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NATURAL DRUGS

IN VITRO ANTI-OXIDATIVE ACTIVITIES AND GC-MS ANALYSIS OF VARIOUS SOLVENT EXTRACTS OF *CASSIA SINGUEANA* PARTS

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Abstract: The present study was conducted to investigate the anti-oxidative activities of different solvent extracts of *Cassia singueana* parts. Our results indicate that all the extracts have reducing power ($Fe^{3+} \rightarrow Fe^{2+}$) and DPPH radical scavenging abilities. However, the ethyl acetate extract of the stem bark has the highest total reducing power whilst the ethanol extract of the stem bark has more potent free radical scavenging activity than all the other extracts. The ethyl acetate extract of the stem bark exhibited more powerful hydroxyl radical scavenging activity than other extracts whilst the aqueous extract of the leaves displayed more potent nitric oxide inhibition activity than other extracts. The GC-MS analysis of the ethyl acetate extract of the stem bark and the ethanol extract of the root and leaves indicated that several aromatic compounds, including phenolics, fatty acids, amino acids and triterpenoids were present in these extracts. Data from this study suggest that the parts of *C. singueana* possessed anti-oxidative activities and can be used as a potential alternative medicine for oxidative stress related non-communicable chronic diseases. Further experimental and clinical studies in this regard are warranted.

Key words: anti-oxidative, *Cassia singueana*, free radicals, GC-MS

Oxidative stress refers to the existence of products called free radicals (molecules possessing an unpaired electron) and reactive oxygen species (ROS) that are formed in normal physiology but become deleterious if they are not quenched by a cascade of antioxidant systems. This can result either from an overproduction of ROS or from the inactivation of the antioxidants (AO), thus shifting the ROS/AO balance in favor of stress (1). Oxidative damage plays a vital pathological role in several non-communicable chronic diseases and metabolic disorders such as arthritis, atherosclerosis, cirrhosis, cancer and diabetes (2).

Current research has confirmed that anti-oxidative agents are the most effective tools to eliminate free radicals which cause oxidative stress and are possible protective agents that can retard the progress of many diseases (3, 4). For this reason, research on therapy and prevention of oxidative stress mediated non-communicable chronic diseases and metabolic disorders has focused attention on the search for agents with anti-oxidative activities that could be used to ameliorate the complications associated with the disease.

The use of medicinal plants for the treatment of various diseases continues to be an important component of health care delivery systems, especially in Africa because the continent has a rich diversity of plants with about 25% of the total number of higher plants in the world where more than 5400 medicinal plants were reported to have over 16,300 medicinal uses (5). The influence of these medicinal plants and natural products upon drug discovery is impressive because a number of clinically active drugs are either natural products or have a natural product pharmacophore (6).

Cassia singueana (Caesalpiniaceae), commonly called golden shower, is native to northern Nigeria. The plant leaves are commonly used in the traditional circle of northern Nigeria to treat diabetes mellitus (7) and to bathe newly delivered mothers. The antipyretic and antiplasmodial activities of the root extract (8) and the anti-ulcer activity of the leaf extract (9, 10) has been reported. Further, the

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methanol extract of the leaves from this plant was found to be safe in a toxicological study (11). Beside that mentioned above, a most recent study reported the preliminary *in vitro* antioxidant potential of the methanolic extract from the leaves of this plant (12). However, in order to completely understand the profile of the antioxidant capacity of any plant, especially as a guide to a future pharmacological study, different solvent extracts from the various parts of the plant need to be investigated for antioxidant activity.

Therefore, we conducted a comprehensive investigation of the stem bark, root and leaves of this plant for anti-oxidative activity by using several models with a view to finding compound(s) that could be used to ameliorate oxidative stress related metabolic disorders. We also analyzed the most highly anti-oxidative extracts by gas chromatography-mass spectrometric (GC-MS) analysis in order to identify the phytochemical components of these extracts.

MATERIALS AND METHODS

Chemicals and reagents

Gallic acid, 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), 2 deoxy-D-ribose and potassium ferricyanide were procured from Sigma-Aldrich through Capital Lab Supplies, New Germany, South Africa. Griess reagent, sodium nitroprusside, thiobarbituric acid (TBA) and Folin Ciocalteau reagent were purchased from Merck Chemical Company, Durban, South Africa.

