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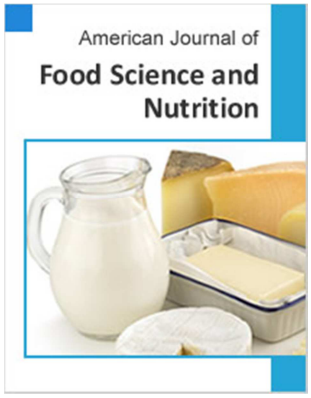

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Antioxidant Properties and the Significance of Testa in *Irvingia* Spp (*Wombolu* and *Gabonensis*)

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Abstract

Physical properties and antioxidant activity of oil extracts from kernels of *Irvingia gabonensis*, *(Ig)* and *Irvingia wombolu (Iw)* were studied. In addition, the effect of testa on antioxidant was analyzed. Total Phenolics (TP), Ferric Reducing Antioxidant Power (FRAP), Total Antioxidant Activity (TAA) by thiocyanate (-SCN) method and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) techniques were used to assay antioxidant activity. Results showed that both oil extracts exhibited high antioxidant activity of between 61 – 93% irrespective of time of investigation. Mean inhibitory concentration, IC_{50} of *Irvingia spp* was 15.4µg/ml compared with those of reference Quercetin and Vitamin C recorded as 4.38 and 1.85 µg/ml respectively. The contribution of testa to inhibit autoxidation was statistically significant ($p<0.05$). IC_{50} of *Irvingia wombolu* with testa, *Iwt* (4.86µg/ml) particularly, was comparable with that of Quercetin. TAA by –SCN method discriminated best both between samples with and without testa. The study, therefore, showed that oil extract of *Irvingia* kernels contain high level of phenolic substances capable of inhibiting lipid autoxidation, and good enough to serve as quality oils in diet of animals and man. The use of intact kernels of *Irvingia* with testa is highly recommended.

1. Introduction

Secondary metabolites from plants have important biological and pharmacological activities, such as anti-oxidative, anti-allergic, antibiotic, hypoglycemic and anti-carcinogenic [1 - 3]. Many disorders in humans and other living organisms such as atherosclerosis, arthritis, Alzheimer disease, cancer etc., may be as a result of increased concentrations of free radicals in an organism. Reactive oxygen species (ROS) and nitrogen (RNS) species are the most frequent pro-oxidants, which either originate from normal metabolism or are induced by UV radiation and different pollutants. Harmful effects of disturbed antioxidant-prooxidant balance can be largely prevented by intake of antioxidant substances [4, 5].

Antioxidants are molecules that can neutralize free radicals by accepting or donating an electron to eliminate the unpaired condition. Typically, this means that the antioxidant molecule becomes a free radical in the process of neutralizing a free radical molecule to a non-free radical molecule. But the antioxidant molecule is a much less reactive free radical than the initial free radical. The antioxidant molecule may be readily neutralized by another antioxidant and/or may have another mechanism for terminating its free radical condition. Free radical damage to LDL cholesterol can result to atherosclerosis,

so antioxidants have the potential to protect against cardiovascular diseases. Similarly, free radicals have been implicated in cancer, Alzheimer`s disease, inflammatory diseases, ischemic-reperfusion injury and several other disease conditions against which antioxidants are of benefit [6].

Primary antioxidants stop the oxidation process by terminating the radical chain reaction by conversion of radicals into more stable compounds. A unique characteristic of antioxidants is that they are effective in very small concentrations, 0.001–0.1%. Exceeding these optimal concentrations can diminish their activity or even enhance pro-oxidation [7].

Apart from primary antioxidants, secondary antioxidants are also distinguishable, because they slow down lipid oxidation as a result of other process than the termination of the auto-oxidation chain reaction. The main mechanism of their actions depends largely on the ability to bind certain metal ions (chelating compounds, *e.g.*, EDTA), and oxygen, as well as on the absorption of UV rays, regeneration of primary antioxidants (*e.g.* ascorbic acid), creation of a protective border surface between oil and air (*e.g.* phospholipids), causing also a decomposition of peroxide to non-radical products or deactivation ("scavenging" or "quenching") of a singlet oxygen (*e.g.* β-carotene) [8, 9].

