European Journal of Applied Sciences 8 (5): 289-296, 2016
ISSN 2079-2077

DOI: 10.5829/idosi.ejas.2016.8.5.1144

Composition and *in vitro* Antioxidant Capacity of Super Bitters

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Abstract: Medicinal plants have been defined as those plants that can be used for the synthesis of useful drugs. Modern pharmacology had its origin in these medicinal plants and till date some drugs are products of active components from plants. Modern science may have widened for some time the differences in terms of medication between orthodox and unorthodox/traditional medicine but, this gap seems to be closing fast as the current trend is that they are both adopting practices from each other. 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. Bitters are made up of a numerous groups of chemical compounds extracted from the herbs and roots, they have the common characteristic of a bitter taste. The results indicate the presence of proteins, saponins, tannins, alkaloids, glycosides/reducing sugar, terpenoids and flavonoids. The chemical composition of these compounds confers the bitter taste that includes a complex pattern of molecular structures. Super bitters contain significant amounts of Na, K, P, Ca, Mg, Zn, Mn, Fe and Cu, while Pb, Cr and Se were not detected in them. The bitters can inhibit the DPPH radical and this confirms the antioxidant capacity of Super bitters.

Key words: Super bitters · Medicinal plants · Antioxidant capacity and pharmacological agents

INTRODUCTION

Unorthodox traditional medicine practice which employs the use of herbs (medicinal plants), have in recent times been gaining much publicity and recognition, for their solution to ailments seemingly elusive to the system of orthodox medical practice. Medicinal plants have been defined as those plants which contain in one or more of their organs, substances that can be used for the synthesis of useful drugs. Medicinal plants have been defined and associated with gods and spiritual invocations. However with the advent of science, civilisation and Christianity, there has been a drastic decline in this practice. Modern pharmacology had its origin in these medicinal plants and till date some drugs are products of active components from plants [1, 2]. 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 aid in digestion and as flavouring in cocktails. Bitters are made up of numerous groups of chemical compounds extracted from the herbs and roots (medicinal plants) that have the common characteristic of a bitter taste and act to increase the vital energy centres in the body [3, 4].

The product to be studied is Super Bitters, a western Nigeria product, composed of four (4) herbal constituents *Hydrastis Canadensis* (Golden Seal), *Garcinia kola* (Bitter Kola), *Buccholzia coricea* (Wonderful Kola), *Allium sativum* (Garlic).

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"Bitters" generally are claimed to be effective in curing all allergic, metabolic and immunological conditions where the diagnosis points to a fault in the digestive process, improves immunity, help in anaemia, wound healing and blood clotting by increasing the population in tissues, of red blood cells, white blood cells and platelets, help with inflammatory conditions of the gastrointestinal tract (Colitis, Crohn's disease, nonspecific inflammation). In addition to the action of bitters on digestive secretions aiding good digestion, they also strengthen the tone of tissues throughout the digestive tract, as well as aid in the healing of damaged mucous membranes. They are generally said to regenerate and heal mucosal lining of the G.I.T especially duodenal and gastric ulcers. This helps resolve conditions ranging from gastroesophageal reflux to ulcers to leaky gut syndrome.

Bitters have been claimed to help heal piles/haemorrhoids and improve sexual function. Enhance blood circulation, purification of blood by the kidneys, blood pressure regulation through arterial dilatation and prevent formation of kidney stones, cleanse the colon of impurities and have also been said to possess anti-tumour properties and especially protects against colo-rectal cancers. They are also said to have anti-inflammatory, antibiotic and antifungal properties.

Bitters have also been said to ensure good digestion of fats and oils and proper functioning of the liver in excretion, reduce accumulated fat (triglycerides) and cholesterol levels thereby confering on it hypolipidaemic properties. They are said to reduce excess body fat and promote healthy weight loss, act as a liver tonic and body detoxifier; being hepatoprotective and enhancing its functions generally and helping in body detoxification. Bitters act on the pancreas and liver, help in cell division and growth of the pancreas thereby helping to normalizing blood sugar and promote the production and release of pancreatic enzymes. Some are even said to have hypoglycaemic properties.

In modern herbal medicine, "bitter principles" occupy a central place in herbal therapeutics, bearing the acrid constituents. Most people consuming herbal medicines complain about the bitterness of the medicines prescribed. This is the only defining attribute of herbal medicine and the only feature to set it apart from other therapies [2, 4]. In times past our traditional diets were not devoid of bitter foods as is presently the case in most modern diets, hence Green [5] desires that we see the medicinal side of bitters in an entirely different light in other that we use it to prevent what he termed the "Bitter Deficiency Syndrome" of our era, which in his opinion is the predisposing factor to many ailments of our time [4, 5]. All these make the study of the constituent and pharmacological effects of present day bitters desirable.

