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Comparative Physicochemical and Microbial Evaluation of Six Herbal Bitters Distributed Within Southwestern Nigeria

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article.

Abstract

Background: Medicinal herbal bitters sold as dietary supplement, digestive stimulant and detoxifier are widely distributed and consumed in Nigeria with similar claims but wide variations in prices.
Objective: Comparative evaluation of the physicochemical properties of six brands of bitters with the aim of proposing possible justification for their interchangeability.
Materials and Methods: The herbal bitters were subjected to physicochemical and phytochemical evaluation; organoleptic test, phytochemical screening, thin layer chromatography (TLC), heavy metal content, total phenolic acid and antioxidant activities as well as microbiological assessment to determine microbial load and antimicrobial properties.
Results: All the bitters were acidic (pH 2.35 - 5.63), of varying colours (orange to brown), characteristic odour and bitter taste with different types and amount of phytochemicals. The TLC profile varied from three to eight spots. The Fe, Zn and Cu content were within acceptable limit. Total phenolic acid content varied from 528.33±33.71 to 1589±11.92mgGAE/ml, while antioxidant properties gave IC_{50} of 4.28±0.01 to 9.54±1.24µg/ml. No direct correlation between the total phenolic contents and antioxidant activity was observed. Microbial evaluation showed absence of bacteria with fungi level within official specifications. Four brands were active on six bacterial clinical isolates, while one brand was totally inactive. Variations in physicochemical parameters does not have any direct correlation with antioxidant and antimicrobial activities, and does not reflect in the variation in the constituent plants, but may be due to possibly processing methods.
Conclusions: The samples were highly distinct from each other, and could not be substituted for each other.

Keywords: Herbal bitters, Total phenolic acid, Antioxidant properties, Heavy metals, Antimicrobial properties

INTRODUCTION

Herbal products have been used since ancient times for the treatment of a wide range of diseases (National Policy for Assessment of Herbal Products, 2007). They usually consist of different plants or plant parts or combinations whether in crude state or as plant preparations. Traditional practice is now being coupled with up to date scientific methodology, and processed dosage forms are now being produced and marketed. Over 90% of people in Africa and 70% in India rely on herbal medicines for some of their primary health care (Wachtel-Galor and Benzie, 2011; Bodeker and Kronenberg, 2002).
There are various types of herbal products of which herbal bitters constitutes a key member of this group of products. Medicinal herbal bitters containing blended ingredients in water or alcohol (tincture) base, proposed for use primarily as digestive stimulants, detoxifiers and antibacterial agents were originally sold as a digestive aids because of their ability to increase the production of saliva and digestive juices (Ales, 2002). Herbal bitters contain various secondary constituents such as alkaloids, flavonoids, phenols and polyphenols responsible for scavenging free radicals; and oils which contribute the "aromatic" taste and odour (WHO, 1998). They are claimed to be formulated to support the immune system and body's ability to resist disease. Thus, they are proposed to effectively maintain the overall health and well-being as well as management of diverse human ailments.
In Nigeria, the use of herbal medicinal products including herbal bitters cuts across the different strata of the economy i.e. from the low income to the elites, as a result of which there have been an increase in the use of these products. The increase in use has led to an increase in the commercialization of the herbal preparations.
The high level of distribution and advertisement of these bitters by the various audio and visual media houses

necessitated the need for comparative evaluation as well as propose possible justification for their use and interchangeability. This study evaluated the physicochemical and microbiological properties of six brands of herbal bitters that are widely distributed within the Southwestern states of Nigeria. Physicochemical properties such as organoleptic properties, pH, phytochemical screening, anti-oxidant potential and antimicrobial activity were evaluated.

Six commonly advertised and used herbal bitters were sourced from drug distribution outlets in Southwestern Nigeria. The herbal bitters investigated are Field Swedish bitters (FSB), Yoyo Cleanser' bitters (YB), Evans healthy bitters (EB), Darasi bitters (DB), Jossy cleanser bitters (JB) and Swedish bitters (SB). The component herb in the various brands, dosage form, price, manufactured date, expiry date, batch number and NAFDAC numbers were noted (Table 1).