Plant materials

The fresh stem bark, root and leaf samples of *Cassia singueana* were collected in the month of January, 2011 from Zaria, Kaduna state, Nigeria. The plant samples were identified and authenticated by the herbarium unit of the Biological Science Department, Ahmadu Bello University, Zaria and a voucher specimen number 6863 was deposited. The stem bark, root and leaves were immediately washed and shade-dried to constant weights for two weeks. The dried samples were ground to a fine powder using a kitchen blender, and stored individually in air-tight zip-loc polyethylene bags for transport to the University of Kwazulu-Natal, Westville campus, Durban, South Africa for subsequent analysis.

Preparation of the plant extracts

Forty grams of the fine powdered plant parts were separately defatted with n-hexane. The defatted materials were sequentially extracted with ethyl acetate, ethanol and water by soaking for 48 h in 200 mL of the relevant solvent followed by a 2 h orbital shaking at 200 rpm. After filtration through Whatmann filter paper (No. 1), respective solvents were evaporated under reduced pressure using a rotary evaporator (Buchi rotavapor II) at 40^{o}C to obtain the solvent extracts. Aqueous extracts were dried on a water bath at 40^{o}C. The solvent extract in each case was weighed, transferred to microtubes and stored in a refrigerator at 4^{o}C until required.

Estimation of total phenolic content

The total polyphenol content of each extract was determined (as gallic acid equivalent) according to the method described by McDonald et al. (13) with slight modifications. Briefly, 200 µL of the extract (240 µg/mL) was incubated with 1 mL of ten-fold diluted Folin Ciocalteau reagent and 800 µL of 0.7 M Na_2CO_3 for 30 min at room temperature. Absorbance values were determined at 765 nm on a Shimadzu UV mini 1240 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). All measurements were done in triplicate.

Ferric (Fe^{3+}) reducing antioxidant power assay

The ferric reducing antioxidant power method of Oyaizu (14) with slight modifications was used to measure the reducing capacity of the extracts. To perform this assay, 1 mL of different extract concentrations (15–240 µg/mL) were incubated with 1 mL of 0.2 M sodium phosphate buffer (pH 6.6) and 1% potassium ferricyanide at 50^{o}C for 30 min. After 30 min incubation, the reaction mixture was acidified with 1 mL of 10% trichloroacetic acid. Thereafter, 1 mL of the acidified sample of this solution was mixed with 1 mL of distilled water and 200 µL of $FeCl_3$ (0.1%) in another test tube and the absorbance was measured at 700 nm in a spectrophotometer. Increased absorbance of the reaction mixture indicates higher reduction capacity of the extracts (15).

Free radical scavenging activity

The total free radical scavenging activity of the tested extracts was determined and compared to that of ascorbic and gallic acids as well as trolox by using a method described by Tuba and Gulcin (16) with slight modifications. In order to perform this assay, a 0.3 mM solution of DPPH was prepared in methanol and 500 µL of this solution was added to 1 mL of the extract at different concentrations (15–240 µg/mL). These solutions were mixed and incubated in the dark for 30 min at room tempera-

ture. The absorbance was measured at 517 nm against a blank lacking the scavenger.

Nonsite-specific hydroxyl radical mediated 2-deoxy-D-ribose degradation assay

The hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and the solvent extracts for hydroxyl radicals generated by the ascorbate–EDTA–H_2O_2 system (Fenton reaction) as described by Hinnerburg et al. (17). The assay was performed by adding 200 µL of premixed 100 µM $FeCl_3$ and 100 µM EDTA (1 : 1, v/v) solution, 100 µL of 10 mM H_2O_2, 360 µL of 10 mM 2-deoxy-D-ribose, 1 mL of different extract concentrations (15–240 µg/mL), 400 µL of 50 mM sodium phosphate buffer (pH 7.4) and 100 µL of 1 mM ascorbic acid in sequence. The mixture was incubated at 50°C for 2 h. Thereafter, 1 mL of 2.8% trichloroacetic acid (TCA) and 1 mL of 1.0% thiobarbutiric acid (TBA) (in 0.025 M NaOH) were added to each test tube. The samples were further incubated in a water bath at 50°C for 30 min to develop the pink chromogen color. The extent of oxidation was estimated from the absorbance of the solution at 532 nm and the hydroxyl radical scavenging activity of the extract is reported as a percentage inhibition of deoxyribose degradation.