MATERIALS AND METHODS

Material: Super bitters were purchased from reputable pharmaceutical stores opposite the University of Benin Teaching Hospital (UBTH), Ugbowo Lagos Road, Benin City, Edo State, Nigeria. The bitters were bought as liquid formulations and stored at room temperature throughout the period of the experiment.

Proximate Analysis: The moisture content of the herbal bitters was determined using the gravimetric method [6]. The ash content was estimated using the method of AOAC [7]. The fat content was determined using the method of AOAC [8]. 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 [9]. The protein content of the bitters was determined from the organic nitrogen content by Kjeldahl method [9]. 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 [9]. The reducing sugar content of the bitters was determined using the dinitrosalicylic acid method [10]. The glucose content was determined using the glucose oxidase method [11]. Triplicate measurements were performed and the mean computed.

Alcohol Composition Analysis: The determination of the alcohol content was done using the AOAC [7] 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 4oC in an air tight container [12]. 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 4oC in an air tight container [12].

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 [12]. The test for glycosides/reducing sugars was carried out using the method described by Onyeike and Osuji [9]. The Borntrager's test was used for the detection of anthraquinones [13].

Quantitative Phytochemical Analysis: The determination of total phenols was by spectrophotometric method [14], while alkaloids was as described by Harborne [15] and Edeoga and colleagues [14]. The determination of tannin was by the Van – Burden and Robinson method [14], while the determination of saponin was as described by Edeoga and colleagues [14]. The determination of flavonoids was by the method of Boham and Kocipai-Abyazan [16], while that of cyanogenic glycosides was by the AOAC [7] method. Elemental Analysis: Extraction and determination of calcium and magnesium in the bitters sample were determined by the titrimetric method using ethylenediaminetetraacetic acid (EDTA), [7]. The sodium and potassium concentrations were determined using the flame photometer (JENWAY PFP 7 model) [7], while phosphorus was determined by Olsen and Sommers [17] and the AOAC [7] 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, [7.]

Determination of the Total Antioxidant Capacity: The ability of the herbal bitters to scavenge 2,2'-azinobis-(3ethylbenzothiazoline-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 [18]. 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) – (ABTS2-). Triplicate measurements were performed and the mean computed. DPPH free radical scavenging activity: DPPH (2,2-diphenyl-1-picrylhydrazyl radical) scavenging activity of the herbal bitters was measured by the spectrophotometric method described by Jain and colleagues [19]. Triplicate measurements were performed and the mean computed. Hydroxyl radical scavenging activity: This was assayed as described by Kunchandy and Rao [20] with a slight modification [21]. 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 shows that Yoyo bitters has a high moisture and alcohol content, no fibre and a higher ash content compared to other food nutrients.

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, phlobatannins, plant sterols (steroids), terpenoids and flavonoids but did not contain cardiac glycosides, glycosides/reducing sugar and anthraquinones. As the key indicates, these phytochemicals were present in varying degrees of slight, moderate and high. The quantitative phytochemical composition of the bitters, indicate saponins as the highest constituent and tannins the least among those determined.

Table 3 summarizes the means of the quantitative elemental composition of the bitters, with magnesium being the highest constituent and manganese 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 1: Proximate and Alcohol composition of the Super Bitters

Proximate Composition	Ash	Moisture	Fibre	Fat & oil	Protein	Carbohydrates	Glucose	Reducing sugar	Alcohol
Quantity in Super Bitters (%)	3.07±0.12	88.38±0.79	0.08±0.01	3.36±0.11	0.96±0.05	4.23±0.13	0.92±0.03	1.06±0.02	8.64±0.68

Table 2: Qualitative and Quantitative Phytochemical Composition of Super Bitters

Parameters	Qualitative composition of Super Bitters: Aqueous extract	Qualitative composition of Super Bitters: Ethanol extract	Quantitative composition of Super bitters (%)
Phytosterol	-	-	ND
Amino acids	+	+	ND
Proteins	+	+	ND
Saponins	+	+	2.56±0.07
Tannins	+	+	0.02±0.00
Alkaloids	+	+	0.13±0.00
Cardiac glycosides	-	-	ND
Glycosides/reducing sugar	+	+	ND
Cyanogenic glycosides	ND	ND	0.13±0.00
Terpenoids	+++	++	ND
Flavonoids	+	+	0.33±0.01
Phlobatannins	-	-	ND
Anthraquinones	-	-	ND
Total phenols	ND	ND	0.05±0.01

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

Table 3: Quantitative Mineral Composition of the Super Bitters

Mineral composition	Ca	Mg	Na	K	P	Fe	Zn	Mn	Cu	Pb	Cr	Se
Quantity in Super bitters (mg/100ml)	40.49±1.20	486.06±10.00	4.10±0.31	190.00±2.89	63.21±1.57	8.95±0.28	21.00±0.50	1.16±0.04	1.47±0.12	<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 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	0.05±0.01	204.64±4.68
Super bitters	22.44±0.03	6.78±0.08	178.70±3.05

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 Super 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 Super bitters.