Table 1: Information on the six brands of herbal bitters investigated

Code	Manufacturer	Country	Batch no	NAFDAC No.	Component herbs	Retail Price (₦)
SB	Dr. Theiss Naturwaren GmbH, Michelinstraβe 10, D-66424 Homburg. For Starling Nigeria Ltd, Surulere, Lagos, Nigeria.	Germany	020811	A7-0125L	*Aloe, Senne, Rhubarb, Zedoary, Manna, Theriac Venez S. Opio, Angelica, Myrrh, Carline Thistle, Camphor, Saffron*	1,500
JB	Aderonsko Global Investment Ltd, Modakeke, Osun State	Nigeria	0004	A7-0530L	*Khaya senegalenisis, Piper guineensis, Aloe vera, Citrullus lanatus, Allium Sativum*	270
YB	Abllat company Nigeria limited	Nigeria	777AD	04-5347L	*Aloe vera, Acinos arvensis, Citrus aurantifolia, Chenopodium murale, Cinamomum aromaticum*	800
FSB	Health from the Fields, Lagos	Nigeria	263COM	A7-0145L	*Cape aloe, Cammiphora molmol, Crocus sativus, Cassia angustifolia, Cinnanomum camphora, Rheum palmatum, Curcuma zedoria, Bamboo mama. Theriac venecian, Angelica archangelica, Artemisia vulgaris*	710
DB	Boss Cognoscenti Ventures, Lagos	Nigeria	000AD2	A7-0589l	*Aloe vera, Alium cepa, Teminalia glauces*	250
EB	Siddhaya Ayrcedic Res. Foundation Pvt Ltd. For Evans Medical Plc, Lagos, Nigeria	India	9114	A7-02491	*Alhagi comelorun, Cassia angustfolia Commiphora myrrha, Andrographis peniculata, Picrohiza kurroa, Tinospora cordifolia, Aloe barbadens, Crocus sativus*	600

Note: All the samples were within their shelf life as at the time of the study

METHODS

Physiochemical analysis of herbal bitters

a. *Visual appearance and organoleptic properties*: the colour, taste, odour and clarity of the samples were noted.

b. *pH determination*: the pH of the samples were determined using a pHmeter (Hanna HI 8521, Portugal).

c. *Thin layer chromatography analysis (TLC):* this was carried out using Silica gel GF_{254} as stationary phase and two mobile phases; M_1 [ethyl acetate: methanol: ammonia (17:6:5)] and M_2 [chloroform: methanol (95:5)]. Visualization was done using daylight, ultraviolet light (254nm and 365nm), and iodine vapour.

d. *Trace element determination:* The samples were analysed for chromium, lead, zinc, iron and copper levels using atomic absorption spectrometry. The trace metals were analysed at various wavelengths as follows; chromium (357nm), lead (283.3nm), zinc (213.9nm), iron (248.3nm) and copper (325nm) (Kebbekus and Mitra, 1998).

e. *Phytochemical screening:* Tests for alkaloids, tannins, saponins, cardenolides and anthraquinones were done according to conventional methods (Sofowora, 2008).

Total phenolic content determination

This was determined using Folin-Ciocalteau method (Singleton and Rossi, 1965). The method involves addition of 100µL of the sample to 100µL of Folin-Ciocalteau reagent (500mg/L), mixed properly and incubated in the dark for 2minutes. This was followed by the addition of 2ml of Na_2CO_3 solution (0.2% w/v) to the mixture after which the mixture was allowed to stand in the dark for 30minutes at 25 ^oC. The absorbance was measured at 750nm using UV-VIS spectrophotometer (Perkin Elmer, Lambda 25, Singapore) against a reagent blank. The total phenolic content was determined using the standard Gallic acid calibration curve and the results expressed as mgGAE/mL. The procedure was repeated in the absence of the sample to obtain the blank reading. The determinations were done in triplicates.

