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Qualitative Phytochemical Screening, GCMS Studies and *In-Vitro* Anti-Oxidative Properties of Aqeuous Leaf Extract of *Gnetum africanum*

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ABSTRACT

This study aimed at carrying out a qualitative phytochemical screening, GC-MS studies and in-vitro antioxidant properties of aqueous leaf extract of *Gnetum africanum*. The qualitative phytochemical screening of the aqueous leaf extract of *Gnetum africanum* was done using standard procedures and revealed the presence of terpenoids, saponins, tannins, steroids, flavonoids, alkaloids, cardiac glucosides and phenols. The GC-MS screening revealed the presence of 14 compounds, 6 out of the 14 compounds were most prominent. The compound with the highest percentage peak area was caffeine with peak area of 96.9%, followed by n-Hexadacanotic acid with peak area of 60.9%, 2-methoxy-4-vinylphenol with peak area of 55.9%, tetradacanoic acid with peak area of 50.3%, cyclopentaneundecanoic acid with peak area of 47.8% and 2-cyclo-penten-1-2-hydroxy with peak area of 43.6% respectively. In-vitro determination of antioxidant property of leaf extract of *Gnetum africanum* was done photometrically using 2,2-dyhenyl-l-picrylhydrazyl (DPPH) assay. The DPPH scavenging ability of the leaf extract (43.2, 60.5, 68.8, and 75.7) was statistically significant at $p<0.05$ when compared with the standard drug ascorbic acid (81.1, 82.6, 85.1, and 90.4) % at 10, 20, 30 and 40 mg/l. In conclusion, the leaf extract of *Gnetum africanum* is loaded with a host of important phytochemicals and has antioxidant properties which increase in potency with increase dose.

Keywords: Phytochemical Screening, GCMS Studies, Anti-Oxidant, *Gnetum africanum*

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INTRODUCTION

Acceptance and use of plant products for health care delivery have continued to gain popularity over the years. Plants are the richest sources of bioactive compounds and have been the basis of many traditional medicines throughout the world for thousands of years[1]. Natural products, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug discoveries because of the unmatched availability of chemical diversity[2].. Clardy and Walsh [3] reported that small molecules from medicinal plants called natural products are still major sources of innovative therapeutic agents for various conditions, including infectious diseases. The medicinal value of the plants useful for healing and cure of human diseases is attributed to presence of phytochemical constituents[4]. Among these phytochemicals are flavonoids, alkaloids, phenols, saponins etc [5,6]. According to Ogbu *et al* [7]., tropical African sub-regions are home to many useful medicinal plants, however, there are many of them whose potentials are yet to be harnessed or domesticated in tropical African Sub-regions.

Gnetum africanum (*G. africanum*) belongs to the Family Gnetaceae of the Order Gnetales[8]. It grows as a wild evergreen climbing plant in the rain forest of Nigeria where it is searched for and highly priced in the regional markets. The seed of G. africanum is oval in shape, small in size with diameter of 0.5cm, green in colour when unripe but turns red with ripening and enclosed in a fleshy envelope[9]. The leaves are decussately opposite, sometimes in a whorl of 3, petiole up to 1cm long with 3 – 6 pairs of strongly curved lateral veins looped near the margin. *Gnetum africanum* is one of the most popular green leafy vegetables in Nigeria, and it is gaining equal popularity as a delicious vegetable in other African countries such as Cameroun, Gabon, Congo and Angola [10]. It is known as “eru, okok, mfumbua or fumbua”in Cameroun, “koko” in Angola and Central African Republic [11]

and "Ntoumou" in Gabon. In Nigeria, it is called "afang" in Ibibio and Efik; "okazi" in Igbo; "yala" in Ogoja and "ajaabaje or ajakotale" in Yoruba[11]..

