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Microbiological Evaluation of Locally Processed Herbal Drugs Sold in Benin City

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ABSTRACT: Plants that are referred to as "herbs" are not used as a food, but are grown and consumed as a garnishment for flavour enhancement, seasoning, and sometimes for alleged healing properties. This study investigates the microbiological quality of common herbal drugs sold in Benin City and antibacterial susceptibility profile of the Isolates. Herbal concoctions were purchased from various traditional medicine sales outlet in Benin City, Nigeria, coded as HA to HJ. Bacterial were cultured on Nutrients Agar while fungi were cultured on Potato Dextrose Agar. The highest bacterial count was $2.90 \pm 0.20 \times 10^4$ cfu/mL (HB and HH) while the lowest was $9.50 \pm 0.20 \times 10^3$ cfu/mL (HF). The fungal counts of herbal concoction samples analysed were found to have the highest value of $1.80 \pm 0.20 \times 10^4$ cfu/mL (HF) and least $6.00 \pm 0.0 \times 10^3$cfu/mL (HI). The identified bacterial isolated from the herbal concoctions were *Bacillus licheniformis, Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus subtilis, Escherichia coli* and *Staphylococcus epidermidi*s. The fungal isolates included *Penicillium* sp, *Aspergillus flavus, Aspergillus niger* and *Rhizopus* sp. *Pseudomonas aeruginosa* had the highest resistance to all tested antibiotics while the most sensitive isolates were *Bacillus licheniformis* and *Staphylococcus epidermidi*s. Bacterial count in the herbal preparations were below the stated acceptable limit, but their presence represents a potential risk of infectious diseases. Therefore, appropriate standards in herbal medicinal preparations and all medicinal plants should be adhered to in order to reduce the risks of infections to consumers in Nigeria.

Keywords: Herbal drugs, Microbial contamination, Antibiotic susceptibility, Plasmid

Introduction

Early humans recognized a need for the dependence on nature for a healthy life and since that time humanity has depended on the diversity of plant resources for food, clothing, shelter, and medicine to cure myriads of ailments (Okunlola *et al*., 2007; Aarland *et al*., 2014). Plants that are referred to as "herbs" are not used as food, but are grown and consumed as garnishment for flavour enhancement, seasoning, and sometimes for alleged healing properties (Douglas *et al*., 2005; Abdullahi 2011). It is generally believed that our Creator puts herbs upon the earth to maintain and restore human and animal health (Stevic *et al*., 2012' Amosu *et al*., 2014). Vanwyk *et al.* (1997) argued that traditional healers commonly called herbalists have broad knowledge of medicinal plants and concentrate on the healing properties of these plants. Esimone *et al*. (2007), advocated for the integration of herbal medicinal products into primary health care system of developing countries. This may be because maintenance of health through conventional medicine is beyond the means of many people in the rural communities arising from the increasing rate of unemployment, rising medicinal cost, and uneasy access to available drugs (Ampofo *et al*., 2012; Kumari and Kotecha, 2016).

The continual usage of herbal products have also given rise to various forms of abuse and adulteration of the products resulting in consumers' and manufacturers' dissatisfaction, and in some instances, fatal consequences (Thillaivanan and Samraj, 2014). The challenge is innumerable and enormous, making the global herbal market unsafe.

Safety, efficacy and quality of these herbal products have been an important concern to health professionals (Okunlola *et al*., 2007). Safety issues related to herbal drugs have continued to be ignored by herbalists whose method of concocting herbal preparations for public use are usually unhygienic leading to microbial hazards (Aarland *et al*., 2014). Some of the adverse effects may be due to some factors such as adulterations, substitutions, contaminations, wrong identification, lack of standardization, incorrect preparations and/or dosage, inappropriate labelling and/or advertisement (Lau *et al*., 2003).

Technical guidelines have been provided to national regulatory authorities and manufacturers by the World Health Organization Good Manufacturing Practice Guidelines in respect of herbal remedies. In Nigeria, the body responsible for control of the quality of herbal medicine and their products is the National Agency for Food, Drugs Administration and Control (NAFDAC) (Okunlola *et al*., 2007).

