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Composition and *in vitro* Antioxidant Capacity of *Paxherbal Bitters*

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Abstract
"Herbal bitters" is a beverage, often alcoholic, flavoured with herbal essences that gives it a bitter taste. This present study aims to ascertain the composition and invitro antioxidant capacity of Paxherbal bitters, with the hope that this will help give some credence to its broad pharmacological claims. Using standard laboratory procedures and assay protocols, its proximate analysis revealed high moisture content, moderate carbohydrate, protein, fat, ash and reducing sugars and no crude fibre. Its qualitative phytochemical analysis revealed amino acids, proteins, saponins, tannins, alkaloids, glycosides, terpenoids and flavonoids, while its quantitative phytochemical and elemental analysis revealed moderate alkaloids, flavonoids, total phenol, saponins, Ca, Mg, Na, K, P, Fe, Zn, Mn, and Cu, with low tannins and cyanogenic glycosides. The alcohol content of Paxherbal bitters is high (44.34±1.13%). The invitro total antioxidant capacity, DPPH (1-diphenyl-2-picrylhydrazyl) free radical scavenging activity and the hydroxyl radical scavenging capacity, indicate that the bitters have inherent antioxidant capacity. This study therefore reveals that Paxherbal bitters contain phytochemical constituents that rightly put it in the class 'herbal bitters'. Relating the results of this study to the already established effects of its constituent phytochemicals and minerals gave evidence to the possibility of Paxherbal bitters, being able to have pharmacological properties ranging from hypolipidaemic, hypoglycaemic and immunity-boosting to choleretic, hepatoprotective, antioxidant, anticarcinogenic, diuretic, vasodilatory and antihypertensive, as well as the ability to prevent cardiovascular diseases.
Keywords: Paxherbal bitters, proximate, phytochemical, antioxidant, pharmacological

Introduction
Modern science may have widened for some time the differences in terms of medication between orthodox and unorthodox/traditional medicine, this gap seems to be closing fast as the current trend is that they are both adopting practices from each other (1, 2). This has led to the resurgence of an ancient remedy for digestive problems in the repackaging of "herbal bitters" and products like it in an "orthodox way".
The term "bitters" as it is used presently, is a beverage, often alcoholic, flavoured with herbal essences that gives it a bitter or bittersweet flavour. The generic term applies to all bitter liquors and herbal bitters. Bitters are produced from herb and root extracts, from the narcotic components of (primarily) tropical and subtropical plants and spices. They are usually dark in colour and valued for their ability to promote appetite and digestion hence their use as patent medicine and as digestive aids and as flavouring in cocktails. Bitters are made up of a numerous groups of chemical compounds extracted from the herbs and roots that have the common characteristic of a bitter taste and act to increase the vital energy centres in the body (3, 4).
Bitters carry out their effect both by their "active principles" and their "bitter principles". Active principles are substances or substance groups definable by chemical analysis that essentially contribute to the therapeutic action of a medicinal herbal preparation (5). Bitter principles on the other hand are substances or substance groups definable by chemical analysis that essentially contribute to the bitterness (and therapeutic action) of a medicinal herbal preparation (3, 4, 5). The bitterness in herbal bitters is often described as being due to a 'bitter principle', but this may be a volatile oil, an alkaloid, iridoid or a sesquiterpene (2, 3). These active or bitter principles are essentially phytochemicals that are found in the herbs used in making the bitters.
According to Anandanayaki(6), "Phytochemicals are chemical compounds formed during the plants normal metabolic processes. These chemicals are often referred to as "secondary metabolities" of which there are several classes including alkaloids, flavonoids, coumarins, glycosides, gums, polysaccharides, phenols, tannins, saponins, terpenes and terpenoids (7, 8, 9, 10).
Bitter herbs are made up of a numerous groups of chemical compounds extracted from the leaves, stem, fruits and roots of plants, they have the common characteristic of a bitter taste. Their phytochemical composition includes a complex pattern of molecular structures. The bitter substances are mostly of terpenoid structures, especially the sesquiterpene lactones, monoterpene iridoids and the secoiridoids (3, 4, 11). The major classes of substances that rightly confer on them the name "bitters" are amino acids, proteins, saponins, tannins, alkaloids, glycosides/reducing sugar, terpenoids and flavonoids (12)
Pax- Herbal Bitters- is locally produced in Edo State, it is composed of forty (40) herbal constituents which includes: *Cymbogon citratus* (Lemon grass), *Aloe vera* (True aloe, Lily of the desert), *Gongronema latifolium* (Utazi), *Zingiber officinale* (Ginger), *Xylopia aethiopica* (Uda), *Vernonia amygdalina* (Bitter leaf), *Tridax*