Phytochemical screening of *Gnetum africanum* leaves revealed the presence of alkaloids, saponin, glucosides and tannins[9, 12, 13]. *G.africanum* leaves have medicinal and great culinary values especially in West Africa. It was reported to show anti-inflammatory, anti-carcinogenic and antioxidant activities [14]. *G. africanum* is employed in Nigerian folk medicine for the treatment of enlarged spleen[15], piles, high blood pressure[16]., sore throats and as a cathartic[11]. In DR Congo it is used to stop nausea and as antidote to some forms of poison and snake bites[11, 17]. In Cameroun the leaves are taken as an enema against constipation and to ease child birth, chewed to mitigate the effects of drunkenness [11]. and used for treating diabetes, boils and fungal infection in the fingers [17].The seeds are used as fungicide for dressing fresh and septic wounds and can also be chewed raw for the management of excessive urination [16].. *G. africanum* leaves are eaten raw as local salad, shredded and used in preparing soups and stews[11]. They are also put to minor use by the Ibos and the Efiks/Ibibios where they are eaten as a salad mixed with dry fish or meat, certain spices and palm oil[18].

Antioxidants are the body's defensive mechanisms that protect the tissues from free radical toxicity through their scavenging ability. Free radicals are highly reactive molecules which arise from the body's biochemical reactions [19] and become toxic when produced in excess quantity[20]. Free radicals are associated with oxidative stress and implicated in the pathogenesis of various chronic and degenerative disorders such as cancer, aging, Alzheimer's disease, diabetes, autoimmune disorders and cardiovascular diseases [21, 22].. A variety of free radical scavenging antioxidants are found in fruits, leaves and other parts of plants and these antioxidants help in converting the free ROS to less reactive species[23].. With increasing oxidative stress related health challenges, it has become very pertinent to explore and use more plant-based products as food. Eating more plant-based foods which are rich in phytochemicals according to Heather and Talcott [24]. prevent oxidative stress in the body and according to reports by [25,26,27], regular intake of plants fruits and vegetables with antioxidative potentials help in the fight against chronic diseases and in enhancing human longevity and well-being.. Studies on the phytochemistry of *Gnetum africanum* have been reported but there is dearth of information regarding its GCMS-based analysis and its anti-oxidative potentials. This study therefore aimed at investigating qualitatively the phytochemicals, GCMS-based compositions and in-vitro antioxidant properties of aqueous extract of *Gnetum africanum.*

MATERIALS AND METHODS

Identification and Collection of Plant materials

Fresh leaves of *Gnetum africanum* plant were harvested from a local garden in Ulakwo, Owerri-North L.G.A of Imo State Nigeria. The plant was identified and authenticated at the department of Plant Science and Biotechnology, Imo State University, Owerri. The leaves were removed from the stalk, thoroughly washed under running tap water to remove unwanted debris and air-dried at room temperature for 14 days. The dried leaves were then pulverized to fine powder with the aid of a mechanical grinder and kept in labeled airtight containers under dry conditions until required for use.

Aqueous leaf extraction of Gnetumafricanum

One hundred and twenty grams (120g) of ground leaf of *Gnetum africanum* was macerated in 1litre of distilled water for 24hrs. This was thereafter filtered with sterile filter paper (Whatman No. 1) and evaporated to dryness at 40°C in a vaccum using a rotary evaporator RE52. Approximate concentrations of the extract were constituted to the required doses for the treatment of the animals using normal saline.

Qualitative phytochemical screening of the aqueous extract of *G. africanum*

Qualitative phytochemical screening of the extract was done to detect the presence or absence of secondary metabolites (phytochemicals) using standard procedures as described below.

Test for terpenoids[28].

Salkowski Test

5 ml of the extract was mixed in 2 ml of chlorofoam, and 3 ml of concentrated H_2SO_4 was carefully added to form a layer. A reddish brown colouration of the interface was formed indicating a positive result for the presence of terpenoid compounds.

Test for saponins[28]

Emulsion test

4ml of distilled water was added to 2 ml of the extract and shaken vigorously for 2 min after which a few drops of olive oil were added. Formation of an emulsion showed the presence of saponins.

Test for tannins[28].