Nitric oxide (NO) radical scavenging assay

Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide (NO), which interacts with oxygen to produce nitrite ions that can be estimated by use of Griess reagent. Scavengers of NO compete with oxygen, leading to reduced production of NO (18). The assay was carried out by incubating 500 µL of 10 mM sodium nitroprusside in phosphate buffer (pH 7.4) and 500 µL of different extract concentrations (15-240 µg/mL) at 37°C for 2 h. The reaction mixture was then mixed with 500 µL of Griess reagent. The absorbance of the chromophore formed during the diazotization of nitrite with sulfanilamide and subsequent coupling with naphthylethylenediamine was read at 546 nm. The percentage inhibition of nitric oxide generated was measured in comparison with the absorbance value of a control (sodium nitroprusside in phosphate buffer).

The scavenging effects of the solvent extracts in the DPPH, hydroxyl and nitric oxide radical scavenging assays were calculated as:

$$\text{Scavening activity } (\%) = (1 - \frac{A_s}{A_c}) \times 100$$

where A_s is the absorbance in the presence of the sample and A_c is the absorbance of the control.

Table 1. Percentage yield and total polyphenol concentrations of various solvent extracts of *Cassia singueana* parts.

Samples %	Yield	Total polyphenol (mg/g GAE)
Stem bark		
EtOAc	4.04	102.36 ± 1.18[a]
EtOH	6.90	91.53 ± 0.96[b]
Aqueous	5.29	81.45 ± 0.15[c]
Root		
EtOAc	0.63	75.65 ± 2.07[d]
EtOH	2.21	77.59 ± 1.36[d]
Aqueous	2.40	13.02 ± 1.93[f]
Leaves		
EtOAc	0.78	5.55 ± 0.77[g]
EtOH	1.34	28.84 ± 0.72[e]
Aqueous	0.48	6.94 ± 0.33[g]

Data are presented as the mean ± SD values of triplicate determinations. [a-g]Different letters along a column are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $p < 0.05$). GAE = gallic acid equivalent.

Gas chromatography-mass spectrometric (GC-MS) analysis

Based on the results of anti-oxidative assays, the EtOAc extracts of the stem bark and the EtOH extracts of the root and leaf samples of the plant were subjected to GC-MS analysis. The analysis was conducted with an Agilent Technologies 6890 Series GC coupled with (an Agilent) 5973 Mass Selective detector and driven by Agilent Chemstation software. A HP-5MS capillary column was used (30 m × 0.25 mm internal diameter, 0.25 µm film thickness). The carrier gas was ultra-

Table 2. Total reducing power (gallic acid equivalent) of solvent extracts from the different part of *Cassia singueana*.

Samples	Concentration (µg/mL)				
	15	30	60	120	240
Stem bark					
EtOAc	37.19 ± 2.39^{gh}	31.38 ± 4.18^{d}	27.11 ± 4.58^{cd}	44.47 ± 6.08^{d}	69.03 ± 16.14^{cf}
EtOH	28.51 ± 3.30^{g}	27.83 ± 5.06^{d}	21.11 ± 2.18^{c}	29.94 ± 4.41^{c}	56.84 ± 1.62^{ce}
Aqueous	0.00 ± 0.00^{a}	6.04 ± 0.97^{a}	3.46 ± 1.02^{a}	8.48 ± 1.91^{ab}	21.56 ± 4.34^{b}
Root					
EtOAc	20.63 ± 2.28^{f}	17.21 ± 2.76^{c}	15.96 ± 1.16^{b}	26.45 ± 2.73^{c}	45.54 ± 4.04^{c}
EtOH	1.99 ± 0.55^{b}	8.14 ± 1.62 ^{a}	15.27 ± 2.13^{b}	32.51 ± 3.68^{c}	56.19 ± 8.53^{c}
Aqueous	3.53 ± 0.97^{c}	10.00 ± 1.84^{ab}	3.49 ± 1.56^{a}	6.82 ± 1.79^{a}	11.93 ± 2.10^{a}
Leaves					
EtOAc	4.43 ± 0.40^{c}	6.00 ± 1.01^{a}	4.44 ± 0.49^{a}	6.97 ± 0.93^{a}	10.70 ± 0.65^{a}
EtOH	7.62 ± 0.56^{d}	6.62 ± 1.19^{a}	5.20 ± 1.02^{a}	9.82 ± 1.07^{b}	15.68 ± 3.59^{ab}
Aqueous	10.23 ± 0.86^{c}	5.65 ± 1.75^{a}	3.56 ± 0.56^{a}	4.57 ± 1.66a	13.28 ± 1.83^{a}
Ascorbic acid	31.50 ± 4.92^{g}	32.66 ± 3.00^{d}	35.62 ± 5.51^{e}	52.56 ± 5.43^{d}	89.95 ± 6.21^{f}
Trolox	17.84 ± 1.86^{f}	20.88 ± 7.33^{c}	24.42 ± 3.95^{c}	29.61 ± 1.96^{c}	52.77 ± 0.90^{cd}

Data are presented as the mean ± SD values of triplicate determinations. $^{a-g}$Different letters along a column are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $p < 0.05$).

