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Full Length Research Paper

OXA-48 type carbapenemase in *Klebsiella pneumoniae* producing extended spectrum β-lactamases (ESBL) in Senegal

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Enterobactericeae producing extended spectrum β-lactamases (ESBL) are likely to express carbapenemases OXA-48 which have hydrolytic activity on carbapenems. The aim of this work was to evaluate prevalence of blaOXA-48 for *Klebsiella pneumoniae* isolates producing ESBL. This work was conducted at the French Reference Center for Antibiotic Resistance with strains from Senegalese Hospital. Standard antibiogram was performed in accordance to CA-SFM/EUCAST 2016 and presence of ESBL was confirmed by synergistic image. Polymerase chain reaction (PCR) was performed to detect systematically blaOXA-1, blaTEM-1, blaCTX-M-1, blaCTX-M-9 and blaOXA-48 gene in case of decrease sensitivity to carbapenem. PCR products were extracted, purified, sequenced and whole-genome sequence (WGS) were used for the analyses. Plasmids extraction was performed by Kado and Liu method. Five isolates harbored a decreased susceptibility to carbapenems. They were positive for blaOXA-48 gene and also expressed blaCTX-M-15. Analysis of the five plasmids by WGS identified a single IncL/M type plasmid of 63 kb and other genes for aminoglycosides and quinolones resistance. Carbapenemase-producing Enterobacteriaceae represent new threat to public health. Decrease in carbapenem susceptibility should be an alert for rapid detection of carbapenemases and to prevent their spread. Phenotypic or molecular methods should be available in many laboratories to take appropriate preventive and therapeutic measures.

Key words: *Klebsiella pneumoniae*, extended spectrum β-lactamases (ESBL), carbapenemase, bla_{OXA-48}, Senegal.

INTRODUCTION

Carbapenems, antibiotics of β-lactam family, are active on most Gram negative bacilli, including Enterobacteriaceae and non-fermenting bacteria such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (Nordmann et al., 2010). Exclusively used in hospitals, they are reserved for treatment of multi-resistant bacterial infections. However, these last two decades was marked by emergence of bacteria producing carbapenemases

able to hydrolyze them. Carbapenemases were increasingly described to constitute threat and universal public health problem. These enzymes have been described in many African countries; however, their prevalence is poorly defined and has not been systematically studied (Manenzhe et al., 2015). Carbapenemases belong to three classes according to Ambler classification (Walther-Rasmussen et al., 2006). Class A and D are referred to as serine because they have serine at their active sites, whereas class B have zinc (zinc dependent) at their active site and are referred to as metallo-β-lactamases. Class A enzymes group together *Klebsiella pneumoniae* carbapenemase (KPC), imipenem (IMI) and Guyana extended-spectrum (GES) and hydrolyze all β-lactams. Class B metallo-β-lactamases are plasmid mediated, or in some cases chromosomal, and the most common enzymes among clinical isolates in this group include Verona imipenemase (VIM), imipenemase (IMP) and New Dehli Metallo-β-lactamase (NDM). These enzymes are able to hydrolyse all β-lactams except for aztreonam, monobactam and their hydrolytic activity is reduced or inhibited *in vitro* by ethylene diamine tetra-acetic acid (EDTA) but not by clavulanic acid. Finally, class D or oxacillinase (OXA-48, OXA-163 and OXA-181), attacks poorly or non third generation cephalosporins and are not inhibited by clavulanic acid or EDTA (Queenan et al., 2007). OXA-48 enzyme was first described in Turkey in 2004 and then rapidly disseminated in several European countries (Carrër et al., 2010). This later was also described in North Africa: Tunisia (Cuzon et al., 2010), Algeria (Agabou et al., 2014), Morocco (Benouda et al., 2010); in West Africa: Senegal (Moquet et al., 2011), Côte d'Ivoire (Jeannot et al., 2013); and in East Africa: Kenya (Pitout et al., 2008).

In Senegal, the first description of OXA-48 was made in 2011, but probably dates back further since imipenem introduction in 2008. Bla_{OXA-48} gene is often associated with presence of ESBL, thus conferred multi-resistance of bacteria. Simultaneous presence of several mechanisms of resistance in a same bacterium associated with absence of new classes of antibiotics makes complex therapeutic monitoring of patients.

Phenotypic or molecular detection of carbapenemases thus becomes indispensable to reduce their spread. Several methods are available, such as phenotype techniques (modified Hodge test), hydrolytic method (carbaNP), mass spectrometry or molecular methods (Burckhardt et al., 2011; Hrabak et al., 2011; Poirel et al., 2015). This research is not easy in countries with limited resource and can better explain underestimated prevalence.

MATERIALS AND METHODS

Bacterial isolates were collected from two senegaleses hospitals and included forty-nine *Klebsiella pneumoniae* from urinary samples. These isolates were transported according to recommendations of biological products for characterization in the National Reference Center (CNR) for resistance of enterobacteria in Clermont-Ferrand, France. After reculture on Trypticase-Soja-Agar (TSA), bacterial identification was confirmed by mass spectrometry VITEK® MS (Biomérieux, La Balme, France). Antibiogram was performed by diffusion method according to the recommendations of CA-SFM-EUCAST 2016 (CASFM, 2016). Presence of ESBL was confirmed by the synergistic image between combination with third generation of cephalosporins (C3G) and amoxicillin-clavulanic acid. The DNA of each isolate was extracted from colonies by thermal shock and then used as a template to search the following genes: bla_{CTX-M1} (CTT CCA GAA TAA GGA ATC; CCG TTT CCG CTA TTA CAA), bla_{CTX-M9} (CTG ATG TAA CAC GGA TTG AC; TTA CAG CCC TTC GGC GAT), bla_{OXA-1} (ATATTATCTACAGCAGCG; TTGGCTTTTATGCTTGATG) bla_{TEM-1} (TAA AAT TCT TGA AGA CG; TCT GAC AGT TAC CAA TGC). For five cases with decrease susceptibility to carbapenems (ertapenem, imipenem), PCR of bla_{OXA}-48 (GGG GAC GTT ATG CGT GTA TT; OXA-48B (GAG CAC TTC TTT TGT GAT GGC) and others genes (*rep*A, *tra*U, *par*A) were performed as previously described (Beyrouthy et al., 2014). PCR products were extracted, purified (UltraClean® Microbial DNA Isolation kit, Quiagen) and sequenced by Sanger method. Transferability of the bla_{OXA-48} gene was studied by a mating-out assay using *Escherichia coli* rifampicin resistant as recipient bacteria. Selection was performed on agar plates supplemented with ticarcillin (32 mg/L) and rifampicin (300 mg/L). The plasmid content of the bacteria and the size of plasmids were determined using plasmid DNA extracted by Kado and Liu method.

Whole genome sequencing (WGS) and genome assembly: Bacterial DNA was extracted from overnight cultures with DNeasyUltraClean Microbial Kit (Qiagen, Hilden, Germany) as recommended by the manufacturer. The whole-genome sequences of isolates were determined by de novo assembly of 2x150-bp paired-end reads generated using Illumina sequencing technology (San Diego, CA, USA). The assemblies were performed using SPAdes (Nurk et al., 2013), the reads were mapped with the Burrows-Wheeler aligner (BWA) (Li et al., 2009), the final polishing of the assembly used Pilon (Walker et al., 2014) and the genome was annotated with RAST (Aziz et al., 2008).

Whole-genome sequence analysis

Isolates were typed in silico using multi-locus sequence typing (MLST) with the *K. pneumoniae* scheme (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html). The antibiotic resistance genes were characterized using the Comprehensive Antibiotic Resistance Database (CARD) and Resfinder (Jia et al., 2017; Zankari et al., 2012). Replicon typing was performed with PlasmidFinder (http://www.genomicepidemiology.org/) using the Enterobacteriaceae database with the detection thresholds set to 95% sequence identity.

RESULTS

Forty-nine isolates of *K. pneumonia* producing ESBL

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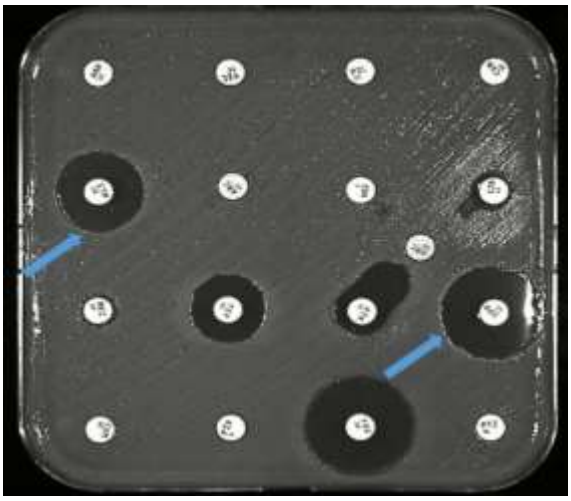

Figure 1. *K. pneumoniae* of reduced sensitivity to imipenem and ertapenem.

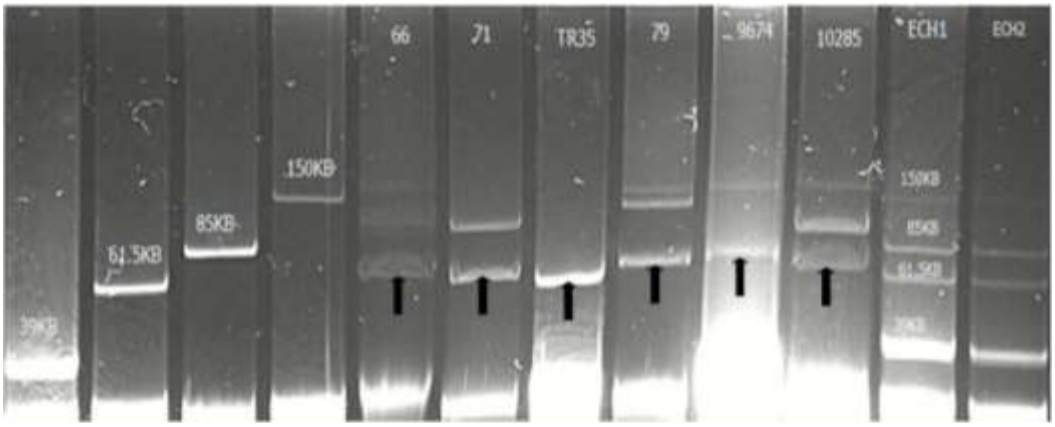

Figure 2. Extraction of plasmids by Kado and Liu.

were tested to research the presence of *bla*$_{CTX-M-1}$, bla$_{CTX-M-9}$, *bla*$_{TEM-1}$ and *bla*$_{OXA-1}$ genes. Forty-six of the isolates (93%) carried a *bla*$_{CTX-M1}$ type gene. The *bla*$_{TEM-1}$ and *bla*$_{OXA-1}$ genes were present in n = 40/49 and n = 38/49 isolates respectively. Five isolates showed a decreased sensitivity to imipenem and ertapenem (Figure 1).

Polymerase chain reaction (PCR) multiplex of carbapenemase was positive for the blaoxa-48 gene in all five isolates, and this was confirmed by the simplex PCR blaoxa-48. The successful plasmid extraction showed two types, one with a PM between 62 and 70 Kb probably associated with the blaOXA-48 gene as shown in the Figure 2.

Transconjugants were obtained from all the clinical OXA-48-encoding strains by a mating-out assay. All of them expressed a β-lactam resistance phenotype compatible with OXA-48 production, and the presence of the *bla*$_{OXA-48}$ gene was confirmed by PCR. Plasmids from clinical isolates and their transconjugants were extracted and hybridized with a probe specific for the *bla*$_{OXA-48}$ gene. The results showed that the *bla*$_{OXA-48}$ gene was located in all isolates on ~ 63 kb conjugative plasmids. Further analysis of these plasmids from the transconjugants revealed similar restriction profiles (data not shown). These results suggested that the plasmids in isolates from Senegal shared the IncL/M pOXA-48a-like backbone. A similar 63,434 bp-long IncL/M plasmid, carried bla$_{OXA-48}$ was identified by WGS in all isolates. The *bla*$_{OXA-48}$ encoding plasmid from Senegal covered 99.98% of the reference plasmid pOXA-48a (JN626286.1). Difference between Senegalese plasmids and pOXA-48a were the presence of the bla$_{OXA-48}$ gene embedded within a Tn1999.2-type composite transposon, which differs from Tn1999 by the insertion of the IS1R insertion sequence in the IS1999 insertion sequence located upstream of blaOXA-48, and the truncation of the

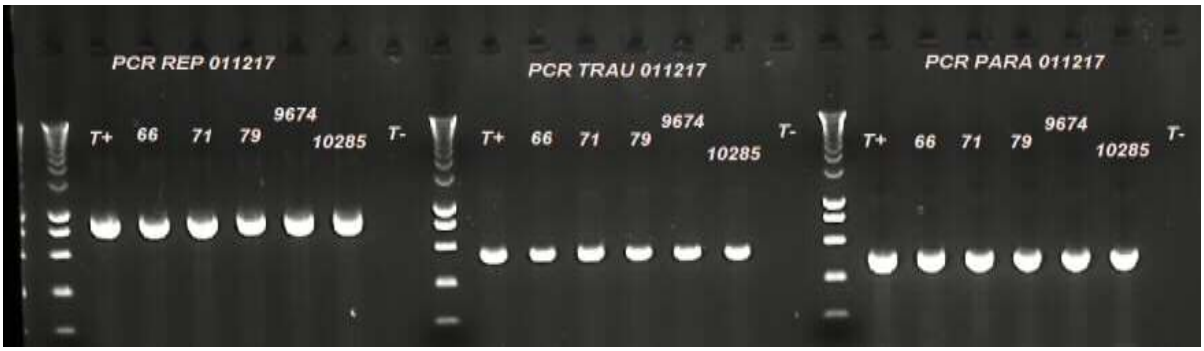

Figure 3. PCR RepA, TraU and parA for isolates expressing blaOXA-48.

Table 1. Genetypic characteritics of bla_{OXA-48} producing *Klebsiella pneumoniae* isolates.

Parameter	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5
MLST genotyping	15	377	193	11	307
Aminosides genes	-aac(3)-IIa -fusA1	-aac(3)-IIa -fusA1 -aadA1	-aac(3)-IIa -fusA1 -strA -strB	-aadA16 -fusA1 -strA -strB	-aac(3)-IIa -fusA1 -strA -strB
β-lactamases	-CTX-M-15 -OXA-1 -OXA-48 -SHV-28	-CTX-M-15 -OXA-48 -SHV-110 -TEM-1	-CTX-M-15 -OXA-1 -OXA-48 -SHV-61 -TEM-1	-CTX-M-15 -OXA-1 -OXA-48 -SHV-11 -TEM-1	-CTX-M-15 -OXA-48 -SHV-28 -TEM-1
Quinolones genes	-QnrB1 -gyrA -parC	-gyrA, -parC	-QnrB1	-QnrB1 -gyrA -parC	-gyrA -parC
Replicon type	*-IncFII* *-IncFIB(K)* *-IncR* *-IncL/M*	*-IncHI1B* *-IncL/M*	*-IncFII(K)* *-IncFIB(K)* *-IncL/M*	*-IncFII* *-IncFIB(K)* *-IncR* *-IncL/M*	*-IncFII* *-IncFIB(K)* *-IncR* *-IncL/M*

hypothetical gene orf25 with an additional copy of the IS1R. Other genes were detected as repA, traU and parA involved in the replication, transport and partition of plasmid pOXA-48a, which further confirms its presence (Figure 3). All of these five isolates expressed simultaneously the $bla_{CTX-M-15}$ gene but also other β-lactamases and markers of resistance to aminoglycosides and quinolones (Table 1).

