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Investigation of the Antioxidant Activity of Aqueous and Ethanol Leaf Extracts of *Ginkgo biloba* from South-East Nigeria

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Abstract

The leaf extracts of *Ginkgo biloba* (GB) have been used extensively over the years in Asia and South America for health beneficial effects to human, however the rarely grown GB in Nigeria is yet to be grossly tapped. In this study, we examined the antioxidant potentials of GB leaf extracts (aqueous and ethanol) from Nigeria. Non-enzymatic and enzymatic methods were used to determine the antioxidant activities while Association of Official Analytical Chemist (AOAC) method was used to determine the β-carotene concentration in both extracts and vitamin C and E composition in the whole dried leaves. The antioxidant analysis showed that ethanol extract exhibited more antioxidant properties than the aqueous extract. However, both extracts were able to show very considerable 2,2-diphenyl-1-picrylhydrazyl (DPPH) and nitric oxide radical scavenging activity, and antioxidant activity observed from β-carotene linoleic acid assay. The antioxidant analysis showed little superoxide dismutase inhibition activity. β-carotene concentrations in aqueous and ethanol extracts were 19.152±0.21mg/g and 22.533±0.82mg/g respectively. High concentrations were observed for vitamins C (79.20±2.56 mg/100g) and E (59.31±2.84 mg/100g). These investigations have revealed the antioxidant potentials of *Ginkgo biloba* leaves grown in Nigeria. The findings therefore, will be useful for medical practice in Nigeria and beyond for maintenance of individuals' good health and management of many diseases caused by oxidative stress.

Keywords

Ginkgo biloba, Antioxidants, Oxidative Stress, β-carotene, Free Radicals, Scavenging Activity

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1. Introduction

Plants have been implicated in the treatment of several ailments but some of these plants may have undesirable effects in another biochemical pathway in the body. It is reported that plant products play crucial roles in drug development in the pharmaceutical industry [1], and that consumption of plant materials improves the health of man [2].

Gingko biloba is ancient Chinese tree, appeared more than 250 million years ago, and the only surviving member of *Ginkgoacea* family. The plant is believed to live for 2,000 to 4,000 years [3]. *Ginkgo biloba* tree is also known as" Maiden hair" tree and it is indigenous to Korea, China and Japan. The different parts of *Ginkgo biloba* plant are reported to have various health benefits like the *Ginkgo biloba* leaf standardized extract termed EGb 761 and *Ginkgo* nuts [4]. *Gingko biloba* leaves is useful and extremely important as regards to their medicinal properties and active ingredients [3]. The leaves have attracted attention as agents for improving circulation, particularly cerebral circulation which may lead to improved mental functions [5]. It is also reported

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that prevention and treatment of asthma, allergic reactions, thrombosis, cataract and heart diseases are among the leaves therapeutic effects [6]. *Ginkgo biloba* leaves therapeutic actions are due to the high concentrations of secondary metabolites it contains [7].

Oxidative stress depicts the existence of products called free radicals and reactive oxygen species (ROS), which are formed under normal physiological conditions but become deleterious when not being eliminated by the endogenous system. Free radical is a chemical compound which contains an unpaired electron spinning on the peripheral layer around the nucleus. Exogenous chemicals and endogenous metabolic processes in human body produce free radicals, especially oxygen derived radicals, which are capable of oxidizing biomolecules, resulting in cell death. Superoxide anion radicals increase under stress conditions such as heavy exercise, certain drugs, infection and various disease states. During normal metabolic processes, human body generates more than 2 Kg of. O^{2-} per year [8].

Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) play important roles in scavenging the free radicals and preventing cell injury [9]. Antioxidants have the property to neutralize free radicals without becoming free radicals themselves. When the antioxidant neutralizes free radicals by receiving or donating an electron, they do not become antioxidants themselves because they are stable in both forms. In other words, antioxidants are chemicals that offer up their own electrons to the free radicals thus preventing cellular damage. These antioxidants are phytochemicals, vitamins and other nutrients that can be sourced from natural food products and medicinal plants.

This paper was designed to evaluate the *in vitro* antioxidant properties of *Ginkgo biloba* grown in Enugu State, South-East Nigeria.

2. Materials and Methods

2.1. Collection of Plant Leaves

Fresh leaves of *Ginkgo biloba* were collected from *Ginkgo biloba* tree at Park Avenue, GRA, Enugu, Enugu State, South-East, Nigeria in a large quantity. The plant was identified and authenticated by Prof Okeke, C. U., a plant taxonomist in the Botany Department of Nnamdi Azikiwe University, Awka. The voucher specimen was deposited in the Hebarium of the Department.

