ORIGINAL RESEARCH

MULTI-DRUG-RESISTANT BACTERIA ISOLATES RECOVERED FROM HERBAL MEDICINAL PREPARATIONS IN A SOUTHERN NIGERIAN SETTING

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ABSTRACT

Objectives: The microbial qualities of some herbal medicinal products circulated within southern Nigeria were evaluated. **Methods:** sampling, isolation and characterization of contaminating microorganisms was carried out using established protocols. Agarose gel electrophoresis was employed for the characterization of possible R-plasmids. **Results:** Out of the 18 herbal preparations 13 (72.2%) showed contamination with either bacteria or fungal microorganisms or both. *Bacillus anthracis* was isolated from five (38.5%) of the contaminated preparations (liquid preparation), *Staphylococcus aureus* from one (7.70%) solid preparation, *Corynebacterium diphtheriae* from three (23.08%) preparations (2 liquid, 1 solid), and *Candida albicans* from 11 (84.62%) preparations (6 liquid, 5 solid). The antimicrobial susceptibility-resistance pattern of the contaminating microorganisms revealed that three out of the five *B. anthracis* strains isolated demonstrated resistance to four out of 12 antimicrobial agents (Ofloxacin, Ciprofloxacin, Pefloxacin, Augmentin, Ampiclox, Erythromycin, Cephalexin, Clindamycin, Ceftriaxone, Gentamicin, Co-trimoxazole, Amoxycillin) employed in the study. *S. aureus* displayed over 50% resistance coverage while the resistance pattern recorded by the *Corynebacterium diphtheriae* isolates appeared to be inconsistent. The various isolates of *C. diphtheriae* recorded 40, 0, and 50 percents respectively. Submerged agarose gel electrophoresis employed for the molecular characterization for possible R-plasmids revealed that one plasmid each was isolated from each of five resistant bacteria strains L_{4a} (*Corynebacterium diphtheriae*), L_{4b} (*Bacillus anthracis*), L_{10a} (*Corynebacterium diphtheriae*), L_{10b} (*Staphylococcus aureus*), S_6 (*Bacillus anthracis*). It also shows that the largest plasmid size (23.05 kbp) was recorded for the *C. diphtheriae* strain L_{4a} while the *C. diphtheriae* strain L_{10a} gave the smallest plasmid size (2.03 kbp). **Conclusions:** The results of the study showed that herbal medicinal preparations are a potential source for the dissemination of multi-drug resistant microorganisms.

Key Words: Plasmids; Resistance factor; Herbal drug; Drug-resistant; bacterial; Nigeria.

INTRODUCTION

Antibiotic resistant bacteria have been a source of an ever-increasing therapeutic problem (Sheikh *et al.,* 2003). Continued mismanagement and resulting selective pressure have contributed towards the emergence of multiple drug resistant bacteria which has been regarded as an inevitable genetic response to antimicrobial therapy (Cohen, 1992). Drug resistant infectious microbes have become an important public health concern warranting organizations in public and private sectors worldwide to work together (Ugwu *et al.,* 2009; NIAID, 2011). Aside from the public health threat, the search for newer microbial-sensitive treatments to overcome resistant microbes is usually very expensive and contributes to the higher costs of health care. Newer treatment regimes use more expensive pharmaceuticals and demand longer hospital stays for infected individuals (NIAID, 2011).

Microbial resistance to antimicrobial agents is usually mediated through resistance gene-coded bacteria plasmids. Plasmids are self-replicating extra-chromosomal DNA molecules found in Gram-negative and Gram-positive bacteria as well as in some yeast and other fungi. These plasmids, called R plasmids, harbor a variety of genes encoding resistance to a wide spectrum of antimicrobial compounds, which include antibiotics, heavy metals, mutagenic agents like ethidium bromide, and even disinfectant agents such as formaldehyde (Foster, 1983; Kummerle *et al.* 1996; Martinez and Baquero, 2002). R plasmids offer resistance to antibiotics and are transmissible from one cell to another by direct cell contact (conjugation). Characterization of resistance plasmids through the use of separation techniques such as agarose gel permeation techniques to determine the size of the plasmids provides insights on the size of the genetic information relating to the resistance to one or more antibiotics. The size of this information may also reflect the extent or amount of the multiple drug resistance.