Microbial contaminations are frequently involved in herbal products; in some cases they are in large amounts and may include pathogenic species (Walther *et al*., 2016). This calls for regular evaluation of the microbial content of herbal remedies. Laboratory studies showed that the more stringent limit for herbal teas indicated that, where the aerobic bioburden exceeded 10^5cfu/g, the prevalence of organisms with potential to constitute a risk to the users increased (Okunlola *et al*., 2007). Increasing prevalence of resistance has been reported in many pathogens over the years in different regions of the world including developing countries (Byarugaba, 2010). This has been attributed to changing microbial characteristics, selective pressures of antimicrobial use, and societal and technological changes that enhance the development and transmission of drug-resistant organisms.

Although antimicrobial resistance is a natural biological phenomenon, it is often enhanced as a consequence of infectious agents' adaptation to antimicrobials exposure in humans or agriculture and the widespread use of disinfectants at the farm and household levels (Walsh, 2000; Thillaivanan and Samraj, 2014). It is now widely accepted that antimicrobial use is the single most important factor responsible for increased antimicrobial resistance (Aarestrup *et al*., 2001; Byarugaba, 2010). Therefore, this study aims at determining microbiological quality of some common herbal concoctions and antibacterial susceptibility profile of the bacterial isolates.

Materials and methods

Collection of samples: Locally produced aqueous herbal drugs were purchased from various traditional medicine sale outlets in Benin City, Nigeria. The concoctions (drugs) were labelled alphabetically and their therapeutic indications are presented in Table 1.

Table 1: Coded herbal drugs and their therapeutic indications obtained from sale outlets in Benin City

Product code	Therapeutic claim
HA	Gonorrhea
HB	Pile
HC	Body pain
HD	Toilet infection
HE	Tuberculosis
HF	Malaria
HG	Diabete
HH	Syphilis
HI	Tooth ache
HJ	Skin rash

Microbial Enumeration of Herbal Samples: Each sample of the herbal drug (HA – HJ) was shaken vigorously and 1 mL was transferred into a test tube containing 9 mL of sterile distilled water, followed with a 10-fold serial dilution of each herbal product. From the aliquots, 0.1 mL was transferred into nutrient agar (NA) supplemented with 50 ug/ml of Nysatin. 0.1 mL was transferred into Potato dextrose agar (PDA) supplemented with 25 ug/mL of chloramphenicol plates. Plates containing NA were incubated at 37 ºC for 48 h while PDA plates were incubated at

28 ± 2 ºC for 72 hrs. The discrete colonies on the Nutrient agar and Potato dextrose agar were selected and counted. The mean colony counts on the nutrient agar and potato dextrose agar plates of each given dilution was used to estimate the total viable count for the samples in colony forming units per millilitre (cfu/mL).

Microbiological identification: From the NA plates, colonies were randomly picked and repeatedly sub-cultured on NA for purification. Purified bacterial isolates were stored in NA slants for further studies. The purified bacterial isolates were characterized by morphology, Gram's reaction and biochemical test using the scheme in Bergey's manual of determinative bacteriology (Holt *et al*., 1994; Cheesbrough, 2000). Fungal isolates were examined macroscopically and microscopically using the needle mounts technique. Their identification was performed according to the procedure of Barnett and Hunter (1972) and Larone (1986).

Antibiotics susceptibility pattern: Antibacterial disc tests of the isolates were performed according to the recommendations of the National Committee Laboratory Standards (NCCLS) (2007) using the following antibiotic discs: Septrin (SXT) 30 µg, amoxicillin (AM) 30 µg, chloranphenicol (CH) 30 µg, gentamicin (CN) 10 µg, ciprofloxacin (CPX), pefloxacin (PEF) 30 µg, Augmentin (AU) 30 µg, Tarivid (OFX), Streptomycin (S) 30 µg and Sparfloxacin (SP) 10 µg. The test organisms were inoculated into sterile nutrient broth in a test tube and incubated at 37 ºC for 24 hrs. From the liquid culture, 0.1 ml was transferred into solidified NA in a Petri dish and a sterile spreader was used to distribute evenly. The plates were allowed to dry for 5 minutes, thereafter the standard antibiotics disc was laid on the inoculated agar. The plates were incubated at 37 ºC for 24 hrs. Clear zones around the discs were measured and interpreted as either susceptible or resistance for the test organisms to the particular antibiotic. Zones of inhibition ≥13 mm were considered sensitive while those ≤12 mm were regarded resistant (NCCLS, 2007). The multiple antibiotic resistance index (MARI) of bacterial isolated was calculated as the ratio between number of antibiotics for which an isolate is resistant and the total number of antibiotics to which the organism was exposed (Akinjogunla and Enabulele, 2010).