DISCUSSION

A prevalence of 10% for the bla_{OXA-48} gene was found in *K. pneumoniae* isolates. All of them hydrolyzed third generation of cephalosporins and aztreonam, and simultaneously expressed $bla_{CTX-M-15}$. The carbapenemase OXA-48 confer resistance to β-lactamase inhibitors and are known to hydrolyze carbapenems and third generation cephalosporins more weakly (Bonnin et al., 2013; Tzouvelekis et al., 2014). However, in some Enterobacteriaceae (*K. Pneumoniae* and *E. coli*), the association of OXA-48 with ESBL (CTX-M or SHV) or lack of membrane permeability increase level resistance to carbapenems (Bonnet et al., 2011; Cuzon et al., 2011). OXA-48 is the main carbapenemase produced by Enterobacteriaceae and most widespread enzyme that has emerged in all countries around Mediterranean and in Africa (Carrër et al., 2008; Nordmann, 2014). It is mainly found in *K. pneumoniae and E. coli*, although other species of Enterobacteriaceae

can produce this enzyme. OXA-48 has distinction of spreading more easily between species of this family compared to other enzymes such as KPC, NDM (Nordmann et al., 2014). In France, among Enterobacteriaceae producing carbapenemases, *K. pneumoniae* remained majority with 59% and OXA-48 enzyme was observed up to 52% (Poirel et al., 2004). Several risk factors are associated with the acquisition of carbapenemase producing bacteria in healthcare settings, such as recent antibiotic therapy, prolonged hospital stay, use of invasive devices and immunosuppression (Jeon et al., 2008).

This study was unable to establish a link between infection with these multidrug resistant bacteria and level of mortality. However, in literature it has been shown that mortality is high with infections caused by bacteria expressing bla_{OXA-48} gene (Doi et al., 2015). All these five isolates also expressed ESBL CTX-M-15; this combination OXA-48/CTX-M-15 leads to a higher level of carbapenem resistance. This association has often been found in other studies (Carrër et al., 2010; Cuzon et al., 2011). In Tunisia, isolates of *K. pneumoniae* that simultaneously expressed carbapenemaseVIM-4, CTX-M-15 and CMY-4 were observed (Ktari et al., 2006). The bla_{TEM-1} and bla_{OXA-1} genes were expressed in four and three strains, respectively. The same association of bla_{OXA-48}, bla_{TEM} and bla_{OXA-1} has been already described in Senegal by Moquet et al. (2011) with nosocomial strains of *K. pneumoniae*. In the same study, a community strain of *K. pneumoniae* expressed only bla_{OXA-48}.

All these OXA-48 isolates expressed other determinants of aminoglycoside and / or quinolone resistance. Coexistence between CTX-M and aminoglycoside and / or quinolone resistance genes has been described in Africa (Alibi et al., 2015; Breurec et al., 2013). In Senegal, Moquet et al. (2011) observed a co-presence of CTX-M15 / AAC'6 at 78.8% and CTX-M15 / QNR at 65%. These different mechanisms of resistance in the same bacteria have a direct consequence on the management which becomes more complex with a reduction of the therapeutic arsenal. The bla_{OXA-48} gene is usually carried by a 62 kb plasmid; analysis of that plasmids by WGS sequencing shows a single plasmid belonged to the incompatibility group IncL of 63 kb. IncL type plasmids are currently detected worldwide in Enterobacteriaceae isolates of different origin and are considered to be epidemic resistance plasmids and contribute to the diffusion of the carbapenemase-encoding genes bla_{NDM-1} and bla_{OXA-48} (Beyrouthy et al., 2014; Bonnin et al., 2013). The presence of the *repA*, *traU* and *parA* genes further confirmed the plasmid support of the bla_{OXA}-48 gene found in these isolates. Therapeutic management of carbapenem-producing enterobacteria makes use of other antibiotics such as aminoglycosides, tigecycline, colistin, fosfomycin or quinolones (Nordmann et al., 2011). Although efficacy of colistin and tigecycline are not sure, activity can be increasing when they are combined (Tzouvelekis et al., 2014). Mortality rate from infections with Enterobacteriaceae producing carbapenemase shows that current therapeutic approaches are insufficient and need to be revised. Nevertheless, mortality may be reduced if patients are treated with a combination containing carbapenem (Tzouvelekis et al., 2014). Mechanisms of resistance are usually combined in the same strain and often only colistin or tigecycline sometimes remain effective (Chassagne, 2012). However, combination of these two antibiotics can reduce mortality (Carmeli et al., 2010). Detection of carbapenemase then remains a necessity in case of decreased carbapenem sensitivity in enterobacteria producing ESBL. However, this task seems difficult in our countries where the majority of laboratories have a limited number of phenotypic methods or molecular techniques.

Conclusion

Carbapenemase OXA-48 expressed by enterobacteria producing ESBL represents a new threat to public health. Surveillance of antimicrobial resistance remains a necessity in both hospital and community. This one must integrate the phenotypic detection and the molecular characterization of the genes involved. This will reduce the spread of the bacteria that harbor them and take appropriate preventive and therapeutic measures.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Bactericidal and brine shrimps toxicity of essential oils from *Aframomum Melegueta* [K. Schum]

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Aframomum melegueta **(Roscoe) K. Schum, family of Zingiberaceae, is a tropical tree with spicy edible fruit. This plant has both medicinal and nutritive values. There is paucity of literature on the toxicity and bioactivity of the essential oils from this plant from Nigeria. Essential oils were extracted from the leaves, stems, roots (rhizomes) and seeds of the plant through hydro-distillation using the Clevenger-type apparatus. The toxicity of the volatile oils was determined using the brine shrimp toxicity assay at concentrations of 10.0, 100.0 and 1000.0 ppm and the median lethal concentration (LC_{50}) was calculated using Finney's probit analysis. The antimicrobial assay was carried out using the cup plate agar diffusion method. Five bacteria consisting of three strains of Gram-positive bacteria (*Staphylococcus aureus, Bacillus subtilis* and *Salmonella typhi*) and Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) were screened. The stems' essential oil displayed the highest toxicity (LC_{50} = 0.2 µg/mL) followed by the seeds' essential oil (LC_{50} = 0.3 µg/mL), while LC_{50} of the essential oil from the leaves offered the least toxicity (LC_{50} = 17.5 µg/mL) in brine shrimp toxicity assay. All essential oils showed moderate inhibition of the tested bacteria at 1mg/mL but showed no inhibition below 125 µg/mL concentration. The high brine shrimps' toxicity and bactericidal activity of the essential oils is indicative of their potentials as sources of pharmaceuticals or natural pesticides.**

Key words: Essential oils, brine shrimps toxicity, bactericidal activity, *A. melegueta*

INTRODUCTION

Essential oils are complex mixtures of natural compounds or secondary metabolites which may contain 20 to 60 components at different concentrations (Ekundayo, 1986; Bakkali et al., 2008; Miguel, 2010; Rubiolo et al., 2010). They often composed of large groups of organic compounds with diverse functionality. However, common constituents are hydrocarbons (terpenes and sesquiterpenes) and oxygenated compounds (Alcohols, aldehydes lactones, acids, phenols, oxides, lactones, ethers, and esters) (Poumortazavi and Hajimirsadeghi, 2007).

It is believed that essential oils take part in plants' chemical defence systems. This suggests that they appeared as plant's reaction to attacks (Bassole and Juliani, 2012). In view of the fact that plants are stationary and could not run from attacks, they have developed alternative methods to fight for their existence. Some produced thorns, hairs, or thick corticle, while others

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produce chemicals that render them toxic, unpalatable or nauseating (Dosumu, 2008). Precisely, essential oils help to protect plants from bacterial, fungal, and other microbial infections. Leaf volatiles serve to dissuade herbivory by marauding insects and animals, while floral volatiles attract pollinators; including animals which are essential for pollination and seed dispersal, and repel others which are harmful. The same compounds that have served plants very well in their chemical warfare also are useful to man in many applications. Thus, essential oils have been used as perfumes, flavours and in medicinal preparations for centuries. Currently, over 1,000 different chemical components have been identified in essential oils. The synergistic effect of all the components make up the peculiar fragrance observed in any essential oil (Bauer et al., 2001). In fact, the absence of one component may alter the odour of oil completely. Such components are the character-impact compounds (George-Nasamanto and Cori, 1971).

Major components of essential oils are found to reflect on the biophysical and biological features. The degree of their effects is dependent on their concentration when tested singly, or comprised in the essential oils used wholly. It is therefore unclear whether the potency of essential oils depends on the synergistic functions of its various components as commonly believed (Ipek et al., 2005). However, there is a strong argument that, the activity of the main components is modulated by other minor constituents (Franzios et al., 1997; Santana- Rios et al., 2001; Hoet et al., 2006). This is expected because all components of the essential oils play a role in defining the density, texture, colour and cellular distribution (Cal, 2006). Thus, for biological purposes, it is more informative to study the entire oil rather than some of its components because the concept of synergism seems more meaningful (Bakkali et al., 2008).

Essential oils are used in cosmetic industry, in sanitary products, dentistry, in agriculture as food preservers and additives, and as natural medicines (Burt, 2004). Moreover, essential oils are used for massages in mixtures with vegetal oil or in baths but most frequently in aromatherapy (Bauer and Garbe, 1985). In all, essential oils play an important role in the protection of plants, and useful to man as fragrance, food and medicine. Most of the people living in rural African settlements rely on medicinal plant preparations use in treatment of any kind of diseases. The preparations are either made by natives themselves or obtained from traditional medicine practitioners. In Nigeria, the alternative therapy, that is, the use of natural herbs for treatment of diseases is now becoming well accepted even among the urban population and the elites as well. In advanced societies, the relatively recent enhancement in the preference for natural products has led to a renewal of scientific interest in essential oils (Nychas, 1995).

The composition of essential oils from a particular species of plant may differ due to geographical sources (Arras and Grella, 1992; Cosentino et al., 1999; Jerkovic et al., 2001; Kokkini et al., 1997). Therefore, there is need for continued research into local plant species from different climes. However, many essential oil-bearing plants in Nigeria have not been investigated for Brine shrimps' toxicity and pharmacological activity. As a result, the bactericidal activity and brine shrimps' toxicity of the essential oils from the leaves, stems, rhizomes and seeds of *Aframomum melegueta* K. Schum grown in Nigeria was investigated.

MATERIALS AND METHODS

General experimental procedures

All chemicals used in this research were of 'analar' grade, obtained from Sigma-Aldrich sales agents in Nigeria. Materials and apparatus used were: an all-glass Clevenger distiller, a 2000 ml heating mantle, 2000 ml round bottom flasks, Pasteur pipette, dimethylsulphoxide (DMSO) and brine shrimps (*Artemia salina*) eggs. The GC-MS spectra were obtained on Agilent 6890N GC coupled with MS-5973-634071 series running on Agilent-Chemstation retention time locking software (Agilent Technologists, USA) as reported earlier (Owokotomo et al., 2014).

Collection of plant materials

A. melegueta plants were harvested at the farm in Akure, Nigeria. Identification and authenticated was achieved at the Herbarium of the Forest Research Institute of Nigeria, Ibadan. Voucher number was *FHI109020.*

Extraction of essential oils and analyses

The leaves, stems and roots of *A. melegueta* were carefully separated and washed. They were then subjected to hydro-distillation separately for 3 to 5 h using a Clevenger-type apparatus. The oil samples were collected into sample vials and kept in the refrigerator before GC-MS analysis.

Essential oils were analyzed by Agilent (USA) 6890N GC coupled with MS-5973-634071 Series. The capillary column used was DB-1 (fused-silica) [30.0m (length) X320.00µm (diameter) X1.00µm (film thickness). Helium was used as the carrier gas at constant flow rate of 1.0 ml/min and average velocity of 37 cm/s; the pressure was 0.78 psi. The initial column temperature was set at 100°C (5 min) then increased to 250°C at the rate of 5°C/min. The injector was the split type (50:1) and volume injected was 1.0 µL. The chromatograms integrated using Chem-Station software and the compositions of the essential oils were ascertained by comparing the GC-MS data with (NIST 02) library spectra and data from literature (Robelo et al., 2003).

Brine shrimps toxicity assay of the extracted essential oils

The brine shrimps toxicity of the essential oils was determined using assay described by Krishnaraju et al. (2005) with minor modifications.

Hatching of *Artemia salina* (Brine shrimps)

A plastic container (aquarium) with two compartments was used as the hatching vessel. Holes were made into divider to allow water

Table 1. Major Bioactive Constituents of *A. melegueta.*

Plant part extract	Predominant bioactive constituent	Percentage occurrence	Terpenoid subclass
Leaf	Myrtenyl acetate	29.06	Monoterpene ester
	Limonene	19.45	Monoterpene olefins
	γ-elemene	8.84	Sesquiterpene
Stem	Caryophyllene oxide	19.70	Sesquiterpene
	Myrtenyl acetate	14.70	Monoterpene ester
	β-eudesmene	10.83	Monoterpene
Seed	α-caryophyllene	48.78	Sesquiterpene
	β-caryophyllene	32.50	Sesquiterpene
Rhizome	Myrtenyl acetate	22.70	Monoterpene ester
	Pinocarvyl acetate	11.50	Monoterpene ester
	Cyperene	8.96	Sesquiterpene
	Caryophyllene	5.97	Sesquiterpene

circulation between the compartments. The container was then flooded with fresh sea water. Two spatulas of brine shrimps were added to one side and covered with a booklet in order to produce a dark environment for proper hatching. Other side of the aquarium was left exposed to light. The aquarium was allowed to stay undisturbed for two days, when the hatched brine shrimps swam across the divider holes to the side is exposed to light.

Sample preparations and stationing of the brine shrimps

Essential oil solutions were prepared by dissolving 20.0 mg of the essential oils separately in 0.3 ml of dimethylsulphoxide (DMSO) and 1.7 ml of sea water (1000.0 ppm). Additional concentrations of 100.0 ppm and 10.0 ppm were prepared through serial dilution.

Sea water (3.0 ml) was transferred into the specimens' vials in triplicates. Then, 0.5 ml of each prepared concentration was introduced into the vials and ten brine shrimps were put into each specimen vial and control vial. All vials were topped with sea water up to 5.0 ml and left open for 24 h.

Statistical analysis

Finney's probit analysis was used to determine the LC_{50} of each essential oil and the percentage mortality calculated using, the equation:

$$\% \text{ Mortality} = \frac{\text{No. of dead } \textit{nauplii}}{\text{Initial No. of live } \textit{nauplii}} \times 100$$

Antimicrobial activity assay of the essential oils

The antimicrobial assay was carried out using cup plate diffusion method (Washington and Sutter, 1980). Five strains of bacteria were used in this study. The bacteria were of three strains Gram-positive bacteria (*Staphylococcus aureus, Bacillus subtilis* and *Salmonella typhi*) and Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*). All microbes were of clinical isolates obtained from the Department of Pharmaceutical Microbiology, University of Ibadan, Nigeria. Nutrient agar (Oxiod Laboratories, UK) was used as a medium of growth of the bacteria. The agar was poured in sterile Petri dishes and was allowed to solidify. Overnight broth cultures of micro-organisms were used to seed different agar plates, one organism per plate.