2.2. Extraction of Plant Leaves

The leaves were dried at room temperature for 3weeks. The dried leaves were later ground into fine powder using local steel grinder. To obtain an aqueous extract, 20g of the ground leaves was mixed with 200ml of distilled water. Twenty (20g) of the ground leaves was mixed with 80% (v/v) ethanol in order to obtain an ethanol extract. Both mixture was refluxed in a water bath at 65°C for 1 hour and filtered using Whatman filter paper No 1 followed by the evaporation of the filtrate using a rotary evaporator. Both extracts were used for *in vitro* antioxidant and β-carotene content analyses. However the remaining ground leaves were used for vitamin C and E analyses.

2.3. In vitro Antioxidant Analysis

2.3.1. Scavenging Activity of DPPH (2,2-diphenyl-1-picrylhydrazyl)

The scavenging activity of DPPH was determined according to the method reported by [10].

Principle: DPPH assay is a stable free radical with specific colour (absorbed at 518nm). A colour change occurs when a DPPH radical is quenched or scavenged by a free radical scavenger that donates a hydrogen atom.

Procedure: Three concentrations (20, 40 and 60µg/ml) of aqueous and ethanol extracts of *Ginkgo biloba* leaves were added to 0.5ml of methanolic solution of DPPH and 0.48ml of methanol in separate test tubes. The mixtures were allowed to react at room temperature for 30 minutes. Ethanol served as the blank and DPPH in methanol without the extracts served as the positive control. After 30 minutes of incubation, the discolouration of the purple colour was measured at 518nm in a spectrophotometer. The radical scavenging activity was calculated as follows:

$$\text{Scavenging activity\%} = \frac{100 - (\text{Absorbance of sample} - \text{Absorbance of blank})}{\text{Absorbance of blank}} \times 100$$

2.3.2. Nitric Oxide Scavenging Activity

The nitric oxide scavenging activity was determined using the method reported by [11].

Principle: Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions. The nitrite ions can be determined by use of griess reaction where diazotization of nitrite ions with griess reagent generates a chromophore that can be read at 540-546nm.

Procedure: Sodium nitroprusside (5mM) in standard phosphate buffer solution was incubated with different concentrations (20, 40 and 60µg/ml) of the aqueous and ethanol plant extract dissolved in phosphate buffer (0.025 M, pH 7.4) and tubes were incubated at 25°C for 5 hours. Control tube without the plant extract, but with equivalent

amount of buffer was maintained in an identical manner. After 5 hours, 0.5ml of the incubated solution was removed and diluted with 0.5ml of griess reagent (1% sulfanilic acid, 5% phosphoric acid, and 0.1% naphthylethylenediamine dihydrochloride). The absorbance of the chromophore formed during diazotization of nitrite ions with suphanilic acid and its subsequent coupling with Napthylethylenediamine was read at 546 nm.

$$NO_2 \text{ Scavenging activity\%} = \frac{\text{Absorbance of Control} - \text{Absorbance of Sample}}{\text{Absorbance of Control}} \times 100$$

2.3.3. Superoxide Dismutase (SOD) Activity Assay

The SOD activity assay was done using the method described by [12].

Principle: The principle involved in this assay is the conversion of Nitroblue tetrazolium (NBT) into NBT diformazan via superoxide radical. SOD utilizes the highly water soluble tetrazolium salt and that produces a water soluble formazan dye upon reduction with a superoxide anion. The superoxide radicals are generated in Phenazine methosulphate (PMS)-NADH system by the oxidation of NADH and assayed by the reduction of NBT.

Procedure: Exactly 156µl NBT was prepared in 10ml of 100mM phosphate buffer (pH 8). 468µM NADH solution was prepared with 10ml of 100mM phosphate buffer (pH 8). One (1ml) of the extracts was put into 0.1ml dimethyl sulphur oxide and 0.9ml phosphate buffer. One (1ml) of NBT solution, 1ml of NADH solution, different concentrations (20, 40 and 60 µg/ml) of the extracts and 0.1ml of PMS solution were added together and incubated at 25°C for 5minutes. After 5minutes, the absorbance was read at 560nm.

$$\text{SOD Inhibition (\%)} = \frac{\text{Sample Optical Density} - \text{Control Optical Density}}{\text{Sample Optical Density}} \times 100$$

2.3.4. β-carotene-linoleic acid Assay

The antioxidant capacity of the aqueous and ethanol leaf extracts of *Ginkgo biloba* was estimated by β-carotene-linoliec acid method following the procedure used by [13].