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procumbens (Tridax daisy, coat buttons), *Capsicum annuum* (Green Pepper), *Carica papaya* seed (Pawpaw seed), *Glycine max* (Soya bean leaves), *Garcinia kola* (Bitter Kola), *Citrus limon* leaves (Lemon leaves), *Uraria picta* (Prishnaparni), *Viscum album* (Mistletoe), *Aloe barteri* (African Aloe), *Citrus aurantifolia* (Lime), *Persea americana* (Avocado pear), *Eucalyptus officinalis* (Thunder Protector/fever tree), *Musa paradisiaca* (French plantain) and *Morinda Lucida* (Brimstone tree), etc, (13). The history surrounding the bitters often make it to be summarized as a product that stimulates the body's metabolism but most especially digestive secretions. It is said to improve digestive functions that results in increased food consumption and digestion in such a way that even sluggish digestion and constipation are ameliorated (3, 14).The present claim that modern bitters remedies are for control and treatment of a broad range of ailments (13) coupled with their increasing popularity in Nigeria that has influenced the present "orthodox" research to determine the composition and *in vitro* antioxidant capacity of one of these herbal bitters called *Paxherbal bitters* that is presently being marketed as a "cure-all"- patent medicine in Nigeria.

MATERIALS AND METHODS

***Material*:** *Paxherbal bitters* was purchased from the manufacturers at the Benedictine Monastery at Ewu-Ishan in Edo State. The bitters were bought as liquid formulations and stored at room temperature throughout the period of the study.

***Proximate Analysis*:** The moisture content of the herbal bitters was determined using the gravimetric method (15). The ash content was estimated using the method of AOAC (16). The fat content was determined using the method of AOAC (17). The crude fibre content was determined by the difference in weight after calcination, following the digestion of the sample in sulphuric acid and sodium hydroxide solutions and the residue being calcined (18). The protein content of the bitters was determined from the organic nitrogen content by Kjeldahl method (18). The carbohydrate content of the bitters was determined by the difference method by adding moisture, fat, protein, and ash content, and the value deducted from 100 (18). The reducing sugar content of the bitters was determined using the dinitrosalicylic acid method (19). The glucose content was determined using the glucose oxidase method (20). Triplicate measurements were performed and the mean computed.

Alcohol Composition Analysis: The determination of the alcohol content was done using the AOAC (16) method.

***Extraction of Bitters for Qualitative Phytochemical Analysis*:** because there may be differential solubility in the constituent of the bitters relative to the polarity of the solvent used the bitters were further extracted in distilled water and ethanol.

Aqueous Extraction: 10ml of bitters sample was added to 90ml distilled water and boiled on slow heat for 2hours. It was then filtered using a Whatmann No. 42 filter paper (125mm); the filtrate was collected, and further concentrated to make the final volume one-fourth of the original volume and stored at 4^{o}C in an air tight container (21).

Preparation of ethanolic extract: 10ml of the bitters sample was taken and put into 50ml of absolute ethanol in a flat bottom flask. The flask was plugged with cotton wool and then kept on a rotary shaker at 190-220rpm for 24hrs. After 24 hours the sample-solvent mix was then filtered using a Whatmann No. 42 filter paper (125mm); the filtrate was collected, and the solvent evaporated to make the final volume one-fourth of the original volume and stored at 4^{o}C in an air tight container (21).