Wells of approximately 5 mm in diameter were made on the agar medium, using a sterile cork borer. The plates were turned upside down and the wells labelled with a marker. Each well was then filled with 0.2 ml of the prepared essential oil solutions. Gentamicin (Oftalmiso Laboratories, Spain) was included as the control drugs for the anti-bacteria activity. The plates were incubated at 37°C for 24 h. Tests were conducted in triplicates and zones of inhibition (mm) were expressed as the mean. Data were analysed using descriptive statistics

RESULTS AND DISCUSSION

Predominant bioactive constituents

The constituents of the essential oils were analysed and reported earlier (Owokotomo et al., 2014). The major constituents of the essential oils of *A. melegueta* are presented in Table 1.

Brine shrimps toxicity

The essential oils of *A. melegueta* (Roscoe) K. Schum were screened for brine shrimps lethality and the toxicity was determined by LC_{50} (median lethal concentration) using Finney probit analysis. The LC_{50} ranges from 0.2 to 17.5 µg/ml (Table 2). The LC_{50} for the essential oils of *A. melegueta* stems and that of the seeds were the lowest (0.2 and 0.3 µg/ml, respectively), while that of the leaves of *A. melegueta* was the highest (17.5 µg/ml). These

Table 2. Toxicity of the essential oils on brine shrimps (*Artemia salina*) (10) in triplicate.

Essential oil	No. of dead Shrimps; Conc.1000 ppm	%Mortality	No. of dead Shrimps; Conc.100 ppm	%Mortality	No. of dead Shrimps; Conc.10 ppm	%Mortality	LC_{50} µg/ml
AFL	6, 5, 7	60	4, 1, 2	23.3	0, 2, 4	20.0	17.5
AFSD	10,10, 10	100	9, 10, 9	93.3	8,7,7	73.3	0.3
AFR	10,10, 10	100	7, 9, 7	76.7	4 ,6 ,4	46.7	9.5
AFSM	10,10,10	100	9,10,9	96.6	8, 8, 7	76.7	0.2

Key: AFL=*A. melegueta* leaf, AFSM = *A. melegueta* stem, AFR = *A. melegueta* rhizome, AFSD = *A. melegueta* seed essential oils.

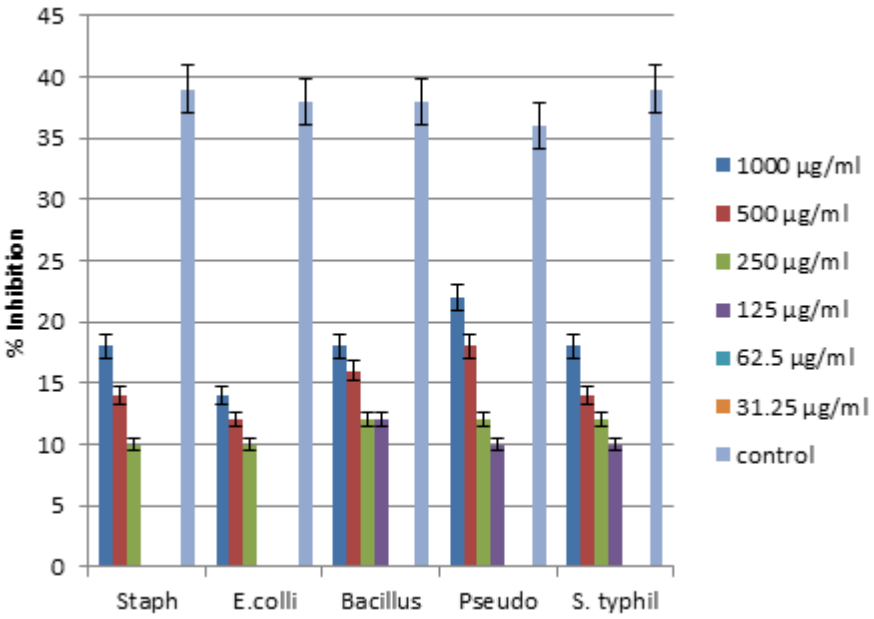

Figure 1. Bacteria growth profile of *A. melegueta* seeds' essential oil.

values were low which indicated that, the essential oils were toxic and thus, contained cytotoxic and pesticidal properties. The degrees of toxicities of the essential oils were directly proportional to the concentration. Maximum mortality (100.0%) of the brine shrimps was recorded at 1000.0 ppm and the least mortality (20.0%) also recorded at 10.0 ppm of solution of the essential oils.

Their toxicity has further corroborated literature reports on the cytotoxicity of plant essential oils (Bakkali et al., 2008). According to Carson et al. (2002), essential oils seem to have no specific mechanism or cellular target because of the high number of compounds present in any sample. They are lipophilic, thus, they pass through the cell wall and cytoplasm membrane, and disrupt the structure of their different layers. Cytotoxicity which appears includes membrane damages.

Antibacterial activity

The plants' essential oils were active against all bacteria strains (*S. aureus, B. subtilis, S.typhi, E. coli,* and *P. aeruginosa*) but inferior compared with inhibition ability of the antibiotic agent, gentamicin (10.0 µg/ml) (Figures 1 to 4). The essential oils were more effective within the concentration range of 1000.0 to 250.0 µg/mL against all micro-organisms. There was marked reduction in the antimicrobial activity of the oils at 62.0 µg/mL. *E. coli* and *S. aureus* seem to be less susceptible when compared to *B. subtilis, S. typhi* and *P. Aeruginosa.*

According to Belletti et al. (Krishnaraju et al.,2005), several essential oils, as well as some of their components such as caryophyllene and caryophyllene oxide, limonene, α-pinene, β-pinene and β-caryophyllene have displayed significant antimicrobial activity against bacteria and yeasts. Therefore, the antimicrobial results observed in this investigation could be related to the presence of myrtenyl acetate, limonene, Caryophyllene oxide, caryophyllene, Pinocarvyl acetate, γ-elemene and Cyperene which are the major constituents of these essential oils. The activities of these compounds were probably modulated by other minor components present

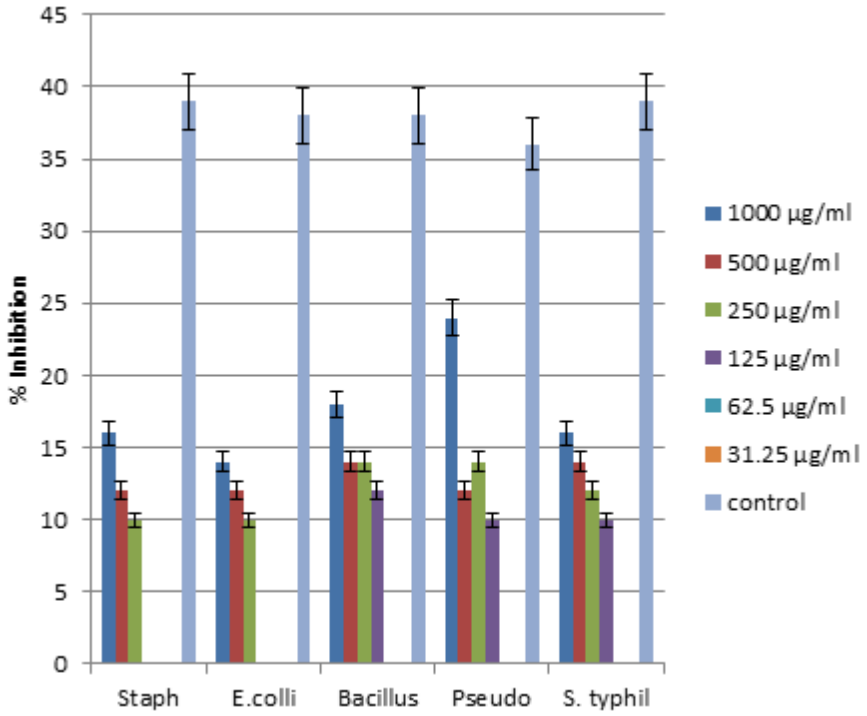

Figure 2. Bacteria growth profile of *A. melegueta* rhizomes' essential oil.

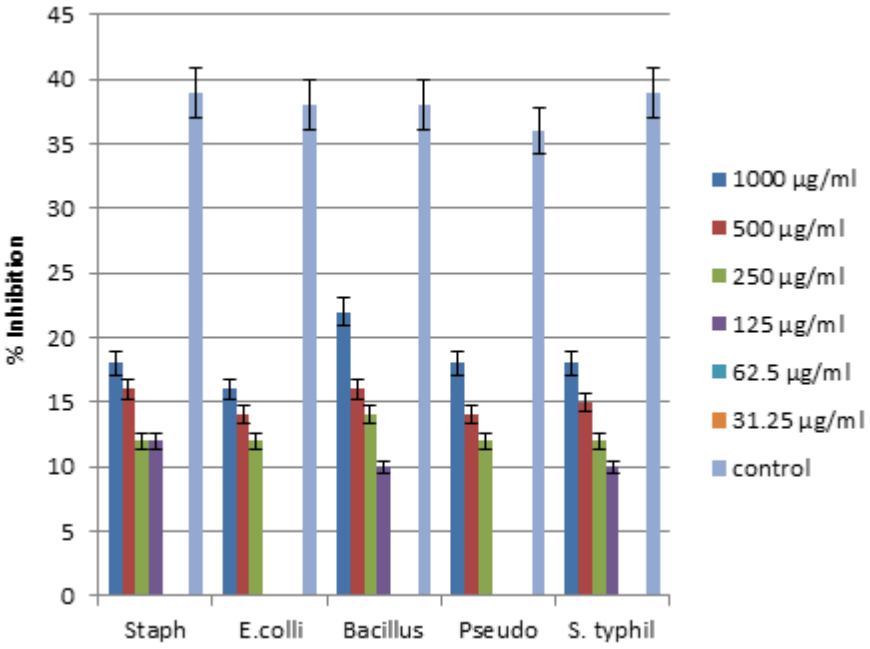

Figure 3. Bacteria growth profile of *A. melegueta* stems' essential oil.

in the essential oils.

Conclusion

Results from this work revealed that essential oils from the leaves, stems, rhizomes (roots) and seeds of *A. melegueta* were lethal to *Artemia salina.* They exhibited 100% mortality at 1000 ppm. *A. melegueta* stem and seed were the most potent with LC_{50} of 0.20 and 0.30 µg/ml respectively, while the leaves essential oil

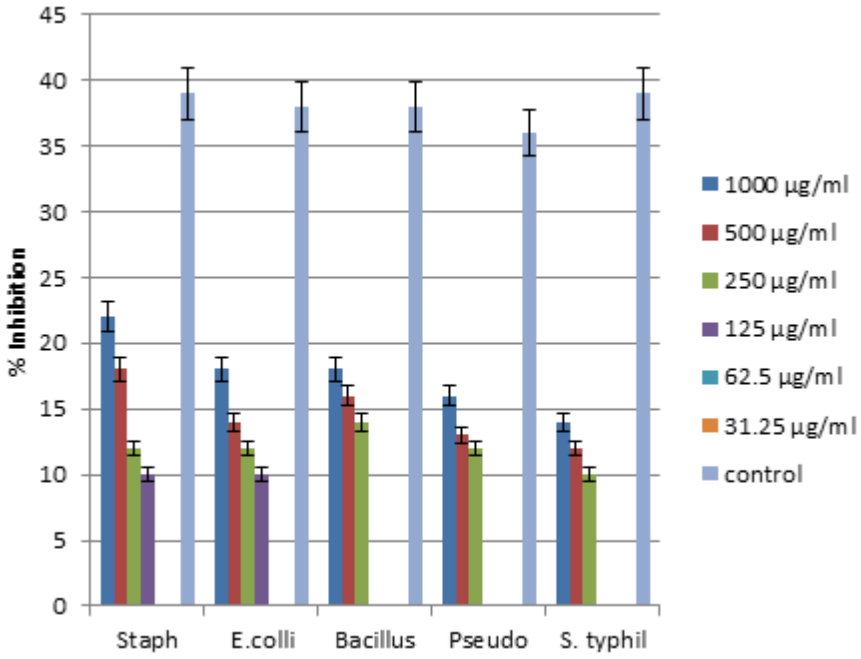

Figure 4. Bacteria growth profile of *A. meleguata* leaves' essential oil.

presented the least brine shrimps toxicity of LC_{50} 17.5 µg/ml.

The antibacterial activity of the essential oils showed that, these were effective against the pathogenic organisms. The activities compared the standard antibiotic and gentamicin, but high enough to support the ethno-medical usage of the plant.

The results suggest that the essential oils of *A. melegueta* (Roscoe) K. Schum may provide a source of natural products, which act as natural antibacterial and pesticidal agents. This research also support and rationalise the use of the plant in African ethno-medicine as used in Nigeria.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Antibacterial activity of selected *Dendrobium* species against clinically isolated multiple drug resistant bacteria

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Dendrobium **species are widely used in traditional medicine as remedies for tonic to nourish the stomach, promote the production of body fluids and decrease fever. In this study, the antibacterial activities of four traditionally used *Dendrobium* species were tested against clinically isolated multiple drug resistant (MDR) bacteria. Hexane, chloroform, acetone, ethanol and methanol extracts of *Dendrobium amoenum, Dendrobium crepidatum*, *Dendrobium moniliforme* and *Dendrobium longicornu* were tested for antibacterial activity against methicillin-resistant *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella typhi* and *Acinetobacter baumannii* by using the well diffusion method. Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) were determined using the serial dilution method. For *D. amoenum*, hexane, chloroform and acetone extracts showed antibacterial activity against *S. aureus* (ZOI: 11.33, 12.00 and 11.00 mm respectively), acetone extract against *A. baumannii* (ZOI: 13.00 mm) and methanol extract against *P. aeruginosa* (ZOI: 12.00 mm). For *D. crepidatum*, hexane, chloroform, acetone, ethanol and methanol extracts showed antibacterial activity against *S. typhi* (ZOI: 10.00, 11.67, 12.00, 9.67 and 12.67 mm respectively), hexane, ethanol and methanol extracts against *P. aeruginosa* (ZOI: 10.00, 9.67 and 15.00 mm respectively), choloroform and acetone extracts against *S. aureus* (ZOI: 9.67 mm) and ethanol extract against *E. coli* (ZOI: 11.00 mm). For *D. moniliforme*, chloroform extract showed antibacterial activity against *K. pneumoniae*, *P. aeruginosa*, *S. typhi*, *A. baumannii* (ZOI: 12.67, 12.00, 11.67 and 13.00 mm respectively), acetone extract against *S. aureus* (ZOI: 11.00 mm) and *A. baumannii* (ZOI: 12.67 mm). For *D. longicornu*, chloroform extract showed antibacterial activity against *A. baumannii* (ZOI: 12.00 mm). MIC and MBC of these extracts of plants showed they have moderate antibacterial activity against the bacterial strains used. In conclusion, these *Dendrobium* species can be used as antibiotic agents.**

Key words: *Dendrobium*, extracts, minimum bactericidal concentration (MBC), multiple drug resistant (MDR) bacteria, minimum inhibitory concentration (MIC), zone of inhibition (ZOI).