Principle: The method is based on the loss of the yellow colour of β-carotene due to its reaction with radicals which are formed by linoleic acid oxidation in an emulsion. The rate of β-catotene bleaching is slowed down in the presence of antioxidants.

Procedure: One (1ml) of β-carotene (0.2mg/ml chloroform), linioleic acid (0.2ml) and tween 20 (0.2ml) were added to 0.2ml of each extracts, standard (α-tocopherol) and control (80% methanol). Thereafter, the chloroform was evaporated to dryness under vacuum using rotary evaporator. After the evaporation, 100ml of deionized water was added into the mixture and shaken vigorously until emulsion was obtained. Two (2ml) of aliquots of the emulsion was pipetted into the test tubes and immediately placed in water bath at 45°C for 2hours. The absorbance was read at 470nm, using UV-visible spectrophotometer.

$$AA\% = \frac{\text{Absorbance of Control} - \text{Absorbance of Sample}}{\text{Absorbance of Control}} \times 100$$

2.4. Estimation of β-carotene

β-carotene content in aqueous and ethanol *Gingko biloba* leaf extracts was analyzed using [14] method.

2.5. Vitamin C and E Analysis

Vitamin analysis to determine the content of Vitamins C and E in the *Gingko biloba* leaves (whole dried) was conducted using [14] method.

3. Results

The data obtained in this study were evaluated using Statistical Package for Social Sciences (SPSS). The values were expressed as a means of triplicate determination ± standard deviation. Analysis of variance (ANOVA) was done at 0.05 level of significance, if P<0.05, there is significant difference.

Table 1. The DPPH Scavenging Activity of Aqueous and Ethanol Extracts of *Ginkgo biloba* Leaf.

Concentrations of sample (µg/ml)	DPPH Inhibition (%)		
	20	40	60
Aqueous Extract	73.319±2.43	82.401±2.82	87.040±0.05
Ethanol Extract	75.210±1.27	87.362±1.41	89.645±0.01

Values are expressed as means + SD of triplicate determination.

Table 2. Nitric Oxide (NO) radical Scavenging Activity of Aqueous and Ethanol Extracts of *Ginkgo biloba* Leaf.

Concentrations of Sample (µg/ml)	No Inhibition (%)		
	20	40	60
Aqueous Extract	45.710±1.70	51.005±0.01	54.763±1.42
Ethanol Extract	48.811±1.97	53.455±0.79	61.441±3.45

Values are expressed as means ± SD of triplicate determination

Table 3. Super Oxide Dismutase (SOD) Assay of Aqueous and Ethanol Extracts of *Gingko biloba* leaf.

Concentration of Samples (µg/ml)	SOD Inhibition (%)		
	20	40	60
Aqueous Extract	1.797±0.16	2.677±0.89	4.421±0.40
Ethanol Extract	6.650±0.42	9.061±1.42	10.453±0.89

Values are expressed as means ± SD of triplicate determination

Table 4. β-carotene-linoleic acid Assay in Aqueous and Ethanol Extracts of *Ginkgo biloba* leaf.

Extracts	Antioxidant Activity (%)
Aqueous	58.152±0.21*
Ethanol	73.331±1.00*
Standard (α-tocopherol)	89.655±0.49

Values are expressed as means ± SD of triplicate determination.*$p<0.05$ when compared with control.

Table 5. β-carotene Concentration in *Ginkgo biloba* Aqueous and Ethanol Leaf Extracts.

Extracts	Concentration (mg/g)
Aqueous	19.152 ± 0.21
Ethanol	22.533 ± 0.82

Values are means ± standard deviation of triplicate determination.

Table 6. Vitamin C and E concentration in *Ginkgo biloba* Leaves.

Vitamins	Concentration (mg/100g)
Vitamin C (Ascorbic acid)	79.20 ± 2.56
Vitamin E (Tocopherol)	59.31 ± 2.84

Values are means ± standard deviation of triplicate determination.

4. Discussion

Table 1 showed the DPPH radical scavenging activity of the aqueous and ethanol extracts of *Ginkgo biloba* leaf. The highest% scavenging activity was observed in the ethanol extract according to their different concentration of 20µg/ml, 40µg/ml and 60µg/ml. The high DPPH scavenging activity of *Ginkgo biloba* leaf extracts is in consonance with the reports of [15]. The extracts were able to donate hydrogen atom to DPPH and change the colour. The increasing intensity of the colour is directly proportional to the inhibition of DPPH. [11] confirmed through his study that increasing concentration of an antioxidant extract increasingly inhibits the activity of DPPH as noticed in this study. Flavonoids and phenolic compounds or phenolic rich plant extracts are capable of carrying out free radical scavenging activities. The leaves may have contained high amount of flavonoids and phenols. This high content of flavonoids and phenols as drivers of antioxidant activities of *Gingko biloba* leaf extracts was observed by the studies of [15] that used *Ginkgo biloba* from Turkey.

