isolated from the herbal samples

Isolate Code	Colonial Characteristic	Cellular Characteristic		Biochemical Test						Probable Organism
		Gram Reaction	Shape	Indole	Cataase	Coagulase	Glucose	Sucrose	Lactose	
L_1E_1	Off white, spready all over the plate	+	Rod	-	+	-	+	+	+	*Bacillus subtilis*
L_2E_1	Off white, spready all over the plate	+	Rod	-	+	-	+	+	+	*Bacillus subtilis*
P_1E_1	Off white, spready all over the plate	+	Rod	-	+	-	+	+	+	*Bacillus subtilis*
P_1E_2	Small umbonate cream colonies	+	Rod	-	+	-	+	+	+	*Corynebacterium* sp.
P_1E_3	Smooth pinkish colonies	-	Rod	+	+	-	+	+	+	*Escherichia coli*
P_1E_4	Mucoid pinkish colonies	-	Rod	-	+	-	+	+	+	*Klebsiella* sp.
P_2E_1	Smooth white colonies	+	Cocci	-	-	-	+	+	+	*Enterococcus feacalis.*
P_2E_2	Elevated cream colonies	+	Cocci	-	+	-	+	+	+	*Staphylococcus epidermidis*
P_2E_3	Smooth pinkish colonies	-	Rod	+	+	-	+	+	+	*Escherichia coli*
P_2E_4	Mucoid pinkish colonies	-	Rod	-	+	-	+	+	+	*Enterobacter* sp.
P_3O_1	Off white, spready all over the plate	+	Rod	-	+	-	+	+	+	*Bacillus subtilis*
P_3O_2	Swarm pale colouration	-	Rod	+	+	-	+	+	-	*Proteus* sp.
P_4O_1	Off white, spready all over the plate	+	Rod	-	+	-	+	+	+	*Bacillus subtilis*
P_4O_2	Smooth pinkish colonies	-	Rod	+	+	-	+	+	+	*Escherichia coli*
P_5O_1	Small umbonate cream colonies	+	Rod	-	+	-	+	+	+	*Corynebacterium* sp.
P_5O_2	Shiny golden yellow colonies	+	Cocci	-	+	+	+	+	+	*Staphylococcus aureus*
P_5O_3	Light pink colonies	-	Rod	-	+	-	+	+	+	*Citrobacter* sp.
$L3B_1$	Off white, spready all over the plate	+	Rod	-	+	-	+	+	+	*Bacillus subtilis*
$L4B_1$	Small umbonate cream colonies	+	Rod	-	+	-	+	+	+	*Corynebacterium* sp.
$L4B_2$	Mucoid pinkish colonies	-	Rod	-	+	-	+	+	+	*Klebsiella* sp.
$L5B_1$	Off white, spready all over the plate	+	Rod	-	+	-	+	+	+	*Bacillus subtilis*

Table 3: Identification of fungal isolates from the herbal samples.

Colonial Appearance	Microscopy	Probable Organism
Black mould	Black spherical conidia, Smooth and colourless Conidiophores	*Aspergillus niger*
Dark green mould	Radiating conidia heads, Conidiophores appears rough	*Aspergillus flavus*
Green spores with white hyphae	Hyphae septate conidia Arranged like mop head	*Penicillium* sp.
Round flat cream Colonies	Large oval budding cells	*Saccharomyces cerevisiae*
Irregular cream Colonies	Ellipsoidal budding cells	*Saccharomyces ellipsoideus*

Table 4: Microorganisms associated with the herbal samples.

Sample Code	Associated Microorganism	
	Bacteria	**Fungi**
L_1E	*Bacillus subtilis*	*Aspergillus niger, Saccharomyces ellipsoideus*
L_2E	*Bacillus subtilis*	*Aspergillus niger, Aspergillus flavus, Saccharomyces cerevisiae*
L_3B	*Bacillus subtilis*	*Aspergillus flavus, Saccharomyces cerevisiae*
L_4B	*Corynebacterium* sp., *Klebsiella* sp.	*Saccharomyces cerevisiae*
L_5B	*Bacillus subtilis*	*Saccharomyces ellipsoideus*
P_1E	*Bacillus subtilis, Klebsiella* sp., *Corynebacterium* sp., *Escherichia coli*	*Aspergillus niger, Aspergillus flavus*
P_2E	*Enterococcus feacalis, S. epidermidis, Escherichia coli, Enterobacter* sp.	*Aspergillus flavus, Saccharomyces cerevisiae*
P_3O	*Bacillus subtilis, Proteus* sp.	*Aspergillus niger*
P_4O	*Bacillus subtilis, Escherichia coli*	*Aspergillus niger, Saccharomyces ellipsoideus, Penicillium* sp.
P_5O	*Corynebacterium* sp., *S. aureus, Citrobacter* sp.	*Aspergillus niger*

Table 5: Antibiotic susceptibility of the bacteria associated with the herbal samples