Plasmid profile of the bacterial isolates: A colony of test bacterial isolates cultured on fresh nutrient agar plates, was picked with the aid of a sterile wire loop and inoculated into sterile test tubes containing 8 ml of fresh nutrient broth and incubated at 37 ºC for 72 hrs. Plasmid DNA extraction was carried out using the alkaline lysis method of Birnboim and Doly (1979). Agarose gel 0.8 % (w/v) was prepared and poured into electrophoresis tank and allowed to solidify. A mixture of 15 µL of the sample and 2 µL of the loading dye was loaded into wells. This was subjected to electrophoresis using a horizontal apparatus-agarose gel unit (HE33; Hoefer, San Francisco) and a constant voltage of 63 V using a power source (P500B; Sigma, St. Louis, MO, USA) for 3 h (Chigor *et al*., 2010). Gels were visualized and photographed using digital photo documentation system (Clinix, Japan) (Agbagwa and Jirigwa, 2015)

Results

The aqueous herbal samples analysed were found to be contaminated with both bacterial and fungal species. The highest bacterial count was obtained in samples HB and HH ($2.90 \pm 0.20 \times 10^4$ cfu/mL) while the lowest value of 9.50 $\pm$ 0.20 $\times$ 10^3 cfu/mL was obtained in sample HC (Table 2). There was no bacterial load in sample HJ.

The fungal counts of samples analysed were found to have the highest value of $1.80 \pm 0.20 \times 10^4$ cfu/mL in HF and least in HI ($6.00 \pm 0.0 \times 10^3$cfu/mL).

The pH values of the herbal concoctions ranged from 2.4 - 5.2 which is indicative of an acidic nature (Table 2).

The various microorganisms isolated from the herbal concoctions after biochemical characterization were confirmed to be *Bacillus licheniformis, Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus subtilis, Escherichia coli* and *Staphylococcus epidermidi*s.

The frequency of occurrence of bacterial isolates is shown in Table 3. *S. aureus* (23.33 %) was the most occurring bacterial isolate while the least occurring (13.33 %) bacterial isolates were *Bacillus licheniformis, Pseudomonas aeruginosa* and *Staphylococcus epidermidi*s.

The fungal isolates included *Penicillium* sp, *Aspergillus flavus, Aspergillus niger* and *Rhizopus* sp. where *Aspergillus flavus* had the highest occurrence of 33.33 % (Table 4).

Results of the antibiotics susceptibility tests performed on isolates showed that *Pseudomonas aeruginosa* had the highest resistance to all the antibiotics with the exception of Gentamycin, Ciprofloxacin, Streptomycin and Ofloxacin. The most sensitive isolates were *Bacillus licheniformis* and *Staphylococcus epidermidi*s (Table 5).

Table 2: Total viable bacterial, fungal counts and pH values of Herbal Concoctions

Samples	pH	Bacterial count (cfu/mL)	Fungal count (cfu/mL)
HA	3.2	$1.30\pm 0.20 \times 10^4$	$1.10\pm 0.20 \times 10^4$
HB	4.0	$2.90\pm 0.01 \times 10^4$	$1.10\pm 0.01 \times 10^4$
HC	3.8	$9.50\pm 0.20 \times 10^3$	$7.50\pm 0.01 \times 10^3$
HD	5.2	$2.00\pm 0.30 \times 10^4$	$7.50\pm 0.30 \times 10^3$
HE	2.4	$1.20\pm 0.20 \times 10^4$	$1.20\pm 0.25 \times 10^4$
HF	3.4	$1.20\pm 0.10 \times 10^4$	$1.80\pm 0.20 \times 10^4$
HG	3.7	$2.00\pm 0.10 \times 10^4$	$9.50\pm 0.10 \times 10^3$
HH	3.4	$2.90\pm 0.20 \times 10^4$	$8.00\pm 0.10 \times 10^3$
HI	3.5	$2.20\pm 0.30 \times 10^4$	$6.00\pm 0.0 \times 10^3$
HJ	.0.9	Nil	$7.50\pm 0.10 \times 10^3$