Determination of the antioxidant activity is one of the ways to biologically and nutritionally evaluate the quality of a fruit. It has been proven that antioxidant activity depends on the type of phenolics present in the fruit, as some phenolic compounds exhibit higher antioxidant activity than others. It is assumed that the ability of plant polyphenols to scavenge reactive oxygen radicals participates in the protective mechanism of plants. Due to the chemical diversity of antioxidants present in fruit, their strictly defined content is unavailable. In spite of the fact that total amount of antioxidants in various fruit types need not represent the total antioxidant capacity, almost all phenolic compounds in fruits demonstrate some antioxidant activity. However, detection of therapeutically active components in a biological matrix is a very complex procedure, and their determination differs in individual studies [10]. The drug formulations which containing natural antioxidant agents are used for treatment of many diseases like Alzheimer's disease, diabetes, stroke, atherosclerosis, and cancer [9]. The antioxidant activity of medicinal plants might be attributed to its phenolic contents especially flavonoids, lignans, catechins and anthocyanin [11].

Irvingia is a genus of African and Southeast Asian trees in the family *Irvingiaceae*, sometimes known by the common names wild mango, African mango, or bush mango. They bear edible mango-like fruits, and are especially valued for their fat and protein rich nuts, known variously as ogbono, etima, odika, or dika nuts. The subtly aromatic nuts are typically dried in the sun for preservation, and are sold whole or in powder form. They may be ground to a paste known variously as dika bread or Gabon chocolate. Their high content of mucilage enables them to be used as thickening agents for dishes such as ogbono soup. The nuts may also be pressed for vegetable oil. The fruit is a large drupe, with fibrous flesh. The trees yield a hard wood, useful in construction.

The wild mango tree is very tall and can reach 50 meters high and 2 meters and half in diameter. Its bark is gray in colour. There are two varieties of wild mango, one is "sweet" (*I. gabonensis*) and the other has a "bitter" skin (*I. wombolu*). The germination rate of these two species is 80%. *I. gabonensis* can be found in the humid forest zone of Cameroon, Nigeria and other West African countries while *l. wombolu* is more localized in the south west of some of these countries. The fruit of the *l. gabonensis* weighs about 200g, while the *l. wombolu* weighs about 85g when harvested [12].

Both varieties do not produce fruits during the same season. *I. gabonensis* produce fruit from April to June, while *I. wombolu* does so from November to March. The *gabonensis* variety has an edible yellowish pulp when ripe, with a turpentine flavour while the *wombolu* variety has a bitter inedible and acrid pulp [13]. The bark of the wild mango fruit tree is used in traditional medicine for the treatment of hernia, diarrhea and yellow fever. It is also used to cure wounds, toothaches and as an antidote [12].

The aim of this research is to study the antioxidant levels of wild mango (*Irvingia spp*) kernels. Also, to determine the effect of testa on the antioxidant activity of both spp.

2. Materials and Methods

2.1. Preparation of Materials

Mature fruits of wild mango (*Irvingia gabonensis* and *Irvingia wombolu)* were plucked from trees at Kogi State University Anyigba, Kogi State, Nigeria. Due to variations in their fruiting seasons, *Irvingia wombolu* was plucked before the *gabonensis* variety. The fleshy pulp was peeled using a stainless steel knife to release the seeds which were then sun-dried for a week. The shells were then cracked manually to obtain the kernels which were creamy and bi-lobed. The kernels were then oven-dried at 105 degrees to storable moisture content and finally ground to powder for commencement of analysis.

Irvingia varieties were divided into two groups, those with testa and those without testa. The groups without testa had theirs deliberately removed. It was noticed that the testa of the *Irvingia* varieties were firmly attached to the grayish creamy kernel which was described by [14] as intact kernel. The percentage moisture content of the kernel samples after drying in the oven to a constant weight was then calculated using: loss in weight/weight of sample x 100

2.2. Chemicals and Reagents

Folin-Ciocalteau reagent from Qualikems Fine Chemicals of India, 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) and Quercetin manufactured by Sigma-Aldrich of Germany,

Sodium carbonate, linoleic acid, Tween-20, Vitamins C and E used were manufactured by BDH Chemicals in UK. Other solvents and reagents used in this study were of analytical grade and available commercially.