Determination of antioxidant activity

The antioxidant capacity was determined using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity assay (Mensor *et al*, 2001). One ml at 0.3Mm DPPH reagent in methanol was added to 2.5ml of the varying concentrations (0, 5, 10, 15, 20, 25, 30, 35µg/ml) of the sample solutions and mixed properly. The mixtures were incubated at room temperature for 30min after which the absorbance were determined at 520nm using a UV/Vis spectrophotometer (Perkin Elmer, Lambda 25, Singapore). The determinations were done in triplicate for each sample preparation. The negative control involves the use of methanol (2.5ml) and 1ml of DPPH. The procedure was repeated with gallic acid and ascorbic acid at similar concentrations. These served as the standards. The DPPH radical-scavenging activity was calculated using the following equation;

% DPPH scavenging = [Abs (control) – Abs (sample) /Abs (control)] x 100

Where Abs (control) is the absorbance of the negative control reaction; containing all the reagents except the test compound, while Abs (sample) is the absorbance of the sample.

Microbial evaluation of herbal preparations

Determination of microbial load: sample solutions (1ml) at serial dilutions 10^{-1}, 10^{-2} and 10^{-3} dilution levels were plated on Nutrient Agar, *Salmonella shigella* agar, Mcconkey Agar and Potato Dextrose Agar in labeled sterile petri dishes. All the agar plates were incubated at 37^oC for 48hour except Potato Dextrose Agar which was incubated for 72hours at 25^oC.

Antimicrobial activity evaluation: Agar-well diffusion method was carried out using overnight broth cultures (10^6cfu/ml) of six (6) clinical isolates of the following microorganisms*;* one strain each for *Klebsiella sp* and *Pseudomonas aeruginosa*, and two strains of *Escherichia coli* and *Staphylococcus aureus*. The herbal samples (30µl) were introduced into labeled agar wells (5mm) bored into the seeded agar plates containing the different microorganisms. Streptomycin was used as a positive control. Zones of inhibition were measured after incubation of the agar plates at 37^oC for 24hours.

Statistical analysis

The results obtained were presented as mean ± SEM and subjected to statistical analysis using SPSS 17.0; student t-test and ANOVA were applied with the Duncans multiple range tests used for the post test where appropriate, with differences considered significant at $p< 0.05$.

RESULTS

The safety and quality of medicinal preparations have become a major concern for health authorities including pharmaceutical industries and the general public (WHO, 2007). All the herbal bitters investigated are liquids, and were within their indicated shelf life as at the time of the study. Organoleptic evaluation revealed a variation in colour from orange to brown with characteristic odour and bitter taste with one brand showing the presence of sediments (Table 2). The colour and odour are quite in line with herbal product, since various types of herbs were combined to achieve the preparation, while the bitter taste corroborates the expected nomenclature of the product; herbal bitters. Most people believe that bitter medicinal products are good for the body system and this may account for the high consumption of herbal bitters. All the herbal bitters under investigation are acidic with pH ranging from 2.35 to 5.63 (Table 2). This may constitute a problem if taken in the absence of food. However this has been taken care of in the dosing instruction which states administration after meals.

Table 2: Organoleptic and Physicochemical properties of the six herbal bitter

Sample	Colour	Odour	Taste	Clarity	pH range	No of TLC Spots	
						M_1	M_2
SB	dark brown	pungent	bitter	slight	5.62-5.63	6	6
JB	Reddish brown	present	bitter	absent	2.86-2.87	5	8
YB	Reddish brown	Slightly present	bitter	absent	3.21-3.22	4	4
FSB	brown	present	bitter	present	5.05-5.07	8	7
DB	wine red	Honey-like	bitter	absent	2.35-2.36	3	5
EB	Golden brown	present	minty bitter	absent	3.29-3.30	3	2

Note:
M_1 – Mobile phase 1 – Ethylacetate: Methanol: Ammonia [17: 6: 5]
M_2 – Mobile phase 2 – Chloroform: Methanol [95: 5]

Thin layer chromatographic (TLC) profiling of all the samples in the different mobile phases showed a wide variation in the number of spots/ components identified which cannot be directly correlated with the number of the component herbs or the medium of the dosage form i.e. alcohol or aqueous. Ethyl acetate: methanol: ammonia (17:6:5) and chloroform: methanol (95:5) showed the highest number of components for most of the samples; FSB, SB and JB samples gave the highest number of spots ranging from six (6) to eight (8) (Table 2). Various phytochemical gives different R_f values in different solvent systems (Talukdar *et al*, 2010).

