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ANTI-OXIDANT PROPERTIES AND MEMBRANE STABILIZING POTENTIALS OF 'ITUGHA': A FERMENTED PRODUCT FROM *IRVINGIA GABONENSIS* SEEDS

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Abstract

This study aimed to investigate the antioxidant potentials and osmotic fragility protection of 'Itugha' (a locally fermented product of *Irvingia gabonensis* seeds). In order to assess the antioxidant effect of Itugha *in vitro*, 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) spectrophotometric and ferric reducing antioxidant power (FRAP) assay techniques were used while in vivo activities were investigated using assay of serum catalase (CAT) activity and reduced glutathione (GSH) concentration) experimental animal models. Osmotic fragility test was conducted using whole blood from treated animals. The DPPH assay showed that Itugha had percentage antioxidant activities comparable to ascorbic acid at 400 µg/ml concentration (88.30 % and 93.7 % respectively). The FRAP results were similar to the DPPH with Itugha at high concentration giving a FRAP value of 2.217 ± 0.06 which is slightly higher than that of ascorbic acid even at 1000 µg/ml. Pre-treatment of albino mice with Itugha increased the activities of CAT (catalase) and protected the integrity of the erythrocyte cell membranes against lysis due to hypertonic environments. This assay revealed that Itugha might prevent reactive radical species from damaging biomolecules such as lipoprotein, DNA, amino acids, sugar, proteins and PUFA in biological and food systems, and also protect cell membranes against attacks by harsh environments.

Keywords: *Irvingia gabonensis, 'Itugha', antioxidants, osmotic fragility, fermentation*

Introduction

Irvingia gabonensis (also known as bush mango or African mango) has become an important commodity to household economies and personal incomes. Since its discovery as possible part of human nutrition, its use in traditional medicine has also been documented for the treatment of gastrointestinal or hepatic disorders, diarrhoea, infections, and as a purgative [1] The dried seeds are known to have short shelf life, which predisposes them to easy contamination by moulds and aflatoxins [2]. In many West African countries, the dried and powdered seed is added to Dika bread to swell the loaf, and it is also added as substitute to cocoa powder in chocolate production to thicken the product [3]. The seed is ground and used as soup thickeners in Nigeria. The powdered dried seed is cheese-like and used in cooking fish and meat to impact an appetizing and characteristic flavour and taste [4]. The seed can be roasted and used as flavouring agent in the preparation of local salads [4]. Recently, the high demand for the cotyledons used in preparing local soups have led to the plant being cultivated and domesticated. *Irvingia gabonensis* seeds have become available on the U.S. market as a dietary supplement and have shown high in vitro antioxidant capacity [5] significant effects on blood lipid decreases and a lowering of plasma glucose [6] in experimental animal or human subject studies

Among the Agoi people of Yakurr ethnicity in Cross River state of Nigeria, the seeds of *Irvingia gabonensis* is fermented and processed into a spread known as "Itugha" (native cheese or butter). In processing Irvingia seeds into "itugha", natives employ a traditional technology involving a sequence of operations: size reduction, pulverization, fermentation and heat treatment. Each unit operations in the process requires a unique working condition in order to obtain the desired product quality which is measured by taste, aroma and flavour [7]. Itugha is highly valued in family circles and its high nutritive value been reported [8].

So many physiological defects, imbalances and disease states have been considered to be caused via oxidative stress mechanisms in living systems [9]. Oxidative damage at cellular level denatures protein affecting their functions as biological catalysts, carbohydrates by changing their structural conformation and lipids via lipid peroxidation [10]. Cell membranes are made up of mostly lipids which are initial targets for invading micro-organisms and chemical agents, radiation and are often destroyed when attacked. This causes oxidative stress or spontaneous proliferation in the cells and animal as a whole. The red blood cell (RBC) is considered a whole, generalized and sensitive tissue in a system and is often employed in the stability studies of a living membrane [11]. The determination of the mean corpuscular fragility, which is the concentration of saline causing 50% haemolysis of the erythrocytes, could give a clear idea on how some pharmacological compounds and plant extracts can maintain membrane stability in hypotonic, isotonic and hypertonic solutions. Or destroy it.