Ferric chloride test

To 1.0 ml portion of the extract, 4.0 ml of distilled water was added and a few drops of 10% ferric chloride solution were also added. The solution was then observed for blue or green precipitate colouration indicating the presence of tannins.

Test for steroid [28]

5 ml of aqueous extract was added to 2 ml chloroform and 3 ml of concentrated H_2SO_4 was added cautiously for a reddish brown intermittent layer, which confirms a positive result.

Test for flavonoids[28]

Lead acetate test

To 2.0 ml portion of the extract, few drops of 10% lead acetate solution were added. A cream or light yellow colouration showed the presence of flavonoids.

Aluminium chloride test

To 2.0 ml portion of the extract, few drops of 1% aluminum chloride solution was added and observed for light yellow colouration. A yellow precipitate indicated the presence of flavonoids.

Test for alkaloid[28]

A few drops of the following reagents were added to each of 2.0 ml of the extract, and observed for colour change:

Dragendorf reagent

A red to orange precipitate indicated the presence of alkaloids.

Wagner's reagent

A reddish or deep-brown precipitate indicated the presence of alkaloids

Test for cardiac glycosides[28]

A known mass of 1 g of sample and 10 ml of water were boiled for 5 minutes. Then 400 µl of equal (v/v) mixture of Fehling's solutions (A and B) was added to 2 ml of filtrate to which 2 ml of dilute ammonia solution (NH_3(aq)) was added and boiled for 5 - 10mins. The filtrate changed to a brick red precipitate, indicating the presence of cardiac glycosides.

Test for phenol

Ferric chloride test

To 2 ml of ethanol, 0.05 g of the extract was added followed by few drops of aqueous solution of ferric chloride. A formation of reddish colour precipitate indicates the presence of phenols.

Gas Chromatography-Mass Spectrometry (GC-MS) analysis of the aqueous extract of *Gnetum africanum*

The aqueous leaf extract of *Gnetum africanum* was subjected to GC-MS analysis on the instrument GCMS-QP2010 PLUS SHIMADZU, JAPAN. The oven temperature was programmed at 60°C for 0 min, was gradually increased to 140°C at 4.0 min and then ending with 250°C at 6 min. A sample volume of 8.0 µl was injected for analysis. Helium gas 99.995% of purity was used as a carrier gas as well as an eluent. The flow rate of helium gas was set to 1.61 ml/min. The sample injector temperature was maintained at 200 ºC and the split ratio was 1.0 throughout the experiment periods.

The ionization mass spectroscopic analysis was done with 70 eV. The mass spectra were recorded for the mass range 35 - 800 m/z for about 25 min. Identification of components was based on comparison of their mass spectra. As the compounds separated on elution through the column, they were detected in electronic signals. As individual compounds eluted from the Gas chromatographic column, they entered the electron ionization detector where they were bombarded with a stream of electrons causing them to break apart into fragments. The fragments were actually charged ions with a certain mass.

The m/z ratio obtained was calibrated from the mass spectrum graph obtained which was the fingerprint of the molecule. Interpretation of GC-MS was conducted using the database of National Institute of Standard and technology (NIST) having more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST Library 2008 WILEY8, FAME. The Name, Molecular weight and structure of the components of the test materials were ascertained.

Determination (In-vitro) of the antioxidant property of the extract using 2,2-dyphenyl-1-picrylhydrazyl (DPPH) photometric Assay

The procedure described by [29] was used in determining the free radical scavenging activity of the *Gnetum africanum* extract. The extract was analyzed by the DPPH assay using a spectrophotometer. 1mL of 0.5mM DPPH (in methanol) in a cuvette was mixed with aqueous extract at concentrations (25,50,100, 200 and 400) µg/ml. After 30 minutes of incubation in a dark room, the absorbance was taken at 517nm. The experiment was done in triplicate and the percentage antioxidant activities were calculated as follows.