INTRODUCTION

Orchids are the largest and most diverse family of angiospermic plants; most orchids are widely used in traditional medicine as remedies for severe diseases (Pant and Raskoti, 2013). Several *Dendrobium* species

are used in traditional medicine as tonics to nourish the stomach, promote the production of body fluids and decrease fever (Ng et al., 2012; Xu et al., 2013). They produce a variety of secondary metabolites, such as phenolic compounds (Hu et al., 2010; Li et al., 2009a; Zhao et al., 2003), bibenzyl derivatives (Bi et al., 2004; Chen et al., 2010; Hu et al., 2008; Li et al., 2009b, 2009c, 2014; Majumder et al., 1999; Majumder and Chatterjee, 1989), lignin glycosides and phenanthrenes (Hu et al., 2008), and alkaloids (Elander et al., 1973; Kierkegaard et al., 1970; Li et al., 2008; Venkateswarlu et al., 2002). Such metabolites are responsible for these species' wide variety of medicinal properties (Chand et al., 2016; Paudel et al., 2015, 2017).

Bacterial infections are a global concern due to development of resistance towards various types of antibiotics. Multiple drug resistant (MDR) pathogens has been increasing, causing nosocomial and community-acquired deadly infections mainly due to the extensive utilization of antibiotics (Köck et al., 2010; Mongalo et al., 2013). Methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella typhi* and *Acinetobacter baumannii* have inherent resistant to most available antibiotics (Chambers, 1997; Kim et al., 2005; Patzer and Dzierzanowska, 2007).

The use of medicinal plant extracts to treat infectious disease is an age-old practice which rely on traditional medicine (Zaiden et al., 2005). According to the World Health Organization (WHO), approximately 80% of the world population rely on plants derived products for their treatment. There are many plants which have been reported to have antibacterial activities. New therapeutic antibacterial agents are greatly needed to treat emerging MDR bacterial infections. Therefore, the present study aimed to evaluate the antibacterial activity of selected *Dendrobium* species: *Dendrobium amoenum*, *Dendrobium crepidatum*, *Dendrobium longicornu* and *Dendrobium moniliforme* extracts against MRSA *S. aureus*, and MDR strains of *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. typhi* and *A. baumannii*.

MATERIALS AND METHODS

Plant materials

Whole stems of selected *Dendrobium* species (*D. amoenum*, *D. crepidatum*, *D. longicornu* and *D. moniliforme*) were collected from Chitlang and Daman of Makawanpur district, central Nepal, and voucher specimens were deposited at the Tribhuvan University Central Herbarium (TUCH), Kathmandu, Nepal (Specimens number: M05, M02, M03, M01 and M04, respectively).

Preparation of plant extracts

Plant materials were prepared by grinding air-dried stems and extracted with hexane, chloroform, acetone, ethanol and methanol in the ratio of 1:10 (w/v) using a Soxhlet extractor (Jones and Kinghorn, 2005). The solvent was evaporated under room temperature to obtain dry extracts. Each extract was dissolved in dimethyl sulfoxide (DMSO) to prepare a 1 mg/ml stock solution.

Microorganisms

The bacterial strains used in this study were clinical isolates and ATCC strains obtained from the National Public Health Laboratory, Kathmandu, Nepal: *S. aureus* (MRSA), *E. coli* (ATCC 25922), *A. baumannii* (ATCC 17978), *K. pneumoniae*, *P. aeruginosa* and *S. typhi*. The standard antibiotic drugs ciprofloxacin (for *S. aureus* and *E. coli*), chloramphenicol (for *K. pneumoniae*), gentamicin (for *P. aeruginosa*), azithromycin (for *S. typhi*) and meropenem (for *A. baumannii*) were used as positive controls.

Antibacterial activity

Individual bacterial strains were streaked on nutrient agar (Hi-Media, India) plates and incubated at 37°C for 24 h. Pure cultures were obtained, transferred to nutrient broth (Hi-Media, India) and incubated in a shaker incubator at 37°C and 120 rpm overnight. The turbidity of bacterial suspensions was adjusted to the 0.5 McFarland standards for antibacterial testing. Single bacterial strains from the appropriate bacterial suspensions were inoculated on Mueller Hinton agar plates with a sterile cotton swab. The antibacterial activities of the plant extracts were determined by using the well diffusion method. Five wells with a diameter of 7 mm were prepared on nutrient agar for each bacterial strain. Each well was filled with 100 µl of plant extract (1 mg/ml), DMSO (the negative control) or the standard antibiotics (the positive control). The plates were incubated overnight at 37°C. After incubation, the clear zone of inhibition around the point of application of each sample solution was measured in millimeters (mm).

Minimum inhibitory concentrations (MICs) were determined by using the serial dilution method (Abu-Shanab et al., 2006). The extracts were serially diluted in nutrient broth medium. Each dilution (1 ml) was inoculated into 1 ml of sterile nutrient broth containing 0.25 ml of the tested bacterial strain and incubated at 37°C for 24 h. Pure plant extracts of each assessed concentration in 1 ml of nutrient broth were used as the positive control, and 0.25 ml of bacteria culture in 2 ml of nutrient broth was used as the negative control. The MIC was equal to the lowest concentration of extract (mg/ml) for which there was no detectable growth of bacteria.

The minimum bactericidal concentrations (MBCs) were determined by sub-culturing test dilutions of the extracts onto fresh medium and incubating for an additional 24 h. The lowest concentration of the extract (mg/ml) that did not result in the appearance of a single bacterial colony on the solid medium was regarded as the MBC (Abu-Shanab et al., 2006).

Statistical analysis

All data are presented as the means ± standard deviation (SD) of triplicate samples. Values for the extracts were tested by using Chi-

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Table 1. Antibacterial effect of different solvent extracts of four *Dendrobium* species and standard antibiotics as positive control at 1 mg/ml concentration against different bacterial strains.

Plant name	Extract	Zone of inhibition (ZOI) (mm) [mean ± SD]					
		***Staphylococcus aureus* (MRSA)**	***Escherichia coli***	***Klebsiella pneumoniae***	***Pseudomonas aeruginosa***	***Salmonella typhi***	***Acinetobacter baumannii***
	Hexane	11.33±1.15	-	-	-	-	-
	Chloroform	12.00±0.00	-	-	-	-	-
D. amoenum	Acetone	11.00±0.00	-	-	-	-	13.00±0.00
	Ethanol	-	-	-	-	-	-
	Methanol	-	-	-	12.00±0.00	-	-
	Hexane	-	-	-	10.00±0.00	10.00±2.00	-
	Chloroform	9.67±0.58	-	-	-	11.67±1.15	-
D. crepidatum	Acetone	9.67±0.58	-	-	-	12.00±0.00	-
	Ethanol	-	11.00±1.00	-	9.67±0.58	9.67±0.58	-
	Methanol	-	-	-	15.00±0.00	12.67±0.58	-
	Hexane	-	-	-	-	-	-
	Chloroform	-	-	12.67±0.58	12.00±0.00	11.67±0.58	13.00±0.00
D. moniliforme	Acetone	11.00±0.00	-	-	-	-	12.67±1.15
	Ethanol	-	-	-	-	-	-
	Methanol	-	-	-	-	-	-
	Hexane	-	-	-	-	-	-
	Chloroform	-	-	-	-	-	12.00±0.00
D. longicornu	Acetone	-	-	-	-	-	-
	Ethanol	-	-	-	-	-	-
	Methanol	-	-	-	-	-	-
	Ciprofloxacin	23.67±1.53	11.33±0.58	-	-	-	-
	Chloramphenicol	-	-	25.00±0.00	-	-	-
Positive control	Gentamicin	-	-	-	13.33±0.58	-	-
	Azithromycin	-	-	-	-	17.67±2.52	-
	Meropenem	-	-	-	-	-	15.00±0.00
	Chi-square	58.39	58.72	58.48	58.13	58.36	58.70
	df	20	20	20	20	20	20
	p	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05

square test with a significance threshold of $p \leq 0.05$.

RESULTS

The antibacterial activity of different solvent extracts of the four *Dendrobium* species were tested by using the well diffusion method. The result of antibacterial activity of four *Dendrobium* species is shown in Table 1. For *D. amoenum*, hexane, chloroform (Figure 1a) and acetone extracts showed antibacterial activity against *S. aureus* (ZOI: 11.33, 12.00 and 11.00 mm respectively), acetone extract against *A. baumannii* (ZOI: 13.00 mm) and methanol extract against *P. aeruginosa* (ZOI: 12.00 mm). For *D. crepidatum*, hexane, chloroform, acetone, ethanol and methanol extracts showed antibacterial activity against *S. typhi* (ZOI: 10.00, 11.67, 12.00, 9.67 and 12.67 mm respectively), hexane, ethanol and methanol extracts against *P. aeruginosa* (ZOI: 10.00, 9.67 and 15.00 mm respectively), choloroform and acetone extracts against *S. aureus* (ZOI: 9.67 mm) and ethanol extract against *E. coli* (ZOI: 11.00 mm). For *D. moniliforme*, chloroform extract showed antibacterial activity against *K. pneumoniae*, *P. aeruginosa*, *S. typhi*, *A. baumannii* (Figure 1b) (ZOI: 12.67, 12.00, 11.67 and 13.00 mm respectively), acetone extract against *S. aureus* (ZOI:

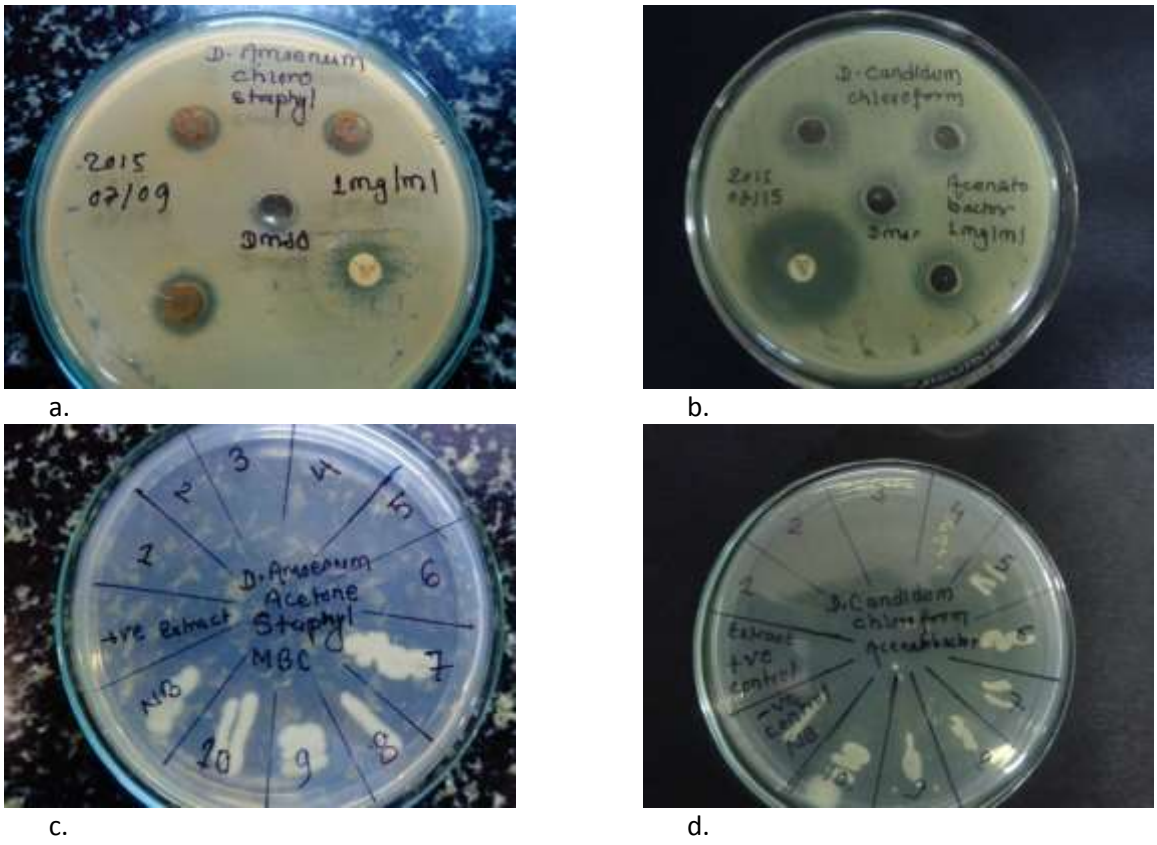

Figure 1. Antibacterial activities of different extracts of *Dendrobium* species against different bacterial strains. a) ZOI of *D. amoenum* chloroform extract against *S. aureus*; b) ZOI of *D. moniliforme* chloroform extract against *A. baumannii*; c) MBC of *D. amoenum* acetone extract against *S. aureus;* d) MBC of *D. moniliforme* chloroform extract against *A. baumannii*

11.00 mm) and *A. baumannii* (ZOI: 12.67 mm). For *D. longicornu*, chloroform extract showed antibacterial activity against *A. baumannii* (ZOI: 12.00 mm).

The antibacterial activity and potencies of the extracts were quantitatively assessed by determining MIC and MBC (Table 2). The values MIC and MBC of the extracts of four *Dendrobium* species indicate that they have moderate antibacterial activity against the bacterial strains used. For *D. amoenum*, acetone extract (Figure 1c) showed lowest MIC and MBC against *S. aureus* (MIC and MBC: 0.39 mg/ml), acetone extract against *A. baumannii* (MIC: 1.56 mg/ml, MBC: 3.12 mg/ml) and methanol extract against *P. aeruginosa* (MIC: 0.78 mg/ml, MBC: 3.12 mg/ml). For *D. crepidatum*, acetone extract showed lowest MIC and MBC against *S. typhi* (MIC: 0.78 mg/ml, MBC: 6.25 mg/ml), hexane extract against *P. aeruginosa* (MIC: 0.78 mg/ml, MBC: 6.25 mg/ml), choloroform extract against *S. aureus* (MIC: 0.78 mg/ml, MBC: 6.25 mg/ml) and ethanol extract against *E. coli* (MIC: 3.12 mg/ml, MBC: 6.25 mg/ml). For *D. moniliforme*, chloroform extract showed moderate antibacterial activity against *K. pneumoniae*, *P. aeruginosa*, *S. typhi*, *A. baumannii* (Figure 1d) (MIC: 3.12, 1.56, 0.39 and 1.56 mg/ml respectively, MBC: 3.12 mg/ml), acetone extract against *S. aureus* (MIC: 0.39 mg/ml, MBC: 6.25 mg/ml) and *A. baumannii* (MIC: 1.56 mg/ml, MBC: 3.12 mg/ml). For *D. longicornu*, chloroform extract showed moderate antibacterial activity against *A. baumannii* (MIC: 0.39 mg/ml, MBC: 6.25 mg/ml).

