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RESEARCH ARTICLE

FREE RADICALS SCAVENGING AND ANTIOXIDANT ACTIVITIES OF ETHANOL LEAF EXTRACT OF SIX NIGERIAN ETHNOMEDICINAL PLANTS: AN IN-VITRO STUDY

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ABSTRACT

Many degenerative diseases are caused by oxidative stress, which is linked to excessive generation of pro-oxidants (free radicals) over antioxidants. Plants have been reported as cheap and rich source of natural compounds with notable bioactivities. This study was therefore designed to assess the free radicals scavenging and antioxidant activities of ethanol leaf extracts of six ethnomedicinal plants from Nigeria using in-vitro methods. This was done by conducting preliminary phytochemical screening, estimating total phenols and flavonoids contents, and determining free radical scavenging/antioxidant activities of the six plants extracts in different models, viz: DPPH radical scavenging, Nitric Oxide (NO) scavenging and Ferric reducing-antioxidant power assays. The results show that PGE and PMEhave highest (51.36±7.44mgGAE/g Extract) and lowest (35.42±5.91mgGAE/g Extract) phenols content respectively, while OGE and SAE have highest (49.71±4.50mg QE/g Extract) and lowest (27.16±5.34mg QE/g Extract) flavonoids contents respectively. All the six plants exhibited significant ($p<0.05$) antioxidant activity, but interestingly, PGE, GLE and OGE respectively produced highest DPPH scavenging (62.68%), NO scavenging (67.95%) and Ferric reducing (69.33%) activities, and thereforecan be exploited as important sources of antioxidant compounds. We therefore recommend for further isolation, characterization of active principles and testing in animal models, with a view to develop natural antioxidants that can used in management of oxidative stress-related disorders.

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INTRODUCTION

Free radicals, which usually bear one or more unpaired electrons and capable of independent existence, are highly unstable chemical species that can cause damage to other molecules by extracting electron from them in order to attain stability[(1,2)]. Free radicals (Reactive Oxygen Specie (ROS) and Reactive Nitrogen Specie (RNS)) are not only present in the environment (exogenous), can also be generated in the body (endogenous) as part of normal aerobic metabolic process [(3,4)]. Although free radicals can be beneficial to the body [(5)], production in excessive amount may lead to various tissue/organ damage and disease[(2)].

Human body is equipped with complex enzymatic and non-enzymatic antioxidant defense system, which in normal physiological state can counteract the harmful effects of free radicals and other oxidants, thereby ensuring well-being[(6,7)]. The enzymatic molecules with antioxidant function include catalase, peroxidase, superoxide dismutase and glutathione, while non-enzymatic antioxidant molecules employed by the body include bilirubin, uric acid and lactoferin among others[(8)]. In disease state, the body's (endogenous) antioxidant system is overpowered by excessively generated free radicals, hence leading oxidative stress-related cellular damage [(9)].Conventional management of oxidative stress involves the use of synthethic antioxidant agents such as Butylated hydroxyanisole (BHA), Butylated hydroxytoluene (BHT) and Propyl gallate (PG) among others[(6)].

Unfortunately, the use of synthetic agents is constrained by adverse effects, unavailability and high cost, hence, prompting a search for alternative compounds from natural plants. Plants apart from having advantage of toxicity consideration based on their long-term traditional use, also serve as a cheap and abundant source of new remedies[10]. Substantial evidences show that natural plants containing antioxidant principles may be of use in retarding the initiation, and/or ameliorating the diseases caused by oxidative stress[11,12]. The present study therefore sought to assess, by in-vitro techniques, the free radicals scavenging and antioxidant activities of six Nigerian edible plants (leaves) widely employed in ethnomedicinal practice in the management of various oxidative stress-related ailments in humans. Summary of the profile of the six Nigerian plants is shown in Table 1 below.