Qualitative Phytochemical Analysis: The test for phytosterol/steroids, amino acids, protein, saponins, tannins, alkaloid, cardiac glycoside, terpenoid, flavonoids, phlobatannins, was determined using the method described in Santhi and colleagues (21). The test for glycosides/reducing sugars was carried out using the method described by Onyeike and Osuji (18). The Borntrager's test was used for the detection of anthraquinones (22).

Quantitative Phytochemical Analysis: The determination of total phenols was by spectrophotometric method (23), while alkaloids was as described by Harborne (7) and Edeoga and colleagues (23). The determination of tannin was by the Van – Burden and Robinson method (23), while the determination of saponin was as described by Edeoga and colleagues (23). The determination of flavonoids was by the method of Boham and Kocipai-Abyazan (24), while that of cyanogenic glycosides was by the AOAC (16) method.

Elemental Analysis: Extraction and determination of calcium and magnesium in the bitters sample were determined by the titrimetric method using ethylenediaminetetraacetic acid (EDTA), (16). The sodium and potassium concentrations were determined using the flame photometer (JENWAY PFP 7 model) (16), while phosphorus was determined by Olsen and Sommers (25) and the AOAC (16) methods. The determination of the trace metals iron, zinc, manganese, copper, lead, chromium and selenium were done using the bulk scientific VGP210 atomic absorption spectroscopy/spectrophotometry, (16).

***Determination of the Total Antioxidant Capacity*:** The ability of the herbal bitters to scavenge 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) radical cation ($ABTS^{+}$) which gives an idea of the total antioxidant capacity, was determined by the improved spectrophotometric version described by Re and colleagues (26).

ABTS radical cation decolourisation assay: In this improved version, $ABTS^{+}$-the oxidant, will be pregenerated by persulfate oxidation of 2,2,-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) – ($ABTS^{2-}$). Triplicate measurements were performed and the mean computed.

DPPH free radical scavenging activity: *D*PPH (2,2-diphenyl-1-picrylhydrazyl radical) scavenging activity of the herbal bitters was measured by the spectrophotometric method described by Jain and colleagues (27). Triplicate measurements were performed and the mean computed.

Hydroxyl radical scavenging activity: This was assayed as described by Kunchandy and Rao (28) with a slight modification (29). The assay is based on quantification of the degradation product of 2-deoxyribose by condensation with TBA. Triplicate measurements were performed and the mean computed.

Statistical analysis: The results are expressed in Mean±SEM. Students t-test was used to compare the means. $P<0.05$ was considered significant.

Results

Table 1: Proximate and Alcohol Composition of the Paxherbal Bitters

Proximate Composition	Ash	Moisture	Fibre	Fat &Oil	Protein	Carbohydrate	Glucose	Reducing Sugars	Alcohol
Quantity in Paxherbal Bitters (%)	1.81±0.13	88.76±2.21	0.00±0.00	5.06±0.20	0.94±0.05	2.41±0.02	0.11±0.01	0.92±0.02	44.34±1.13

The results were determined in triplicates and the values are expressed as Mean±SEM.

Table 1 shows that Paxherbal bitters has a high moisture and alcohol content, no fibre and a higher fat content compared to other food nutrients.

Table 2: Qualitative and Quantitative Phytochemical Composition of Paxherbal Bitters

	Qualitative Composition of Paxherbal Bitters		Quantitative Composition of Paxherbal Bitters (%)
Parameters	Aqueous Extract	Ethanolic Extract	
Phytosterol	-	-	ND
Amino acids	+	+	ND
Proteins	+	+	ND
Saponins	+	+	2.90±0.15
Tannins	++	++	0.11±0.00
Alkaloids	+	+	0.20±0.02
Cardiac Glycoside	-	-	ND
Glycosides/Reducing Sugar	-	+	ND
Cyanogenic Glycoside	ND	ND	0.18±0.01
Terpenoids	+	++	ND
Flavonoids	+	+	0.84±0.03
Phlobatannins	-	-	ND
Anthraquinones	-	-	ND
Total Phenol	ND	ND	0.62±0.02