DISCUSSION

Drug-resistant bacterial infection treatment has been stimulated by investigations on natural compounds as alternative medicine (Abu-Shanab et al., 2004; Adwan and Mhanna, 2008; Denis et al., 2009). In the present study, analyses of growth inhibition activity performed by using the well diffusion method showed that all the tested plants, which are commonly used by traditional medical practitioners, exhibited moderate activity against clinical isolates of bacterial strains at extract concentrations of 1 mg/ml. The results are in agreement with those obtained in prior studies that have investigated medicinal plants for antibacterial activity (Abu-Shanab et al., 2004, 2006; Adwan and Mhanna, 2008; Aliyu et al., 2008; Habib et al.,

Table 2. MIC (mg/ml), MBC (mg/ml) and ZOI (mm) of different solvent extracts of four *Dendrobium* species against different bacterial strains.

Plant names	Extracts	*Staphylococcus aureus* (MRSA)			*Escherichia coli*			*Klebsiella pneumoniae*			*Pseudomonas aeruginosa*			*Salmonella typhi*			*Acinetobacter baumannii*		
		MIC	MBC	ZOI	MIC	MBC	ZOI	MIC	MBC	ZOI	MIC	MBC	ZOI	MIC	MBC	ZOI	MIC	MBC	ZOI
	Hexane	1.56	6.25	12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Chloroform	0.39	6.25	15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D. amoenum	Acetone	0.39	0.39	16	-	-	-	-	-	-	-	-	-	-	-	-	1.56	3.12	18
	Ethanol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Methanol	-	-	-	-	-	-	-	-	-	0.78	3.12	15	-	-	-	-	-	-
	Hexane	-	-	-	-	-	-	-	-	-	0.78	6.25	15	1.56	6.25	13	-	-	-
	Chloroform	0.78	6.25	14	-	-	-	-	-	-	-	-	-	1.56	6.25	13	-	-	-
D. crepidatum	Acetone	1.56	3.12	18	-	-	-	-	-	-	-	-	-	0.78	6.25	13	-	-	-
	Ethanol	-	-	-	3.12	6.25	11	-	-	-	1.56	6.25	13	3.12	6.25	13	-	-	-
	Methanol	-	-	-	-	-	-	-	-	-	3.12	6.25	17	3.12	12.5	12	-	-	-
	Hexane	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Chloroform	-	-	-	-	-	-	3.12	3.12	13	1.56	3.12	17	0.39	3.12	13	1.56	3.12	19
D. moniliforme	Acetone	0.39	6.25	13	-	-	-	-	-	-	-	-	-	-	-	-	1.56	3.12	14
	Ethanol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Methanol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Hexane	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Chloroform	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.39	6.25	15
D. longicornu	Acetone	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Ethanol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Methanol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

2011; Nitta et al., 2002; Voravuthikunchai and Kitpipit, 2005).

Infections caused by multiple drug resistance, including resistance to β-lactam, are among the most difficult infections to treat (Abu-Shanab et al., 2004, 2006; Kim et al., 2005). In this study, the growth of *S. aureus, E. coli, K. pneumoniae, S. typhi* and *A. baumannii* were markedly inhibited by some extracts of *D. amoenum, D. crepidatum, D. moniliforme* and *D. longicornu*. Therefore, it appears likely that the antibacterial compound(s) extracted from these *Dendrobium* species (data not shown here) may inhibit bacteria via a different mechanism than that associated with currently used antibiotics and may have therapeutic value as antibacterial agents against bacterial strains with multiple drug resistance.

Overall, the screening of extracts from all tested plants showed that Gram-positive bacteria were more susceptible than Gram-negative bacteria. This phenomenon may be attributable to a distinctive feature of Gram-negative bacteria: the presence of a thick outer murein membrane. This outer membrane prevents certain drugs and antibiotics from entering the cell. In contrast, Gram-positive bacteria may be more susceptible to the tested extracts because the peptidoglycan

that constitutes the outer layer of such bacteria is not an effective permeability barrier to bioactive compounds. Thus, Gram-negative bacteria have more complex cell wall than Gram-positive bacteria; this distinction partially explains why Gram-negative bacteria are generally more resistant to antibiotics than Gram-positive bacteria (Behera et al., 2013; Zaiden et al., 2005). Extracts of the tested plants do not have the types of antibiotic compounds that can readily enter the cell and kill Gram-negative bacteria.

The MIC and MBC values obtained for the extracts against the bacterial strains support the findings obtained by using the well diffusion method. One concern is that, for the extracts obtained in this study, MIC values were lower than MBC values, thus suggesting that these extracts are bacteriostatic at lower concentrations and bactericidal at higher concentrations (Abu-Shanab et al., 2006; Nitta et al., 2002; Ross et al., 2011).

In conclusion, some of the extracts of the selected plants showed moderate antibacterial activity against the bacteria tested. The findings indicated that these species that are frequently used in traditional medicine and as tonic may be potential sources of antimicrobial agents.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Effect of processing methods on the chemical composition and microbiological quality of vegetable drink extract of african bush mango (*Irvingia gabonensis*)

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The effect of processing methods on the chemical composition, proximate, mineral, vitamin and microbiological quality of vegetable drink extract of *Irvingia gabonensis* was studied. The processing methods included drying (shade and solar drying), blanching (at 0, 2, 4 and 6 min) as well as blanching and drying of the leaves. Aqueous extracts were obtained from the leaves and the analysis carried out using standard methods. The result showed that whereas some parameters analyzed varied with processing, others remained unaffected. The moisture, protein and fat content did not vary significantly ($p<0.05$) with treatments while shade drying increased ash content significantly. Shade drying also increased the phytochemicals from 17.50 to 72.50% for total phenol; 4.53 to 6.72% for flavonoids and 2.98 to 3.86% for alkaloid. It also reduced steroids and saponin significantly ($p<0.05$). Phytate content was non-significantly ($p<0.05$) reduced by shade and solar drying whereas the other processing methods increased it to the range of 5.38 to 14.33 mg/100 g. Six minutes blanching reduced oxalate from 8.15 to 7.25 mg/100 g and tannin content from 0.023 to 0.019 mg/100 g. Cyanide content of 0.03 mg/100 g was increased by all the processing methods though minimally by blanching and solar drying. Carotenoid content of 164.56 IU was reduced significantly ($p<0.05$) to the ranges of 92.83 to 151.90 IU for blanched and 16.88 to 59.07 IU for dried vegetable drink extracts. Vitamin A content of 987.34 IU was significantly ($p>0.05$) reduced by all the processing methods to the range of 101.27 to 632.91 IU. Vitamin C, on the other hand, was increased significantly ($p<0.05$) by all the drying methods. The rest of the vitamins and also minerals were inconsistent with the processing methods. Total plate count was negligible in blanched drink extracts while shade drying gave the highest count (1.30×10^3 cfu/g). Total coliforms were reduced in all the drinks from the processed leaves. The mould count in the blanched drink extract remained relatively low, but increased mould counts occurred in drink extract from shade dried leaves.

Key words: Blanching, *Irvingia gabonensis* leaf, shade drying, solar drying, vegetable drink extract.

INTRODUCTION

The key fruits and vegetables in human nutrition both in the developed and developing countries of the world are the yellow corn, spinach, green leafy vegetables, fruits, tomato, watermelon, pink-fleshed guava, red-fleshed

papaya, red pepper, mango, orange, carrot, melon, yellow and orange-fleshed sweet potato, nuts, tea, wine, flaxseed, sesame seed, grapes, peanuts, cereals and pulses. Evidences of the benefits to human-health associated with their consumption have caused an increase in the demand for fresh-like fruits and vegetables (Oms-Oliu et al., 2012). Hence, the need for the identification and exploitation of other novel plants to fulfill the growing need of plant-based chemicals. The emphasis therefore shifted to the underutilized plant foods which as a result of lack of attention from research and development has meant that their potential value to human well-being and income is underexploited (IPGRI, 2002). While such crops continue to be maintained by sociocultural preferences and the ways they are used, they remain inadequately documented and neglected by formal research and conservation (IPGRI, 2002). *Irvingia gabonensis* (Family: Irvingiaceae) commonly known as the African bush mango, Dika nut, bush mango or wild mango, and *Manguier sauvage* in French is one of such crops, with the leaves only known to be used as food for livestock by farmers (Ayuk et al., 1999).

African bush mango (*I. gabonensis*) belongs to the Irvingiaceae plant family (Lamorde et al., 2010). It is a wild forest tree 15 to 40 m with a bole slightly buttressed, possessing dark green foliage and yellow flowers (Kuete et al., 2007). The ripe fruit is green while the edible mesocarp is soft, juicy, and bright orange. The fruits are sometimes referred to as 'Mangoes' (hence the synonym of African Bush Mango) although they are unrelated, since the true Mango fruits are borne from the plant *Mangifera indica* of the plant family Anacardiacea (Bally, 2006). The pulp of this fruit is eaten fresh and can also be used for the preparation of juice, jelly and jam as well for a good quality wine (Akubor, 1996). The mango-like fruits of bush mango are especially valued for their dietary-fiber, fat- and protein-rich seeds (Jianghao and Pei, 2012). The bark of the *I. gabonensis* tree (rather than the seeds, which are commonly used as fibrous supplements) appears to be traditionally used by the Mende tribe of Africa for pain relief (Okolo et al., 1995). Studies have shown that the seeds of *I. gabonensis* could be effectively used as an ingredient in health and functional food to ameliorate certain disease states such as diabetes (Dzeufiet, 2009). And the leaves of plants have been shown to be richer in chemical compostion than the fruit pulps and seeds (Nwofia et al., 2012). Bush mango leaf/root extracts have documentary inhibitory activity against several bacteria and fungi. Aqueous leaf extracts significantly protected mice against diarrhoea induced experimentally by castor oil in terms of severity and onset and the population of animals with diarrhoea (Abdulrahman et al., 2004). As reported by Kuete et al. (2007), the aqueous maceration of the leaves is used as anti-poison. And when taken in combination with palm oil, the leaves are also used to stop hemorrhages for pregnant women in Cameroon. Thus the focus of this work was to determine the effect of processing methods on the chemical, nutrient, vitamin, mineral content and microbiological quality of vegetable drink extract of African bush mango.

Research on the effect of processing methods on the chemical composition and microbiological quality of the vegetable drink extract of *I. gabonensis* would add to the existing knowledge of the processing methods to adopt to meet specific and desired needs. Also, the knowledge of the microbiological quality would guide in the selection of process controls to apply to vegetable drink extracts prior to consumption.

The general objective of this research was to produce and evaluate the effect of processing methods on the chemical composition and microbiological quality of vegetable drink extract from leaves of African bush mango (*I. gabonensis*).

MATERIALS AND METHODS

Fresh leaves of *Irvingia gabonensis* were plucked from a single plant in a farm in Alor-Uno community in Nsukka. The plant leaves were authenticated by a botanist of the Herbarium section in the Department of Plant Science and Biotechnology, University of Nigeria, Nsukka, Enugu State. All the reagents/chemicals used for analysis were of analytical grade.

Sample preparation and extraction of vegetable drink from the leaves of *I. gabonensis*

The freshly plucked leaves were divided into five groups of 100 g each for the various processing methods. The samples were processed, and the vegetable drink extracted as outlined in the flow diagram (Figure 1). The groups were processed follows:

Group A (Vegetable drink extract from fresh leaves as control designated as sample 4): One hundred grams of the fresh leaves were wet milled with 200 ml of clean drinking water using a household blender (Kenwood model, China) and thereafter filtered using a filter cloth. A transparent plastic container was used to store the drink extract in the refrigerator at the temperature of 4°C for further analysis.

Group B (Vegetable drink extract from shade dried leaves designated as sample 5): One hundred grams of the freshly plucked leaves were air dried at room temperature of an average of 28°C for 7 days (shade drying) followed by milling using a hammer mill. The obtained powder used for extraction of drink was packaged in air-tight plastic container to avoid rehydration and stored in a cool, dry and clean area away from direct light. The extract was obtained by reconstituting leaf powder (100 g) with 200 ml of clean drinking water and filtered using a filter cloth.

Group C (vegetable drink extract from solar dried leaves designated

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Figure 1. Flow diagram for the preparation of vegetable drink extract from from raw (control treatment), raw/dried, blanched (2, 4 and 6 min), blanched/shade-dried (2, 4 and 6 min), leaves of *Irvingia gabonensis*.

as sample 6): One hundred grams of the freshly plucked leaves were solar dried at 35°C for 18 h followed by milling using a hammer mill. The resulting powder for vegetable drink extract formation was packaged in air-tight plastic container to avoid rehydration and stored in a cool, dry and clean area away from direct light. The extract was obtained by reconstituting leaf powder (100 g) with 200 ml of clean drinking water and filtered using a filter cloth.

Group D (Vegetable drink extract from blanched leaves designated as sample 1 to 3): Four portions of leaves weighing 100 g each were separately sliced and steam blanched at 100°C (boiling water) for 0 (same as control), 2, 4 and 6 min (designated as samples 3, 2 and 1) respectively. Blanching for varying times helped to determine the blanching time that ensured maximum nutrient retention. The blanched leaves were wet milled with 200 ml of water and thereafter filtered using a filter cloth. Transparent plastic containers were used to store the drink extracts in the refrigerator at the temperature of 4°C for further analysis.

Group E (vegetable drink extract from blanched and shade dried leaves designated as samples 7 to 9): four portions of leaves weighing 100 g each were separately sliced and steam blanched at 100°C (boiling water) for 2, 4 and 6 min (designated as samples 9, 8 and 7) respectively (while the unblanched sample served as the control). The blanched leaves were shade dried followed by milling using a hammer mill. The obtained powders used for vegetable drink extract formation were packaged in air-tight plastic containers to avoid rehydration and stored in a cool, dry and clean area away from direct light. The extract was obtained by reconstituting powder (100 g) with 200 ml of clean drinking water and filtered using a filter cloth.

Analysis of the vegetable drink extract

The analyses were carried out using appropriate analytical methods.

Proximate analysis

Moisture, protein, crude fat, ash and carbohydrate contents were determined according to the standard methods of AOAC (2010).

