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Antioxidant Evaluation, Acute Toxicity Screening and Heavy Metal Analysis of a Poly Herbal Mixture

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ABSTRACT

The persistent exposure of plants to contaminants is a great public health concern. This makes it pertinent to determine the safety levels of herbal extracts especially for the presence of heavy metals. RUZU bitters is a polyherbal plant extract with demonstrated antidiabetic activity. It is composed of the medicinal plants; *Uvaria chamae*, *Curculigo pilosa* and *Colocynthis citrullus.* This study is aimed at investigating the acute toxicity, antioxidant activity and heavy metal contents of RUZU bitters. Phytochemical screening and polyphenolic content were done using standard methods. The acute toxicity study was carried out using modified Lorke's method while the heavy metals analysis was carried out using Atomic Absorption Spectrophotometer. Antioxidant potential of the extract was evaluated using 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging method. The results of the phytochemical screening revealed the presence of carbohydrates, reducing and deoxy sugars, saponins, phenolics, flavonoids and tannins. The extract showed significant polyphenolic content with total phenol content of 30.22 ± 3.42 mgGAE/g and total flavonoid content of 43.5 ± 2.17 mgQE/g. Further, it demonstrated low radical scavenging activity with IC_{50} of 236.32µg/mL. The metal content showed 1.09mg/L for Cr, 0.10 mg/L for Zn, 2.10mg/L for Fe, 0.23mg/L for Mn, 0.20mg/L for Cu, 0.06mg/L for Cd, 6.50mg/L for Na, 37.50mg/L for K, 32.00mg/L for Mg, 8.90mg/L for Ca and nil for Pb and Cd. RUZU bitters has little radical scavenging activity, hence low antioxidant potential. The little or no presence of heavy metals showed that it is safe for daily consumption.

Keywords: Antioxidants, Heavy metals, Polyherbal mixture, Toxicity

INTRODUCTION

The use of medicinal plants as herbs for health care purposes dates back to the origin of man. They are known to contain biologically active compounds such as alkaloids, flavonoids, terpenoids, saponins, essential oils and tannins with curative properties (Ogbeide and Akhigbe, 2019). According to WHO, about three quarters of the world's population depend upon traditional medicines (mainly herbs) as an alternative to pharmaceuticals. In Africa for example, about 80% of the population depends on traditional medicine to be a quality, safe and efficacious alternative for treatments thereby contributing to the goal of ensuring that all people have access to health care (WHO, 2018).

Herbal mixture is a mixture of plants or plant parts that are used to maintain health and to prevent, alleviate or cure diseases. The herbal mixture under study (Ruzu bitters®) is a Polyherbal(PHB) plant extract constituted by three (3) medicinal herbs; *Uvaria chamae* of family Annonaceae (bush banana or finger root), *Curculigo pilosa* of family Hypoxidaceae (squirrel groundnut), and *Citrullus colocynthis* of family Cucurbitaceae (bitter apple, desert gourd or eguisi) (Hussain *et al*., 2014; Emordi *et al*., 2018; Karigidi *et al*., 2019).

U. chamae has been reported to be used in treating severe abdominal pains, diarrhoea, sickle cell anaemia, cough, diabetes, urinary tract and cerebral infections and also possesses hepatoprotective activity (Oluremi *et al*., 2010). The leaves of the plant are used for the treatment of malaria (Lagnika *et al*., 2016). Phytochemical analysis of its root, leaves and stem revealed varying degrees of alkaloids, glycosides, saponins, lipids and oils, carbohydrates, tannins, flavonoids, terpenoids and acids (*Okwuosa et al*., 2012; Ebi *et al*., 1999).

C. pilosa have been used traditionally to treat impotence, arthritis, gastrointestinal and heart diseases (Nie *et al*., 2013). The phytochemical screening of the plant has revealed the presence of alkaloids, traces of anthraquinones, cardenolides, saponins and tannins (Gbadamosi and Egunyomi, 2010). Results for the phytochemical analysis of *C. colocynthis* have shown the presence of saponins, sterols, steroids, terpenoids, flavonoids, tannins and alkaloids in different proportion in the tree parts

(fruit, leaf and root) of the plant (Nora *et al*., 2015; Uma and Sekar, 2014). It is reported to be widely applied in the management of diabetes, leprosy, common cold, cough, asthma, bronchitis, jaundice, joint pain, cancer, toothache, wound, mastitis and gastrointestinal disorders (Hussain *et al*., 2014).