Thus, the essence of this study is to investigate the antioxidant potentials of itugha and its possible effects on osmotic fragility of red blood cells.

Methods

Sample Preparation

The was done as described by Ekpe et al [7]. With the aid of a ceramic pestle and mortar, cotyledons (without the hull) from the seeds of *Irvingia gabonensis* were pounded for about 1 h on the first day and again, daily 5 days by which time the gummy component of the mashed Irvingia mass was lost. Post each day's pounding, the mash was stored away in a native gourd (Lagenaria sicereria). At the end of six (6) days, (when the "drawness" was completely lost), the mash was moulded manually into round or oblong shapes of convenient sizes. These products were wrapped with *Piper umbellantum* leaves and placed over the fire place for 2- 3 days to smoke dry. This sample was collected and stored 4 °C until when used for analysis or treatment

In vitro antioxidant activities of Itugha

Antioxidant capacity of Itugha with 1, 1- diphenyl-2-picrylhydrazyl radical (DPPH) photometric assay

The method of Mensor et al. was adopted [12]. Two (2) ml of test sample at concentrations ranging

from 10 µg/ml - 400 µg/ml was each mixed with 1 ml of 0.5 mM DPPH (in methanol). Absorbance at 517 nm was taken after 30 minutes incubation in the dark at room temperature. The concentrations were prepared in triplicates. The percentage antioxidant activity was calculated as follows:

% antioxidant activity [AA] =

100 – ([absorbance of sample-absorbance of blank] * 100)

Absorbance of control

1ml of methanol plus 2 ml of the extract was used as blank while 1ml of 0.5 mM DPPH solution plus 2 ml of methanol was used as control. Ascorbic acid was used as reference standard.

Ferric reducing/antioxidant power (FRAP) assay

The total antioxidant potential of sample was determined using a ferric reducing ability of plasma (FRAP) assay of Benzie and Strain [13] as a measure of "antioxidant power". FRAP assay measures the change in absorbance at 593 nm owing to the formation of a blue coloured Fe11-tripyridyltriazine compound from colourless oxidized Fe111 form by the action of electron donating antioxidants. Standard curve was prepared using different concentrations (100-1000 µmol/L) of $FeSO_4 \times 7H_2O$. All solutions were used on the day of preparation. In the FRAP assay the antioxidant efficiency of the extract under the test was calculated with reference to the reaction signal given by an Fe^{2+} solution of known concentration, this representing a one-electron exchange reaction. The results were corrected for dilution and expressed in µmol Fe11/L. Vitamin C was measured within 1 h after preparation. The sample to be analysed was first adequately diluted to fit within the linearity range. All determinations were performed in triplicate.

FRAP value of sample (µM) =

changes in absorbance from 0-4 min x FRAP value of std changes in absorbance of standard 0 min - 4 min

Animal Experiments

Albino mice of both sexes weighing between 22-35 g were used. They were obtained from the Laboratory Animal Unit of the Faculty of Basic Medical Sciences, University of Calabar, Calabar, Nigeria. The animals were housed in cages at room temperature and under a light period of 16-18 h daily for a period of 2 weeks prior to the commencement of the experiment. Standard commercial rat pellets (Vital feed®) and water were provided ad-libitum. The laboratory animals were used in accordance with laboratory practice regulation and the principle of laboratory animal care as documented by Zimmerman [14].

Acute Toxicity Study

Five groups of albino mice of both sexes with each group containing five rats were used. Four groups were treated orally with varying doses of the Itugha at 250, 500, 1000 and 2000 mg/kg respectively. Group 5 was given an equivalent volume of distilled water to serve as control. The animals were observed for toxic signs like excitability, dullness, diarrhoea, in-appetence and death over 72 h.

Experimental design

Twenty-five (25) albino mice were used for the experiment. They were randomly grouped into five groups of five animals each. Animals in group I, II and III were treated daily with the Itugha dissolved in distilled water at doses of 200, 400 and 800 mg/kg respectively. Animals in group IV were treated daily with ascorbic acid (standard antioxidant drug). Group V animals were given distilled water daily. All animals were treated for 28 days. Blood was collected from the mice on Day 28 of treatment through the media canthus of the eye for antioxidant assays.