Quantitative evaluation of phytochemicals

Determination of total phenol content

The total phenol content of the vegetable drink extract was determined by the spectrophotometric method (Obadori and Ochuko, 2001). Two grammes of the test sample was weighed into a flask and defatted with petroleum ether. The residue was allowed to stand for five minutes to air dry and thereafter boiled with 50 ml of diethyl ether for 15 min to extract the phenol. Five milliliter of the aliquots of the boiled sample was transferred into test tube and 2 ml of NH_4OH solution and 5 ml of amyl alcohol were added and allowed to stand for 30 min for colour development. The blank and the standard were prepared. The total phenol was calculated using the formula:

$$\text{Phenol (mg/100 g)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \frac{\text{Concentration of standard}}{1}$$

Determination of flavonoid

The flavonoid was determined using the method of Harborne (1973). Ten grammes of the sample was weighed and extracted repeatedly with 100 ml of 80% aqueous methanol at room temperature. The solution was filtered using Whatman No. 42 (125 mm) filter paper and the filtrate transferred into a weighed crucible. The filtrate was evaporated into dryness and the crucible reweighed to a constant weight. The percentage flavonoid was calculated using the formula:

$$\text{Flavonoid (\%)} = \frac{\text{Weight of flavonoid}}{\text{Total weight used}} \times \frac{100}{1}$$

Determination of alkaloid content

The gravimetric method of Harbone (1973) was used. Five grammes of the sample was weighed out and dispersed into 50 ml of 10% acetic acid solution in ethanol. The mixture was shaken very well and then allowed to stand for 4 h before filtering. The solution was concentrated and the alkaloids precipitated with concentrated NH_4OH by adding it drop wise. This was allowed to cool and the precipitate filtered with a weighed filter paper. The precipitate in filter paper was dried in the oven at 60°C and reweighed to obtain the weight of the filter paper and the alkaloid.
The percentage alkaloid was calculated using the formula:

$$\text{(Alkaloid (\%)} = \frac{\text{(weight of filter paper + alkaloid) – (weight of filter paper)}}{\text{weight of sample}} \times \frac{100}{1}$$

Determination of tannins

The Folin-Dennis colorimetric method as described by AOAC (2010) was used to determine tannin content. One gram of the test sample was weighed out and 10.0 ml distilled water added. This was shaken at 5 min intervals for 30 min and then centrifuged to get the extract. Two and a half milliliter of the supernatant was transferred into a test-tube. Also, 2.5 ml of standard tannic acid solution was added into a 50 ml flask and 1.0 ml Folin-Denis reagent added into the flask, followed by 2.5 ml of saturated Na_2CO_3 solution. The solution was made up to the mark and the absorbance read after 90 min incubation at room temperature. Percentage tannin was calculated as shown:

$$\text{Tannin (\%)} = \frac{A_n}{A_s} \times C \times \frac{100}{W} \times \frac{V_f}{V_a}$$

Where, A_n = absorbance of test sample; A_s = absorbance of standard solution; C = concentration of standard solution; W = weight of sample used; V_f = total volume of extract; V_a = volume of extract analyzed.

Determination of saponin content

The method of Obadori and Ochuko (2001) was used to determine the saponin content of the sample. Twenty grammes of the sample was weighed into a conical flask and 100 ml of 20% aqueous ethanol added. The sample was heated over hot water bath for 4 h with continuous stirring at about 55°C. This was filtered and the residue re-extracted with another 200 ml of 20% ethanol. The extract was concentrated to 40 ml in the water bath at 90°C and 20 ml of diethyl ether added and shaken vigorously to separate. The aqueous layer was recovered and 60 ml of n-butanol added and then washed twice with 10 ml 5% aqueous sodium chloride. The extract was evaporated in the oven and the weight of saponin quantified in percentage using the formula:

$$\text{Saponin (\%)} = \frac{\text{weight of dry extract}}{\text{weight of sample}} \times \frac{100}{1}$$

Determination of steroid content

This was carried out as described by Subhadhirasakul and Pechpongs (2005). Two grammes of the sample was weighed out and 20 ml of chloroform added. This was allowed to stay for 2 h and filtered. The chloroform layer was the steroid and it was dried in the oven and the percentage steroid was calculated using the formula:

$$\text{Steroids (\%)} = \frac{\text{final weight - initial weight}}{\text{weight of sample used}} \times \frac{100}{1}$$

Determination of phytate content

The method of Oberleas(1973) was used to determine the phytate content. The solutions used were: Solution 2: A 0.2 g ammonium iron III sulphate in 100 ml 2 N HC1 made up to 1000 ml with distilled water; Solution 3: A 10 g bipyridrine and a 10 ml thioglycollic acid in distilled water made up to 1000 ml. One milliliter of the extract was pipetted into a test tube and 2 ml of solution (2) added. The test tube was covered and heated in a boiling water for 30 min and cooled to room temperature. Four milliliter of solution (3) was added and mixed well. The absorbance was measured at 519 nm and the phytate content calculated using the formula:

$$\text{Phytate (mg/100 g)} = \frac{\text{absorbance of sample}}{\text{absorbance of standard}} \times \frac{\text{concentration of standard}}{1}$$

Determination of oxalate content

Oxalate content was determined by the method of Kirk and Sawyer

(1998). A 2.0 g of the sample was weighed into 300 ml flask and 20 ml of 30% HCl added and allowed to stand for 20 min. A 4.0 g of ammonium sulphate was added and allowed to stand for 30 min. The solution was filtered and the filtrate made up to 50 ml with 30% HCl. A 10 ml of the filtrate was transferred into a 100 ml flask and 30 ml diethyl ether added. The pH was adjusted to 7.0 with NH_4OH and the solution titrated with 0.1 M $KMnO_4$. Oxalate content was calculated from the formula:

$$\text{Oxalate (\%)} = \frac{\text{Titre} \times \text{mol } KMnO_4 \times \text{dilution factor} \times 100}{\text{Weight of sample used}}$$

Determination of cyanide content

The method of Wangaffin as described in Harborne (1973) was used for cyanide determination.

Cyanide extraction

Cyanide extraction was done by weighing 5 g of the sample into a clean 250 ml flask and adding 50 ml of deionized water. This was allowed to stay overnight, filtered and the filtrate used for the cyanide determination.

Preparation of alkaline picurate solution

Alkaline picurate solution was prepared by dissolving 1 g of picrate and 5 g of sodium carbonate in warm water and the volume made up to 200 ml with deionized water.

Procedure for cyanide determination

A 4 ml alkaline picrate was added to 1 ml of the sample filtrate in a test tube and allowed to stand for 5 min. The absorbance was read at 490 nm after colour development (redish brown colour). The absorbance of the blank and the standard was also read and the cyanide content of the test sample extrapolated from cyanide standard.

Estimation of mineral elements

The sodium and potassium contents were determined by Flame photometry (Jenway Ltd Dunmow, Essex, UK). Estimation of iron, zinc, magnesium, and calcium contents were by atomic absorption spectrophotometry as described in Buck scientific operations manual (model 220DGB).

Estimation of sodium contents

A 2.0 g of sample was ashed in a furnace at the temperature of 450°C for 3 h. The ashed sample was dissolved with 5 ml of 30% HCl before the solution was made up to 50 ml with deionized water in a volumetric flask. This was filtered and aspirated into the enablizer of the flame photometer and the electron emission reading taken at 539 nm wavelength. The standard was prepared using sodium salt, and the concentration of the test sample extrapolated from the standard reading (7 = 10 ppm).

Estimation of potassium contents

A 2.0 g of sample was ashed in a furnace at the temperature of 450°C for 3 h. The ashed sample was dissolved with 5 ml of 30% HCl before the solution was made up to 50 ml with deionized water in a volumetric flask. This was filtered and aspirated into the enablizer of the flame photometer and the electron emission reading taken at 767 nm wavelength. The standard was prepared using potassium salt, and the concentration of the test sample extrapolated from the standard reading (16 = 20 ppm).

Estimation of iron contents

Iron content was determined by Buck Scientific atomic absorption spectrophotometry (Model 210 VGP). The first step in the procedure was digestion of sample. It involved mixing 0.2 g of sample with 15 ml of aquaregia (30% HCl and 10% HNO_3) inside a Teflon crucible and heating in the oven at 130°C for 2 h. This was allowed to cool and then filtered using Whatman No. 42 filter paper and the filtrate made up to 250 ml in a 250 ml volumetric flask. This was transferred to a clean container that was used for the analysis. Prior to the analysis, the equipment was calibrated using three different working standards of iron which were prepared from the stock standard (a concentrated solution of iron prepared in the laboratory with an assayed reference compound). The working standards for iron used were:

5 ppm (5 ml of stock + 100 ml distilled water);
3 ppm (3 ml of stock + 100 ml distilled water);
1 ppm (1 ml of stock + 100 ml distilled water).

The above working standards were used to plot a graph of absorbance against concentration to obtain a straight line graph (calibration curve).
Absorbance, A α Concentration

$$A = \log_{10} \frac{Io}{It}$$

Where: A = absorbance; Io = incident light; It = transmitted light

The digested sample was aspirated into the equipment and the various concentrations (in parts per million, ppm) were displayed according to Pearson (1976).

Estimation of zinc contents

Zinc content was determined by Buck Scientific atomic absorption spectrophotometry (Model 210 VGP). The digestion of sample was as described above. Also prior to the analysis, the equipment was calibrated using three different working standards of zinc prepared from the chloride of the salt (ZnCl). The working standards for zinc used were:

2 ppm (2 ml of stock + 100 ml distilled water);
1 ppm (1 ml of stock + 100 ml distilled water);
0.2 ppm (0.2 ml of stock + 100 ml distilled water).

The above working standards were used to plot a graph of absorbance against concentration to obtain a straight line graph (calibration curve).
Absorbance, A α Concentration

$$A = \log_{10} \frac{Io}{It}$$

Where: A = absorbance; Io = incident light; and It = transmitted light. The digested sample was aspirated into the equipment and the various concentrations (in parts per million, ppm) were displayed.

Estimation of magnesium contents

Magnesium content was determined by Buck Scientific atomic absorption spectrophotometry (Model 210 VGP). The digestion of sample was as described previously. Also prior to the analysis, the equipment was calibrated using three different working standards of magnesium prepared from the chloride of the salt ($MgCl_2$). The working standards for magnesium used were:

1.5 ppm (1.5 ml of stock + 100 ml distilled water);
1 ppm (1 ml of stock + 100 ml distilled water);
0.5 ppm (0.5 ml of stock + 100 ml distilled water).

The above working standards were used to plot a graph of absorbance against concentration to obtain a straight line graph (calibration curve).
Absorbance, A α Concentration

$$A = \log_{10} \frac{Io}{It}$$

Where: A = absorbance; Io = incident light; It = transmitted light
The digested sample was aspirated into the equipment and the various concentrations (in parts per million, ppm) were displayed.

Estimation of calcium contents

Calcium content was determined by Buck Scientific atomic absorption spectrophotometry (Model 210 VGP). The digestion of sample was as described previously. Prior to the analysis, the equipment was also calibrated using three different working standards of calcium prepared from the chloride of the salt ($CaCl_2$). The working standards for calcium used were:

6 ppm (6 ml of stock + 100 ml distilled water);
3 ppm (3 ml of stock + 100 ml distilled water);
1 ppm (1 ml of stock + 100 ml distilled water).

The above working standards were used to plot a graph of absorbance against concentration to obtain a straight line graph (calibration curve).
Absorbance, A α Concentration

$$A = \log_{10} \frac{Io}{It}$$

Where: A = absorbance; Io = incident light; It = transmitted light
The digested sample was aspirated into the equipment and the various concentrations (in parts per million, ppm) were displayed.

Determination of vitamins

Vitamin A

The procedure of Jakutowicz et al. (1977) was used. One gram of the sample was weighed. Then the proteins were first precipitated with 3 ml of absolute ethanol before the extraction of vitamin A with 5 ml of heptane. The test tube containing this was shaken vigorously for 5 min. On standing, 3 ml from the heptane layer was taken up in a cuvette and read at 450 nm against a blank of heptane. The standard was prepared, read at 450 nm and vitamin A calculated using the formula:

$$\text{Vitamin A (IU)} = \frac{\text{Absorbance of sample} \times \text{Concentration of standard}}{\text{Absorbance of standard} \times \text{weight of sample used}}$$

Vitamin C

Vitamin C analysis was carried out by the method describbed by Olokodona (2005).

Reagents:

1. Freshly prepared standard Indophenol's solution: A 0.05 g of 2,6-dicrophenol-indophenol was dissolved in water, diluted to 100 ml and filtered.
2. Standard ascorbic acid solutions: A 0.05 g of pure ascorbic acid was dissolved in 60 ml of 20 % metaphosphoric acid and diluted with water to 250 ml.
3. 20% meta-phosphoric acid
4. Acetone

Standardization of indophenol's solution

A 10 ml of standard ascorbic acid solution was pipetted into a small flask and titrated with indophenol's solution until a faint pink colour persisted for 15 s. The concentration was expressed as mg ascorbic acid equivalent to 1 ml of dye solution (10 ml ascorbic acid solution= 0.002 g ascorbic acid).

Procedure for analysis

A 5 g of sample was weighed into a 100 ml volumetric flasks, and 2.5 ml of 20 % meta-phosphoric acid added as stabilizing agent and diluted to volume with distilled water. Ten milliltre of the solution was pipetted into a small flask and 2.5 ml of acetone added. The solution was titrated with the indophenol solution until a faint pink colour persisted for 15 s and the vitamin C content in the sample was calculated as mg/100 ml.

Determination of riboflavin

Riboflavin was extracted with dilute acids after removing the interfering substances by treatment with $KMnO_4$ (Jakkutowicz et al., 1977). A 5 mg weight of the sample was taken, and 50 ml of 0.2 NHCl added and boiled on a water bath for an hour. This was afterwards cooled and the pH adjusted to 6.0 using NaOH. A 1 N HCl was added to lower the pH to 4. 5 and then filtered in a 100 ml measuring flask and the volume made up to mark. A 10 ml of aliquot was taken from the 100 ml volume and 1 ml of acetic acid (glacial) added to each tube and mixed. Then 0.5 ml of 3% $KMnO_4$ solution was added. This was kept away for 2 min and then 0.5 ml of 3% H_2O_2 added and mixed well before taking the reading at 470 nm. Riboflavin content was calculated using the formula:

$$\text{Riboflavin (mg/100 ml)} = \frac{\text{Absorbance of sample} \times \text{Concentration of standard}}{\text{Absorbance of standard} \times \text{weight of sample used}}$$

Determination of thiamin

Thiamin complex was extracted with dilute HCL and the resultant solution treated with phosphatase enzyme to liberate free thiamin (Jakkutowicz et al., 1977). A 1 g of the sample was weighed into a flask and 100 ml of 0.2 N HCl added and all heated to boil for 30 min on a water bath. This was cooled, and 5 ml of phosphatase

enzyme added and incubated at 37°C. This will be filtered and 3 g of anhydrous Na_2SO_4 added. A 5 ml of the solution was put into 5 ml stopped flask and 3 ml of 15 % NaOH added. The absorbance was taken at 435 nm wavelength and thiamin calculated using the formula:

$$\text{Thiamin} = \frac{\text{Absorbance of sample} \times \text{Concentration of standard}}{\text{Absorbance of standard} \times \text{weight of sample used}}$$

Determination of carotenoid

The method of AOAC (2010) was used for the determination of total carotenoid in the samples. Five grams (5 g) of the sample was mixed with hexane and ethanol (40:60 ml) to extract the carotenoid. The mixture was transferred into a separating funnel and swilled vigorously after adding 2% Nacl solution. It was thereafter allowed to settle and the aqueous layer ran off. The top layer which contained the carotenoid was collected. It was diluted and optical density determined in a spectrophotometer at 460 nm. The result was compared with carotenoid standard curve prepared using carotenoid stock solution of 0.8 mg/ml of hexane. The content of carotenoid in the sample was calculated using the formula:

$$\text{Mg carotenoid/100 g sample} = \frac{\text{standard curve reading} \times \text{dilution factor} \times 100}{\text{weight of sample}}$$

Determination of pH

pH determination was by the use of the pH meter (Hanna HI96107, Italy). The pH meter was standardized using buffer solutions of pH 4 and 9. The electrode was rinsed with de-ionized water and dipped into 20 ml of the sample, allowing sufficient time for stabilization before taking the reading.