Table 3. Hydroxyl radical scavenging activity of extracts from various parts of *Cassia singuena*.

Samples	Concentration (µg/mL)				
	15	30	60	120	240
Stem bark					
EtOAc	59.30 ± 14.42^{a}	78.06 ± 0.31^{a}	81.37 ± 1.46^{a}	84.07 ± 1.54^{a}	82.24 ± 0.53^{a}
EtOH	-5.81 ± 2.60^{b} -15.	20 ± 4.46^{b}	-30.67 ± 1.67^{b}	-52.47 ± 1.78^{b}	-100.00 ± 0.00^{b}
Aqueous	-25.14 ± 7.42^{c}	-45.39 ± 1.48^{c}	-66.61 ± 0.81^{c}	-100.00 ± 0.00^{c}	-100.00 ± 0.00^{b}
Root					
EtOAc	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	-13.94 ± 1.59^{d}	-33.08 ± 0.84
EtOH	62.23 ± 3.38^{a}	71.01 ± 2.04^{e}	74.47 ± 6.57^{a}	84.56 ± 0.71^{a}	87.60 ± 0.56^{d}
Aqueous	42.50 ± 3.26^{c}	37.37 ± 0.38^{f}	35.18 ± 3.57^{e}	35.49 ± 2.98 ^{e}	29.17 ± 0.69^{c}
Leaves					
EtOAc	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	0.00 ± 0.00^{f}	20.10 ± 4.62^{f}
EtOH	54.84 ± 1.17^{a}	68.48 ± 1.01^{e}	73.85 ± 0.76^{a}	77.53 ± 0.93^{g}	84.68 ± 2.23^{d}
Aqueous	11.85 ± 1.69^{f}	18.40 ± 2.82^{g}	21.37 ± 3.97^{f}	27.99 ± 0.75^{h}	35.77 ± 3.40^{g}
Trolox	57.32 ± 2.95^{a}	73.11 ± 1.44^{e}	76.04 ± 2.05^{a}	80.09 ± 3.93^{ag}	79.82 ± 3.50^{d}

Data are presented as the mean ± SD values of triplicate determinations. $^{a-g}$Different letters along a column are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $p < 0.05$).

Table 4. Nitric oxide scavenging activities of extracts from various parts *Cassia singueana*.

Samples	Concentration (µg/mL)				
	15	30	60	120	240
Stem bark					
EtOAc	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	16.12 ± 0.85^{b}	23.70 ± 1.66^{b}	30.93 ± 4.20^{b}
EtOH	-46.61 ±5.34^{b}	-100.00 ± 0.00^{f}	-100.00 ± 0.00^{j}	-100.00 ± 0.00^{h}	-100.00 ± 0.00^{f}
Aqueous	8.99 ± 1.31^{c}	12.70 ± 0.59^{c}	37.56 ± 0.75^{d}	44.91 ± 7.16^{d}	50.24 ± 1.88^{c}
Root					
EtOAc	20.63 ± 2.28^{f}	17.21 ± 2.76^{c}	15.96 ± 1.16^{b}	26.45 ± 2.73^{c}	45.54 ± 4.04^{c}
EtOH	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
Aqueous	6.89 ± 0.89^{c}	17.20 ± 2.09^{d}	77.83 ± 5.26^{h}	80.87 ± 2.89^{g}	34.07 ± 1.31^{b}
Leaves					
EtOAc	56.01 ± 2.30^{e}	52.93 ± 13.42^{e}	61.67 ± 2.48^{f}	58.06 ± 1.84^{e}	65.39 ± 4.17^{e}
EtOH	46.51 ± 1.20^{f}	37.66 ± 10.97^{e}	67.60 ± 0.39^{g}	65.33 ± 2.60^{f}	44.15 ± 1.94^{c,d}
Aqueous	9.19 ± 2.91^{c}	16.36 ± 0.89^{d}	24.97 ± 2.81^{c}	26.03 ± 0.37^{c}	56.22 ±4.69^{e,e}
Ascorbic acid	47.81 ± 0.51^{e}	50.97 ± 1.35^{e}	52.82 ± 1.35^{e}	56.72 ± 3.69^{e}	64.08 ± 4.25$^{e\ f}$
Galicic acid	4.43 ± 2.69^{c,d}	7.52 ± 1.11^{b}	25.74 ± 0.44^{c}	27.66 ± 1.91^{c}	31.70 ± 2.51^{b}
Trolox	66.30 ± 1.23^{g}	65.51 ± 1.27^{e}	63.39 ± 1.84^{f}	59.53 ± 1.81^{e}	52.21 ± 2.47^{c}