Resistant bacteria strains may develop almost anywhere particularly in a pressurized environment containing previously non-resistant bacteria strains as contaminants. One of such environments can be a herbal medicinal product (HMP). HMPs have been previously implicated as a

pool for such contaminations (Peter, 1999; Esimone *et al.*, 2007). The use of HMPs as a form of complimentary medicine is becoming increasingly popular in both developing and developed countries (Eisenberg *et al.* 1998). About 70% to 80% of the world's population particularly in the developing world has been shown to depend on the primary source of herbal drug regimen for their health needs (Akerele, 1993; WHO, 1998). It is of utmost importance to both monitor and ascertain the microbial purity of HMPs given the huge medical and economic implications of any such microbial contamination especially with multiple drug-resistant strains. Such surveillance will both help to identify microbial contamination of herbal products and slow down or prevent the emergence of drug-resistant strains. In the present study selected HMPs from southern Nigeria were evaluated for presence of contaminating microorganisms which were later subjected to susceptibility studies to establish their resistance profiles. The resistance plasmids from the drug-resistant strains were thereafter isolated and characterized.

METHODS
Drugs and reagents
The following materials were used, TBE buffer (540g Tris, 225g Borate, 415g EDTA in water), Agarose gel 1%, TENS solution (10mM Tris-HCl pH 7.5, 1mM EDTA, 0.1N NaOH, 0.5% SDS). Antibiotic disks used were as follows Augmentin 30µg, Ofloxacin 5µg, Ciprofloxacin 5µg, Tetracycline 30µg (Biological Limited, Liverpool).

Sample collection
HMPs used were collected randomly from different herbal practitioner selected across the Niger delta states of Rivers, Cross Rivers, Delta and Akwa Ibom all located within southern Nigeria. Eighteen different samples of herbal drug preparations were obtained. Of the 18 herbal preparations sampled, eight (44.4%) were liquid preparations while 10 (55.6%) were solid preparations. The samples were kept at room temperature and used within two weeks of collection.

Isolation and identification of contaminating bacteria
The HMPs each were serially diluted and plated on nutrient agar and sabouroud dextrose agar plates in triplicate and were incubated at room temperature (30°C) for 24 and 96 hours, respectively, to allow for bacterial and fungal growth. The ensuing colonies were further purified, isolated and characterized using standard methods (Cowan *et al.*, 1993; Baron and Finegold, 1990).

Susceptibility studies on the bacteria isolates
The standard method described by Okore (2005) was employed. Briefly, the following antimicrobial disks Augmentin 30µg, Ofloxacin 5µg, Ciprofloxacin 5µg, Tetracycline 30µg (Biological Limited, Liverpool) were placed onto nutrient agar plates seeded with the bacteria strains isolated from the herbal drug samples. The plates were incubated for 48 h and any microorganism that showed resistance (Lalitha, 2004) to any of the antibiotics was isolated for further resistance plasmid isolation studies.

Isolation of plasmids
The isolation of plasmids from each of the bacteria culture was done using previously established protocols (Kraft *et al.* 1998). Briefly, the pure colonies of drug-resistant bacteria isolated from the susceptibility studies above were grown in nutrient broth for 24 h and thereafter harvested by centrifugation (1000 rpm). The supernatant were discarded and the residue containing the bacteria cells were harvested. The bacteria cells were re-suspended in 300µl of TENS solution and 3M sodium acetate to lyse, spun for 2 min to pellet cell debris and chromosomal DNA. Supernatant containing plasmid was transferred onto another sterile test-tube, spurned for 2 min to pellet plasmid DNA, and thereafter supernatant discarded while the pellets (residue) was rinsed twice with 1ml of 70 % ethanol, dried under vacuum and re-suspended in 30µl of Tris EDTA buffer. The plasmid-containing preparation was then stored in a refrigerator prior to their characterization. This was done for all isolated resistant bacteria strains.