The use of herbs and herbal extracts to prevent, treat and manage diseases is highly prevalent in Africa. This is due to their claimed efficacy and safety, high availability at little or no cost which is further encouraged by high cost of modern medicinal drugs. However, the biological, chemical and physiological effects of heavy metals which is sourced mainly from the advent of industrialization has led to constant exposure of plants. This has become a worrisome concern to the society, researchers, and regulators worldwide due to their potential to accumulate and ultimately enter the food chain especially through plants (Shahid *et al*., 2014; Whiteside *et al*., 2010; Sarma *et al*., 2011; An *et al*., 2012; Schreck *et al*., 2012). Moreover, a more common effect of heavy metal toxicity in plants is increased production of reactive oxygen species (ROS). The increased level of ROS can disrupt the redox status of cells, resulting in oxidative stress to exposed body cells, leading to pathological conditions which may include cardiovascular disease, cancer, neurological disorders, diabetes, ischemia/reperfusion and ageing (Dalle-Donne *et al*., 2006; He *et al*., 2011; Carrasco-Gil *et al*., 2012; Chen *et al*., 2012).

Hence, the objective of this study was to determine the antioxidant properties, safety of the polyherbal extract (RUZU bitters) due to its daily consumption for medicinal purposes and determination of heavy metals present in RUZU bitters in order to validate its ethnomedicinal use, therapeutic efficacy and safety.

MATERIALS AND METHODS

Sample Collection

A 200 mL bottle of RUZU bitters was bought from a Pharmacy in Benin city, Edo state, Nigeria. The sample was poured into plates and freeze dried, after which it was stored in an air-tight container until they were ready for use.

Phytochemical Screening

Simple chemical tests to detect the presence of alkaloids, terpenoids, flavonoids, phenols, tannins, saponins, carbohydrates, reducing sugars and proteins were carried out using standard methods (Evans, 2002; Sofowora, 1982; Stahl, 1973).

ACUTE TOXICITY TEST

Animals

Twenty (20) adult Swiss albino mice with an average weight of 25g were obtained from the Animal house, Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Benin, Nigeria. The animals were kept under 12-hour light/dark cycle in plastic cages with saw dust as beddings and given food and water *ad libitum*. The animals were fed with standard rodent pellets (Bendel feeds and flower mill, Ewu, Nigeria). The animals were maintained and cared for in accordance with the international guidelines for the use and maintenance of experimental animal (OECD, 2001).

Experimental

Acute toxicity test of RUZU bitters was carried out using modified Lorke method (Lorke, 1983). The study was carried out in two phases. In phase one of the study, nine mice were randomized into three groups of three mice each and were given 10, 100 and 1,000 mg/kg body weight of the bitters orally. The mice were observed for signs of toxicity which include but not limited to paw licking, salivation, stretching of the entire body, weakness, sleep, respiratory distress, coma and death in the first twenty-four hours and subsequently daily for seven days. In the second phase of the study, another fresh set of three mice were randomized into three groups of one mouse each and were given 1600, 2900, and 5000 mg/kg body weight of the bitters orally based on the result of the first phase. These were observed for signs of toxicity and mortality for the first twenty-four hours and thereafter daily for seven days. The oral median lethal dose was calculated using equation 1 (Igbe *et al*., 2010);

$$LD50 = \sqrt{minimum\ toxic\ dose\ X\ maximum\ tolerated\ dose} \quad (1)$$

Determination of Polyphenolic content

Total Phenol

Total phenol content in the extracts was determined by the method previously described by Kim and Byzova, (2014). The bitters solution (0.5mL) of concentration 1mg/mL was added to 4.5mL of distilled water, and 0.5mL of Folin-Ciocalteu's reagent (previously diluted with water 1:10, v/v) was added to the solution. After mixing the tubes, they were maintained at room temperature for 5 minutes followed by the addition of 5mL of 7% sodium carbonate and 2mL of distilled water. After mixing the samples, the samples were incubated at room temperature for 90 minutes. The absorbance was measured by spectrophotometer at 750nm. The total phenolic content was expressed as milligrams of gallic acid equivalent per gram of extract (mg GAE/g extract). The standard curve was prepared using gallic acid in six different concentrations (12.5, 25, 50, 75, 100 and 150 mg/L).