A trace element is a dietary mineral that is needed in very minute quantities for the proper growth, development, and physiology of the organism [Bowen, 1966]. The use of agrochemical products such as fertilizers and pesticides during the cultivation of the herbal component plants can be a source of physicochemical contaminants such as trace metals (Anim *et al*, 2012). The trace metal content of the herbal bitters under investigation showed the absence of chromium and lead in all the samples, while three of the samples were devoid of copper (Table 3). The levels of zinc was far below the 50mg/Kg allowable limit for herbal products (WHO, 2005; WHO, 2006). The WHO is yet to determine the limits for iron and copper (Dghaim, et al, 2015). Trace metals are well noted for their wide environmental dispersion, their tendency to accumulate in selected tissues of the human body and their potential to be toxic at relatively lower levels of exposure (Anim *et al*, 2012).

Table 3: Trace metal content of the six herbal bitters

Samples	Trace metal (mg/L)		
	Fe	Cu	Zn
SB	2.99±0.00	-	0.35±0.00
JB	2.68±0.00	-	0.44±0.00
YB	2.14±0.00	-	0.25±0.00
FSB	0.89±0.00	0.09±0.00	0.25±0.00
DB	1.38±0.00	0.07±0.00	0.12±0.00
EB	2.16±0.00	0.04±0.00	0.28±0.00

Discovery of novel drugs from plants is based on the essential information regarding the chemical constituents which are generally provided by the quantitative phytochemical screening of plant extract. These chemical substances known as plant secondary metabolites are known to possess a wide range of pharmacological activities (Sofowora, 2008). Phytochemical investigation of all the herbal products showed the presence of alkaloids, cardenolides and anthraquinones. Saponins were detected in all the samples except one, while tannins were absent in two samples (Table 4).

Total phenolic content is an indicator of the antioxidant behavior and contributes significantly to the total antioxidant activity of medicinal and aromatic samples (Wang *et al*, 1996). The total phenolic content of the various brands of herbal preparation using the Gallic acid calibration curve ($y = 0.001x + 0.09$, $R^2 = 0.979$) as determined by Folin-Ciocalteu method ranged from 528.33±11.47mg GAE/ml to 1589.70±11.92mg GAE/ml.

There was a significant difference in the obtained values from the different brands ($p<0.05$); three brands had similar value while the remaining three brands were statistically different ($p<0.05$) (Table 5).

The antioxidant assay using 2,2-diphenyl-1-picrylhydrazyl (DPPH) revealed varying scavenging activities at 5 – 35 (µg/ml) for the six herbal bitters and the controls (gallic and ascorbic acid), with four of the samples showing significantly higher scavenging activities than the controls, while one sample was lower (Fig. 1, Table 5). The half maximal inhibitory concentration ($1C_{50}$) is defined as the concentration of substrate that causes 50% loss of the DPPH activity (Molyneux, 2004). The IC_{50} obtained in this study ranged from 4.28±0.01 to 9.54±1.24 µg/ml. The higher the percent (%) inhibition of DPPH the lower the free radical scavenging activity and antioxidant power (Quian and Nihorimbere, 2004). The antioxidant activity of plants has been related to the bioactive principles.