***In vivo* antioxidant activities of Itugha assays**

Assay of catalase (CAT) activity

CAT activity was measured according to the method of Aebi [15]. A given volume (0.1 ml) of the serum was pipetted into cuvette containing 1.9 ml of 50 mM phosphate buffer of pH 7.0. Reaction was started by the addition of 1.0 ml of freshly prepared 30 % (v/v) hydrogen peroxide (H_2O_2). The rate of decomposition of H_2O_2 was measured spectrophotometrically from changes in absorbance at 240 nm. The enzyme activity was expressed as units/ml protein.

Assay of reduced glutathione (GSH) concentration

Reduced glutathione concentration was determined by the method of Ellman [16]. A volume (1.0 ml) of serum was treated with 0.5 ml of Ellman's reagent (19.8 mg of 5, 5- dithiobis-2-nitrobenzoic acid (DTNB) in 100 ml of 0.1% sodium nitrate) and 3.0 ml of phosphate buffer (0.2 M, pH 8.0). Then 0.4 ml of distilled water was added. The mixture was thoroughly mixed; absorbance was read at 412 nm and expressed as units/ml

Osmotic fragility of Red Blood cells in rats treated with Itugha

Blood was collected from mice into heparinized tubes. For each concentration of the ethanol extract (20, 10 and 5 mg/ml), test tubes were numbered from 1 to 17. Five ml of buffered NaCl ranging from 0.1 to 0.85% was placed in tubes 1 through 16. Five ml of distilled water was placed in tube 17.

0.1 ml of blood was added to each tube and mixed gently and then incubated at 37°C for 1 h. The tubes were centrifuged at 2000 rpm for 10 min to sediment any intact red cells. The haemoglobin content of the supernatant was measured at 540 nm with a spectrophotometer using the 0.85%-saline tube as a blank and the distilled water tube as the 100%-haemolysis as standard. The highest value of optical density which corresponded to an incubation concentration of 0.1 % NaCl was taken as 100 % haemolysis.

Percentage lysis was calculated using the formula:

$$\text{Haemolysis (\%)} = \frac{\text{Abs of test}}{\text{Abs of standard}} \times 100$$

% haemolysis was plotted against % NaCl concentrations. The mean corpuscular fragility (MCF), which is the concentration of NaCl producing 50% lysis was extrapolated. Analysis was done using the student t-test was applied at 5% confidence level [11].

Statistical analysis

All data were expressed as Mean ± SEM. Data were analysed using one-way analysis of variance (ANOVA) at 5% level of significance. Dunnet's test was used to detect the difference among the treatment groups.

Results

Acute toxicity

No death was recorded in the rats treated orally with varying doses (250, 500, 1000 and 2000 mg/kg) of Itugha. It was well tolerated by the mice without any overt signs of toxicity.

Antioxidant potentials

The results of the antioxidant activities (DPPH and FRAP) are shown in Figure 1 and 2 respectively, while the in vivo antioxidant potentials (serum catalase and reduced glutathione) are represented in Table 1. The result showed that Itugha had percentage antioxidant activities comparable to ascorbic acid at high concentrations.

Discussion

This article goes to maintain that the methods used by the locals for production of Itugha is safe, since the acute toxicity test did not produce any mortality even at 2000mg/kg. Fermentation, which is the slow decomposition of organic matter induced by micro-organisms such as yeast, bacteria and moulds has also been implicated in the production of itugha from fresh *Irvingia gabonensis* seeds, thereby classifying itugha as a fermented food [17].

In the traditional preparation method, mashed fresh *Irvingia gabonensis* seeds are covered and kept away for a day, this is repeated every day until the Irvingia mash loses its elastomeric property. Without this protocol the final product fails and itugha is not obtained. The implication being that the fermentation process has to be controlled. The production process abhors water as both cleaning, pounding and storage has to be in dry environment [18]. Thus, this is a solid-state fermentation in which microorganisms intrinsic to *Irvingia gabonensis* are involved in initiating the process and the Irvingia mass providing the solid support on which successive microorganisms grow depending on the metabolites generated. The fermenting solid medium comprises both the substrate and the solid

support (mash) on which the fermentation takes place [19].

