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Comparative studies of phytochemical contents and antioxidant potentials of selected nuts consumed in eastern part of Nigeria

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Abstract
The study investigated and compared the phytochemical contents and antioxidant potentials of tiger nut, groundnut, cashew nut and walnut. Phytochemical contents and the antioxidant studies of the nut extracts were done following standard methods. The result of this study showed that the nut extracts had varied concentration of the phytochemical contents. The nut extracts scavenged DPPH with EC_{50} of (2.00 ± 0.00, 3.51 ± 0.01, 3.00 ± 0.01 and 2.00 ± 0.0 µg/ml) for tiger nut, groundnut, cashew nut and walnut respectively compared to ascorbic acid standard (1.25 ± 0.02 µg/ml). The extracts also reduced hydroxyl radical (OH^-), EC_{50} (1.74 ± 001, 2.60 ± 0.01, 1.92 ± 0.01 and 1.48 ± 0.00 µg/ml) compared to ascorbic acid standard EC_{50} (1.14 µg/ml). Reduction of ferric ion to the ferrous form and the total antioxidant capacity which measures the water-soluble and fat-soluble in an extract shows a concentration dependency indicating antioxidant potentials of the nut extracts.

Keywords: nuts, phytochemicals and antioxidants

Introduction

Nut consumption has risen in Western countries in recent years as a result of both the inclusion of this food category in many healthy eating recommendations and widespread media coverage of recent evidence linking nut consumption to a variety of health benefits. The scientific evidence for nuts as cardio-protective foods stems from both epidemiological observations indicating a consistent inverse association between the frequency of nut intake and the development of choronal heart disease (CHD) [1, 2] and numerous short-term clinical trials showing beneficial effects of nut intake on the lipid profile [1, 2]. The explanation for these beneficial effects is most likely the synergistic association of the several bioactive constituents of nuts, all of which could have a positive impact on human physiology. Thus, nuts contain a lot of vegetable protein and fat, mainly unsaturated fatty acids [3]). They are also high in dietary fiber vitamins (e.g., folic acid, niacin, tocopherols, and vitamin B6), minerals (e.g., calcium, magnesium, potassium), and many other bioactive constituents such as phytosterols [4]. Reactive oxygen species (ROS) are chemically reactive molecules formed by living organisms as a consequence of normal cellular metabolism. At low to moderate concentrations, they function in physiological cell processes. Though, when present in high concentrations, they create adverse modifications to cell components, such as lipids, proteins, and DNA [5]. Antioxidant compounds are radical scavengers since they stop or delay the oxidation of substrate by radicals thus resulting in significant hindrance of lipid peroxidation in biological systems. Polyphenolic and Phenolic compounds comprise the core class of normal antioxidants present in foods, plants, and beverages. This study is aimed at comparing the phytochemical contents and antioxidant potentials of nuts consumed in south eastern part of Nigeria.

Materials and Methods

Materials

Samples collection and preparation

Cashew nuts (*Anacardium occidentale*), Tiger nuts (*Cyperus esculentus*), Ground nuts (*Arachis hypogaea),* Walnuts (*Tetracarpidium conophora),* were all bought from Eke Awka market in Awka, Anambra State, Nigeria. Some of the samples (Tiger nuts, cashew nuts and ground nuts) were already in their processed form ready for consumption when bought while the walnut samples were de-husked and sun dried for four days. After sun drying the samples, were ground using manual grinder and stored in an air tight container prior to use.

Methods

Quantitative phytochemical screening

Flavonoid

Flavonoid content was determined by the method of Boham and Kocipai-Abyazan [6]. Exactly 10 g of the nut samples were extracted with 100 ml of 80 % methanol at room temperature and allowed to stand for like 5 to 10 minutes. The whole solutions were filtered through whatman filter paper No. 42 (125 mm). The filtrate were later transferred into a crucible and evaporated to

dryness and weighed to a constant weight. The percentage flavonoid was calculated by difference.

$$\% \, flavonoids = \frac{W_2 - W_1}{Weight \, of \, sample} \, x \, 100$$

Where, W_1 = Weight of empty crucible. W_2 = Weight of crucible + residue.