Data are presented as the mean ± SD values of triplicate determinations. $^{a-j}$Different letters along a column are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $p < 0.05$).

Table 5. IC_{50} values of different extracts of *C. singueana* parts in different anti-oxidative models.

Samples	IC_{50} (µg/mL)		
	DPPH	Hydroxyl	Nitric oxide
Stem bark			
EtOAc	1.76	1.56	1116.40
EtOH	1.20	P	P
Aqueous	34.05	P	188.78
Root			
EtOAc	31.78	P	115.51
EtOH	14.57	3.12	Nil
Aqueous	2.58	2.05	87.73
Leaves			
EtOAc	44.23	161016.65	4.57
EtOH	35.88	6.47	29.56
Aqueous	53.09	1439.25	2.81
Ascorbic acid	2.56	ND	26.28
Gallic acid	1.27	ND	1026.45
Trolox	5.04	2.610	599.21

ND means not determined and P means the extract showed pro-oxidative properties in the experimental model.

pure helium at a flow rate of 1.0 mL/min and a linear velocity of 37 cm/s. The injector temperature was set at 250°C. The initial oven temperature was at 60°C which was programmed to increase to 280°C at the rate of 10°C/min with a hold time of 3 min at each increment. Injections of 1 µL were made in splitless mode with a split ratio of 20 : 1. The mass spectrometer was operated in the electron ionization mode at 70 eV and electron multiplier voltage at 1859 V. Other MS operating parameters were as follows: ion source temperature 230°C, quadrupole temperature 150°C, solvent delay 4 min and scan range 50-700 amu. The compounds were identified by direct comparison of the mass spectrum of the analyte at a particular retention time to that of a reference standard found in the National Institute of Standards and Technology (NIST) library. The Applied GC/MS method was validated according to the ICH Harmonised Tripartite Guideline: Validation of Analytical Procedures: Text and Methodology, Q2(R1), Geneva, 2005, which can be accessed at http://www.ich.org.

Statistical analysis

Data are presented as the mean ± SD of triplicates determination. Data were analyzed by SPSS statistical software (version 18) using Tukey's multiple range *post-hoc* test. Values were considered significantly different at $p < 0.05$.