Different methods have been adopted to evaluate in vitro antioxidant activities so as to allow rapid screening of substances since substances that have low antioxidant activity in vitro, may likely show little activity in vivo [20]. Free radicals play enormous roles in a wide variety of pathological manifestations. Antioxidants neutralize the free radicals and prevent them from causing diseases. This action is accomplished either by scavenging the reactive oxygen species or by protecting the antioxidant defence mechanisms [21]. The electron donation ability of natural products can be measured by 2, 2-diphenyl-1- picrylhydrazyl radical (DPPH) purple-coloured solution bleaching [22]. The method is based on scavenging of DPPH through the addition of a radical species or antioxidant that decolorizes the DPPH solution. The degree of colour change is proportional to the concentration and potency of the antioxidants. A large decrease in the absorbance of the reaction mixture indicates significant free radical scavenging activity of the compound under test [23].

Antioxidant activity of plant extracts have formerly been described using the DPPH radical as described by Iwalewa [24], which is similar to the one used in this experiment. The percentage antioxidant activity of Itugha increased considerably up to 400 µg/ml concentration where it produced its optimum effect. The percentage antioxidant activity of Itugha is below the values for ascorbic acid although comparable significantly ($p<0.05$) (Fig. 1). These findings demonstrate that Itugha may possess antioxidant properties. Antioxidants neutralize the free radicals and prevent them from causing diseases. Also, the FRAP values of Itugha at 10, 50, 100, 200, and 400 µg/ml were significant at $p<0.05$. There was concentration dependent increased in the FRAP values of Itugha (Fig. 2). Since FRAP assay is easily reproducible and linearly related to molar concentration of the antioxidants present, thus it can be reported that Itugha may act as a free radical scavenger, capable of transforming reactive free radical species into stable non radical products.

Reduced activities of catalase (CAT) seen in the serum analysis might have been as a result of their utilization from the decomposition of superoxide anion generated by lipid peroxidation (Table 1). Lowered activities of these enzymes in normal animals may result in a number of deleterious effects. Pre-treatment with Itugha increased the activities of CAT. Reduced glutathione (GSH) is an intracellular reductant and protects cells against free radicals, peroxides and other toxic compounds. GSH is a naturally occurring substance that is abundant in many living creatures (Table 1); GSH depletion increases the sensitivity of cells to various aggressions leading to tissue disorder and injury [25].

The osmotic fragility of red blood cells and the body cells in general reflect their ability to take up water without lysis and also withstand hypotonic environments [11]. Osmotic fragility is experimentally denoted by MCF values which was significantly higher in 20 mg/ml thus, the red blood cells in this group was able to retain more water volume in a hypotonic solution before 'stretching' of the membrane that progresses to lysis (Table 2). This implies that the red blood cells in these groups were able to retain more water volume in a hypotonic solution before 'stretching' of the membrane that progresses to lysis. Previous studies have shown that the cytoskeletal proteins of red blood cells from sick patients are heavily glycosylated and that spectrin is oxidatively damaged [26]. Also, several lipids (free cholesterol, sphingomyelin, and phosphatidylcholine) on the outer surface of the phospholipid bilayer is significantly decreased [27]. Sujatha and Srivinas showed that aqueous extracts inhibited lipid peroxidation (LPO) in human erythrocyte membrane [28]. The efficacy of treatment regimens on the integrity, cell shape, and health status of red blood cells should be tracked, as these cells' health status is crucial to the overall wellness of a disease infected individual [29].

Conclusion

The current evaluation indicated that Itugha possess antioxidant properties by scavenging free radicals, and increasing the endogenous blood antioxidant enzymes levels. It was also able to protect the integrity of erythrocyte membranes from haemolysis.