Characterization of plasmids using agarose gel electrophoresis
Submerged horizontal agarose electrophoresis was employed to determine the size and fragments of the isolated plasmids according to a previously established method (Jeyaseelan, 1987). Briefly, prepared plasmid mixture was electrophoresed using 1% agarose gel concentration in TBE running buffer (540g Tris, 225g Borate, 41.5g EDTA in water). A Hind III digest of lambda DNA was used as a molecular weight marker, bromophenol was employed as the tracking dye. Electrophoresis was carried out at 60 mA and 220 V for 2 h. The gel was viewed on ultraviolet plate using protective goggles. Photomicrograph of the gel was generated to show the size, lane and profile of the plasmids. The molecular size of each plasmid was determined by comparison with the Hind III digest, and distances travelled.

RESULTS AND DISCUSSION
The profile of the therapeutic indications and organoleptic properties of the HMPs is shown in Table 1. Of the 18 HMPs sampled, eight (44.4%) were liquid preparations while 10 (55.6%) were solid preparations. All herbal preparations were indicated for serious medical problems, and four (22.2%) were meant for use against microbial agents in infectious diseases while the remaining 14 (77.8%) were targeted against non-infectious diseases. Medicinal products designed for the purpose of chemotherapeutic and pharmacological benefits should be effective against the target medical condition. Several factors could compromise this goal, one of them being possible contamination with pathogenic and non-pathogenic microorganisms (Okunlola *et al.*, 2007). Apart from possible microbial degradation of the active constituents contained in the herbal preparations, the presence of these contaminating microorganisms could constitute a source of infection and serious health risk to the consumers of the herbal preparations who were probably already overwhelmed by the serious medical conditions for which the herbal drugs were initially indicated (Mangram *et al.*, 1999; Bowler *et. al.*, 2001).

Table 1: Therapeutic indications of the 18 herbal medicine products (HMP).

Sample	Therapeutic indication
L₁	Anti-*Staphylococcus aureus*
L₂	Anti-typhoid
L₃	Bumps, whitlow, toothache, split heel cure
L₄	Anti-malarial
L₅	Pile and bloody stool cure
L₆	Waist pain, arthritis and rheumatism
L₇	Pile
L₈	Stomach noise/bite
S₁	Itching
S₂	Stomach acne
S₃	Hypertension
S₄	Anti-syphilis
S₅	Premature ejaculation
S₆	Anti-fibroid
S₇	Purgative
S₈	Appendicitis and hernia
S₉	Diabetes
S₁₀	Anti-poison/ Pre-fibroid therapy

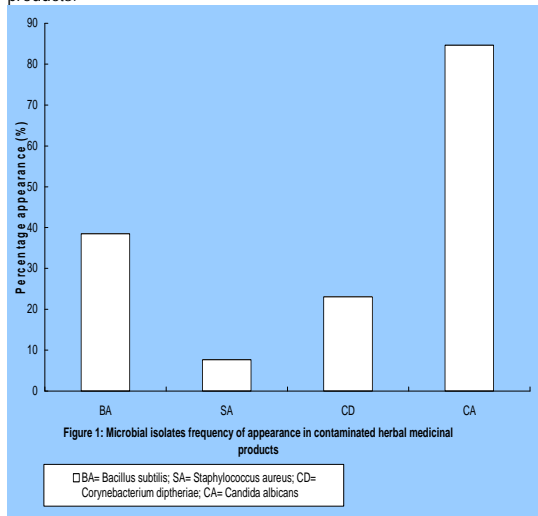L= Liquid herbal preparation; S= Solid herbal preparation

Table 2 shows the isolated and identified microorganisms from the herbal preparations. From the table it can be seen that out of the 18 HMPs 13 (72.2%) showed contamination with either bacteria of fungal microorganisms or both. Of the 13 contaminated HMPs one (7.7%) showed contamination by one bacteria type, six (46.2%) products by *Candida albicans* (yeast/fungi) only, one (7.7%) products by two (mixed) bacteria types alone, four (30.8%) products by a fungus (*C. albicans*) plus simultaneously a bacteria, while for one (7.7%) product alone *C. albicans* plus two bacteria types occurred together. The frequency of microorganism types isolated showed that *Bacillus anthracis* was isolated from five (38.5%) of the contaminated preparations (liquid