MATERIALS AND METHODS

Chemicals and Drugs: Sodium Phosphate Dibasic Heptahydrate (Sigma Aldrich Chemie, Germany), Sodium Phosphate Monobasic Monohydrate (Sigma Aldrich Chemie, Germany), Sodium Carbonate (Bhagwati Chemicals, Gujarat, India), Gallic acid (Merck, Darmstadt, Germany), Aluminium Chloride (Merck, Darmstadt, Germany), Sodium Nitrite (Nilkant Organics, Mumbai, India),Ethylacetate (Rankem, Mumbai, India), Sodium Nitroprusside (Suvchem, India), Quercetin (Manus AkttevaBiopharma LLP, India) Hydrochloric acid (Nice Laboratories Reagent, Kevala, India),Sulphanilamide (CDH Fine Chemicals, India) Sodium Tetraoxocarbonate IV (Sigma Aldrich Chemie, Germany), Tetraoxosulphate VI acid (Hi Media Laboratories Pvt Ltd, India), Phosphoric acid (Nippon Chemical Industrial Co, Ltd, Tokyo, Japan), 96% Ethanol (GungsdongGuandgua Chemical Factory ,China), Sodium Hydroxide (Rankem Mumbai, India),Naphthylethylenediamine (Sigma Aldrich Chemie, Germany) Ferric Chloride (Super Tek Chemical, Germany). Glacial Acetic Acid (Sigma Aldrich Chemie, Germany), Potassium Ferricyanide (Hemadri Chemicals, Maharashtra, Mumbai, India), Trichloroacetic acid (Vizag Chemical, Mumbai, India), Ascorbic acid (Merck, Darmstadt, Germany), 1,1-diphenyl-picrylhydrazine (DPPH) (Sigma Chemical Co, USA).

Plant Material Collection and Authentication: Matured fresh leaves of the six plants were collected from their natural habitat and forwarded to the Herbarium of Department of Plant Science and Biotechnology, University of Port Harcourt, Rivers State, Nigeria, for taxonomic identification and authentication. The six plants were identified as *Piper guineense, Pterocarpus mildbraedii, Solanum aetiopicum, Ocimum gratissimum, Millettia aboensis* and *Gongronema latifolium*. The assigned voucher specimen number of individual plant is listed in Table 1

Extraction of Plant Material: Fresh leaves of individual plant were thoroughly washed with tap water and dried under shade until a uniform weight was attained. The dried leaves (2.0 kg) were milled into coarse powder, and about 500 g of the individual powder was separately macerated in 1.5 liter ethanol (80%) for 72 hours with vigorous agitation every 6 hours. At the end of maceration period, the solution was filtered and the marc was re-macerated and re-filtered. Both filtrates were pooled together and evaporated to dryness in a hot-air oven at 40^{O} C. The extraction process was conducted in triplicate and yield of each cycle was calculated, as stated in our previous work [10]. The dried extracts of the six plants were stored in separate containers, labeled as PGE (*Piper guineense* extract), PME (*Pterocarpu smildbraedii* extract), SAE (*Solanum aethiopicum* extract), OGE (*Ocimum gratissimum* extract), MAE (*Millettia aboensis* extract), GLE (*Gongronema latifolium* extract),and kept in a refridgerator until when needed.

Phytochemical Screening: Leaf extracts from the six plants were subjected to phytochemical screening using the method described by[36], to test for the presence or absence of various phytoconstituents. Intensity of color and/or precipitate formation indicated the abundance of phytoconstituents present.

Estimation of Total Phenol Content (TPC) of Plant Extracts: The amount of total phenol content of the extracts was determined using Folin-Ciocalteu's assay technique as described by[37], with slight modification. In the present study, 2 ml solution (500 mcg/ml) of test samples (i.e. individual plant extract) was mixed with equal volume (i.e. 2 ml) of Folin-Ciocalteu-phenol reagent, and the resulting mixture was made up to 9 ml with deionized water. Sodium carbonate solution (I ml; 20%w/v) was added, and the resulting reaction mixture (10 ml) was incubated at room temperature for 20 minutes. Thereafter, the solution was centrifuged at 1500 rpm for 5 minutes, and the absorbance of the supernatant was measured at 765 nm using u-v spectrophotometer. Standard calibration curve was generated using gallic acid, and total phenol content was expressed as milligram gallic acid equivalent per gram of each extract (i.e. mg GAE/g of extract). The experiment was conducted in triplicate.

Estimation of Total Flavonoid Content (TFC) of Plant Extracts: This was done by Aluminium Chloride Colorimetric Assay as described by[38], with slight modification. To a 25 microliter solution (500 mcg/ml) of individual test sample, 125 microliter of deionized water and 7.5 microliter of $NaNO_2$ (5%) were added and mixed thoroughly. Then, a 15 microliter of $AlCl_3$-ethanol (5%) was added and the incubated for 60 minutes, thereafter, 50 microliter of NaOH (1.0M) was added for color development.