Alkaloids

Five (5) grams of the nut samples were placed in a 250 ml beaker and 200 ml of 10 % acetic acid (CH_3CO_2H) in ethanol (C_2H_5OH) was added. The mixture was covered and allowed to stand for 4 hours at 25°C i.e. at room temperature. It was then filtered with filter paper No. 42 and the filtrate was concentrated on a water bath until it reaches a quarter of its original volume. Concentrated NH_4OH was added drop wise until precipitation was complete. The mixture was allowed to settle and the precipitate collected on a weighed filter paper and washed with dilute NH_4OH. The precipitate, alkaloid, was dried and weighed. The percentage alkaloid was calculated by difference [7, 8].

$$\% \, Alkaloids = \frac{W_2 - W_1}{Weight \, of \, sample} \, x \, 100$$

Where, W_1 = Weight of empty filter paper. W_2 = Weight of filter paper + Alkaloid.

Saponins

Saponin was determined by the method described by Obadoni and Ochuko [8]. Exactly 20 g of sample was weighed into a conical flask and 100 ml of 20 % ethanol was added. The sample was subjected to a hot water bath at 55 °C for 4 h with continuous stirring. The mixture was filtered and the residue re-extracted with another 200 ml 20 % ethanol. The combined extract was concentrated to 40 ml over water bath at about 90 °C. The concentrate was transferred into a 250 ml separatory funnel and 20 ml of diethyl ether added and shaked vigorously. This was allowed to settle and the aqueous layer collected while the ether layer was discarded. The purification process was repeated with 60 ml of n-butanol. The combined n-butanol extracts were washed twice with 10 ml of 5 % aqueous sodium chloride. The remaining solution was heated on a water bath to evaporate the solvents and dried in the oven to a constant weight. The saponin content was calculated as a percentage.

$$\% \, Saponins = \frac{W2 - W1}{Wt \, of \, sample} \, x \, 100$$

Where, W_1= Weight of filter paper. W_2= Weight of filter paper + residue.

Phenols

The fat free sample was boiled with 50 ml of diethylether $(CH_3CH_2)_2O$. A 5 ml of the boiled extract was pipetted into a 50 ml flask, then 10 ml of distilled water was added. After the addition of distilled water,2 ml of ammonium hydroxide solution and 5 ml of concentratedamylalcohol ($CH_3(CH_2)_3CH_2OH$), were also added. The samples were made up to markand left to react for 30 min for colour development. This was measured at 505 nm [9].

$$Conc. of \, Sample \, (mg/l) = \frac{Absorbance \, of \, Sample \, x \, Conc. \, of \, Sample}{Absorbance \, of \, Standard}$$

Tannin

The Follin Denis titrating method as described by Pearson [10]. To 20 g of the crushed sample in a conical flask was added 100 ml of petroleum ether and covered for 24 hours. The samples were then filtered and allowed to stand for 15 minutes allowing petroleum ether to evaporate. It was then re-extracted by soaking in 100 ml of 10 % acetic acid in ethanol for 4 hours. The samples were then filtered and the filterate collected. 25 ml of NH_4OH were added to the filtrate to precipitate the alkaloids. The alkaloid were heated with electric hot plate to remove some of the NH_4OH still in solution. The remaining volume was measured to be 33 ml. 5 ml of this was taken and 20 ml of ethanol was added to it. It was titrated with 0.1 M NaOH using phenolphthalein as indicator until a pink end point is reached. Tannin content was then calculated in % ($C_1V_1 = C_2V_2$) molarity. Where, C_1 = Conc. of tannic acid, C_2 = Conc. of base, V_1 = volume of tannic acid and V_2 = Volume of base.