DISCUSSION AND CONCLUSION

Discussion: Bitters are made up of a 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. Super bitters of this study contain the major classes of substances that rightly confer on them the name "bitters" as they contain amino acids, proteins, saponins, tannins, alkaloids, glycosides/reducing sugar, terpenoids and flavonoids. The chemical composition of these compounds that confer the bitter taste 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, 22]. Iridoids are responsible for the chief bitter constituents of the plant family Gentianaceae, *Cichorium intybus* (chicory), Dandelion, *Valeriana officinalis* (valerian), wild lettuce (*Lactuca virosa*) and quassia bark. Sesquiterpenes account for the bitter taste of the Artemisia plants, or wormwood genus, *Cnicus benedictus* (blessed thistle) and *Ginkgo biloba* (ginkgo) [4 and 23]. Other components which add to the bitterness are diterpene bitters, found in columbo root (*Jateorrhiza palmata*) or white horehound (*Marrubium vulgare*). Triterpenoids are the cause of bitterness for the Curcubitaceae family of plants, which includes pumpkin, cucumber, colocynth, marrows and the bryonies [4, 22, 23].

Many alkaloids also contribute to the bitter taste as in the protoberberine isoquinoline alkaloids of golden seal (*Hydrastis canadensis*) and Berberis, the morphine alkaloids, the quinoline alkaloids of quinine and angostura and the purine alkaloids (in coffee). In addition to this, many miscellaneous compounds like ketones and amino-acids are responsible for the bitterness, as found in hops [3, 4, 23].

The results of these studies show that Super bitters were all found to have high moisture content of 88.38%, ash content of 3.07%, has the highest carbohydrate and reducing sugar contents of 4.23% and1.06% respectively and the only bitters that contains some crude fibre. 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 [24]. Important in the selection of herbs and herbal products for nutritive value are the quality and quantity of its nutrient content [25]. Depending on the medical needs of the patient, the level of the proximate constituents in the herbal bitters outlined in results can therefore be taken into cognizance when deciding which of the bitters to prescribe.

The aqueous and ethanolic extracts of the bitters studied indicate that generally they contain in varying degrees amino acids, proteins, saponins, tannins, alkaloids, glycosides/reducing sugar, terpenoids, flavonoids, phytosterols and phlobatannins. It is however of note that the terpenoids where present not just in both extracts of all the bitters but in relatively higher amounts when compared to other phytochemical constituents. Super bitters were highest in their terpenoid, saponin and flavonoid contents. Super bitters also have appreciable amounts of alkaloids, tannins, total phenols and cyanogenic glycosides. Compared to other phytochemical constituents super bitters seem to have saponins as their highest constituent. This study is in agreement with that of Awa and James [26], which showed that herbal bitters contain appreciable amounts of the phytochemicals tannin and phenol.

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 [27, 28 and 29]. Phytochemicals are present in a variety of plants utilized as important components of both human and animal diets. These include fruits, seeds, herbs and vegetables [30]. Diets containing an abundance of fruits and vegetables are protective against a variety of diseases, particularly cardiovascular diseases [29 and 31]. Herbs and spices are accessible sources for obtaining natural antioxidants [28]. In addition to these substances, plants contain other chemical compounds. These can act as agents to prevent undesirable side effects of the main active substances or to assist in the assimilation of the main substances [29, 32].

The qualitative and quantitative estimation of the phytochemical constituents of a medicinal plant is considered to be an important step in medicinal plant research [33]. The presence of these secondary metabolites in plants probably explains the various uses of plants for traditional medicine [34]. Herbal bitters are claimed to have so many medicinal properties, since this study reveals they have appreciable amounts of secondary metabolites such as alkaloids, tannins, flavonoids, total phenols, saponins and cyanogenic glycosides, its claimed medicinal uses is therefore not surprising. In relation to the known medicinal uses of the identified phytochemical constituents of the herbal bitters and depending on the medical needs/ailment of the patient and the level of the phytochemical constituents in the herbal bitters, the most appropriate bitters can easily be prescribed.

Alkaloids and their derivatives have been certified to have important biological functions, physiological and medicinal effects in man and herbal bitters being a general body tonic can be said to be imparting some of its effects because of the presence of appreciable amounts of alkaloids in them which mimic the actions of the various alkaloids and their derivatives.