% antioxidant activity (AA) = 100 – [{(sample abs-blank abs) x 100}/abs of control] Where abs = absorbance

One millilitre of methanol plus 2.0mL of the test extract was used as the blank while 1.0 mL of the 0.5 nM DPPH solution plus 2.0 mL of methanol was used as the negative control. Ascorbic acid (Vitamin C) was used as reference standard.

Statistical Analysis

The data generated in this study was entered, cleaned and coded in excel sheets and was statistically analyzed using the SPSS/IBM version 21 software. Means and standard errors of mean were calculated. Statistical differences between the experimental and control groups were determined using ANOVA and values were considered significant at $p<0.05$. Descriptive statistics (percentages and signs) were also used.

RESULTS

The results are presented in tables and figures (graphs). The qualitative phytochemical screening and GC-MS studies are presented in tables while the antioxidant properties are presented in figures.

Table 1: Qualitative phytochemical screening of aqueous leaf extract of Gnetumafricanum

Phytochemical	Method of Test	Presence/ Absence	Indicator
Terpenoids	Salkowski test	+	Reddish brown colour
Saponins	Emulsion test	+	Emulsion formation
Tannins	Ferric chloride test	+	Blue or Green ppt colour
Steroids	Trease and Evans, 1996	+	Reddish brown intermittent layer
Flavonoids	Lead Acetate and Aluminium chloride test	+	Yellow colour
Alkaloids	Dragendorf Reagent and Wagner's Reagent	+	Red to Orange ppt and deep brown ppt
Cardiac glucosides	Fehling's solution (A and B)	–	Brick red ppt
Phenols	Ferric chloride test	+	Reddish colour

Table 1 shows qualitative phytochemical screening of aqueous extract of *Gnetum africanum*. Presence of phytochemical is shown with a + sign while absence is shown with a – sign. All the phytochemicals investigated are present in the leaf extract of *Gnetum africanum* except cardiac glucoside which was found to be absent.

Table 2: Composition of aqueous leaf extract of *G. africanum* based on GC-MS analysis

S.N.	Name of Compound	Molecular Formula	Molecular Weight (MW)	Peak Area
1	2-cyclopenten-1-2-hydroxy	$C_5H_6O_2$	98	43.6%
2	Estragole	$C_{10}H_{12}O$	148	32.8%
3	2-methoxy-4-vinylphenol	$C_9H_{10}O_2$	150	55.9%
4	5-chloropentanoic acid 4-methylpentyl ester	$C_{11}H_{21}ClO_2$	220	9.39%
5	1,3 Benzodioxole, 4-methoxy-6-(2-propenyl)	$C_{11}H_{12}O_3$	192	17.3%
6	Tetradacanoic acid	$C_{14}H_{28}O_2$	228	50.3%
7	Hexyl amine	$C_{13}H_{25}N$	195	17.0%
8	Caffiene	$C_8H_{10}N_4O_2$	194	96.9%
9	3-P-methyl-d-glucose	$C_7H_{14}O_6$	194	12.2%
10	n-Hexadacanotic acid	$C_{16}H_{32}O$	256	60.9%
11	Phytol	$C_{20}H_{40}O$	296	39.0%
12	Linoelaidic acid	$C_{18}H_{32}O_2$	280	20.2%
13	Cyclopentaneundecanoic acid	$C_{16}H_3O_2$	254	47.8%
14	Cis, cis, cis-7-10-13-Hexadecatrienal	$C_{16}H_{26}O$	234	8.6%

Table 2 shows 14 compounds obtained from the aqueous leaf extract of *Gnetum africanum*, their MW and their percentage (%) peak areas using GCMS method. Out of these 14 compounds, six (6) were most prominent as indicated by their percentage (%) peak areas. The prominent compounds include caffeine (96.9%), n-Hexadacanotic acid (60.9%), 2-methoxy-4-vinylphenol (55.9%), Tetradacanoic acid (50.3%), Cyclopentaneundecanoic acid (47.8%), 2-cyclopenten-1-2-hydroxy (43.6%). Three (3) were less prominent while the remaining five (5) were only present in minute amounts.