Cardiac glycoside

To 1 g of the samples in a beaker, 5 ml of methanol was added and allowed to stand for 10 minutes. From the mixture, 1 ml of the extract was taken to a 100 ml beaker and 1 ml of 2 % solution of 3,5-DNS (Dinitro salicylic acid) and 1 ml of 5 % aqueous NaOH were added. The samples were boiled for 2 minutes in a water bath at 95-100 °C until brick red precipitate was observed. An empty Whatman filter paper No. 42(125 mm) was weighed and was used to filter the boiled samples. The filter paper with the absorbed residue was taken and dried in an oven at 50 °C till dryness and the filter paper reweighed. The percentage of cardiac glycoside was calculated.

$$\% \text{ Cardiac Glycosides} = \frac{(\text{Wt of filter paper + residue}) - (\text{Wt of filter paper})}{\text{Wt of sample}} \text{ x } 100$$

Phytate

The phytate content was determined using the method of Young and Greaves (1940) as adopted by Lucas and Markakes [11]. A 0.2 g of the samples were weighed into different 250 ml conical flasks. Each sample was soaked in 100 ml of 2 % conc HCl for 3hrs. The sample was then filtered. 50 ml of each filtrate was placed in 250 ml beaker and 100 ml distilled water added to each sample. Also 10 ml of 0.3 % ammonium thiocyanate solution was added as indicator and titrated with standard iron (III) chloride solution which contained 0.00195 g iron per 1 ml.

Oxalate

This determination involves digestion, oxalate precipitation, and permanganate titration [12].

- **Digestion**

Two grams (2 g) of the samples were suspended in 190 ml of distilled water in a 250 ml volumetric flask,10 ml of 6M HCL was added and the suspension digested at 100°C for 1 hour, cool and then make up to 250 ml mark before filtration.

- **Oxalate precipitation**

Duplicate portions of 125 ml of the filtrates were measured into beakers and 4 drops of methyl red indicator added. This was followed by the addition of NH_4OH solution (dropwise) until the test solution changes from salmon pink colour to a faint yellow colour (pH 4-4.5). Each portion was then heated to 90 °C, cooled and filtered to remove precipitate containing ferrous ion. The filtrate was again heated to 90 °C and 10 ml of 5 % $CaCl_2$ solution was added while being stirred constantly. After heating, it was cooled and left overnight at 25 °C. The solution was then centrifuged at 2500 rpm for 5 minutes. The supernatant was decanted and the precipitate completely dissolved in 10 ml of 20 % (v/v) H_2SO_4 solution.

- **Permanganate titration**

At this point, the total filtration resulting from digestion of 2 g of flour was made up to 300 ml. Aliquots of 125 ml of the filtrate was heated until near boiling and then titrated against 0.05 M standardized $KMNO_4$ solution to a faint pink colour which persists for 30 sec. The calcium oxalate content was then calculated.

Steroids

Quantities of (0.5 g) of samples were weighed into a 100 ml beaker and were extracted with 20 ml of chloroform-methanol (2:1) for about 30 mins. The samples were filtered using a whatman filter paper (No.1) and was dried using 100 ml flask. The analysis was carried out repeatedly until the samples were free from steroid. About 1ml of the extract was pipette and 5 ml KOH was added and the mixture was shook properly to ensure homogeneous mixture. The mixture was placed in a water bath at 40 ^{0}C for about 90 mins, after that the sample was cooled at room temperature and 10 ml of petroleum ether was added followed by 5 ml of distilled water. This mixture was evaporated to dryness on a water bath. About 6 ml of Liebermann-Burchard reagent was added to the residue and the absorbance was read at 620 nm [13]. The standard steroids concentraton of 0-4mg/ml was prepared and the sample was treated similarly. To calculate the percentage steroid we used the formula:

$$\% \text{ Steriod} = \frac{Absorbance \times Gradient\ Factor \times Dilution\ factor}{Sample\ weight\ in\ grams \times 10000}$$