Microbiological analysis of the processed vegetable drink extract

Determination of total plate count

The pour plate method as described by Harrigan and McCance (1976) was used to determine the total plate count. One milliliter of the solution (extract) was mixed with 9 ml of the diluent (Ringer's solution). The first of such dilution from the stock gives 10^{-1} while the next of the dilution made from 10^{-1} gives 10^{-2} dilution. One milliliter solution from each of the dilutions (up to 10^{-6}) was seeded into petri dishes (in duplicate) and 15 ml of sterile nutrient agar poured and mixed thoroughly with the inocula by gently rocking the plates. The plates were incubated at 37°C for 24 h after which the colonies formed were counted and expressed as colony forming unit per gram of sample (cfu/g) using the formula:

$$\text{cfu/g} = \frac{\text{Number of colonies} \times \text{original dilution}}{\text{Dilution factor volume of innoculum}}$$

Determination of total coliforms

Total coliform was determined as outlined by Sharma (2009). About 1 ml from each of the samples was inoculated into lactose broth and incubated at 37°C for 48 h. Serial dilutions of 10^{-1} and 10^{-2} were made from the resulting cultures and about 1 ml of solution from each of the dilutions used to inoculate eosin methylene blue (EMB) agar plates (in duplicates) using the pour plate technique. The plates were incubated at 37°C for 48 h and the characteristic metallic green colonies counted and expressed as colony forming unit per gram of sample (cfu/g) as stated above.

Determination of mould count

The pour plate method as described by Harrigan and McCance (1976) was used to determine the mould count. A volume of 1 ml of inoculum of sample serial dilutions was deposited in the empty petri dishes and covered with 15 ml of sterile Sabouraud Dextrose Agar (SDA) and thoroughly mixed by gently rocking the plates. The inoculated plates were incubated at 25°C for three days. The observed colonies were counted using the Quebec counter and the counts obtained expressed as colony forming units per gram of sample (cfu/g) as stated above.

Data analysis/experimental design

The experiment was performed in duplicates and the results expressed as the average. One-way analysis of variance (ANOVA) in Split plot design was performed using Cropstat (version 7.2) for the determination of significant differences in the parameters for the various processing treatment groups. Significance was accepted at ($p<0.05$) probability (Steele and Torrie, 1980).

RESULTS AND DISCUSSION

Effect of treatment methods on the proximate composition of vegetable drink extract from *Irvingia gabonensis* (African bush mango) leaves

The effect of treatment methods on the proximate composition of vegetable drink extract from *Irvingia gabonensis* is presented in Table 1. The moisture contents are, however, higher in vegetable drink from steam blanched leaves which could be due to the absorption of moisture by the leaves during the blanching process. According to Yousif et al. (1999), during the long hours of drying, heat was conducted from the surface to the interior of the leaves and the rate of evaporation of water on the surface of the leaves was faster than the rate of diffusion to the surface. The protein content is low in all the vegetable drinks, ranging from 0.02 to 0.06%. Similar observation was made by Dauthy (1995), that most fresh vegetables and fruits are high in water content, low in protein, and low in fat. The seeming increase in protein content with the various drying treatment methods may be due to the concentration of nutrients by loss of water (Finglas and Faulks, 1985). The highest value (0.06 %) was obtained in the vegetable drink from leaves dried after 6 min of blanching. The fat content of the vegetable drink from the unprocessed leaf was 2.02%. Leafy vegetables are known to be poor sources of lipids, thus the low fat content. However, the values compares with that of Baobab (1.68%) (Ihekoronye and Ngoddy, 1985). The six minutes leaf blanching resulted in a significant ($p<0.05$) increase in fat value while the rest of the processing methods led to a significant ($p<0.05$) reduction in fat content.

Although many studies have shown fat to increase with

Table 1. Effect of different treatment methods on the proximate composition of *I. gabonensis* vegetable drink extract.

Treatments	Moisture (%)	Protein (%)	Fat (%)	Fibre	Ash (%)	Carbohydrate (%)
1	96.61	0.03*	2.88	Trace	0.48	0.52
2	96.71	0.03*	1.25	Trace	0.46	2.02
3	96.80	0.03*	0.61	Trace	0.44	2.88
4	96.10*	0.02*	2.02	Trace	0.49*	2.94*
5	94.33	0.04*	0.78	Trace	1.33*	3.63*
6	95.31	0.03*	1.00	Trace	0.46	4.07*
7	95.06	0.06*	1.38	Trace	0.93*	2.72
8	94.11*	0.04*	0.60	Trace	0.44	4.97*
9	95.10	0.02	1.00	Trace	0.46	2.97
Mean	95.57	0.033	1.28	-	0.61	2.96
LSD	1.85	0.67-05	0.19	-	0.17	0.12
CV (%)	0.80	0.00	6.50	-	12.20	1.70

Any two means having difference greater than LSD (%) is significantly different at 5% level of probability. *Significant difference between processing method and control. 1 = 6 min blanched/raw; 2 = 4 min blanched/raw; 3 = 2 min blanched/raw; 4 = Control; 5 = shade dried/raw; 6 = solar dried/raw; 7= 6 min blanched/dried; 8 = 4 min blanched/dried; 9 = 2 min blanched/dried

Table 2. Effect of different treatment methods on the phytochemical composition of *I. gabonensis* vegetable drink extract.

Treatments	Total phenol (%)	Flavonoids (%)	Alkaloids (%)	Carotenoids (IU)	Saponins (%)	Steroids (%)
1	15.00	4.73	1.90	105.49*	9.36	0.86*
2	22.50*	3.54	3.24	92.83*	5.09*	0.46*
3	25.00*	4.44	2.68	151.90*	4.34*	0.94*
4	17.50*	4.53*	2.98	164.56*	9.71*	1.51*
5	72.50*	6.72*	3.86	37.97*	2.82*	0.94*
6	32.50*	5.87	2.99	59.07*	5.31*	0.48*
7	30.00*	7.00*	3.61	16.88*	7.80*	0.46*
8	47.50*	6.71*	3.40	25.32*	4.83*	0.48*
9	37.50*	6.44*	2.69	29.54*	4.57*	0.47*
Mean	33.33	5.55	3.04	75.95	5.98	0.73
LSD	4.43	1.55	1.63	2.73	1.20	0.12
CV (%)	5.8	12.1	23.2	1.6	8.7	7.0

Any two means having difference greater than LSD (%) is significantly different at 5% level of probability. *Significant difference between processing method and control. 1 = 6 min blanched/raw; 2 = 4 min blanched/raw; 3 = 2 min blanched/raw; 4 = Control; 5 = shade dried/raw; 6 = solar dried/raw; 7= 6 min blanched/dried; 8 = 4 min blanched/dried; 9 = 2 min blanched/dried.

different processing methods, the finding from this work agrees with that of Tsado et al. (2015) who in their work reported that all the methods they used in processing *Vernonia amygdalina* caused reduction in lipid. The ash content of vegetable drink from fresh leaf was low (0.49), an indication of a low mineral content. However, this was significantly ($p<0.05$) increased in the drink from shade dried (treatment 5) and 6 min blanched and dried leaves (treatment 7). The trace amount of crude fiber detected in the vegetable drink could be as a result of dilution effect and filtration process that removed most of the fiber (Kennedy, 1993). The variation in the carbohydrate content did not follow a particular trend. However, the highest increase was obtained from vegetable drink from dried leaves, attributable to the effect of drying which removed volatile moisture thereby concentrating the nutrient. Thus, the optimal blanching condition was 6 min blanched and fresh leaf extracts.

Effect of treatment methods on the phytochemical composition of vegetable drink extract from *I. gabonensis* (African bush mango) leaves

Table 2 shows the effect of treatment methods on the phytochemical composition of vegetable drink from the leaves of *I. gabonensis*. The total phenols in the vegetable drinks produced from the unprocessed leaf

Table 3. Effect of different treatment methods on the antinutrients in *I. gabonensis* vegetable drink extract.

Treatments	Tannin (mg/100 g)	Phytate (mg/100 g)	Oxalate (mg/100 g)	Cyanide (mg/100 g)
1	0.037*	9.52*	7.25*	0.04
2	0.008*	5.38	9.55*	0.04
3	0.040*	14.33*	8.25	0.04
4	0.023*	5.00*	8.15	0.03*
5	0.031	4.71	10.85*	0.08*
6	0.025	4.52	13.55*	0.05
7	0.019	11.25*	13.90*	0.10*
8	0.010*	10.33*	10.50*	0.09*
9	0.030	9.04*	10.05*	0.07*
Mean	0.02	8.68	10.21	0.06
LSD (%)	0.01	1.02	0.12	0.03
CV (%)	17.2	5.1	0.5	20.8

Any two means having difference greater than LSD (%) is significantly different at 5% level of probability. *Significant difference between processing method and control. 1 = 6 min blanched/raw; 2 = 4 min blanched/raw; 3 = 2 min blanched/raw; 4 = Control; 5 = shade dried/raw; 6 = solar dried/raw; 7= 6 min blanched/dried; 8 = 4 min blanched/dried; 9 = 2 min blanched/dried

(control).was 17.5%, which is much higher than what has been reported for some commonly consumed green leafy vegetables in Nigeria (Oboh et al., 2005). The total phenols in the drinks from processed leaves, however, varied significantly ($p<0.05$) from that of the fresh leaves, with the highest increase of 72.5% occurring in vegetable drink from the shade dried leaves. Similarly, Nayak et al. (2015) in their review, reported that processing and storage can promote or enhance the stability and quantity of phenolic antioxidants in the processed foods. In most of the cases, processing diminishes the natural antioxidants, while forming new compounds with potential antioxidative properties. These newly formed antioxidative compounds compensate the total antioxidant capacity of processed foods.

Antioxidant activity is correlated with the occurrence of phytochemicals including phenolics, flavonoids, and anthocyanins in foods (Sun et al., 2002). Phenols have antioxidant capacities that are much stronger than those of vitamins C and E (Oloyede et al., 2014). The flavonoids in the vegetable drink from fresh leaf was 4.53% and this increased significantly ($p<0.05$) with the drying methods except solar drying which gave a non-significant ($p<0.05$) increase. Similar results were reported by Mudau and Ngezimana (2014) in bush tea, who concluded that shade and freeze drying methods were shown to be more useful for retention of phytochemicals. The alkaloid content of vegetable drink from the variously treated leaves varied non-significantly ($p<0.05$) from that obtained from the fresh leaf. This result is almost in consonance with the report of Ebuehi et al. (2005) that alkaloids are relatively stable when food is subjected to heat treatment. Odufuwa et al. (2013), however, reported that the effect of processing on alkaloids content in plants and its product is controversial and inconclusive. The level of carotenoid in the vegetable drink from the fresh leaf of *Irvingia gabonensis* was found to be 164.56 IU. A significant ($p<0.05$) reduction occured in all the processed groups. However, there is much greater retention of carotenoid in the blanched fresh leaves than in the dried leaves; also among the dried groups, the solar dried had the highest retention of carotenoid. The saponins content in the vegetable drink from fresh leaf is 9.71%. The closest value of 9.36% was obtained with 6 min blanching while the rest of the treatment methods gave a significant ($p<0.05$) reduction in the saponin content. The percentage steroids in the vegetable drink from the fresh of *I. gabonensis* is 1.51% and this was reduced significantly ($p<0.05$) for all the vegetable drinks from the treatment groups.

Effect of treatment methods on anti-nutrients in vegetable drink extract from (African bush mango) *I. gabonensis* leaves

The data for the effect of treatment methods on the anti-nutrients in the vegetable drink extract of *I. gabonensis* is presented in Table 3.

The tannin content is quite low in the drink from fresh leaf (0.023 mg/100 g) and variations from the treatment groups were inconsistent. Foods rich in tannins are considered to be of low nutritional value because they precipitate proteins, inhibiting digestive enzymes and iron absorption and affect the utilization of vitamins and minerals from meals (Tinko and Uyano, 2001). The tannin contents of the vegetable drink extracts in the present study were found however to be too low to pose any danger to the users. The phytate content was slightly reduced in the vegetable drink extracts from the shade

Table 4. Effect of different treatment methods on the vitamin content of *I. gabonensis* vegetable drink extract.

Treatments	Vitamin A (IU)	Vitamin C (mg/100 g)	Vitamin E (mg/100 g)	Vitamin B_1 (mg/100g)	Vitamin B_2 (mg/100 g)
1	632.91*	0.32	6.00	7.80	3.45
2	556.96*	0.40	6.00	7.80	1.91
3	911.39*	0.37	6.25	11.00*	1.00
4	987.34*	0.35*	8.50*	8.00*	2.64*
5	227.85*	0.88*	2.75*	15.80*	6.64*
6	354.43*	0.90*	13.75*	9.00	4.73*
7	101.27*	0.83*	11.45	10.70*	9.00*
8	151.90*	0.86*	10.25	5.80	3.55
9	177.22*	0.78*	11.25	10.80*	8.36*
Mean	455.70	0.63	9.58	9.63	4.59
LSD	16.38	0.11	3.46	2.18	2.06
CV (%)	1.60	7.50	15.60	9.80	19.50

Any two means having difference greater than LSD (%) is significantly different at 5% level of probability. *Significant difference between processing method and control. 1 = 6 min blanched/raw; 2 = 4 min blanched/raw; 3 = 2 min blanched/raw; 4 = Control; 5 = shade dried/raw; 6 = solar dried/raw; 7= 6 min blanched/dried; 8 = 4 min blanched/dried; 9 = 2 min blanched/dried

dried and solar dried leaves. Significant ($p<0.05$) increases occurred in vegetable drink extracts from the 2 and 6 min blanched and from the 2, 4 and 6 min blanched and dried leaves. With the drying methods without blanching pretreatment (shade and solar drying), there was howevver, non-significant ($p<0.05$) decrease in phytate contents. The above trends are consistent with the results of other research findings. In a study on the effect of thermal processing on antinutrients in common edible green leafy vegetables (Udousoro et al., 2013), heating caused the phytate levels to decrease in all the samples analyzed. Yet in some other studies, phytate levels remain unchanged or increased after heat treatments (Yagoub and Abdalla, 2007; Martin-Cabrejas et al., 2009; Embaby, 2011). Likewise for the oxalates, there were significant increases ($p<0.05$) in the vegetable drink extracts from all the dried leaves as well as from the 4 min blanched leaf. Only the vegetable drink extract from the six 6 min blanched leaves gave a significant ($p<0.05$) reduction in the oxalate content. The cyanide in the vegetable drink extracts from fresh leaf was also low (0.03 mg/100 g). However, it is not clear why there was increased level of cyanide in all the processed groups. Significant increase ($p<0.05$) was obtained with shade drying.