2.3. Oil Extraction

Each sample type was subjected to solvent extraction using Soxhlet method. *Irvingia* kernel powder (100g) each of the species was placed in a cellulose paper (thimble), placed in the extraction chamber and extracted using ethanol as solvent. The extraction was carried out for six (6) hours and the resulting extract was evaporated under reduced pressure by the aid of a rotary evaporator to remove the solvent. The specific gravity of the oils was determined using a 2.5ml specific gravity bottle as described by [15]. The ambient temperature on this occasion was 29°C. Refractive index was determined using an Abbe table refractometer Model RG 701, Officine Galeleo, Italy, [14]. The melting points of the oils/fats were determined using Thiele-Dennis tube. All values of analyses were reported as mean of three (3) determinations.

The total phenolic contents of the extracts were determined using the modified method of [16] using Folin-Ciocalteu's phenol reagent (FCR). Briefly, each of the sample extract of varied concentration (20, 40, 60, 80 and 100mg/l) in 25ml volumetric flaks containing 9ml of distilled water, was added 1ml FCR and vortexed. After 5minutes, 10ml of 7% sodium carbonate solution was added and incubated for 90 minutes at room temperature. Standard solutions of gallic acid were prepared in the same way. Absorbance was measured at 765nm and the results of the samples were expressed as milligrams of gallic acid equivalent per 100g of fresh weight (mgGAE/100g fresh).

Ferric reducing antioxidant power FRAP assay was determined as described by [17]. Briefly, Acetate buffer (300mM) at pH 3.6 was prepared by dissolving 3.1g of sodium acetate tri-hydrate in 16ml of glacial acetic acid and making the volume to 1L with distilled water. 2,4,6-tripyridyl-s-triazine (TPTZ) (10mM) was dissolved in 40mM of HCl and 20mM of $FeCl_3.6H_2O$. The working FRAP reagent was prepared by mixing the three (3) solutions in the ratio of 10:1:1 respectively at the time of use. L- Ascorbic acid was used as the standard. Samples were mixed with 3ml of working FRAP reagent, vortexed and absorbance (593nm) was measured at 0 minute. The samples were then placed in the water bath at 37°C and absorbance was again measured after 4 minutes. The absorbance of standard was processed in the same way. The results were calculated as follows.

$$\text{FRAP value of sample } (\mu M) = \frac{\text{Change in absorbance of sample from 0 to 4 minute}}{\text{Change in absorbance of standard from 0 to 4 minute}} \times \text{FRAP value of standard}$$

FRAP value of standard (Ascorbic acid) is given as 2. [17].

The antioxidant activities in a linoleic acid emulsion system of the samples were determined using the thiocyanate method as modified by [16]. Briefly, Samples (10mg) were dissolved in 10ml of water and various concentrations (50, 100, 250 and 500µg/ml) were prepared and added to the linoleic acid emulsion system and homogenized. {The emulsion system was prepared by mixing 0.2804g linoleic acid, 0.2804g tween 20 and 50ml of 40mM potassium phosphate buffer (pH 7.0)}. The emulsion system (2.5ml), 2ml of the phosphate buffer was added to prepared sample solutions. The final volume of the emulsion system was adjusted with the phosphate buffer to 5ml. The control was prepared by mixing 2.5ml of the emulsion system and 2.5ml of the phosphate buffer. The reaction mixture was incubated in the dark at 37°C in a glass flask in the oven for 60 hours. The incubated samples (1ml) was removed at 12 hour intervals, 0.1ml 20mM $FeCl_2$ and 0.1ml 30% NH_4SCN were added. After the mixture was rested for three (3) minutes, the peroxide value was determined by monitoring absorbance at 500nm until the absorbance of control reached the maximum. The degree of linoleic acid peroxidation (antioxidant activity) was calculated using the following formula:

$$\text{Antioxidant activity} = \frac{A_c - A_s}{A_c} \times \frac{100}{1}$$

Where Ac is the absorbance of control and As is absorbance of sample.

The free radical scavenging activity of the samples were evaluated using the stable radical, 2, 2-diphenyl-1-picrylhydrazyl (DPPH*) according to method described by [18]. To 1ml of various concentrations of the samples or the reference compounds, Quercetin and vitamin C, (31.25, 62.50, 125.00, 250.00 and 500µg/ml) in test tubes were added 1ml of 0.3mM DPPH* in methanol. Absorbance was measured after 30 minutes at 517nm against a DPPH* control containing only 1ml of methanol. The percentage scavenging activity was calculated by the following formula:

$$\%\ \text{scavenging activity} = \text{Absc} - \frac{\text{Abss}}{\text{Absc}} \times 100$$

Where Absc is absorbance of control and Abss is the absorbance of test sample.