Final volume adjustment of the reaction mixture to 250 microliter was done using deionized water. The absorbance at 490 nm of the resulting solution was measured using u-v spectrophotometer. Using the equation generated from quercitin standard calibration curve, total flavonoid content of each of the test samples was expressed in milligram of quercitin equivalent per gram of extract (i.e. mg QE/g of extract) The experiment was conducted in triplicate.

In-vitro Antioxidant Assay

DPPH Radical Scavenging Activity: This test was conducted according to method described by[39], with slight modification. In the present study, a 1.0 ml solution (500 mcg/ml) various test samples (i.e. individual plant extract or ascorbic acid) was mixed with equal volume of freshly prepared ethanolic solution of 1,1-diphenyl-picrylhydrazine (DPPH). The mixture was vigorously agitated, and incubated in the dark room temperature for 30 minutes. Thereafter, the absorbance of the mixture against the blank (ethanol)

was measured at 517 nm using u-v spectrophotometer. Ethanolic DPPH solution without the test samples (i.e. plant extract or ascorbic acid) was used as control. The test was performed in triplicate and mean absorbance was determined. Decreased absorbance of the reaction mixture is an indication of greater DPPH scavenging activity. Percent DPPH scavenged/inhibited was calculated formula[40]:

% DPPH Scavenged = $((Ab_c - Ab_s)/Ab_c) \times 100$
Where, Ab_c = absorbance of the control at 517 nm
Ab_s = absorbance of the sample at 517 nm

Nitric Oxide Scavenging Activity: In this test, Nitric Oxide (NO) generated by decomposition of Sodium nitroprusside in aqueous solution at physiological pH (7.2), was estimated by Griess reaction as described by[41], with slight modification. The reaction mixture (3 ml) was constituted by dissolving 2 ml of 10 mM Sodium nitroprusside in 0.5 ml freshly prepared phosphate buffer solution and then mixed with 0.5 ml solution (500 mcg/ml) of various test samples (extracts or ascorbic acid). The mixture was incubated at 25° C for $2^{1/2}$ hours. Thereafter, 0.5 ml of the incubated solution was withdrawn and mixed thoroughly with Griess reagent (1.0 ml Sulfanilic acid (2% sulfanilamide in 5% phosphoric acid) mixed with 1.0 ml naphthylethylenediamine dihydrochloride (0.2% w/v),then allowed to stand for 5 minutes at room temperature for complete diazotization). The mixture was incubated for 30 minutes at room temperature during which a solution that changes color from yellow to blue and then pink, was formed. The absorbance at 546 nm of the pink chromophore was measured and compared with that produced by control (i.e. solution without samples). The experiment was performed in triplicate. The percent of nitric oxide radical scavenged/inhibited was calculated using the formula:

% Nitric Oxide (NO) scavenged $=((Ab_c - Ab_s)/Ab_c) \times 100$
Where, Ab_c = absorbance of the control at 546 nm
Ab_s = absorbance of the sample at 546 nm

Ferric Reducing Antioxidant Activity: This test was performed using potassium ferricyanide reducing method as described by[42],with slight modification. The following solutions were freshly prepared: 1.0% Potassium Ferricyanide, 0.1% Ferric Chloride, 0.2M Phosphate buffer (pH 6.6) and 10% Trichloroacetic acid. One milliliter (1.0 ml) solution (500 mcg/ml) of various test samples (extracts or ascorbic acid) was mixed with 2.5 ml of Phosphate buffer and 2.5 ml of Potassium Ferricyanide. The resulting mixture was agitated vigorously and incubated at 50°C for 20 minutes. Thereafter, 2.5 ml of Trichloroacetic acid was added to the mixture and then centrifuged at 3000 rpm for 10 minutes. To 2.5 ml of the supernatant, 2.5 ml deionized water and 0.5 ml Ferric Chloride were added, and the reaction mixture was incubated at 30° C for 10 minutes. The absorbance at 700 nm, of the reaction mixture was measured using u-v spectrophotometer. Increased absorbance was interpreted as increased reducing power[43]. The experiment was repeated in triplicate. Phosphate buffer solution was used as negative control. Percent Ferric reducing-antioxidant power of the test samples was calculated using the formula:

% Ferric reducing-antioxidant activity = $((Ab_s - Ab_c)/Ab_s) \times 100$

Where, Ab_s = absorbance of the sample at 700 nm
Ab_c = absorbance of the control at 700 nm

RESULTS

Phytochemistry: Phytochemical analysis in Table 2 shows that while large amounts of flavonoids are contained in PGE, OGE, MAE and GLE, they are present in small amount in PME. Phenols are present in large quantities in PGE and OGE, while it is absent in MAE, and moderately present in PME.