Effect of treatment methods on vitamins content of vegetable drink extract from *I. gabonensis* (African bush mango) leaves

Table 4 shows the effect of treatment methods on the vitamins content of the vegetable drink extracts from *I. gabonensis* leaf. The drink extract from fresh leaf had an appreciable level of vitamin A (987.34 IU). Vitamin A belongs to the classes of vitamins known to be soluble in fat and fat solvents. They are not easily lost by ordinary cooking as is the case with the result obtained in this study with steam blanching. However, pronounced losses were observed with the drying methods. The losses were more pronounced in the drink extract from dried leaves, 227.85 and 101.27 IU for shade dried and 6 min blanched and dried respectively. Vitamin C had slight variations with blanching. However, there were significant increases in vitamin C content with drying, the highest (0.90%) occurring in vegetable drink extract from solar dried leaves followed by that from shade dried leaves (0.88%). This result does not fully agree with the findings of Babalola et al. (2010) on the effect of processing on the vitamin C content of seven Nigerian leafy vegetables. Their result showed that the vitamin C values were generally high for all the raw vegetables when compared with their processed counterparts. However, their observation that sun drying had the least effect on vitamin C content when compared with other processing methods compares with the findings in this work except that theirs did not involve shade drying. In another work that involved shade drying, Onoja (2014) in her result showed that the dried leaf curd and the sun-dried sample had similar ascorbate (0.16 and 0.18 mg/100 g) while the shade dried and fresh leaves had similar ($p>0.05$) levels (0.28 mg/100 g respectively).

Vitamin E content of vegetable drink extract from fresh leaf (control) was 8.50 mg/100 g. This was non-significantly reduced in vegetable drink extract from blanched leaf but increased with all the drying methods. Significance ($p<0.05$) was however only observed in vegetable drink extract from solar dried leaves (13.75 mg/100 g) and shade dried leaves (12.75 mg/100 g). Thiamin (vitamin B_1) and riboflavin (vitamin B_2) contents of the vegetable drink extract from fresh leaf were 8.0 mg/100 g and 2.64 mg/100 g, respectively. These values

Table 5. Effect of different treatment methods on the mineral composition of *I. gabonensis* vegetable drink extract.

Treatments	Sodium (ppm)	Potassium (ppm)	Calcium (mg/100 g)	Magnesium (mg/100 g)	Zinc (mg/100 g)	Iron (mg/100 g)
1	2.57*	6.25*	17.30*	246.00	0.00*	0.00*
2	2.57*	6.25*	22.70*	186.00	0.01	0.04
3	1.43	6.25*	19.65	218.00	0.01	0.08
4	1.43*	2.5*	19.45*	201.00	0.01*	0.08*
5	1.43	11.25*	25.80*	139.00	0.00*	0.17*
6	0.71*	10.00*	31.75*	126.00	0.02*	0.52*
7	0.71*	11.25*	33.15*	170.00	0.00*	0.58*
8	1.43	12.25*	25.05*	150.00	0.02*	0.59*
9	0.71*	13.75*	23.85*	199.00	0.01	0.41*
Mean	1.44	8.86	24.30	168.22	0.01	0.27
LSD	0.17	0.45	0.35	127.50	0.00	0.06
CV (%)	5.00	2.20	0.60	32.90	0.01	8.7

Any two means having difference greater than LSD (%) is significantly different at 5% level of probability. *Significant difference between processing method and control. 1 = 6 min blanched/raw; 2 = 4 min blanched/raw; 3 = 2 min blanched/raw; 4 = Control; 5 = shade dried/raw; 6 = solar dried/raw; 7= 6 min blanched/dried; 8 = 4 min blanched/dried; 9 = 2 min blanched/dried

are much higher than were obtained in selected Tanzanian vegetables (0.62 - 1.71 and 0.09 - 0.30 mg/100g for ascorbic and thiamine respectively) according to Mosha et al. (1995). Also, result from the present study shows that drying led to further increase (significant at $p<0.05$) in thiamin and riboflavin in the vegetable drink from the leaves of *Irvingia gabonensis* contrary to their report on the decrease in the vitamins resulting from sun/shade drying for all vegetables they examined (Mosha et al., 1995). The only exception is with drying preceding 4 min blanching.

Effect of treatment methods on the mineral content of vegetable drink extract from *I. gabonensis* (African bush mango) leaves

The effects of processing treatments on mineral contents of vegetable drink extracts are given in Table 5. The vegetable drink from fresh leaf contains a high level of magnesium (201 mg/100 g). Thus the drink is a good source of magnesium as the majority of good magnesium sources contain only about 10 % or less of recommended daily amounts (200 - 350 mg/day) (DRI, 1997). In line with report from earlier researches, there was reduction of magnesium with processing although this was non-significant ($p<0.05$). Calcium level of the vegetable drink is also appreciable and in line with other studies (Joshi and Mehta, 2010), increased significantly ($p<0.05$) with drying but variably with blanching. A comparison of the levels of potassium and iron in the vegetable drink extract from the dried leaves with that present in fresh leaves also showed significant increases ($p<0.05$). For iron, a non-significantly reduction ($p<0.05$) was observed in the drink from steam blanched leaves.

This result, however, does not agree with the report of Yan et al. (2002) that iron bioavailability increases when certain vegetables are cooked. Blanching equally caused significant ($p<0.05$) increase in potassium content but the increase was more with drying processes. The zinc content of the vegetable drink extract was quite low. But that notwithstanding, solar drying and 4 min blanching prior to drying increased zinc content significantly ($p<0.05$). The variation of sodium with processing appeared inconsistent.

Effect of treatment methods on the microbiological quality of vegetable drink extract from *I. gabonensis* (African bush mango) leaves

The effect of treatment methods on the microbiological status of vegetable drink extract from the leaves of *I. gabonensis* is presented in Figures 2 to 4. In the figures, the vegetable drink extract from fresh leaves had a total viable, coliform and mould counts of 13×10^2, 4.2×10^2 and 5.1×10^2 cfu/ml, respectively. The main microbial contamination of plant materials, in general, are attributed to total aerobic mesophilic, enterobacterial, yeast and mould (Kneifel et al., 2002). These derive from contamination from air, soil, water, insects, animals, workers, and harvesting and transportation equipment (ICMSF, 1998). The figures obtained in this study however, falls below WHO specification for plant material for use as teas and infusions, not more than 10^7 cfu/g for total aerobic microorganisms and 10^5 cfu/g for the yeasts and moulds

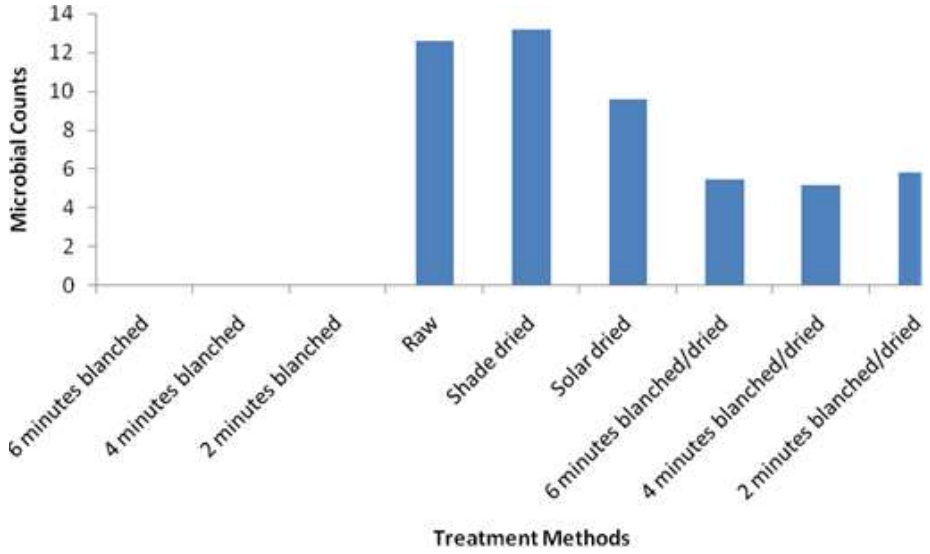

Figure 2. Effect of treatment methods on the total plate count (cfu × 10^2) of *I. gabonensis* vegetable drink extract.

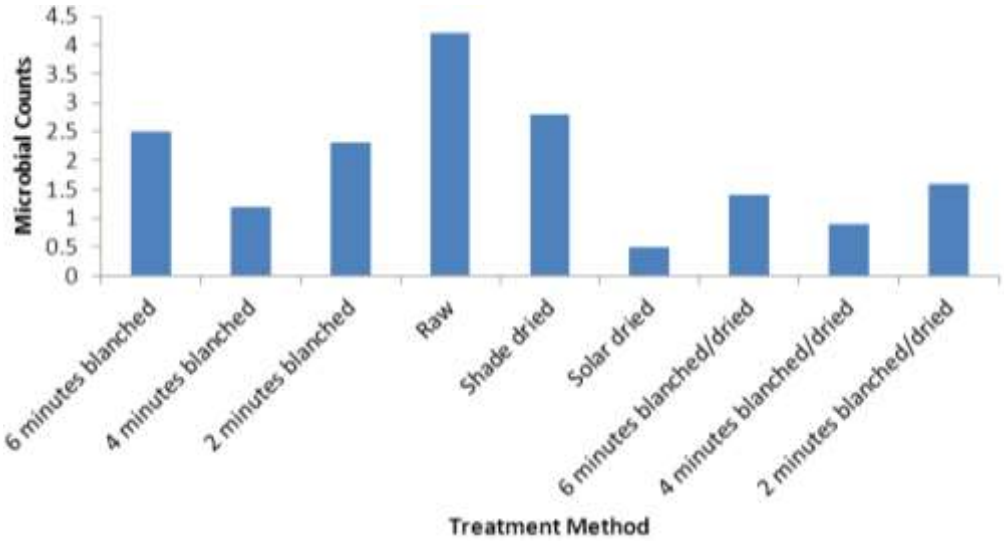

Figure 3. Effect of treatment methods on the total coliforms (cfu × 10^2) of *Irvingia gabonensis* vegetable drink extract.

(WHO, 1998). For the total plate count, negligible growth was observed in the vegetable drink extract from the steam blanched leaves. This could be as a result of the heat treatment as the reduction of plant enzyme activity and inactivation of microorganisms achieved by heating (Kneifel et al., 2002). The highest count occurred in vegetable drink extract from shade dried leaf which can be explained by the fact that the drying period would have exposed them to more contaminations, unlike the solar drying method which is faster and also provides isolated atmosphere that minimizes the contamination of the plant material (Kassem et al., 2006).

The total coliform count, as shown Figure 3, was highest in the vegetable drink from the raw unprocessed leaf (control). This finding agrees with the findings of Saranraj et al. (2012) that among the groups of bacteria commonly found on plant vegetation are mainly coliforms or precisely faecal coliforms, such as *Klebsiella* and Enterobacter (Saranraj et al., 2012). This count was however reduced by all the processing methods, with the lowest count (cfu/ml) of 0.5 × 10^2 occurring with solar drying. In Figure 4, the effect of processing methods on

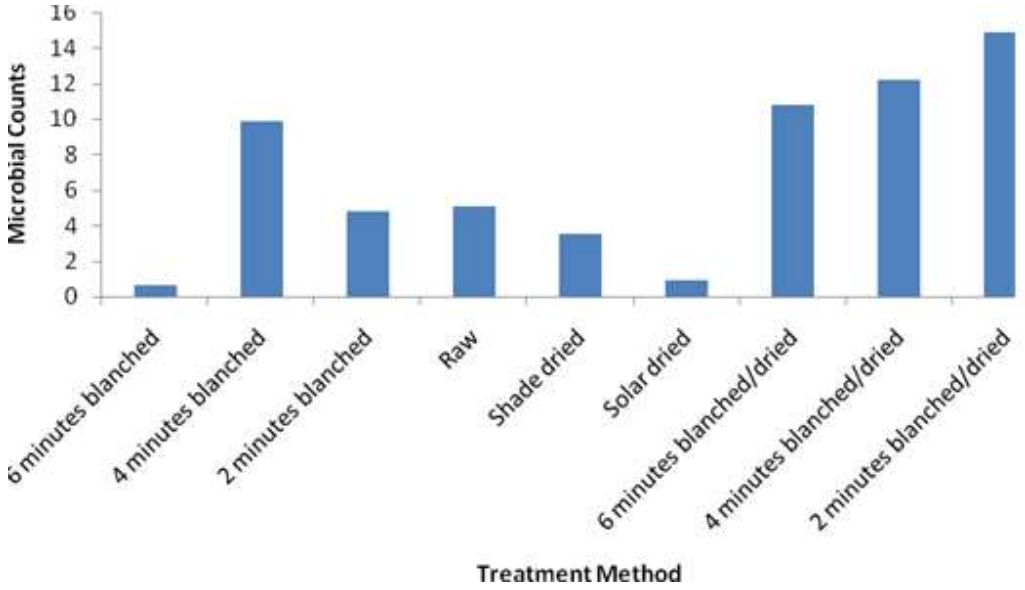

Figure 4. Effect of treatment methods on the mould counts (cfu × 10^2) of *Irvingia gabonensis* vegetable drink extract.

mould counts is shown. The highest counts occurred in vegetable drink extract from shade dried leaves while the least occurred in drink extract from solar dried leaves.

Conclusion

The present study found that moisture was the major component of the vegetable drink extract of the leaves of *I. gabonensis* and that this was fairly stable in all the aqueous products. The drink extract was also seen to have an appreciable level of phytonutrients which is an important nutritional indicator of the usefulness of this plant leaves in producing vegetable drink extract. Amongst these, total phenol, flavonoids and alkaloids were most enhanced by shade drying.while carotenoids, saponins and steroids were decreased by same. The concentrations of anti-nutrients in the vegetable drink extracts were low to significantly interfere with nutrients utilization and the effect of the different processing methods on their concentrations appeared inconsistent. For instance, the oxalates were stable with blanching but increased with drying; cyanides increased in all the processed groups while the variations of the concentrations of tannins and phytates with processing lacked a trend. The vegetable drink also had appreciable levels of vitamins A, E and B_1. Vitamin A is necessary for a broad range of bodily function, including production of vision pigments, resistance to infectious agents and maintenance of health in many epithelial cells. Vitamin E is essential for absorption of iron, slowing of the ageing process, and fertility. It is a powerful antioxidant which helps protect cells from damage by free radicals. Vitamin B complexes are necessary for converting blood sugar to energy, keeping the nervous system healthy; B_1 promotes growth. Steam blanching had a high retention of vitamin A while solar drying gave the highest increase of vitamin E. Shade drying gave the highest increase of vitamin B_1 but the least retention of vitamin E. The vegetable drink extract was also seen to be rich in calcium and magnesium which are in the group of the essential minerals that cannot be synthesised by the body and must, therefore, be ingested as part of the diet. The microbial contaminants detected in the fresh vegetable drink extract, which included the aerobic mesophiles, the moulds and the coliforms were all reduced by all the processing methiods. The leaves of *I. gabonensis* has been shown to contain important bioactive substances (phenols, alkaloids and carotenoids), minerals (K, Ca and Mg), vitamins (A, E, B_1, B_2); low in anti-nutrients (tannins and cyanide). It is therefore recommended for use in production of vegetable drink extract. Following the results that processing was not detrimental to the nutritional value of the vegetable drink extract from the leaves of *I. gabonensis*, it is being recommended that the leaves be processed so as to enhance their availability out of season. However, the selection of a processing method for use should be guided by the compounds of interest to the food processor. It is also recommended that shade drying be carried out in a clean environment, if possible in an enclosure since it has been observed from the result of this study that there could be possible exposure of the sample matrerials to greater chances of contamination than when the solar drier was used.Further research is recommended to ascertain the effect of these treatmentson the toxicological properties of the vegetable

drink extract.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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