Gram-Positive Bacteria	**Zone of Inhibition Diameter (mm)**								
	PEF	GEN	APX	AM	CPX	S	STX	R	Z
Bacillus subtilis	R	18	18	15	R	R	14	R	R
Enterococcus faecalis	R	15	R	12	20	R	16	10	10
Staphylococcus aureus	18	16	14	R	18	R	12	12	R
Gram-Negative Bacteria	AUG	GEN	AM	OFL	SP	CPX	CH	STX	PEF
Escherichia coli	15	R	14	18	R	13	R	R	16
Klebsiella sp.	14	8	10	12	13	11	15	10	12
Proteus sp.	R	11	15	17	10	15	R	R	12

Keys: AU = Augmentin, GEN = Gentamycin, AM = Amoxicillin, OFX = Tarivid SP = Sparfloxacilin, CPX = Ciprofloxacin, CH = Chloramphenicol, STX = Septrin, S = Streptomycin, R= Rocephin, Z=Zinnacef, PEF = Pefloxacin

Discussion

This study reveals the presence of various microorganisms in the locally made herbs sold in Ilaro. The samples showed presence of bacteria; *Bacillus subtilis, Staphylococcus aureus, Staphylococcus epidermidis, Enterococcus faecalis, Corynebacterium* sp., *Escherichia coli, Enterobacter* sp*., Klebsiella* sp. and *Proteus* sp., More so, *Aspergillusniger, Aspergillus flavus, Penicillum*sp*., Saccharomyces cerevisiae* and *Saccharomyces ellipsoideus*were isolated form the samples. The presence of these microorganisms might be due to improper processing technique or post-production contamination. It might also be due noncompliance with quality control measures laid down by WHO and NAFDAC to minimize microbial contamination. More so some of the organisms might be normal flora of plants that depends on several environmental factors (Akerele, 1993). The present study agree with the findings of Odedara and Memuletiwon (2014) on similar herbal medicines. The similarity could due similar handling technique and environmental factors. The use of unhygienic packaging materials may contribute to the contamination of the products; most times pet bottles picked up from different locations (dump sites or outdoor parties) are used for their packaging or storage.

High prevalence of *Bacillus* sp.recorded in most of the herbal samples could be due to its ubiquitous in nature. They are persistent in the environment (soil, water, air and dust) and as endospore forming organisms, they are resistant to heat treatment which necessitate their dominance in herbal preparations or medicines (Charnock*,* 2004). The presence of *Bacillus subtilis*in the herbal medicines might also be due to availability of favourable factors such as high moisture content and storage temperature, most especially in liquid herbal medicines. (Ayepola, Ugboko, Abu and Olorunshola, 2017). *Bacillus subtilis*is known to produce exotoxins that are detrimental to man health.*Bacillus subtilis* has been implicated in food-borne infection. The presence of Bacillus has also been reported in non-sterile orthodox pharmaceuticals (De la Rosa, Mosso, Garcia and Plaza, 1993).

More so, the presence of S*taphylococcus* sp. in herbal medicines is an indicative of possible human contamination; *Staphylococcusaureus* is normal flora with the ability to produce enterotoxin which can cause serious gastroenteritis (Coker, 2005). This study agrees with the findings of Igbeneghu and Lamikanra (2016), this might be due to similar source of contamination.The presence of coliforms in herbal preparation is an indicative of possible faecal contamination of the plants and water used in preparation or washing of the herbs (Czech, Kneifel, & Kopp, 2001). Contamination of the products with coliform might be due to proper handling of the herbal medicine or contamination of the plants right from the field. The presence of *Escherichia coli* and other coliforms in the samples is of public health concern. *Escherichia coli*is a well-known enteropathogen and is the most common causative agent of diarrhea of bacterial origin as reported by Bonkoungou, Haukka,Osterblad, Hahanen, Traore… &Siitonen, (2013).

The herbal samples recorded high fungal contaminants, thisagrees with the study of Ayepola*etal*. (2017). The similarity in the two studies might be due to sporulation and ubiquitous nature of the fungal isolates. *Aspergillus*isolated from the herbal preparationsmay endanger the health of consumers. Strains of *Aspergillus flavus* are known for their toxin producing ability; the mycotoxinsproduced by these moulds are very harmful and carcinogenic in nature.

Antibiotics sensitivity tests performed on some of the bacterial isolates revealed varied degrees of susceptibility. Although the isolates were susceptible to gentamycin, pefloxacin, ciprofloxacin and amoxicillin, some of the carry multiple antibiotic resistance genes that can be transferred between organisms of same genus or taken up by transformation. The presence of these organisms with multiple antibiotic resistant genes portraits a bad omen for consumers of traditional herbal medicines. The antibiotic resistant ability of some of the isolates might be chromosomal and plasmid mediated. The presence plasmid and chromosomal mediated antibiotic resistant bacteria has been earlier reported in herbal medicines by Oshoma&Dijeh (2017).

Conclusion

In conclusion the herbal medicines sold in Ilaro showed presence of bacteria and fungi that are of public health concern. The microbial loads of the samples were generally high. Some of the bacteria also showed multiple antibiotic resistant. The presence of these organisms could pose a great danger to traditional health seekers. Since herbal medicine is an important part of African culture, and also play vital role in primary health care in developing countries, therefore there is need for the methods of preparation and storage to be standardized in order to guide against microbial contamination.

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