GAS-Chromatography Mass Spectrometry Graph

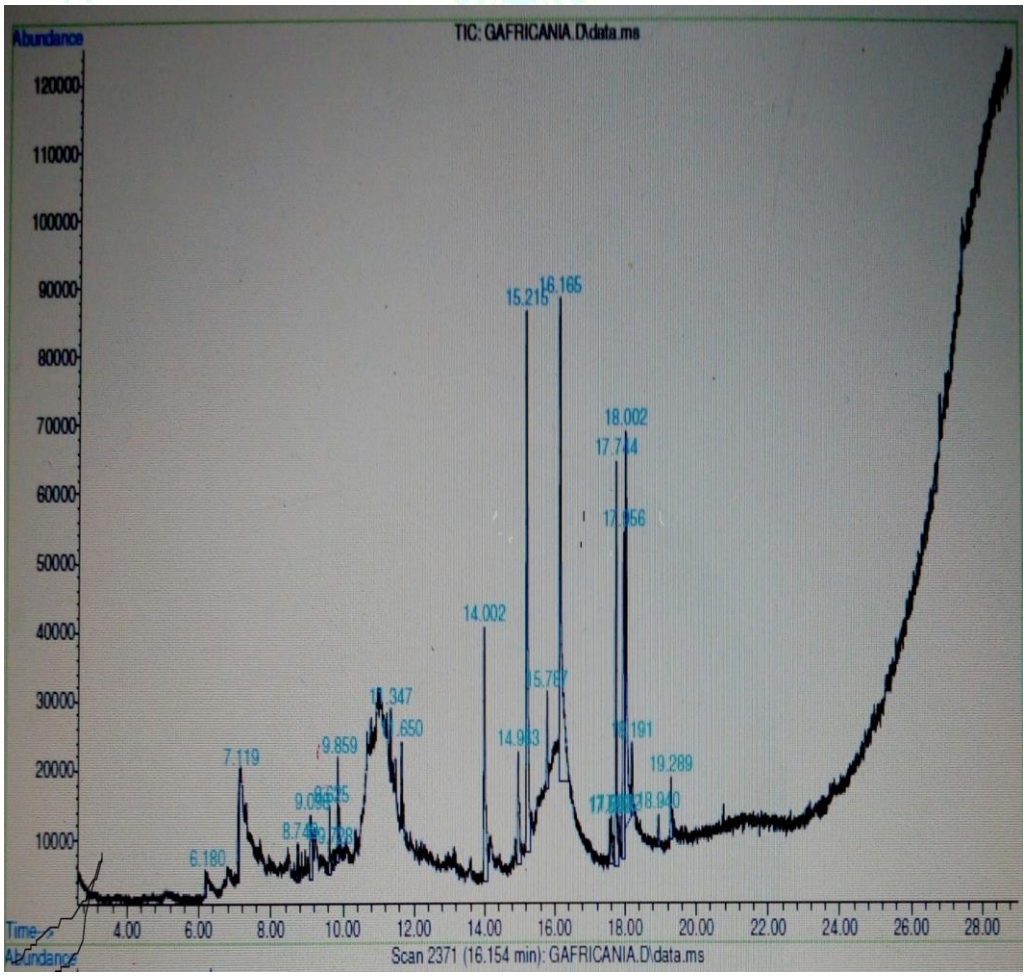

Figure 1: GAS-Chromatography Mass Spectrometry Graph

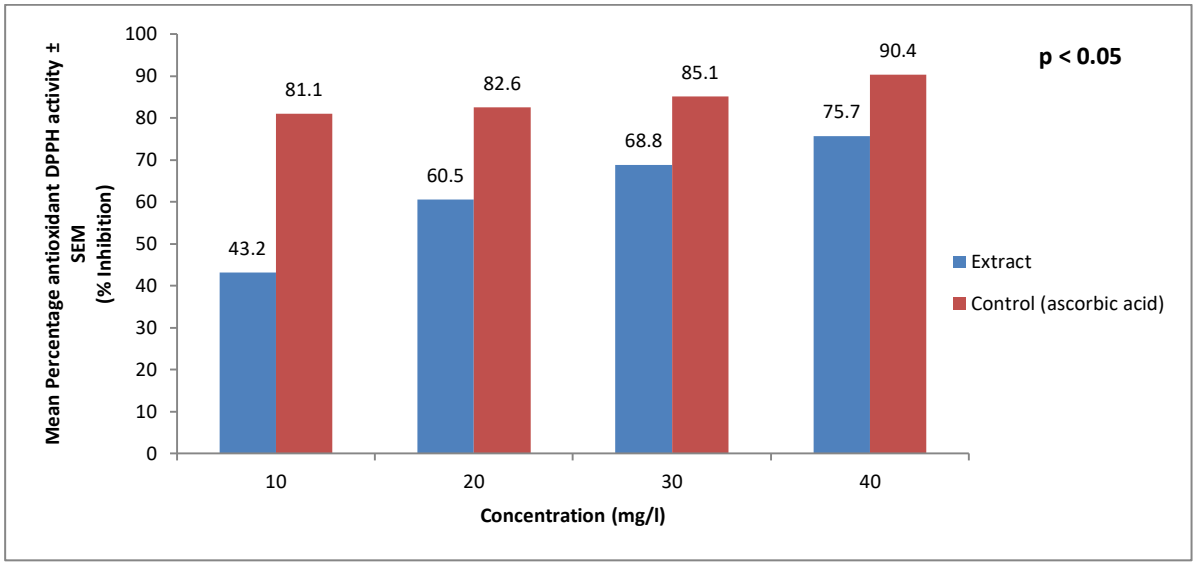

Figure 2: 2,2-Diphenyl-1-picryhydrazyl (DPPH) radical scavenging photometric assay.

Figure 2 shows the graphical representation of the mean DPPH radical scavenging activity obtained by photometric assay. The horizontal axis represents the concentration of leaf extract of *Gnetum africanum* and a standard drug ascorbic acid measured in mg/l while the vertical axis represent the mean percentage antioxidant activity of both the standard ascorbic acid and seed extract.

The legend shows leaf extract in blue colour and ascorbic acid in red colour. From the graph it can be deduced that the extract possesses good DPPH radical scavenging activity (43.2, 60.5, 68.8, and 75.7)%which is statistically significant at $p<0.05$ when compared with the standard ascorbic acid (81.1, 82.6, 85.1, and 90.4) % at 10, 20, 30 and 40 mg/l. It can also be deduced from the graph that the DPPH radical scavenging activity of the leaf extract of *Gnetum africanum* is dose dependent.

DISCUSSION AND CONCLUSION

Phytochemicals are natural bioactive compounds found in plants that work with nutrients and dietary fiber for disease protection [30]. The medicinal value of the plants useful for healing and cure of human diseases is attributed to presence of phytochemical constituents[4] . The leaves of plants according to Owolabi *et al* [31] and Egba *et al.* [32] provide both nutritional and medicinal benefits principally due to their nutrient composition and secondary bioactive metabolites which are known to possess antioxidant, anti-bacterial, anti-inflammatory, anti-sickling, hypoglycaemic and immunomodulatory properties.

Qualitative Phytochemical screening of aqueous extract of *Gnetum africanum* in the present study revealed the presence of terpenoids, saponins, tannins, steroids, flavonoids, alkaloids and phenols. The phytochemical constituents of *G. africanum* obtained in this study are not in total agreement with the reports of Dike[12] and Okerulu and Onyema[9]. Dike [12] reported the presence of alkaloids, saponins, flavonoids and tannins while Okerulu and Onyema[9] reported the presence of alkaloids, Glycosides, saponins and tannins. The little discrepancies in these findings may be due to differences in the methods of extraction and differences in species. The present study was done on domestic species while that of Dike; Okerulu and Onyema were each done on wild species of G. africanum.