Total Flavonoids

Total flavonoids content was estimated using the method previously described by Olajire and Azeez, (2011). Briefly, 0.5mL of the bitters (1 mg/mL) was mixed with 1.5mL of water and then, 0.1 mL of 10% aluminium chloride was added,

followed by 0.1mL of 1 M potassium acetate and 2.8mL of distilled water. The mixture was incubated at room temperature for 30 minutes. The absorbance was measured by a spectrophotometer at 415nm. The results were expressed as milligrams quercetin equivalent per gram of extract (mg QE/g extract). The standard curve was prepared by quercetin in six different concentrations (12.5, 25, 50, 75, 100 and 150 mg/L).

ANTIOXIDANT ACTIVITY ASSAY

DPPH Radical Scavenging Assay

The radical scavenging activity was evaluated using the method previously described with slight modification (Huang *et al.*, 2005). A solution of 0.2 mM DPPH in methanol was prepared, and 1.0 mL of this solution was mixed with 3.0mL of the bitters in water containing 0.001 - 0.2 mg/mL of the extract. The reaction mixture was mixed thoroughly and left in the dark at room temperature for 30 minutes. The absorbance of the mixture was measured spectrophotometrically at 517nm. Ascorbic acid was used as reference standard. The ability to scavenge DPPH radical was calculated by the following equation 2;

$$\text{DPPH Radical Scavenging Activity}\,(\%) = \frac{Ao-A1}{Ao} X\ 100 \quad (2)$$

Where;
A_o = Absorbance of DPPH radical in methanol,
A_1 = Absorbance of DPPH radical + sample extract (or standard)

The 50% inhibitory concentration value (IC_{50}) is indicated as the effective concentration of the sample that is required to scavenge 50% of the DPPH free radical (Jain *et al*., 2008).

Determination of heavy metal content

The concentrations of Pb, Cr, Zn, Fe, Mn, Cu,Cd, Ni and Mg were determined using Bulk scientific VGP 210 Atomic Absorption Spectrophotometer equipped with a flame autosampler (Model 240), a deuterium continuum lamp correction and variant Giant Pulse (VGP) correction and a hollow cathode lamp(wavelength 280nm for Mn, 285nm for Mg, 232nm for Ni, 214nm for Zn,327 nm for Copper, 248nm for Fe, 228nm for Cd, 358nm for Cr, and 283nm for Pb).

Statistical analysis

Data were expressed as means ± standard error of mean (SEM) of three replicates. Comparison between means was done using one-way analysis of variance (ANOVA). P-value less than 0.05 were regarded as significance at 95% confidence interval.

RESULTS AND DISCUSSION

Phytochemical Screening

The phytochemical screening carried out revealed the presence of the following phytoconstituents; carbohydrates, reducing and deoxy sugars, saponins, phenolics and flavonoids as shown in Table 1. Flavonoids and phenolics are a major group of compounds that act as primary antioxidants or free radical scavengers (Polterait, 1997). This could be responsible for the mixture's antibacterial, antiflammatory and antioxidant properties (Sofowora, 1993). Pamplona-Roger, (1999) earlier reported that plant extracts containing compounds with antibacterial properties have been useful in treating bacterial and fungal infections. The presence of saponins is in line with other studies of the presence of saponins in plant. (e.g *Mucunapruriens*) (Okoko, 2011). The work indicated that saponins have the properties of precipitating proteins. The presence of saponins might be responsible for the mixture's antifungal property (Delmas *et al*., 2000; Wang *et al*., 2000), cholesterol lowering (Potter *et al*., 1993; Southon *et al*., 1988), hypoglycemic (Petit *et al*., 1993) and antiprotozoal activities (Traore *et al*., 2000). Thus, saponins present in the mixture can help the body fight against infections and microbial invasions.