***In-vitro* Antioxidant Assay**

***In-vitro* DPPH (2, 2-Diphenyl-1-picrylhdrazyl) Radical Scavenging assay**

The method of Gyamfi *et al* [14]. was used, one millilitre (1 ml) of 3mM DPPH in methanol was added to 2.0 ml of the extract at concentrations of 15.63, 31.32, 62.50, 125.00 and 250.00 µg/ml. The mixture was shaken vigorously and allowed to stand in the dark at room temperature for 25 minutes. One millilitre (1.0 ml) of methanol served as blank solution while the negative control was 1.0 ml of 3mM DPPH in methanol. L-Ascorbic acid was used as the reference standard. The absorbance of the assay mixture was read at 518 nm and blank used to zero the spectrophotometer. Percentage inhibition of DPPH was calculated using the equation:

$$\% \text{ inhibition of DPPH} = \frac{A_0 - A_S}{A_0} \times 100$$

A_o is absorbance of the control and A_s is absorbance of the test sample.

***In-vitro* hydroxyl radical (·OH) scavenging assay**

The method reported by Aruoma and Halliwell [15] was used for the assay. The reaction mixture in a final volume of 1.0 ml contained, 500 µl of the extract in various concentrations of 15.63, 31.32, 62.50, 125.00 and 250.00 µg/ml, 100 µl of (2.5 µM of 2-deoxyribose in 20 mM potassium phosphate buffer pH 7.4), 200µl of (100 µM) $FeCl_3$ and (104 µM) EDTA (1:1, v/v), 100 µl of (1 mM) H_2O_2 and 100 µl of (100 µM) L-ascorbic acid. The mixtures were incubated for 1 h at 37 ^{o}C, followed by addition of 1.0 ml of 1% (w/v) TBA in 0.05 M NaOH and 1.0 ml of 2.8% (w/v) TCA. The resulting mixture was heated for 15 min at 100 ^{o}C. After cooling on ice, the absorbance was read at 532 nm. A blank containing deoxyribose and buffer was used to zero the spectrophotometer while the control contained the same volume of the reaction mixture without the sample. Inhibition of 2-deoxyribose degradation expressed in percentage was calculated as per the equation:

$$\% \text{ inhibition} = \frac{A_0 - A_S}{A_0} \times 100$$

***In-Vitro* ferric reducing antioxidant power assay (FRAP)**

The method of Oyaizu [16] was use to determine the reducing power of the extracts. Two milliliters (2.0 ml) of the extracts at different concentrations of 15.63, 31.32, 62.50, 125.00 and 250 µg/ml were differently mixed with 2.0 ml of 10 mg/l potassium ferricyanide (0.1% w/v).

The mixtures were incubated in water bath at 50 ^{0}C for 20 minutes. Following this, 2.0 ml of 100 mg/L trichoroacetic acid solution (10% w/v) was added.

Two milliters (2.0 ml) of distilled water was then added to an aliquot of 2.0 ml of the mixture.

After which 0.4 ml of 0.1 % ferric chloride ($FeCl_3.6H_2O$) solution was added to the solution and mixed thoroughly. The absorbance of the reaction mixture was read at 700 nm after 10 minutes of reaction. The blank contained distilled water.

Total antioxidant capacity assay (TAC)

Total antioxidant capacity assays of the extracts were done using the method described by Dildar *et al* [17]. A 0.1 ml aliquout of different concentrations of the extract 15.63, 31.32, 62.50, 125.00 and 250 µg/ml and ascorbic acid was mixed with 1 ml of reagent solution containing 600 mM sulphuric acid, 28 mM sodium phosphate and 4 Mm ammonium molybdate at 1:1:1 ratio. The test tubes were covered with aluminium foil and incubated in water bath at 95 ^{0}C for 90 minutes. After the solutions were cooled at room temperature; the absorbances of the mixtures were read at 765 nm.

The blank contained 1 ml of the reagent solution while the control contained the same volume of the reaction mixture without the extract.

Ascorbic acid was used as the standard.

The assay was carried out in triplicate. The antioxidant capacity (TAC) is expressed as equivalent of ascorbic acid.