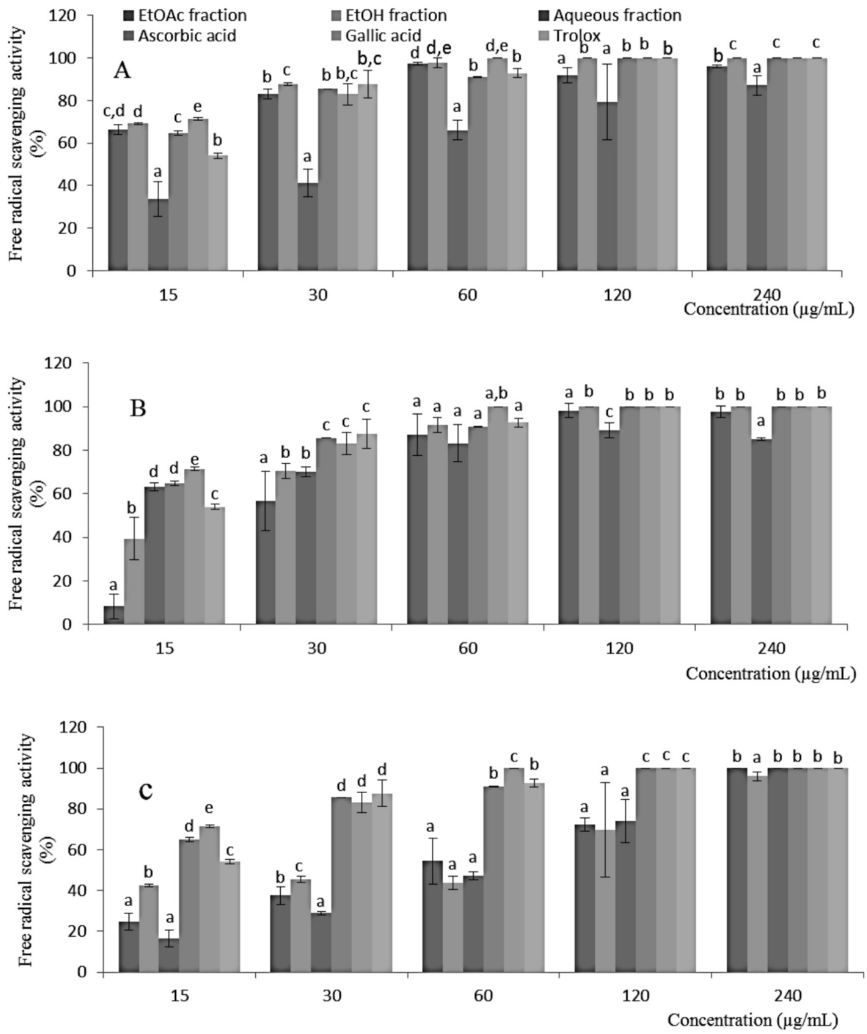

Figure 1. DPPH radical scavenging activities of various solvent extracts from stem bark (**A**), root (**B**) and leaves (**C**) of *Cassia singueana*. [a-e]Values with different letters over the bars for a given concentration for each extract are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $p < 0.05$)

Table 6. Identified compounds of EtOAc extract of stem bark and EtOH extracts of root and leaves of *Cassia singueana* by GC-MS.

No.	Compounds	Retention time (min)	Molecular mass	Relative abundance (%)
	EtOAc extract of stem bark			
1	2,3-Dihydrobenzofuran	7.67	120.15	4.08
2	Resorcinol	8.52	110.10	54.03
3	4-Propylphenol	9.74	136.19	7.24
4	Dehydroxylevodopa	12.59	179.10	ND
5	3-Methylanthralin	19.85	240.05	1.71
6	2,3,5,7-Tetramethylpyrrolo[2,3-f] quinolin-9-ol	21.12	240.00	ND
7	2,2'-(*m*-Phenylene)dithiophene	21.40	242.10	ND
8	6-Dehydroestradiol	22.41	270.36	2.62
9	1-(4-Methoxyphenyl)-2-(2-hydroxy-4-methylphenyl)-diazene	22.58	242.10	ND
10	Methyl 3-(1-formyl-3,4-methylenedioxy) benzoate	23.42	284.06	9.28
	EtOH extract of root			
2	Resorcinol	8.51	110.10	ND
11	*p*-Hydroxyphenylpyruvic acid	9.74	180.15	ND
5	3-Methylanthralin	19.86	240.10	ND
12	5,6-Dimethyl-4-phenyl-3-cyanopyridine-2-thione	21.13	240.10	ND
13	1-Methylchrysene	21.48	242.10	ND
14	9-Benzylidenexanthene	22.41	270.10	ND
15	3',7-Dihydroxy-4-methoxy-flavonol	23.43	284.10	ND
	EtOH extract of leaves			
16	2-Methoxy-4-vinylphenol	9.02	150.06	2.67
17	1-(3,6,6-Trimethyl-1,6,7,7-tetrahydrocyclo-penta[c]pyran-1-yl) ethanone	10.16	191.10	ND
18	4-Methyl-2,5-dimethoxybenzaldehyde	12.17	180.10	ND
19	Coniferyl alcohol	14.21	180.20	2.72
20	*Long chain aromatic ester	15.52	194.20	ND
21	Palmitic acid	16.50	256.42	5.50
22	Phytol	17.95	296.53	23.70
23	Linolenyl alcohol	18.20	264.44	3.92
24	Stearic acid	18.39	284.48	1.73
25	Linolenic acid ethyl ester	18.46	306.48	2.24
26	Squalene	23.94	410.72	7.28
27	Eicosane	24.88	282.55	3.32

The compounds presented in the Table are those which matched similar compounds in the NIST library software and which contained the molecular ion of the matching compound. *Compound had the fragmentation pattern characteristic of long chain aromatic esters. The aliphatic chain had a good hit with the library. ND means not determined while # refers to the annotated peak number.