Table 1: Phytochemical composition of Ruzu bitters

Phytochemicals	Inference
Alkaloids	-
Flavonoids	+
Saponins	+
Phenolics	+
Carbohydrates	+
Reducing sugar	+
Deoxysugars	+
Tanins	-
Proteins	-
Terpenoids	-

+ indicates presence of the component, - indicates absence of the component

Acute Toxicity study

In the acute toxicity study, no animal died within 24 hours of administration of the polyherbal mixture. Lorke suggested that LD_{50} > 5000 mg/kg body weight is thought to be safe. Similarly, according to the acute toxicity grading standards (Aniagu *et al*., 2005; Duan and Liang, 2011), if the LD_{50} is greater than 5000 mg/kg, the drug is considered as practically non-toxic. The LD_{50} of the polyherbal mixture was found to be higher than 5000mg/kg. The absence of death among the rats in all the doses administered seems to support this claim. Hence, the oral administration of the polyherbal mixture can be said to be practically non-toxic.

Evaluation of the Polyphenolic Content
Phenolic and Flavonoid Contents

The total phenolic content of the extract, calculated from the calibration curve (R^2 = 0.9941; Figure 1), was 30.22 ± 3.42 gallic acid equivalents/g (Table 2), while the total flavonoid content (R^2 = 0.9981; Figure 2) was 43.50 ± 2.17 quercetin equivalents/g (Table 2). Phenolic compounds have redox properties, which allow them to act as antioxidants. The antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers. They may also have a metal chelating potential (Kumarasamy *et al*., 2007; Subhan *et al*., 2008). Their hydroxyl groups facilitate their free radical scavenging ability; the total phenolic concentration could be used as a basis for rapid screening of antioxidant activity. It might be suggested that phenolic compounds in the mixture were the major contributor to its antioxidant activity. However, thorough phytochemical analyses should be done to identify the active phenolic and flavonoid components.

Antioxidant Activity

The antioxidant activity of Ruzu bitters was carried out using the DPPH radical scavenging activity. The DPPH free radical scavenging activity is based on the ability of 1, 1-diphenyl-2-picryl-hydrazyl (DPPH), a stable free radical, to be decolorized in the presence of antioxidants (Kumarasamy *et al*., 2007). The DPPH radical contains an odd electron, which is responsible for the absorbance at 515-517 nm and also for visible deep purple colour. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized which can be quantitatively measured from the changes in absorbance (Yang *et al*., 2007). The DPPH radical scavenging activity result obtained (Figure 3) showed that Ruzu bitters has weak DPPH scavenging effect with the 50% inhibitory concentration (IC_{50}) of 236.32µg/mL. This was significantly higher compared to the standard antioxidant agent ascorbic acid with IC_{50} value of 4.71µg/mL (Table 3).

Total phenolic activity represent chemical reaction based on the binding of a phenolic compound that contains free oxygen, but not a free radical, while total antioxidant activity displays the presence and conversion of free radicals present in a biological system. The sensitivity of Folin-Ciocalteu reagent covers a broad range of phenolic compounds whereas the DPPH free radicals show different sensitivity to various antioxidants. The Folin-Ciocalteu reagent react with both free phenolics and bound phenolics in extracts and other samples, but the DPPH assay determine the free antioxidants and phenolics (CAC, 1993). Therefore if the bound phenolics and antioxidants exist, they may not contribute to the radical scavenging activity in the DPPH assay. This may explain why there is high amount of polyphenols but weak antioxidant activity.