Results

Table 1: Phytochemical composition of four selected food nuts consumed in south east Nigeria

Composition (%)	Tigernuts	Ground nuts	Cashew nuts	Walnuts
Flavonoid	7.80±0.03[a]	3.40±0.01[b]	3.00±0.00[d]	3.20±0.01[c]
Alkaloid	1.60±0.01[a]	1.20±0.00[b]	1.20±0.01[b]	0.80±0.00[c]
Saponin	4.80±0.00[c]	5.20±0.01[b]	2.80±0.01[d]	8.20±0.02[a]
Glycosides	10.00±0.02[b]	7.00±0.01[d]	15.00±0.03[a]	8.12±0.01[c]
Tannins	726.13±2.34[a]	696.30±2.54[c]	672.75±3.04[d]	712.10±2.14[b]
Phenols	970.24±5.32[b]	992.98±4.65[a]	273.64±2.01[d]	886.42±3.76[c]
Phytate	0.10±0.00[b]	0.10±0.00[b]	0.12±0.00[b]	0.62±0.01[a]
Oxalate	0.51±0.00[c]	1.01±0.01[b]	1.54±0.01[a]	0.24±0.00[d]
steroids	1.50±0.01[b]	0.50±0.00[c]	0.00±0.00[d]	2.68±0.02[a]

Values are mean ± Standard deviation of triplicate determinations.
Values within the same row bearing the same superscript letters are not statistically significant at $P<0.05$

Table 2: 2, 2 Diphenyl-1- picrylhydrazyl (DPPH) radical scavenging capacity of four selected nuts commonly consumed in south east Nigeria

Conc (ug/ml)	Tigernuts	Groundnuts	Cashewnuts	Walnuts	Ascorbic acid
62.50	33.56±0.03	10.78±0.02	17.12±0.02	38.16±0.03	59.22±0.03
125.00	58.98±0.02	13.69±0.01	19.94±0.03	60.54±.02	69.64±0.02
250.00	77.99±0.04	26.45±0.02	28.16±0.02	70.62±.01	75.34±0.02
500.00	79.19±0.02	29.96±0.02	44.09±0.02	75.14±0.03	78.21±0.03
EC_{50}	2.00±0.00	3.51±0.01	3.00±0.01	2.00±.0.00	1.25±0.02

Values are mean ± Standard deviation of triplicate determinations.

Table 3: Hydroxyl (OH) radical scavenging capacity of four selected nuts commonly consumed in south east Nigeria

Conc (ug/ml)	Tigernuts	Groundnuts	Cashewnuts	Walnuts	Ascorbic acid
62.50	52.47±0.02	14.01±0.01	42.99±0.02	45.16±0.02	57.00±0.03
125.00	60.98±0.02	39.15±0.02	60.03±0.02	62.52±-.01	66.21±0.02
250.00	88.87±0.03	42.03±0.02	68.68±0.03	72.14±-.02	68.34±0.02
500.00	89.56±0.02	50.27±0.03	83.79±0.02	76.28±0.03	70.42±0.03
EC_{50}	1.74±0.01	2.60±0.01	1.92±0.01	1.48±.0.00	1.14±0.02

Values are mean ± Standard deviation of triplicate determinations.

Table 4: Ferric reducing antioxidant power (FRAP) of four selected nuts commonly consumed in south east Nigeria

Conc (ug/ml)	Tigernuts	Groundnuts	Cashewnuts	Walnuts	Gallic acid
62.50	0.01±0.00	0.03±0.01	0.13±0.02	0.01±0.00	0.01±0.00
125.00	0.02±0.00	0.04±0.01	0.36±0.01	0.03±0.01	0.02±0.00
250.00	0.04±0.01	0.46±0.02	0.52±0.02	0.14±-.01	0.10±0.01
500.00	0.07±0.02	0.57±0.02	0.76±0.02	0.26±0.01	0.13±0.01

Values are mean ± Standard deviation of triplicate determinations.