Table 4: Phytochemical content of the six brands of herbal bitters

Phytochemicals	Samples					
	JB	EB	YB	SB	DB	FSB
Alkaloid						
Dragenduff's reagent	+	+	+	+	+	+
Mayer's reagent	-	+	-	-	-	-
Wagner's reagent	-	+	-	-	-	-
Cardenolide						
Keller-killiani	+	+	+	+	+	+
Kedde	+	+	+	+	+	+
Anthraquinone	+	+	+	+	+	+
Saponin	+	+	-	+	+	+
Tannin	+	+	-	+	-	+

+ = Present
- = Absent

Table 5: Total phenolic acid (TPA) content and IC_{50} of the six brands of herbal bitters

Sample	TPA (mgGAE/mL) (Mean± S.E.M)	IC_{50} (µg/L) (Mean± S.E.M)
SB	850.33±19.46	4.60±0.02
FSB	528.33±11.47	6.40±0.02
YB	566.00±16.92	4.82±0.02
DB	827.33±3.38	4.28±0.01
EB	771.67±18.41	5.16±0.01
JB	1589.70±11.92	9.54±1.24
Gallic acid	-	6.21±0.00
Ascorbic acid	-	6.35±0.02

Antioxidants are compounds that can delay or inhibit the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidizing free radical reactions (Velioglu *et al*, 1998). Phenolic compounds and flavonoids have been reported to be associated with antioxidative action in biological systems, acting as scavengers of singlet oxygen and free radicals (WHO, 2007).

Plant phenolics are a major group of compounds acting as primary antioxidants or free radical scavengers (Rice-Evans et al, 1997; Seo et al, 2004; Ayoola et al, 2008).

The results obtained from this study did not indicate direct correlation between the total phenolic contents and antioxidant activity for most of the herbal preparations. The sample with highest total phenolic contents gave the highest IC_{50} value indicating the least scavenging activity. This shows that phenolic compounds were not the only major contributors to the antioxidant activity for such herbal preparations. The relationship between the antioxidant activity and the phenolic compounds have been reported to depend on several factors such as chemical structure of individual components, synergistic interaction among them and specific conditions applied in the different assays (Huang *et al*, 2005; Ciz et al. 2010).

Table 6: Fungi load (cfu/mL) and Zones of inhibition (mm) of the six brands of herbal bitters on bacterial clinical isolates

Herbal Bitter	Antibacterial activity						Fungi load (cfu/mL)
	Zone of inhibition (mm)						
	Klebsiella sp	*Ps. aeruginosa*	*E. coli-I)*	*E. coli-II)*	*St. aureus-I*	*St. aureus-II*	
SB	6.7±0.3	6.7±0.3	7.6±0.3	9.7±0.3	9.7±0.3	9.7±0.3	11.3±0.9
JB	3.8±0.2	NA	NA	NA	NA	2.0±0.0	10.7±1.4
YB	NA	NA	NA	NA	NA	NA	80.0±0.2
FSB	9.3±0.3	12.7±0.3	9.7±0.3	8.3±0.3	8.0±0.0	8.7±0.3	11.7±0.3
DB	11.7±0.3	11.7±0.3	19.3±0.3	19.0±0.6	15.0±0.0	13.7±0.3	87.0±3.2
EB	8.3±0.3	6.8±0.2	19.7±0.3	7.7±0.3	6.3±0.3	8.3±0.3	18.3±0.9
Streptomycin (Positive control)	41.3±2.8	36.3±0.3	42.7±0.3	20.7±0.3	24.7±0.3	41.0±0.6	-

Note: NA – No Activity

The presence of microorganisms including moulds have been adduced to failure to control the moisture levels of herbal medicines during transportation and storage, as well as from failure to control the temperatures of liquid forms and finished herbal products (WHO, 2007). This may indicate poor quality of production and harvesting practices. The microbial evaluation of the samples showed absence of bacterial contamination, while fungi level obtained for all the samples ranged from 10.67±1.45 to 87.00±3.22 cfu/ml (Table 6). A significant variation in the level of contamination with fungi was observed; two samples were similar and significantly higher in comparison with the remaining four samples which were lower and not significantly different from each other. The acceptable limit of yeasts and moulds for herbal products for internal use is 10^3 per gram (American Herbal Products Association (AHPA), 2015; WHO, 2007) which was not exceeded by any of the samples.

Antimicrobial activity evaluation of the herbal bitters showed that four samples were active against all the organisms tested with varied zones of inhibition, while one sample was not active on any of the organisms. However none of the products gave comparable activity to the streptomycin used as positive control (Table 6).