preparation), *Staphylococcus aureus* from one (7.7%) preparation (solid preparation), *Corynebacterium diphtheriae* from three (23.1%) preparations (2 liquid, 1 solid), and *Candida albicans* from 11 (84.6%) preparations (6 liquid, 5 solid) (Figure 1). The presence of microorganisms in HMPs clearly portends dangers given the fact that each of the isolated organisms have been known to be implicated in serious infection-induced diseases which could assume high level of morbidity and mortality (Hugo and Russell, 2004). *Bacillus anthracis* is a spore forming gram-positive bacteria, and is the known causative agent of anthrax disease which can be lethal. *Bacillus spp.* were also the most frequently found in these medicaments because they are widely distributed in the soil, dust and air and because they are resistant to many environmental destructive factors (Devleeschouwer and Dony, 1979; Garcia-Arribas et al., 1986; Wayne et al., 2000). A number of reports have described other serious human infections caused by members of the genus Bacillus (Sliman et al., 1987; Kramer and Gilbert, 1989; Mahmood, 2005). *Staphylococcus aureus* on the other hand is the causative agent of a variety of skin and soft tissue infections as well as severe infections of the reproductive and alimentary tract (Lowry, 1998; Klevens et al., 2007). *S. aureus* could have originated from people who handled the medicinal products, as the human skin is its habitat. Infections by toxic *Corynebacterium diphtheria* strains have been previously reported (Rockhill *et al.*, 1982). *C. albicans*, a fungus (yeast) is commonly implicated in oral candidiasis, and is also known to colonize the vaginal leading to vaginal candidiasis. The presence of the identified microorganisms in these herbal medicines can potentially be a source for their transmission.

Table 2: Isolation and identification of microbes present in the herbal samples

Sample	Bacteria isolated	Counts (cfu/ml)(x10⁴)	Fungi isolated	Counts (cfu/ml)(x10⁴)
L₁	No bacteria growth	-	*Candida albicans*	1
L₂	*Bacillus anthracis*	10	*Candida albicans*	1
L₃	No bacteria growth	-	No fungal growth	-
L₄	*Corynebacterium diphtheriae, Bacillus anthracis*	20	*Candida albicans*	1
L₅	No bacteria growth	-	*Candida albicans*	10
L₆	*Bacillus anthracis*	10	No fungal growth	10
L₇	*Bacillus anthracis*	10	*Candida albicans*	-
L₈	*Corynebacterium diphtheriae*	10	*Candida albicans*	1
S₁	No bacteria growth	-	*Candida albicans*	20
S₂	No bacteria growth	-	*Candida albicans*	1
S₃	No bacteria growth	-	*Candida albicans*	1
S₄	No bacteria growth	-	No fungal growth	-
S₅	No bacteria growth	-	No fungal growth	-
S₆	*Bacillus anthracis*	20	*Candida albicans*	1
S₇	No bacteria growth	-	*Candida albicans*	10
S₈	No bacteria growth	-	No fungal growth	-
S₉	No bacteria growth	-	No fungal growth	-
S₁₀	*Corynebacterium diphtheriae, Staphylococcus aureus*	20	No fungal growth	-

Figure 1: Microbial isolates in contaminated herbal medical products.

Figure 1: Microbial isolates frequency of appearance in contaminated herbal medicinal products

□BA= Bacillus subtilis; SA= Staphylococcus aureus; CD= Corynebacterium diptheriae; CA= Candida albicans

The antimicrobial susceptibility-resistance pattern of the contaminating microorganisms is recorded in Table 3. From the table it can be seen that three out of the five *B. anthracis* strains (L_2, L_6, L_7) demonstrated zero resistance coverage being sensitive to all of the antimicrobial agents (Ofloxacin, ciprofloxacin, pefloxacin, augmentin, ampiclox, erythromycin, cephalexin, clindamycin, ceftriaxone, gentamicin) employed in the study. The remaining other two isolates L_{4b} (33.3%) and S_6 (16.7%) were resistant to ampiclox, augmentin, co-trimoxazole, and clindamycin while being sensitive to the others. *S. aureus* displayed more than 50% resistance and the antibiotics covered were Cephalexin, cotrimoxazole, clindamycin, ampiclox, pefloxacin, augmentin. This trend of high resistance