Conflict of interests

The authors declare no competing financial interest. The authors solely funded the project

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Table 1. Effect of Itugha on antioxidant parameters

Groups	Treatment (mg/kg)	Serum CAT (U/ml)	Serum GSH (U/ml)
Group I	250	0.63±0.02*	8.14±0.41*
Group II	500	0.65±0.04*	9.45±045*
Group III	1000	0.79±0.03*	13.24±0.41*
Group IV	Ascorbic acid	0.87±0.06*	14.41±0.59*
Group V	Distilled water	0.44±0.04	4.14±0.38

* indicate significant ($p<0.05$) increases compared to normal control. Values are mean±SEM. n = 5 in each group.

Table 2. Median Corpuscular Fragility (MCF) values and % stabilization activities of Itugha

	Concentration (mg/ml)	MCF (% [NaCl])	% stabilization
Itugha	20	0.361±0.011	61.88
	10	0.287±0.025	28.70
	5	0.244±0.031	9.42
control	-	0.223±0.017	

The MCF values and their corresponding % stabilization were increased in the treatment groups as compared to the negative control. The highest concentration (20 mg/ml) gave highest % stabilization.

Figure 1. 2, 2-diphenyl-1-picrylhydrazylradical (DPPH) spectrophotometric assay for Itugha

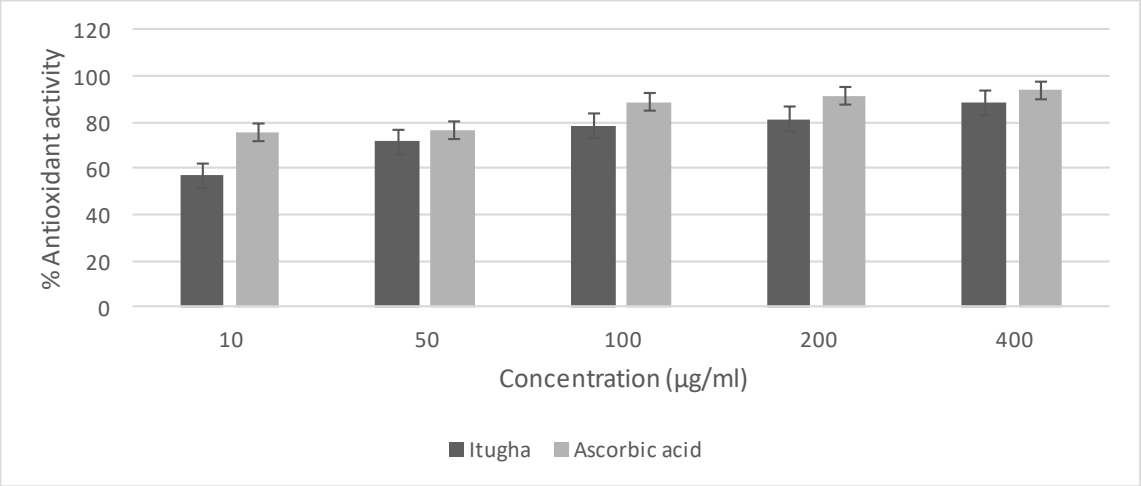
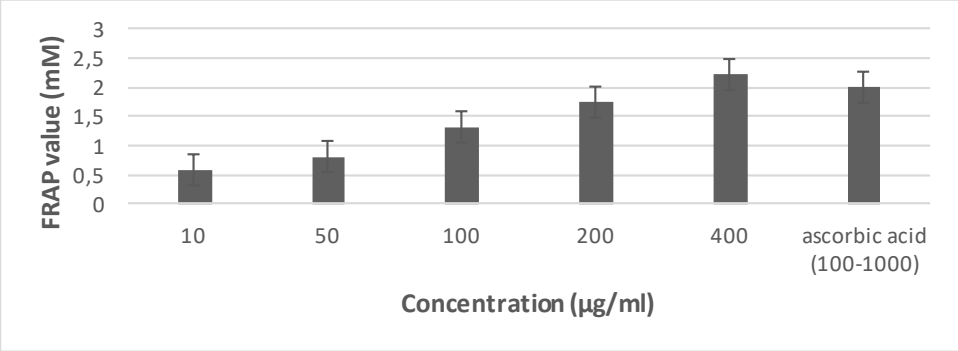


Figure 2. Ferric reducing antioxidant power (FRAP) of Itugha



The FRAP of Itugha assayed showed a significant ($p<0.05$) effect