Table 2: Polyphenolic Content of Ruzu Bitters

Polyphenolic Content	Values
Total phenolic content	30.22 ± 3.42 mgGAE/gExt
Total flavonoid content	43.50 ± 2.17 mgQE/g Ext

Table 3: IC_{50} Values for the Antioxidant assay

DPPH Scavenging Activity	IC_{50} Values
Ascorbic Acid	4.71µg/mL
Ruzu Bitters	236.32µg/mL

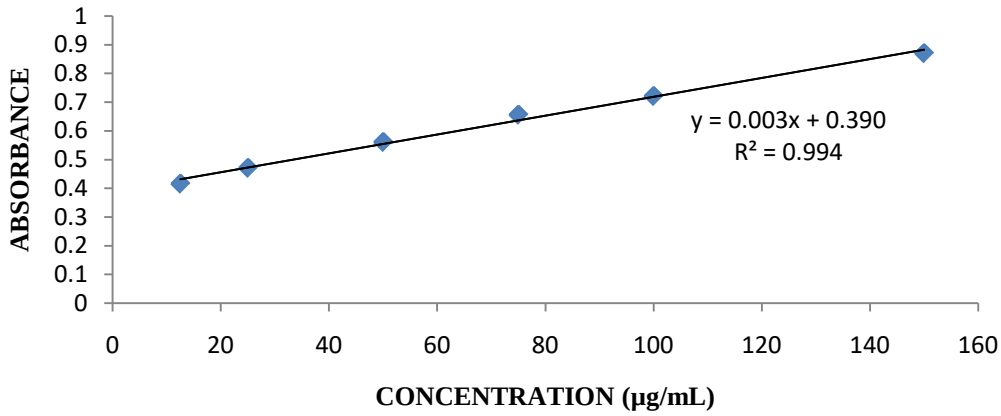

Figure 1: Gallic acid calibration curve

The total phenolic contents obtained from the equation of the calibration curve of Gallic acid Y= 0.003x + 0.390, R^2= 0.994

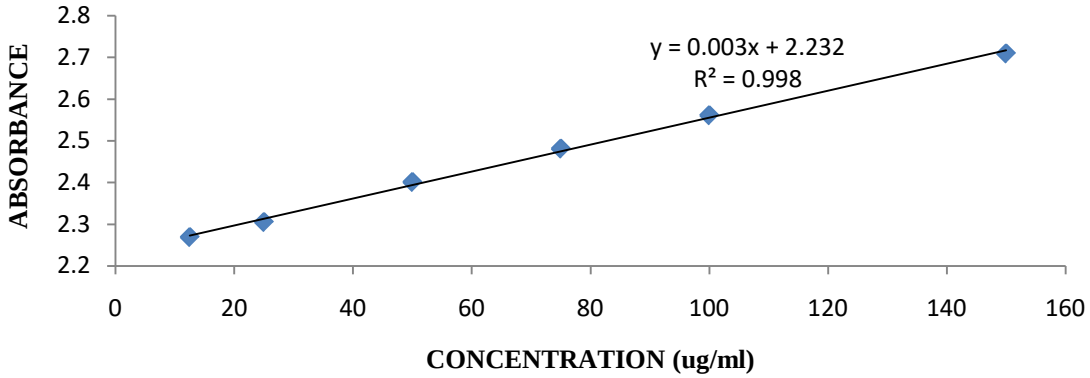

Figure 2: Quercetin calibration curve

The total flavonoid content obtained from the equation of the calibration curve of quercetin Y= 0.003x + 2.232, R^2= 0.998