The use of herbal bitters as digestive stimulants, detoxifier and antibacterial agents is corroborated by the results obtained for almost all the samples. The variations in the various parameters evaluated in this study are a reflection of the variation in the constituent plants and possibly processing methods. Also, the variation in dosage and

price does not have any direct correlation with the evaluated antioxidant and antimicrobial activities. Ironically, DB which was the most acidic sample (pH of 2.35 – 2.36) and which contains the least number of plants gave the highest antioxidant activity (IC_{50} of 4.28µg/ml) with antimicrobial activity against all the microorganisms, and was also the cheapest. Furthermore, the higher antioxidant properties of some of the preparations when compared with those of gallic and ascorbic acids used as standards may indicate the need for dosage limits for these preparations.

It can be concluded from the outcome of this study that the six herbal bitters were highly distinct from each other and could not be substituted for each other.

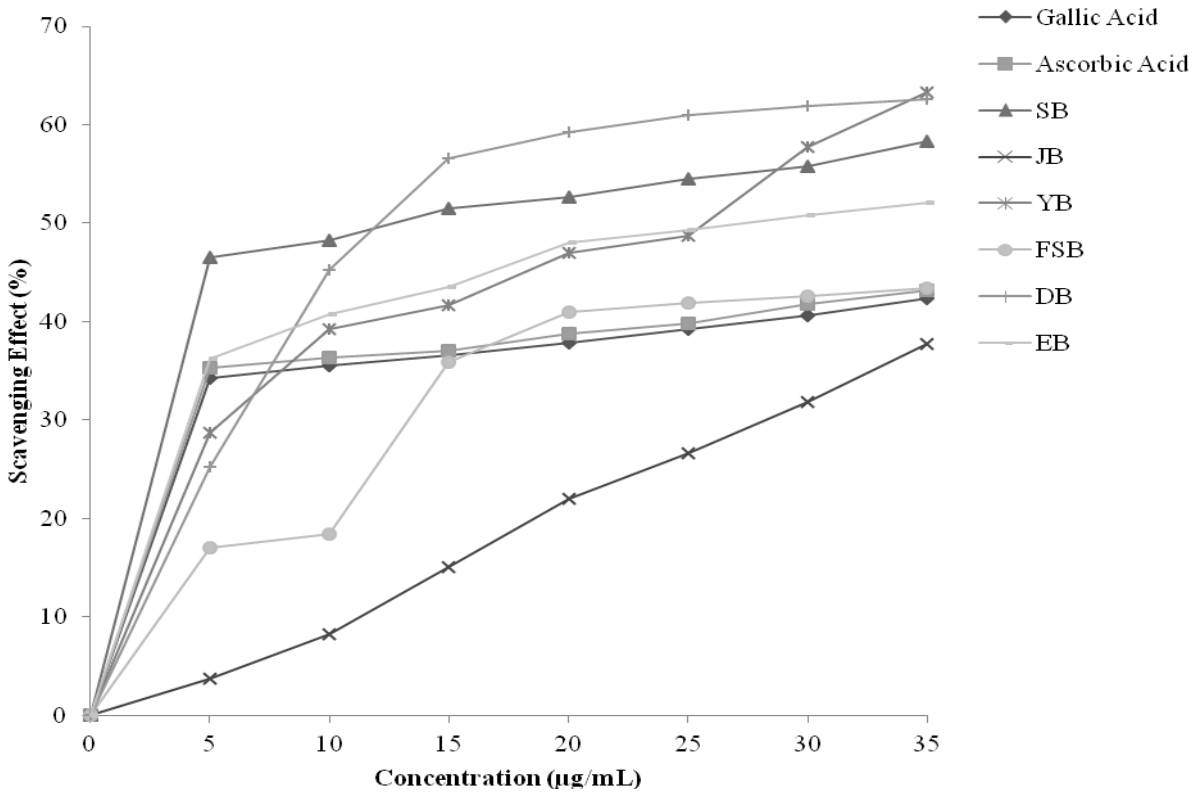

Figure 1: Percent inhibition of DPPH at different concentration of the herbal bitters and standards (ascorbic and gallic acid)

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