Key: +++ = Highly Present; ++ = Moderately Present; + = Slightly Present; - = Absent; ND = Not Determined

Table 2 shows that considering both the aqueous and ethanolic extracts of the bitters, the bitters used in this research contains amino acids, proteins, saponins, tannins, alkaloids, glycosides/reducing sugar, terpenoids and flavonoids but did not contain plant sterols (steroids), cardiac glycosides, phlobatannins and anthraquinones. As the key indicates, these phytochemicals were present in varying degrees of slight and moderate. The quantitative phytochemical composition of the bitters, indicate saponins as the highest constituent and tannins the least among those determined.

Table 3: Quantitative Mineral Composition of the Paxherbal Bitters

Mineral Composition	Ca	Mg	Na	K	P	Fe	Zn	Mn	Cu	Pb	Cr	Se
Quantity in Paxherbal Bitters (mg/100ml)	50.10 ±0.95	1215.00 ±20.82	3.10± 0.26	185.00 ± 2.65	57.19± 1.54	9.13± 0.09	5.00± 0.58	3.12± 0.16	3.25± 0.14	<DL	<DL	<DL

Where <DL means less than detection limit. Triplicate measurements were performed and the mean computed. The values are expressed as Mean±SEM.

Table 3 summarizes the means of the quantitative elemental composition of the bitters, with magnesium being the highest constituent and sodium the lowest. The results show that the bitters contain significant amounts of Na, K, P, Ca, Mg, Zn, Mn, Fe, and Cu, while Pb, Cr, and Se where not detected.

Table 4: Antioxidant Capacity by IC_{50} Values of the Control and Test Bitters using various methods.

Groups	IC_{50} (mg/ml) By Total Antioxidant Capacity	IC_{50} (mg/ml) By DPPH Scavenging Ability	IC_{50} (mg/ml) By Hydroxyl Radical Scavenging Ability
Standard*	0.08 ± 0.00^{a}	0.05 ± 0.01^{a}	204.64 ± 4.68^{a}
Paxherbal Bitters	11.33 ± 0.15^{b}	$4.97\pm0.01^{\mathbf{b}}$	656.91 ± 62.41^{b}

Values are expressed as Mean±SEM. Values in the same column with different superscript letters differ statistically significantly (P<0.05) from one another.
**Standard for total antioxidant capacity and DPPH scavenging ability was ascorbic acid while that of hydroxyl radical scavenging ability was mannitol.*

Table 4 shows that Paxherbal bitters has the ability to inhibit the ABTS radical, scavenge DPPH and the hydroxyl radical, hence it has antioxidant capacity. Statistical evaluation however shows that the concentration required for 50% inhibition of ABTS and DPPH by ascorbic acid (IC_{50}) and 50% inhibition of the OH radical by mannitol (IC_{50}) is significantly ($P<0.05$) lower than that of Paxherbal bitters.

Discussion

Though herbal bitters are presently being marketed as a "cure-all"- patent medicine in Nigeria, a lot of them have never been subjected to scientific scrutiny. Hence this study was aimed at ascertaining the composition and *in vitro* antioxidant capacity of one of such bitters, with the hope that this will help give some credence to its broad pharmacological claims. The results of table 1 show the proximate composition of Paxherbal bitters has high moisture content and small quantities of some essential food nutrients. The results of this research is in agreement with the findings of Awa, *et al.,* (2013) (30), who worked on Swedish bitters and found that they contained carbohydrates, proteins, lipids, ash and a high moisture content, though with some variance in terms of the amount of these constituents compared to the amount of the same constituents found in the Paxherbal bitters of this study. Proteins, fats and carbohydrates are essential for life and studies have indicated that life is sustained by nutrient mixtures in which every component is definable chemically and soluble in water (31). Important in the selection of herbs and herbal products for nutritive value are the quality and quantity of its nutrient content (32). Depending on the medical needs of the patient, the level of the proximate constituents in the herbal bitters can therefore be taken into cognizance when deciding to prescribe the bitters.