Table 2 showed the nitric oxide assay of aqueous and ethanol leaf extracts of *Ginkgo biloba*. The% inhibition of the nitric oxide (NO) was seen to be higher in the ethanol extract at 20, 40 and 60µg/ml concentration respectively than in the aqueous extract at same concentration level. Nitric oxide is a free radical which is formed from sodium nitroprusside and it react with oxygen to form nitrite. The ability of the ethanol and aqueous extracts of the leaf to react with oxygen, nitric oxide and other nitrogen compounds to inhibit the nitrite formation indicated the essential use of *Gingko* leaves as an antioxidant. This result is similar to those of [16] who screened the antioxidant activity of standardized *Ginkgo biloba* extract.

Table 3 showed the SOD assay of aqueous and ethanol extracts of *Ginkgo biloba* leaf. The% inhibition of superoxide by the extracts is actually low with the ethanol extract having higher percentage than the aqueous extract according to the three concentrations of 20, 40 and 60µg/ml. The inhibition of both extracts increases with increase in the extract concentration. Superoxide dismutase (SOD) is an important enzyme in an antioxidant defense system. It converts the superoxide anion into hydrogen peroxide, thus reducing the toxic effect. Therefore, percentage of inhibition of superoxide by SOD will certainly reduce cellular damage and impairment. [17] reported that the *Ginkgo biloba* extracts from Netherland inhibits the activity of xanthine oxidase, which uses molecular oxygen as an electron acceptor to produce superoxide ions. Hence the extracts activity inhibits the formation of these oxygen reactive species and prevents cellular damages.

Table 4 showed the mean antioxidant activity based on β-carotene bleaching rate of aqueous and ethanol extracts of *Ginkgo biloba* leaf. It was the extract with lowest β-carotene degradation rate that exhibited the highest antioxidant activity. Both extracts had significant lower antioxidant

activity than the standard (α-tocopherol). In this assay, linoleic acid produces hydro peroxides as free radicals during incubation and attacks the β-carotene molecules that cause reduction in the absorbance at 470nm. The β-carotene in the system undergoes rapid discolouration in the absence of antioxidant and vice versa in its presence. The linoleate free radicals and other free radicals formed in the system can possibly delay the extent of β-carotene bleaching by neutralizing the radicals [18]. Therefore, the degradation rate of β-carotene-linoleate depends on the antioxidant activity of the extracts. The results of this study likens to those of [13] who observed the high antioxidant activity of *Ginkgo biloba* leaf from Turkey by β-carotene linoleic acid system.

The β-carotene levels in the aqueous and ethanol extracts of *Ginkgo biloba* leaf was analyzed as shown in Table 5. Its level is higher in ethanol extract than in aqueous extract, though they exhibited appreciable amount for maintenance of good health when used as pharmacologically or as dietary supplement (nutraceutical). β-carotene exhibits a very high antioxidant properties and had remained a matter of interest to anyone who seeks to improve their skin and sight condition. Due to its antioxidative properties, carotenoids are involved in the prevention of severe conditions such as cancer, heart disease, macular degeneration and most importantly cataract [19].

The results of Table 6 showed appreciable vitamins C and E contents in the plant leaves. Vitamin C and E are potent natural antioxidants that scavenge free radicals and ameliorate their deleterious effects. They are normally referred to as *in vivo* antioxidants; however care must be taken because vitamins are heat labile and are easily oxidized.

5. Conclusion

In conclusion, this study has revealed that aqueous and ethanol leaf extracts of *Ginkgo biloba* from Nigeria possess antioxidant properties that can mob up highly reactive radicals that may cause many health disorders and manage many diseases and situations that cause oxidative stress. The importance of antioxidants cannot be over emphasized; therefore greater awareness should be vigorously pursued to effectively appropriate the rich antioxidant potentials of *Ginkgo biloba* found in Nigeria.

6. Recommendation

Ginkgo biloba tree may not be abundant in Nigeria and West Africa environment unlike Asia and South America, however it represents one of nature's invaluable gifts to man as proven by this study (observed antioxidant activities) but which unfortunately has remained grossly untapped here in Nigeria.

It is therefore recommended that intensified field research in the search of this plant tree in many regions of Nigeria and West Africa for further comparative studies including *In vivo* antioxidant and toxicological studies, as it is believed that the plant still exists in inner forest regions and old open parks of many countries especially in warm temperate regions.

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