RESULTS

The yield of the various extracts collected from the plant indicated that higher yields are apparently obtained from the stem bark extracts. Furthermore, the stem bark extracts contained significantly ($p < 0.05$) higher amount of total phenolics than extracts from other parts of the plant. Within the stem bark, the ethyl acetate extract contained a significantly ($p < 0.05$) higher amount of the total phenolics than the other solvent extracts (Table 1).

The total reducing power (in terms of percentage gallic acid equivalent) of the various extracts of *C. singueana* was compared to ascorbic acid and trolox (Table 2). While all the solvent extracts from the various parts of *C. singueana* demonstrated $Fe^{3+} \rightarrow Fe^{2+}$ reductive ability, the EtOAc extract of the stem bark exhibited a consistently higher reducing ability compared to all other extracts as well as a standard, trolox, though the difference was not significant ($p > 0.05$) compared to the EtOH extract of the same plant part at lower concentrations. With the exception of the EtOAc extract of the root, the total reducing power of the other root and leaf extracts were significantly lower than the standards used in these assays (Table 2).

Figure 1 shows the DPPH radical scavenging activities of the various solvent extracts of *C. singueana* parts. All the extracts showed a tendency to quench DPPH free radicals as manifested by the concentration dependent increase in the percentage inhibitions. The EtOH extracts of the stem bark and root had a consistently higher DPPH radical scavenging ability than other extracts in these parts,

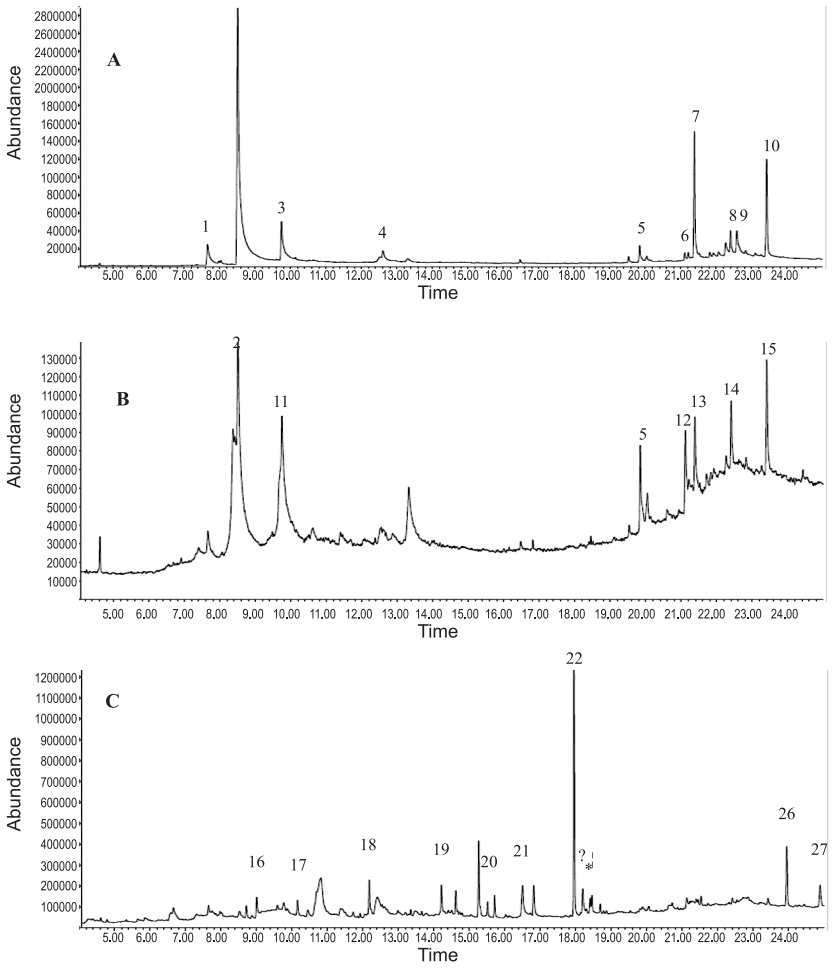

Figure 2. GC-MS chromatogram of EtOAc extract of the stem bark (**A**) and EtOH extracts of the root (**B**) and leaves (**C**) of *C. singueana*. Symbols *8*,* and ◆ refer to annotated peak numbers 23, 24 and 25, respectively

although the difference in some cases was insignificant. There was no difference between the free radical scavenging activity of most of the stem bark and root extracts and the standard antioxidant used (Figure 1). On the contrary, the leaf extracts showed significantly lower free radical scavenging activity compared to the standards used in this assay.