The results presented in table 1 also indicate that Paxherbal bitters has a relatively high alcohol content of approximately 44%. The high alcohol and the final presentation/nature of the product and its packaging may be the reason for bitters been prone to abuse as reported by Salaam and Brown (2012) (33).

The aqueous and ethanolic extracts of the bitters studied indicates that generally it contain in varying degrees amino acids, proteins, saponins, tannins, alkaloids, glycosides/reducing sugar, terpenoids and flavonoids. The locally produced Paxherbal bitters was found to be moderate in its content of tannins and terpenoids as these were its highest constituents.

Bitters are made up of numerous groups of chemical compounds extracted from the herbs and roots, they have the common characteristic of a bitter taste. The chemical compounds that confer this bitter taste have been classified into those that are glycosides, alkaloids, terpenoids (especially the diterpenes, triterpenes and

sesquiterpenes), and those that are flavonoids and tannins (12). Table 2 indicates that the bitters of this study contain the major classes of substances that rightly confer on them the name "bitters" (4, 11, 34).

The qualitative and quantitative estimation of the phytochemical constituents of a medicinal plant is considered to be an important step in medicinal plant research (35). The presence of these secondary metabolites in plants probably explains the various uses of plants for traditional medicine (36). Herbal bitters are claimed to have so many medicinal properties. In relation to the known medicinal uses of the identified phytochemical constituents of the herbal bitters (table 2) and depending on the medical needs/ailment of the patient, Paxherbal bitters can easily be prescribed if it has the constituent that will help improve the ailment. Saponins, flavonoids and total phenols are in significant quantity and relatively higher than other phytochemicals quantitatively determined and this may be associated with the antioxidant properties of Paxherbal bitters.

Alkaloids and their derivatives have been certified to have important biological, physiological and medicinal effects in man. Paxherbal bitters having some appreciable amounts of alkaloids in them may well impart some of these effects.

Tannins as a group of compounds have received a great deal of attention in recent years, since it was suggested that the consumption of tannin-containing beverages, especially green teas and red wines, can cure or prevent a variety of ills (47). Many human physiological activities, such as stimulation of phagocytic cells, host-mediated tumour activity, and a wide range of anti-infective actions, have been assigned to tannins (48, 49, 50, 51, 52, 53). Some of these same claims associated with Paxherbal bitters may be traced to its tannin content.

Flavonoids are regarded as having anti-inflammatory (inhibit inflammatory metabolites and granulation tissue formation), anti-allergic (inhibit histamine release), and anti-oxidant effects. They are well known for strengthening and protective effect on fragile capillary and venous structures. Flavonoid often exhibit their therapeutic function through an ability to inhibit enzyme system (eg lipooxygenase, cyclo-oxygenase, elastase and aldose reductase) as well as free radical scavenging and co-factor activity for the anti-oxidant , vitamin C (54, 55). Other actions demonstrated by different flavonoids include hepatoprotective, anti-spasmodic, hypo-cholesterolaemic, diuretic, anti-viral and anti-bacterial effects (54, 55). Some of these same claims associated with Paxherbal bitters may be traced to its flavonoid content.

Phenols and phenolic compounds may be used as anti-microbial agents as they have antibacterial and antifungal properties (56, 57). They are also said to be antioxidant, immune enhancers, anti-clotting and hormone modulators (56, 57). Some of these same claims associated with Paxherbal bitters may also be traced to its phenol content.

Saponins when present in high concentration in the plants as compared to other phytochemicals, will likely indicate that extracts from these plants are used in wound healing and bleeding treatment (57, 58). Saponins have properties of precipitating and coagulating red blood cells and they also have cholesterol binding properties (hence their hypolipidaemic/hypocholesterolaemic property), formation of foams in aqueous solutions and haemolytic activity (57, 58). Some of these same claims associated with Paxherbal bitters may therefore be traced to their saponin content.

Our results show that the cyanogenic glycoside content of Paxherbal bitters (0.18%) is safe and too low to cause any toxicity in the prescribed dosage the bitters are normally consumed; instead the range is enough to harness any positive effect known in the medicinal use of cyanogenic glycosides.