Table 3: Occurrence of the bacterial isolates

Bacterial isolates	% Occurrence
Pseudomonas aeruginosa	13,33
Staphylococcus aureus	23.33
Staphylococcus epidermidis	13.33
Bacillus licheniformis	13,33
Bacillus subtilis	20.00
Escherichia coli	16.66

Table 4: Occurrence of the fungal isolates

Fungal isolates	% Occurrence
Aspergillus niger	28.57
Aspergillus flavus	33.33
Mucor sp.	19,05
Rhizopus sp.	19.05

Table 5: Antibiotic susceptibility pattern of the bacterial isolates

Gram negative	**SXT**	**CH**	**SP**	**CPX**	**AM**	**AU**	**CN**	**PEF**	**OFX**	**S**
P. aeruginosa	R	R	R	S	R	R	S	R	S	S
E. coli	R	R	S	S	S	S	S	R	S	S
Gram positive	**PEF**	**CN**	**APX**	**Z**	**AM**	**RO**	**CPX**	**S**	**SXT**	**E**
B. licheniformis	S	S	R	S	S	S	S	S	R	S
B. subtilis	S	S	R	S	S	S	S	S	R	S
S. aureus	R	S	R	S	R	S	S	S	R	R
S. epidermidis	S	S	R	S	S	S	S	S	R	S

Key: PEF: Pefloxacin, CN: Gentamycin, APX: Ampiclox, Z: Zinnacef, CPX: Ciprofloxacin, S: Streptomycin, SXT: Septrin, E: Erythromycin, AM: Amoxicillin, RO: Rocephin, CH: Chloramphenicol, SP: Sparfloxacin, AU: Augmetin, OFX: Ofloxazin S: susceptible (Zones of inhibition $\geq$ 13 mm), R: resistant (Zones of inhibition $\leq$ 12 mm).

Plasmid analyses revealed that there were detectable plasmids in the bacterial isolates (Fig. 1). All the isolates were found to possess single band of plasmid with the exception *Pseudomonas aeruginosa* that had multiple plasmid bands.