Table 5: Total antioxidant capacity (TAC) of four selected nuts commonly consumed in south east Nigeria

Conc (ug/ml)	Tigernuts	Groundnuts	Cashewnuts	Walnuts	Ascorbic acid
62.50	0.12±0.00	0.29±0.01	0.33±0.00	0.26±0.00	0.02±0.00
125.00	0.14±0.00	0.55±0.01	0.57±0.01	0.32±0.01	0.12±0.01
250.00	0.28±0.01	0.81±0.02	0.58±0.02	0.41±-.01	0.13±0.01
500.00	0.52±0.02	0.59±0.01	0.87±0.01	0.46±0.00	0.22±0.00

Values are mean ± Standard deviation of triplicate determinations.

Discussion

Phytochemicals have been shown by different researchers to have various pharmacological effects. Alkaloids are utilized in drugs for decreasing cerebral pain and fever. These are ascribed to their antibacterial and pain relieving properties [18, 19]. Tannin compounds have antimicrobial exercises and are liable for forestalling and treating urinary tract infections and other bacterial diseases. The result of this study shows that tiger nut has the higher quantity of flavonoids and alkaloids (7.80 ± 0.03 and 1.60 ± 0.0) respectively compared to the other nuts. Walnut showed the highest quantity of saponin (8.20 ± 0.02) while cashew nut had the highest quantity of glycosides compared to the nuts assayed. Tiger nut had the highest concentration of tannin (726.12 ± 234) while the phenols were higher in groundnut (992.98 ± 4.65), walnut showed the highest concentration of phytate (0.62 ± 0.01) while tiger nut had the highest concentration of oxalate (0.51 ± 0.00) compared to other nuts examined. The highest quantities of steroids were seen in walnut. The presence of these metabolites proposed that these nuts may be of restorative significance [20]. Presence of these optional

metabolites may add to its antioxidant and pharmacological potentials.

The addition of the extracts to diphenylpicrylhydrazine (DPPH) resulted to the discoloration of DPPH from purple to yellow, in a concentration dependent manner, which could be as a result of the antioxidant compounds in the nuts. Tiger nut and walnut reduced DPPH radical EC_{50} (2.00 ± 0.00 and 2.00 ± 0.00 µg/ml) more than groundnut and cashew nut EC_{50} (3.51 ± 0.01 and 3.00 ±0.01 µg/ml) respectively compared to the ascorbic acid EC_{50} (1.25 ± 0.02 µg/ml) used as standard (Table 2).

Hydroxyl radical (OH^-) is the most active in the reactive oxygen species, and can cause most harm to organisms. Hydroxyl radicals reacts with molecules such as polypeptides, proteins, nuclear acids and lipids, to cause biomolecules oxidative damages and cell mutations or necrosis [21, 22, 23]. The extract also scavenged hydroxyl radical in a concentration dependent manner with tiger nut and walnut EC_{50} (1.74 ± 0.01 and 1.48 ± 0.00 µg/ml) scavenging OH^- more than groundnut and cashew nut EC_{50} (2.60 ± 0.01 and 1.92 ± 0.00 µg/ml) respectively, compared to ascorbic acid EC_{50} (1.14 ± 0.02 µg/ml).

Ferric iron (Fe^{3+}) is reduced, by electron-donating antioxidants present in the extracts, to its ferrous form (Fe^{2+}). The extracts showed a concentration dependency in the reduction of ferric ion to ferrous ion Table 3 which indicates their antioxidant potentials.

Total antioxidant capacity evaluates both water-soluble and fat-soluble antioxidants (total antioxidant capacity) and it's based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of green phosphate/ Mo(V) complex at acid pH. The nut extracts showed a concentration dependency in the reduction of Mo(VI) to Mo(V) Table 5 indicating antioxidant potentials compared to ascorbic acid which has been used as a reference standard with which plant extracts with potential antioxidants are compared [24].

Conclusion

The nut extracts scavenged DPPH, hydroxyl radical, reduced ferric iron to ferrous ion.

The extracts showed concentration dependency in reduction of Mo (VI) to Mo (V) indicating high content of water and fat soluble antioxidants.

The result also showed that walnut and tiger nut scavenged the radicals than other nut extracts, this could be as a result of high phenolic compound Table 1 in the nut extracts compared to the other nuts extract.

The addition of these nuts to food regime will be beneficial in other to ameliorate disease caused by free radicals.

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