The % scavenging activity was plotted against the log concentration of the extracts (µg/ml) to determine the concentration of extract that reduced DPPH activity by 50% (IC_{50}). All determinations were performed in triplicates.

The statistical analysis was carried out using Graphpad instat 3 statistical solution software. Results were presented as mean value + standard deviation of three replicate determinations. Statistical analysis among treatments were determined at the significance level of $P < 0.05$.

3. Results and Discussion

The average moisture content of the *Irvingia* kernels was 26% and 6.4% for *Ig* and *Iw* respectively. The testa of

Irvingia kernels contributed 4% to the observed result. Since the moisture content of the *Ig* kernels was above equilibrium moisture content of 14.1% as reported by [14], this could suggest that these kernels could easily deteriorate from mould growth as well as early sprouting. However, all kernel types, with or without testa, were given similar treatment.

Table 1. *Some physical Properties of Oil Extract of Irvingia Varieties (wombolu and gabonensis).*

Kernel types	% Oil extracted (w/w)	Specific gravity (SG)	Refractive Index (RI)	Melting point (ºC)
Iwt	27.30+0.80	0.67+0.01	1.4425+0.41	38 – 40
Iwt_o	14.40+1.20	0.65+0.02	1.3940+0.03	37 – 40
Igt	34.02+ 1.40	0.92+0.01	1.3606+0.31	35 – 38
Igt_o	32.00+0.90	0.91+0.01	1.3443+0.00	36 – 38

Values are mean+SD of triple determinations at ambient temperature of 29°C.
Iwt = Irvingia wombolu kernel with testa; Iwt_o = Irvingia wombolu kernel without testa; Igt = Irvingia gabonensis kernel with testa; Igt_o = Irvingia gabonensis kernel without testa

Physical properties including percentage oil extracted, specific gravity, refractive index and melting point were determined and reported in Table 1. The results shows that percentage oil extracted were in the range of 14 – 34%. This indicated that the *Irvingia gabonensis* variety particularly, was a richer oil seed than *Irvingia wombolu* variety. [19] Reported that *Irvingia* seed kernels are richer in lipids than other conventional oil seeds such as cotton seeds, soybean, rapeseed and palm fruit. It was further reported that *Irvingia gabonensis* oil content varies between 34.3 and 62.7%. The value of 34.02% in this study is in the lower range. Differences in literature values could be due to geographical location.

The *Irvingia* varieties with testa had a difference of about 13% oil content and were relatively higher than those without testa, and which affirms the earlier work of [14]. By the thiocyanate method, TAA, all the extracts show high antioxidant activity of between 61 – 93% irrespective of time of investigation [20]. Furthermore, *Irvingia* varieties with testa inhibited linoleic acid peroxidation the most when compared with the other oils. This was ascribed to a proportion of about 11% polyphenolic constituents in the testa [14].

Table 2. *Phenolic content and Mean Antioxidant Property of the Kernel Oil Extracts.*

Oil extract	TP (mgGAE/100g)	FRAP (µM)	TAA (% inhibition of peroxides)
Iwt	$30.40 + 2.1^a$	$0.34 + 0.02^a$	$75.00 + 4.4^b$
Iwt_o	$28.80 + 2.0^a$	$0.33 + 0.03^a$	$68.09 + 2.0^a$
Igt	$36.80 + 2.0^b$	$0.11 + 0.02^a$	$83.92 + .9^c$
Igt_o	$36.20 + 1.9^b$	$0.52 + 0.03^b$	$70.00 + 2.0^a$
SEM	3.75	0.24	2.87

Values are mean of triplicate measurements +SD.
FRAP = Ferric Reducing Antioxidant Power assay. TAA = Total Antioxidant Activity TP = Total Phenolic Contents. SEM= Standard Error of Mean. Values with the same superscripts in a particular column are not significantly different ($p>0.05$) (Bonferroni multiple comparisons test).