coverage recorded by the *S. aureus* isolate is worrisome but not entirely surprising given the fact that other related studies involving clinical strains of *S. aureus* have equally reported multi-antibiotic resistance (Richard, 2007; Ugwu *et al.*, 2009). It is also worthy to note that the relatively wide ranged resistances displayed by some of the bacteria strains especially the *S. aureus* against penicillins (ampiclox, augmentin) and lower generation cephalosporin cephalexin may be due to the activity of beta-lactamase enzymes possibly produced by the *S. aureus* isolate. The resistance pattern recorded by the *Corynebacterium diphtheriae* isolates appears inconsistent. The various isolates (L_{4a}, L_8, S_{10a}) recorded 40%, 0%, and 50% respectively. The isolate L_8 which is usually found to be a fully sensitive strain may serve to reflect the possible dynamics of resistance (R-factor) gene transfer between species among microbial populations. It may be plausible to suggest that while L_8 is yet to acquire resistance, L_{4a}, and S_{10a} may have acquired their resistances through R-factor transfer from other bacteria species present within the herbal medicinal product. The likely bacteria cause may be the isolated *S. aureus* strain which has also displayed similar resistance pattern as the *C. diphtheriae* isolate S_{10a}. Moreover, intergeneric transfer of resistance among different genera with *Staphylococci spp* playing a prominent donor role has been reported (Schaberg and Zerros, 1986). Although *C. albicans* was isolated from eleven out of the thirteen contaminated herbal products no susceptibility-resistance test was carried out against it because our present evaluation is focused on the bacteria pathogens which are most often involved in the frequently observed multiple-drug resistance behavioural pattern. However, it is instructive to note that the presence of *C. albicans* in the screened herbal products portends potential dangers to the users of these products especially in paediatric and immune-compromised patients.

Table 3: Resistance-susceptibility pattern of the microorganisms isolated from the herbal preparations

Microorganism isolated	Sensitive (+) to	Resistant (-) to
Bacillus anthracis (L_2)	OFL, CIP, PEF, AUG, AMP, ERY, CEP, CLI, CEF, GEN,	None
Corynebacterium diphtheriae (L_{4a})	PEF, CEP, OFL, CEF, GEN, ERY	AUG, AMP, CLI,
Bacillus anthracis (L_{4b})	PEF, CEP, OFL, CEF, GEN, ERY, CLI, CIP	AMP, AUG, COT,
Bacillus anthracis (L_6)	OFL, CIP, PEF, AUG, AMP, ERY, CEP, CLI, CEF, GEN, COT	None
Bacillus anthracis (L_7)	OFL, CIP, PEF, AUG, AMP, ERY, CEP, CLI, CEF, GEN	None
Corynebacterium diphtheriae (L_8)	OFL, CIP, PEF, AUG, AMP, ERY, CEP, CLI, CEF, GEN	None
Bacillus anthracis (S_6)	OFL, CIP, PEF, AUG, ERY, CEP, CEF, GEN, COT	CLI, AMP
Corynebacterium diphtheriae (S_{10a})	OFL, CIP, PEF, ERY, CEF, GEN,	CLI, AUG, AMP, CEP, COT,
Staphylococcus aureus (S_{10b})	OFL, CIP, PEF, ERY, CEF, COT,	AUG, AMP, CEP, GEN, COT, CLI

OFL= Ofloxacin, CIP= Ciprofloxacin, PEF= Pefloxacin, AUG= Augmentin, AMP= Ampiclox, ERY= Erythromycin, CEP= Cephalexin, CLI= Clindamycin, CEF= Ceftriaxone, GEN= Gentamicin, COT= Co-trimoxazole, AMO= Amoxycillin.

Table 4: Profile of plasmids isolated from drug-resistant bacterial strains

Bacteria isolate	No. plasmids isolated	Plasmid size (kbp)
Corynebacterium diphtheriae (L_{4a})	1	23.05
Bacillus anthracis (L_{4b})	1	2.32
Corynebacterium diphtheriae (S_{10a})	1	2.03
Staphylococcus aureus (S_{10b})	1	2.21
Bacillus anthracis (S_6)	1	6.42