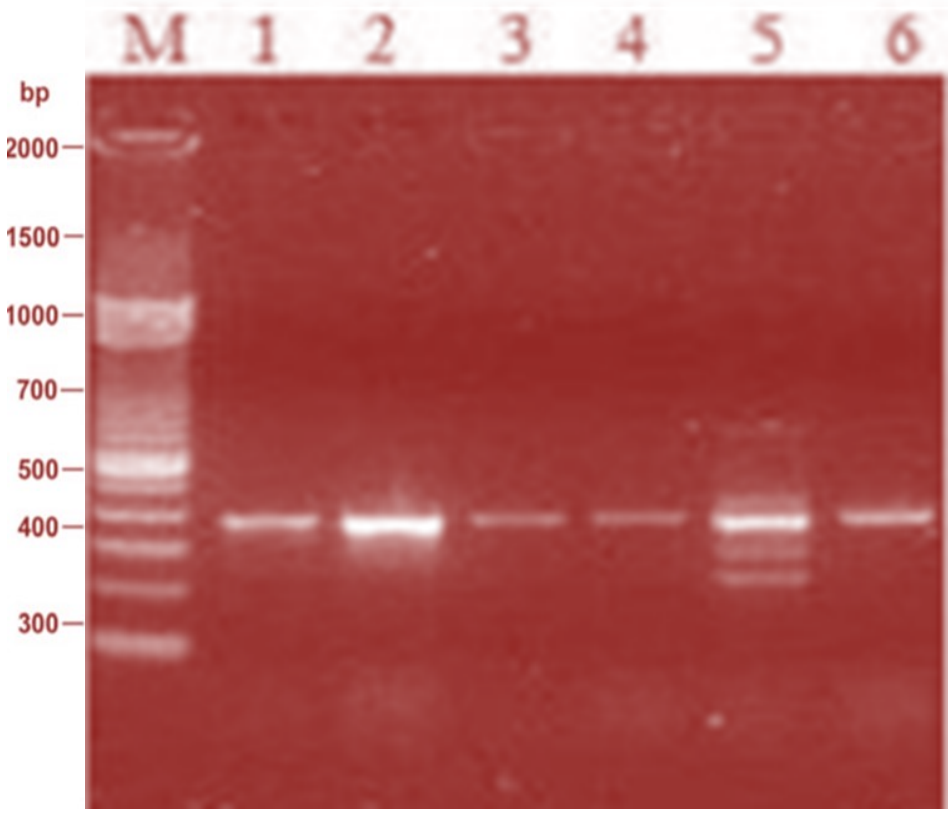

Figure 1: Gel electrophoresis for plasmid profiles of bacterial isolates
Key: M: DNA molecular marker. Lane 1: *Staphylococcus epidermidis,* Lane 2: *Staphylococcus aureus,* Lane 3: *Escherichia coli,* Lane 4: *Bacillus licheniformis,* Lane 5: *Pseudomonas aeruginosa,* Lane 6: *Bacillus subtilis*

Discussion

Microorganisms have existed in their natural environment and are normal floras of the tree; they could be sources of infections when in contact with human as susceptible host. The microbiological background of herbal medicines depends on several environmental factors and exerts an important impact on the overall quality of the herbal products and preparations (Akerele, 2007). Drugs from herbs contain mostly bacteria and moulds contaminated from soil and atmosphere. Soil, harvesting, drying, conditions of storage and poor handling of herbal plants can influence the microbial quality of the herbal medicinal products (Okunlola *et al*., 2007; Stevic *et al*., 2012). Microbial contamination or presence of pathogens of the non-sterile herbal drugs can inactivate or reduce the therapeutic activity of the drugs and may have potential adverse effect to consumers (Noor *et al*., 2013). This can be viewed as a serious public health issue resulting to mortality. The challenge is that the use of highly pathogen-contaminated natural materials has been associated with several fatal infectious outbreaks (Noor *et al*., 2013).
The total microbial count shows the conditions obtained when herbal drugs is prepared in the industry. It is recognized that an improvement in the cleanliness of equipment reduces the level of contaminants found in herbal drugs especially with organism which grow at 20-30 ºC (Mudgil *et al.*, 2004; Yadav *et al.,* 2011). According to Okunlola *et al*. (2007) the contamination acceptable limits are 10^5 cfu/g for bacteria and 10^3 cfu/g for yeast and moulds. The herbal drugs analysed in this study were found to have bacterial contamination less than the acceptable contamination limits. This shows that the bacterial load of the herbal drugs was within the acceptable limit. Also, the low bacterial counts in some of the herbal concoctions may be probably due to low pH value of the herbal concoction (Yadav *et al.,* 2011).

Although the bacteria count was within acceptable limit, the presence of some potential pathogenic bacteria detected in the samples (*E. coli, S. aureus* and *P aeruginosa*) is a major source concern. The presence of these pathogenic bacteria in the herbal drugs constitutes a health risk mostly with *Escherichia coli* which may be causative agent of harmful diseases (Enayalifard *et al*., 2010). *Staphylococcus aureus* has been implicated in serious health risk issues as contaminants of herbal drugs. The bacterium is a normal flora of the skin and causes a lot of illnesses ranging from pimples, impetigo, cellulitis, scalded skin syndrome, abscesses, *et cetera* to life threatening sicknesses like pneumonia and meningitis. It is still a major source of nosocomial infection (Nester *et al*., 2007). *P. aeruginosa* is often isolated in water and damp areas and its infection may be mild as in external otitis and severe as in wound infections. However, high morbidity rate of infection is associated with immuno-compromised individuals or in cases of health challenges such as diabetes (Eze *et al*., 2013). The presence of these bacteria may be due to the preparation methods, equipment and materials used in manufacturing the herbal drugs (Abba *et al*., 2009). Other contamination sources are introduction of bacteria by the personnel during processing (Noor *et al*., 2013).