Yield Total Phenols and Flavonoids Contents: From the result in Table 3, the yield of the extracts is low when compared to the amount (500 g) of macerated plant material. PGE and SAE produced the highest (24.61±5.14 g) and lowest (19.37±3.26 g) yield respectively. While PGE and PME respectively possess highest (51.36±7.44) and lowest (35.42±5.91) mg GAE/ g extract of phenol content, OGE and SAE have highest and lowest flavonoids content of49.71±4.50 and27.16±7.48 mg QE/g extract respectively.

Antioxidant Activities: The result of antioxidant activity (Table 4) shows that PGE, GLE and OGE respectively exhibit highest DPPH scavenging (62.68%), NO scavenging (67.95%) and Ferric reducing activities (69.33%).

DISCUSSION

Phytochemical screening of the leaves of the six plants tested in this study reveals the presence of a number of phytoconstituents, with phenols, flavonoids and tannins being present in large amounts. A report has shown that phytoconstituents are responsible for a broad range of bioactivities[44]. Phenols and flavonoids have been reported to possess redox and/or antioxidant abilities that can counteract the effects of free radicals[45-49], thereby shielding cells from destruction. The result of chemical analysis (Table 2) shows that total phenols and flavonoids contents vary in the six plants, and this can be attributed to many internal and external factors including phonological stage, genetic profile, environmental abiotic and biotic factors such as growing site, light intensity, temperature, radiation, soil drought and salinity [50]. There are various methods for evaluation of antioxidant activity of compounds[51,52]. The result obtained with different methods may vary, therefore, accounting for different sensitivity of each method[53]. The present study employed three methods, namely 1,1-diphenyl-2-picrylhydrazine (DPPH) radical scavenging, nitric oxide (NO)radical scavenging and ferric reducing power assays.DPPH, because of its cheapness, is the most frequently used method in the evaluation of antioxidant potentials of compounds from natural plants[54-56]. In DPPH method, compounds are considered to possess antioxidant activity if they can scavenge and/or reduce DPPH radicals in-vitro[6], i.e, possess the ability to donate hydrogen to DPPH radicals[57]. DPPH, on accepting hydrogen, decolorizes from blue to yellow, resulting to changes in absorbance that can be quantitatively measured at 517 nm. In the present study, various plant extracts exhibited varying degree of antioxidant activity (absorbance), which may be attributed to the fact that the antioxidant compounds are present in these plants in different amounts and complexity. Test concentration (500 mcg/ml) of each of the extracts demonstrated significant ($p<0.05$) antioxidant activity when compared to control as shown in Table 4.

Table 1. Profile of Six Nigerian Ethnomedicinal Plants Tested for Antioxidant Activity

Name of plant	Family	Plant part	Herbarium number	Local name	Reported activities
Piper guineense	Piperaceae	Leaf	UPH/P/251	Igbo: Uziza Hausa: Mansoro Yourba: Iyere	Antiulcer [13] Antioxidant [14] Hepatoprotective[15] Antibacterial [16]
Pterocarpus mildbraedii	Fabaceae	Leaf	UPH/P/442	Igbo: Oha Hausa:Madobiyar rafi Ijaw:Geneghar Edo:Urube	Antiinflammatory[17] Hepatoprotective [17]
Solanum aethiopicum	Solanaceae	Leaf	UPH/P/443	Igbo;Akwukwo anara Hausa:Gayan gauta Yourba: Igbagba	Antioxidant [18] Insecticidal [19,20] Hepatoprotective[21] Anticancer [22]
Ocimum gratissimum	Lamiaceae	Leaf	UPH/P/444	Igbo;Nchu-anwu Hausa:Daidoya Yourba:Efinrin	Antidiabetic [23] Antiinflammatory[24,25] Hepatoprotective [26] Antioxidant [27]
Millettia aboensis	Rabaceae	Leaf	UPH/P/1470	Igbo;Uturekpa Efik:Odudu Edo:Erurumesi	Antioxidant [28] Hematopoietic [29] Antiinflammtory[30] Antimicrobial[31]
Gongronema latifolium	Asclepiadeceae	Leaf	UPH/P/1471	Igbo; Utazi: Yourba: Arokeke	Antioxxidant[32] Antimalarial [33] Immunomodulator[34] Antiinflammatory [35]

Table 2. Phytochemical Analysis of Extracts from Six Nigerian Ethnomedicinal Plants