One of its derivatives, Isoquinoline alkaloids find many applications, including (but not limited to): anaesthetics; dimethisoquin is one example; antihypertension agents, such as quinapril, quinapirilat and debrisoquine (all derived from 1,2,3,4-tetrahydroisoquinoline); antifungal agents, such as 2,2'Hexadecamethylenediisoquinolinium dichloride, which is also used as a topical antiseptic; disinfectants, like N-laurylisoquinolinium bromide and vasodilators, a well-known example being papaverine [35].

Super bitters contain significant amounts of Na, K, P, Ca, Mg, Zn, Mn, Fe and Cu, while Pb, Cr and Se were not detected in them. Recent promotion of herbs as health foods commonly includes reference to their mineral contents. Unfortunately, little consideration is generally given to the fact that only five mineral elements are considered essential for metabolism in substantial amounts (calcium, magnesium, potassium, phosphorus and sodium), while ten others (chromium, cobalt, copper, fluorine, iodine, iron, manganese, molybdenium, selenium and zinc) are important in trace amounts only; of these, probably only selenium, molybdenuim, manganese, chromium and fluorine are essential [36]. Controversy exists over metabolism need versus optimal intake. Toxic levels are often very near the required dosages for "normal diets" and minerals like lead (Pb), arsenic (As), mercury (Hg), silver (Ag) and cyanide (CN^-) are toxic and of no significant use in the human body [37]. In case of the Pb concentration, the suggested concentration in plant species that is "safe" is 0.2 to 0.6 mg/100ml, however, WHO recommendations for Pb level in humans, is that it should not exceed 10 ppm [38]. It is noteworthy that the bitters of this study are not contaminated with lead (Pb) so there is no danger of Pb toxicity from them. There has been some speculation that mild deficiencies of minerals may be beneficial. Dietary deficiencies are common with iron, calcium, iodine and fluorine [36]. So the bitters used in this study 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, [37] their presence in bitters may as well explain these same roles said to be played by bitters. Contribution of Phosphates to overall health by its involvement in energy transfer, phosphorylation/dephosphorylation reactions and lipid metabolism by being a constituent of lipoproteins and acid-base balance and enzyme action [37] may as well explain the claim of bitters contribution to overall health and lipid metabolism. 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 [39], so the presence of calcium in the bitters may well explain these same claims about bitters. Concentration on which Zn affects human health ranges from 100 to 500 mg/l (10-50mg/100ml) [40].

The presence of zinc in the plants could mean that the plants can play valuable roles in the management of diabetes, which result from insulin malfunction [39], so apart from the claim of bitters playing a role in glucose metabolism, the presence of Zinc may well contribute to the claim that bitters play a role in free radical scavenging activity, improved growth, sexual activity and wound healing.

That Super bitters have IC_{50} values for 50% ABTS inhibition of 22.44mg/ml indicate that the bitters can be said to have a relatively high antioxidant capacity as claimed by the producers of the bitters. Herbal products have been said to have phytochemical constituents that confer on them antioxidant properties [41, 42], this is in agreement with this research as the fact that these bitters can inhibit the ABTS radical confirms the antioxidant capacity of the herbal bitters. The potential of the phytochemicals of the plant/herbal materials 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 coronary heart diseases, cancer, anti-carcinogenic and anti-mutagenic effects [41, 42].

Herbal products have been said to have phytochemical constituents that confer on them antioxidant properties [41, 42], this is in agreement with our findings as the fact that these bitters can inhibit the DPPH radical confirms the antioxidant capacity of the herbal bitters. The fact that the phytochemicals of the plant/herbal materials can have large scale pharmacological and biological implications, have been proven by the finding that its antioxidant constituents (hydrolysable tannins, phenolic acid and flavonoids) have been proven to be effective for the care of health and protection from coronary heart diseases, cancer, anti-carcinogenic and anti-mutagenic effects [41, 42].

Antioxidant constituents can delay or inhibit the oxidation of lipids and other compounds by inhibiting the propagation of oxidation chain reaction [43, 44]. Primarily, antioxidant effect is due to phenolic compounds such as phenolic acid, flavonoids and phenolic diterpenes and their mode of action as antioxidant compounds is due to their redox reaction properties which can absorb and neutralize free radicals by quenching singlet and triplet oxygen [44, 45].

CONCLUSION

The biochemical assay results of this study with inferences derived from some of the already established effects of some of the phytochemical and mineral constituents of these bitters, gave some evidence that the herbal bitters of this study may be said to have the potential or possibility of having the following pharmacological properties -hypocholesterolaemic, hypoglycaemic, anti-anaemic and anti-inflammatory, stimulant and immuno-modulatory, hepatoprotective and antihepatotoxic, *invivo* and *invitro* antioxidant capacity and by extension antineoplastic, as well as diuretic and antihypertensive properties and the ability to prevent coronary artery disease and cardiovascular diseases generally.

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