This investigation also revealed that all the herbal drugs (HA to HJ) were all above the acceptable fungal limit. Among the fungal isolates, *Aspergillus* and *Pencillium* were predominant species which corroborate the report of Aiko and Mehta (2016). Stevic *et al*. (2012) also reported that the highest contaminants of herbal drugs in terms of microbiological quality were mould and that the load exceeded the acceptable limit. The presence of these fungal isolates in the herbal drugs not only reduces their quality and usefulness, but could result in the production of toxic metabolites like mycotoxins (Noor *et al*., 2013). Mycotoxins are thermostable and cause carcinogenic and mutagenic effects to the patients. The detrimental potentiality of mycotoxigenic *Aspergillus* and *Penicillium* spp showed that necessary precautions should be put in place due to the danger of mycotoxins production (Aiko and Mehta 2016).

The ability of some of the antibiotics (Ciprofloxacin and Streptomycin) applied in the sensitivity tests to resist the growth of the two opportunistic pathogens such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* indicated the potency of these conventional antibiotics against such bacteria and might be the drugs of choice for the herbal consumer in the event of probable infection after herbal consumption. Since applications of herbal medicines for curative purposes is on the increase, there is need for conduct Risk Assessment of microbial load of the medicinal plants at critical control points during processing. Relevant agencies of the Nigerian government charged with herbal consumption control also need to introduce some standards that must be met by every herbal processor and seller. There is also a need for good sanitation training exercises and high maintenance standards for all herbal processors so as to safeguard the health of herbal consumers.

Ciprofloxacin, Gentamicin, Streptomycin and Ofloxacin were the most effective antibiotics in *in vitro* testing followed by Amoxicillin which was effective against all the pathogens except *S. aureus*. All the isolates were resistance to Septrin. Similar results with aminoglycosides, betalactams and quinolones have been reported by other authors (Gupta *et al*., 1999; Goswami *et al*., 2011). All isolates were resistant to Septrin, Ampiclox and Chloramphenicol. This may be due to wide and frequent use of these antibiotics, as many people in this geographical area are in the habit of engaging in self-medication in the treatment of infectious diseases even before visiting hospitals for treatment (Akoh *et al*., 2013).

Plasmid profiles have been reported to be useful in tracing the epidemiology of antibiotic resistance (Meyer, 1988). In this study, plasmid profiles were detected which indicates that plasmid profiling can also be used as an epidemiological tool for typing bacteria (Meyer 1988). Son *et al*. (1998) stated that generally, epidemiologically unrelated isolates contain different plasmid profiles whereas related isolates could also display variation in plasmid profiles. The more plasmids exist in an organism, the more specific is the plasmid profile as a marker for a single isolate. Bacterial antibiotics resistance patterns are sometimes associated with the presence of large plasmids and ability of plasmids for conjugation process (Alitheen *et al*., 2009). The analysis of isolates plasmid profile makes evaluation on the dependence between antibiotic susceptibility and the presence of plasmid in an isolate possible. Therefore, plasmid mediated antibiotic resistance has been connected to differences observed in treatment by health workers (Agbagwa *et al*., 2015). However, for other isolates that had no plasmid, they also showed the multiple antibiotics resistance patterns with high number of antibiotics which indicates that resistance to most of these antibiotics is of chromosomal origin or on mobile genetic elements that may help in the dissemination of the resistant genes to other bacteria of human clinical significance (Son *et al*., 1998; Esimone *et al*., 2007). According to Carattoli (2003) and Yah *et al.* (2007), the antibiotic resistance in those isolates that seem not to possess plasmids was associated with chromosome and/or transposons instead of being plasmid-mediated.

Conclusion

In conclusion the results of this present study revealed the presence of bacterial and fungal contaminants in herbal concoction sold in Benin metropolis. Although the count of bacterial load in the herbal preparations falls below the

stated limit howbeit, that of the fungal load did not. However, their presence represents a potential risk of infectious diseases. Considering the worldwide increase in the use of herbal products as alternative medicines and the risk associated with acquiring natural products contaminated with microbes suspected to be pathogenic, it is necessary to set appropriate standards for herbal medicinal preparations to reduce the risks of infection to consumers in Nigeria. Manufacturers of herbal products should be compelled to adhere to strict quality control measure in order to ascertain the quality, safety and efficacy of their finished products.

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