Phytoconstituent	PGE	PME	SAE	OGE	MAE	GLE
Aikaloids	++	+	+++	++	++	+++
Saponins	++	++	++	+++	+++	++
Flavonoids	+++	+	++	+++	+++	+++
Tannins	++	++	+++	+++	+++	+++
Glycosides	+++	+	+	++	++	++
Phenols	+++	++	+	+++	-	+
Terpenoids	+	-	+++	++	++	++
Sterol	++	++	+	++	-	+

- = Absent +=Present in small Amount ++=Present in moderate Amount +++ =Present in large amount

Table 3. Yield, Total Phenols and Total Flavonoids of Extracts from Six Nigerian Ethnomedicinal Plants

Plant Extract	Yield (g/500 g Dry Powder)	Total Phenols (mgGAE/g Extract)	Total Flavonoids (mgQE/g Extract)
PGE	24.61±5.14	51.36±7.44	40.24±6.50
PME	22.42±6.03	35.42±5.91	34.53±9.18
SAE	19..37±3.26	43.18±8.54	27.16±5-34
OGE	23.55±7.20	47.84±4.75	49.71±4.50
MAE	20.36±4.55	39.63±6.61	38.84±6.19
GLE	21.09±8.17	41.72±6.87	42.65±7.22

Values represent ± SEM (n=3),

Table 4. Antioxidant Activities of Extracts from Six Nigerian Ethno medicinal Plants

Plant Extracts (500mcg/ml)	DPPH Scavenging Activity		NO Scavenging Activity		Ferric Reducing Activity	
	Absorbance (517 nm)	% scavenged	Absorbance (546 nm)	% scavenged	Absorbance (700 nm)	% Antioxidant
PGE	0.53±1.35*	62.68	0.28±0.47*	64.10	0.55±2.11*	58.18
PME	0.94±1.09*	33.80	0.51± 1.14*	34.62	0.41±0.66*	43.90
SAE	0.62 ±.69*	56.34	0.37±0.79*	52.56	0.46±1.16*	50.00
OGE	0.60±1.01*	57.74	0.40±1.20*	50.67	0.75±1.53*	69.33
MAE	0.96±0.98*	32.39	0.45±1.28*	42.31	0.49±0.80*	53.06
GLE	0,58±2.07*	59.15	0.25±1.45*	67.95	0.66±1.02*	65.15
Ascorbic Acid	0.30±1.41*	79.58	0.22±1.33*	71.80	1.08±0.94*	78.70
Control	1.42±0.56	-	0.78±1.26	-	0.23±.71	-

Values represent ±SEM (n=3), *p<0.05 is significant compared to control

Nitric oxide (NO) is a reactive nitrogen specie (RNS) that can react with superoxide anion radical (O_2*) to form a stronger oxidant called peroxynitrite[58], which can oxidize a broad range of bio-molecules[59]. Natural antioxidants of plant origin can compete with nitric oxide for superoxide oxygen, hence, preventing the formation of peroxynitrite[58,59]. The result of this study (Table 4) has demonstrated that the extracts from the six plants when compared to the control, produced significant ($p<0.05$) of nitric oxide activity with concomitant antioxidant activity. Ferric reducing antioxidant activity is based on the principle that reductants (antioxidants) can convert Fe^{3+}/Ferricyanide into Fe^{2+}/Ferrocyanide that can be examined by measuring absorbance of resultant Prussian blue color solution at 700 nm[60]. Higher absorbance indicates stronger reducing capacity of a compound [61].

The result of this study (Table 4) shows that various plant extracts exhibited significant ($p<0.05$) reducing power, at the tested concentration **(**500mcg/ml**)**. This finding corroborates with the report by (6) on other plants like *Caesalpina volkensii*, *Vernonia lasiopus* and *Acacia hockii.* Ferric reducing antioxidant activity observed with the six plants studied in this work may be attributed to the presence of phenols and/or flavonoids which has been reported to break the free radical chain by hydrogen atom transfer, thereby converting Fe^{3+} into Fe^{2+}(52,58)

CONCLUSION AND RECOMMENDATION

The ethanol leaf extract of the six plants exhibited free radical scavenging activities and can be exploited as important sources of natural antioxidant compounds. We therefore recommend for further isolation and characterization of active principles, and testing in animal models, in view to developing natural antioxidants that can used in management of oxidative stress-related disorders.

CONFLICT OF INTEREST: The authors declare no conflict of interest among them

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