The assessment of antioxidant properties of the various oil types by different techniques are presented in Table 2. There appeared to be freer gallic acid equivalents in *Irvingia* oil extracts. Significant difference ($p<0.05$) was observed between the varieties of *Irvingia* species. By the FRAP technique, *Irvingia gabonensis* without testa was more potent when compared with the *Irvingia wombolu* oil samples. The TAA technique was more sensitive to discriminate between kernel types and effect of testa. That is, TAA distinctly showed the significant ($p<0.05$) contribution of testa to inhibit autoxidation.

The oil of the *Irvingia* species had an IC_{50} of 15.4µg/ml on the average. The *Irvingia* variety with testa showed more potency by having relatively lower IC_{50} values than those without testa. The *wombolu* variety irrespective of the treatment given, had an average IC_{50} of 11.1µg/ml compared with the *gabonensis* variety with an average of 20.0µg/ml indicating that the *wombolu* variety was more potent in scavenging free radicals than the *gabonensis* variety. *Iwt*, was particularly the most potent of the oil extracts, implicitly identifying the role of testa in reducing the rate of autoxidation of oils. *Figure 1* shows the inhibition activity curve using DPPH technique.

Table 3. *IC_{50} Values of Oil Extracts ±Testa and Standards Using DPPH Technique.*

Extracts	IC_{50} (µg/ml)
Iwt	4.86
Iwt_o	17.30
Igt	17.12
Igt_o	22.41
Standards Quercetin	4.38
Vitamin C	1.85

When compared with the standards used, the oil extracts possessed lower potencies than the standards (Quercetin and Vitamin C). The standard, Vitamin C was found to have the lowest IC_{50} value of 1.85µg/ml, making it the most potent reducing substance to scavenge free radicals. Also, when comparing between the oil extracts, those with testa were found to have lower IC_{50} values making them more potent radical scavengers than their non-testa counterparts.

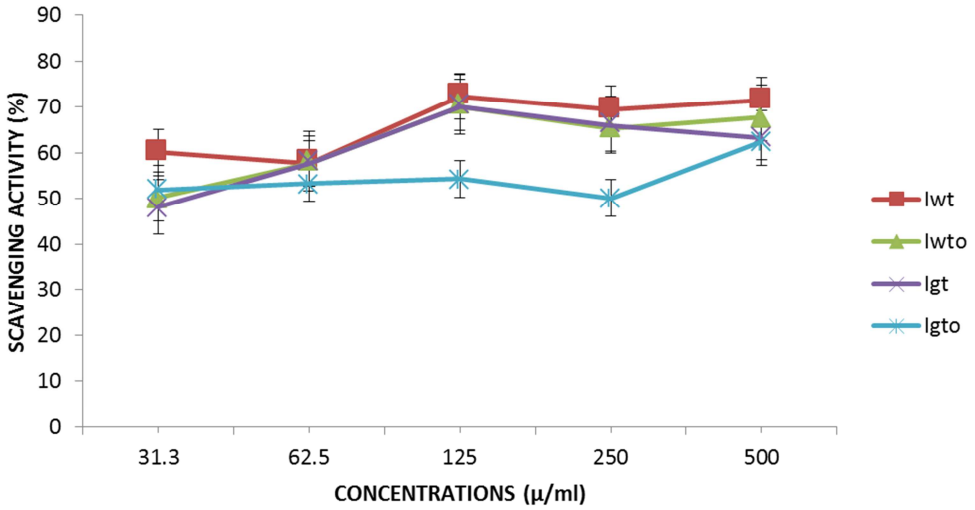

Figure 1. *Inhibition Activity of the Extracts using DPPH technique.*

4. Conclusion

This study has shown that the oil extracts of *Irvingia wombolu* and *Irvingia gabonensis* with testa proved to contain effective and potent antioxidants capable of reducing oxidative stress in animals and man. Furthermore, their antioxidant content can also prevent oxidative rancidity of oils. These beneficial effects support the earlier work of [14, 20, 21] that the testa of these kernels contributed about 11% by weight to the intact kernels and containing equivalent level of reducing substances, polyphenolic compounds inclusive [22, 23]. The antioxidant activity of polyphenols is directly related to their structure. Studies on the structure–antioxidant potential relationship have demonstrated that the effectiveness of conjugation and the number and position of hydroxyl groups on the phenolic rings are parameters determining antioxidant capacity [24, 25].

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