G. africanum leaves can therefore be said to be good reservoirs of a host of important phytochemicals that promote wellbeing and also capable of combatting certain disease conditions.Terpenoidshave both antimalarial and hypoglycaemic effects[33, 34] while Saponins have been shown to have hypotensive, hypocholesterolaemic, and cough depressant activities [35] According to[34] , saponins are active agents against fungal infections. Tannins possess antiviral, antibacteria and also potent against degenerative diseases. Report also has it that tannins play major role as anti-diarohoea and anti-haemoharragic agents[36] . Steriods promote nitrogen retention in osteoporosis, have therapeutic application as cardiac drugs and play important roles in the functions of sex hormones [37] . Flavonoids are known for their antioxidant activities and can help protect the body against Reactive Oxygen Species (ROS) which are involved in either the initiation or progression of carcinogenesis and other degenerative diseases. Reactive oxygen species cause oxidative damage by peroxidation and oxidation of cellular lipids, proteins and deoxyribonucleic acids[38] . Flavonoids act as hydrogen peroxide scavengers as they are oxidized by peroxidase[39] . Alkaloids have anticancer activity, CNS stimulatory effects and are used as anasthetics in ophthalmology[40]. Phenols act as natural antioxidants and are used as nutraceuticals for their anticancer and cardioprotective potentials[40] .

GC-MS analysis of the aqueous extract of *G. africanum* in this present study identified fourteen (14) compounds with six (6) of them being most prominent and including caffeine (96.9%), n-Hexadacanotic acid (60.9%), 2-methoxy-4-vinylphenol (55.9%), Tetradacanoic acid (50.3%), Cyclopentaneundecanoic acid (47.8%), 2-cyclopenten-1-2-hydroxy (43.6%). These compounds possess antioxidant, hypocholestrolemic, nematicide, pesticide, hemolytic, anti-inflammatory properties and lubricant activities. Phytol with percentage peak area of 30% is a product of chlorophyll metabolism in plant and has the ability to reduce the risk of cardiovascular disease, stroke and heart attack. 0

Antioxidants are powerful substances that prevent oxidation and delay or inhibit the damaging effects of reactive oxygen species (ROS) to a target molecule. They have the ability to trap or scavenge free radicals thus protecting the body from free radical induced oxidative stress[41] . Oxidative stress affects all cell macromolecules including DNA, proteins and lipids causing mutation, protein inactivation and lipid

peroxidation respectively[42] .This study showed a high free radical scavenging activity of the extract on DPPH at almost all concentrations but most especially at concentrations 20mg/l, 30mg/l and 40mg/l when compared with that of ascorbic acid. This result is in tandem with the findings of [18]) where high DPPH free radical scavenging ability of raw and cooked forms of *Gnetum africanum* was demonstrated. The high DPPH free radical scavenging ability of *Gnetum qafricanum* leaf extract must be attributed to its constituent phytochemicals alkaloids, flavonoids and tannins (polyphenols).There exist a strong positive relationship between total phenolic contents and antioxidant activity which appears to be the trend in many plant spece [43] . Rice-Evans *et al.*, [40] reported that under experimental conditions, the antioxidative potentials of plant phenolics are always linked to their electron donation, reducing power, and metal-chelating ability. Polyphenols have the ability to donate hydrogen atoms or electrons and to capture free radicals[44] and this explains the antioxdative property of the extract.

The percentage inhibition of the free radical was dose dependent and the higher the concentration of the extract, the higher the percentage inhibition. Vitamin C which is a potent and safe antioxidant in medicine has a higher antioxidative ability than the extract.This may be due to the fact that vitamin C is a refined drug.

In conclusion, the leaf extract of *Gnetum africanum* is loaded with a host of important phytochemicals and has antioxidant properties which increase in potency with increase dose. We therefore recommend regular consumption of *Gnetum africanum* as this will promote wellbeing and if properly harnessed, may be applied in the management and treatment of various chronic and degenerative disorders which oftentimes are oxidative stress related.

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