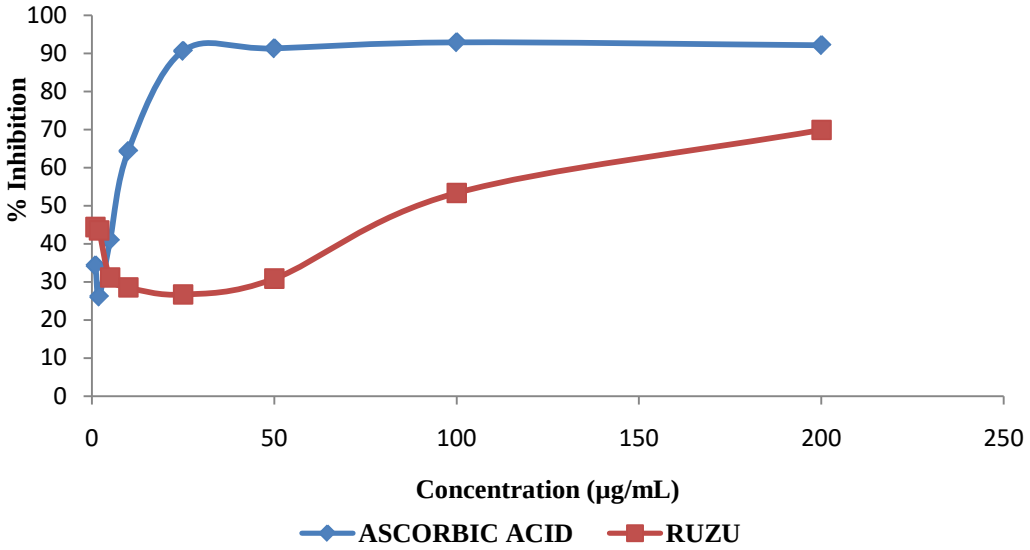

Figure 3: DPPH radical scavenging activity of Ruzu bitters compared with standard (ascorbic acid)

Metallic Content of the Herbal Extract

Heavy metals are natural components of the earth's crust. They cannot be degraded or destroyed. To a small extent they enter our body via food, drinking water and air. As trace elements, some heavy metals (e.g. copper, selenium, zinc) are essential to maintain the metabolism of the human body. However, at higher concentrations they can lead to poisoning.

The three most pollutants heavy metals; Lead, Cadmium, and Mercury are generally considered the most toxic because of the adverse human health effects linked with exposure to them even at low concentration (Morais *et al*., 2012). High levels of exposure to lead metal may result in toxic biochemical effects in humans which in turn cause problems in the synthesis of haemoglobin, effects on the kidneys, gastrointestinal tract, joints and reproductive system, and acute or chronic damage to the nervous system (Morais *et al*., 2012). Long-term exposure to cadmium is associated with renal dysfunction. High exposure can lead to obstructive lung disease. Cadmium may also produce bone defects (*osteomalacia*, *osteoporosis*) in humans (Castro-González and Méndez-Armenta, 2008; WHO, 2004; WHO, 2006.Exposure to high levels of metallic, inorganic, or organic mercury can permanently damage the brain, kidneys, and developing fetus (ATSDR, 2003). The general mechanism involved in heavy metal - induced toxicity is recognized to be the production of reactive oxygen species resulting oxidative damage and health related adverse effects (An *et al*., 2012; Morais *et al*., 2012). Thus utilization of heavy metal contaminated food and herbal mixtures is resulting in high morbidity and mortality rates all over the world.

The WHO recommends a recommended dietary allowance (RDA) for Ca; 1.3 g, Na; 1.5 g, Mg; 0.42 g, Cr; 35 µg, Cu; 0.9 mg, Fe; 18 mg, Mn; 2.3 mg, Zn; 11 mg, Ni;<1mg, and K; 4.7 g. As shown in Table 4, the polyherbal extract is safe for daily consumption as it has no lead and cadmium. The study showed that the levels of heavy metals are generally below the safe limits (CAC, 1993; WHO, 1989; IARC, 1984), and within the recommended dietary limits (Press, 1985).

Table 4: Metallic Content of the Polyherbal Extract

Metal	Concentration (mg/L)
Lead (Pb)	0.00
Chromium (Cr)	1.09
Zinc (Zn)	0.10
Iron (Fe)	2.10
Maganese (Mn)	0.23
Copper (Cu)	0.20
Candium (Cd)	0.00
Nickel (Ni)	0.06
Sodium (Na)	6.50
Potassium (K)	37.50
Magnesium (Mg)	32.00
Calcium (Ca)	8.90

CONCLUSION

In this study, phytochemical screening, antioxidant potential, acute toxicity and metallic content of Ruzu mixture was carried out. The study shows that the antioxidant potential of the mixture could be associated to its polyphenol content. The polyherbal mixture is considered safe for daily consumption as it LD_{50} > 5000mg/kg and the heavy metal content are within the permissible limits. Further studies are recommended for the isolation of compounds and to understand the mechanism of their actions. Also, sub-acute and chronic toxicity studies for Ruzu bitters could be done to determine its long-term effect.

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