Submerged horizontal agarose electrophoresis was employed for the molecular characterization of the R-plasmids. Using this, the sizes of the DNA fragments and plasmids were separated and characterized on the basis of their comparative molecular weights and speed of travel through the electrophoretic agarose system (Jeyaseelam, 1987). These results are presented in Table 4. The presence of some plasmid DNA in the isolates corresponding to the reference standard DNA fragments suggests that their antimicrobial resistance is possibly plasmid-mediated. In that case they may possibly be

referred to as Resistance plasmids (R-factor). One plasmid each was isolated from each of five resistant bacteria strains L_{4a} (*Corynebacterium diphtheriae*), L_{4b} (*Bacillus anthracis*), L_{10a} (*Corynebacterium diphtheriae*), L_{10b} (*Staphylococcus aureus*), S_6 (*Bacillus anthracis*). From the table the largest plasmid size (23.05 kbp) was recorded for the *C. diphtheriae* strain L_{4a} while the *C. diphtheriae* strain L_{10a} gave the smallest plasmid size (2.03 kbp). The isolated plasmids may only inconclusively be responsible for possibly mediating some or all of the expressed resistances of the microorganisms. Moreover, it should be expected that the amount of the plasmid genetic information coding for the expression of specific antibiotic resistances which again can be correlated to the extent of multiple antibiotic resistance coverage, would be reflected in the size of the bacteria plasmids. Interestingly, there appears to be little correlation between these two parameters (Table 3 and 4) thus raising the likelihood that chromosomal determinants other than the extrachromosomal plasmids, may be involved in the observed resistance profiles of the resistant microorganisms (Sheikh *et al.*, 2003; Esimone *et al.*, 2007). Further studies including resistance gene curing and actual sequencing of the isolated plasmid genomes would be required to establish this link. In bacteria, antimicrobial resistance is facilitated through their ability to quickly adapt to new environments, and along with their ability to replicate very quickly comes the aptitude to mutate their DNA acquired from other drug-resistant bacteria (NIAID, 2000). Therefore, the acquisition of resistance may be due to chromosomal mutations or through plasmids that are often capable of transfer from one strain of organism to another, even across the species barrier. Plasmids transfer within and across species is further enhanced through the activities of transposons which are mobilizable genetic elements conferring resistance determinants (O'Brien and Acar, 1987). The ability of transposons to integrate into either conjugative plasmids or the organisms' chromosome enhances the transferability of a given resistant determinant. This process is a natural phenomenon exacerbated by the abuse, overuse and misuse of antimicrobials in the treatment of human illness and in animal husbandry, aquaculture and agriculture (O'Brien and Acar, 1987; Stohr, 2000; Lexchin, 2000).

The importance of surveying resistant environmental strains is that under favourable situations, they may transfer their resistance plasmids to pathogens (O'Brien and Acar, 1987; Burns *et al.*, 1989; Ugwu *et al.*, 2009). If such organisms are present in medicaments, such as herbal medicinal products they could behave as opportunist pathogens and initiate an infection, particularly in immuno-compromised patients as well as lead to transfer of antibiotic resistance traits to hitherto sensitive microorganisms co-habiting within the consumers of those products. Given the increasing rate of development of resistant bacteria strains, our challenge is to slow the rate at which resistance develops and spreads. In order to decrease the spread of resistance among antibiotics, physicians, pharmacists, researchers and consumers alike need to be more aware of the selective pressures driving these bacteria to decrease their susceptibility (Gershman, 1997). These selective pressures include the abuse, overuse and misuse of antimicrobials in therapy, improperly manufactured and mishandled HMPs

(Lexchin, 2000; Esimone *et al.*, 2007) as well as other numerous socioeconomic factors that govern the development of multi-drug resistant bacteria strains (Toebe, 2001). In such circumstances, a collective and concerted effort towards the prevention of development of resistant bacteria strains through rational antimicrobial use policy, right practices and intensive research leading to novel and alternative drugs therapies would help put under check the emergence of multiple-drug-resistant bacteria strains.

Conclusion
The results of the present work show that 13 out of 18 herbal medical products evaluated were contaminated with one or more microbial organisms' type, and that five of the products harboured resistant bacteria strains. These products were sold and utilized in Niger Delta of Nigeria for the treatment of various medical conditions. The high rate of resistance to antimicrobial agents of strains isolated from these herbal preparations may indicate a widespread antibiotic resistance among microorganisms from different sources. It is important therefore that quality assurance be built into the whole process of HMPs manufacturing, beginning from the selection of propagation material to the final product reaching the consumer. Thus, there is need for constant monitoring and control of the microbial standards of herbal medicines available on the market.

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