Evidence suggests that cyanogenic glycosides may have beneficial effects in animals that consume them. These compounds do have anticarcinogenic activity invitro according to recent research (59, 60, 61, 62).

Cyanogenic glycosides have also been said to have sedative and expectorant effect in the respiratory tract. (59, 60, 61, 62). They have also been said to have a cooling effect as hydrocyanic acid cuts down heat at a cellular level. Examples include- peach, apple and pear which are all cooling fruits that contain cyanogens. (59, 60, 61, 62).

Most of the claims of Paxherbal bitters may therefore be attributed to its phytochemical constituent.

Table 3 shows that Paxherbal bitters contain significant amounts of Na, K, P, Ca, Mg, Zn, Mn, Fe, and Cu, while Pb, Cr, and Se where not detected. Recent promotion of herbs as health foods commonly includes reference to their mineral contents (63). So the Paxherbal bitters can be of help in mineral deficiencies especially in cases of Na, K, P, Ca, Mg, Zn, Mn, Fe, and Cu deficiencies.

Sodium (Na) and Potassium (K) play significant roles in acid-base balance, fluid balance, nerve function and proper neuromuscular and cardiac activity/function (64). Phosphates contribute to overall health by its involvement in energy transfer, phosphorylation/dephosphorylation reactions, lipid metabolism and acid-base balance and enzyme action (64). Normal extracellular calcium concentrations are necessary for blood coagulation and for the integrity of intracellular cement substances, and integrity and proper functioning of nerves, skeletal muscle and heart/smooth muscle (65). The presence of zinc in Paxherbal bitters could mean that they can play valuable roles in the management of diabetes, which result from insulin malfunction (65, 66) as well as explain the claim that the bitters play a role in free radical scavenging activity, improved growth, sexual activity and wound healing (64, 65). Infact the presence of these mineral elements (Na, K, P, Ca, Mg, Zn, Mn,

Fe, and Cu) in Paxherbal bitters may well explain some of the said physiological and pharmacological claims associated with this bitters.

Paxherbal bitters can be said to have a low IC_{50} value as they inhibit 50% of ABTS, 50% DPPH and 50% OH Radical at 11.33mg/ml, 4.97mg/ml and 656.91mg/ml respectively. The fact that this bitters inhibit these free radicals at relatively low doses, indicate that the bitters can be said to have a relatively high antioxidant capacity as claimed by its manufacturers. Herbal products have been said to have phytochemical constituents that confer on them antioxidant properties (5, 67). This is in agreement with this our study as the fact that Paxherbal bitters can inhibit the ABTS, DPPH and OH radicals, lends credibility to the fact that the phytochemical constituents of Paxherbal bitters confers antioxidant capacity to it. The potential of the phytochemicals have large scale pharmacological and biological implications, for example its antioxidant constituents (hydrolysable tannins, phenolic acid and flavonoids) have been proven to be effective for the care of health and protection from cardiovascular/coronary heart diseases and cancer (anti-carcinogenic and anti-mutagenic effects) (5, 67, 68, 69, 70).

Conclusion: The results of this study showed that Paxherbal bitters may as a general body tonic, be responsible for some of the physiological and pharmacological effects ascribed to it. Making inferences from the already established effects of some of the phytochemical and mineral constituents of this bitters, the results of this study gave some evidence that Paxherbal bitters may be said to have the following pharmacological properties- hypolipidaemic/hypocholesterolaemic, hypoglycaemic, anti-anaemic and anti-inflammatory, stimulant and immunity-boosting/immuno-modulatory, choleretic/hepatoprotective and antihepatotoxic, invitro antioxidant capacity and by extension anticarcinogenic/antineoplastic/ antimutagenic/antitumour as well as diuretic/vasodilatory and antihypertensive properties and the ability to protect against/prevent coronary artery disease and cardiovascular diseases generally. This initial positive evidence on the possible pharmacological properties of Paxherbal bitters, should therefore encourage further research to confirm them.

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