The results of the hydroxyl radical inhibition of the solvent extracts of *C. singueana* parts indicated that all the other extracts inhibited hydroxyl radicals generated by Fenton's reaction except the EtOH and aqueous extracts of the stem bark as well as the EtOAc extract of the root which showed pro-oxidative tendencies (Table 3). However, the EtOAc extract of the stem bark and EtOH extracts of the roots and leaves demonstrated significantly higher ($p < 0.05$) anti-$OH^{\cdot}$ activity than other extracts within the plant parts. The IC_{50} values of 1.56, 3.12 and 6.47 µg/mL were obtained for the EtOAc extract of the stem bark and EtOH extracts of the roots and leaves, respectively, whereas 2.61 µg/mL was found for trolox (Table 5).

The NO inhibition activities of the different solvent extracts of *C. singueana* parts are presented in Table 4. Apart from the EtOH extracts of the stem bark and root, all other extracts were found to exhibit NO scavenging activities. The EtOAc and aqueous extracts of the root as well as the EtOAc and EtOH extracts of the leaves exhibited a non concentration dependent NO inhibition effect. Aqueous extracts were found to possess lower IC_{50} values in the NO scavenging model than other extracts within the plant parts.

Based on the high anti-oxidative activities, the EtOAc extract of the stem bark and the EtOH extracts of the root and leaves were analyzed by GC-MS (Figure 2). The EtOAc extract of the stem bark was found to contain phenolic compounds such as resorcinol and 4-propylphenol, amino acids such as dehydroxylevodopa, sterols such as 6-dehydroestradiol and aromatic esters such as the methyl benzoates. The ethanol extracts of the roots was found to contain polyphenols such as resorcinol, anthralin, and a polyhydroxylated flavonoid, precursors to polyphenol compounds such as hydroxyphenylpyruvic acid and aromatics such as benzylidenexanthene and methylchrysene. The EtOH extract of the leaves contained phytol along with benzyl alcohols, fatty acids and their esters and precursors to triterpenoids such as squalene (Table 6).

DISCUSSION

Cassia singueana is used in the traditional management of diabetes mellitus in Nigeria whose pathogenesis has, in part, been linked to the development of oxidative stress. Apart from now, only a few authors have reported the antioxidant activities of the plants within the *Cassia* genus (19-21). This study investigated the complete anti-oxidative profile of various solvent extracts from different parts of this plant as a prelude to finding agent(s) that could be used to ameliorate oxidative stress-associated complications. We found some of the *C. singueana* extracts to possess exceptionally high anti-oxidative activities, at least with the experimental models used.

The different antioxidant compounds act through different mechanisms and therefore, no single method can fully evaluate the total antioxidant capacity. For this reason, studying the complex antioxidant activities often used is a multi-method approach (22). It was suggested that the electron donating capacity, reflecting the reducing power of bioactive compounds, is associated with antioxidant activity (16, 23). Antioxidants can be reductants, and inactivation of oxidants by reductants can be described as redox reactions in which one species is reduced at the expense of the oxidation of the other. The presence of reductants, such as antioxidant substances in the samples, causes the reduction of the ferric to the ferrous form, which can be monitored by measuring the formation of Perl's prussian blue at 700 nm. The FRAP assay, therefore, provides a reliable method to study the antioxidant activity of various extracts and/or compounds. In our experiment, the higher reducing power of the EtOAc extracts of the stem bark and root in most of the concentrations used suggests that the phytochemical constituents with high redox potential are more extractable with EtOAc.

The DPPH radical has been used widely as a model system to investigate the scavenging activities of several natural compounds including phenolic compounds, solvent extracts or crude mixtures of plants. The effect of antioxidants on DPPH was thought to be due to their hydrogen donating ability (24). Hence, DPPH is usually used as a substrate to evaluate anti-oxidative or free radical scavenging activity of antioxidants agents *in vitro* (15). The high DPPH radical scavenging activities of the various solvent extracts which are comparable to standard antioxidants used suggest that the extracts have compounds with high proton donating ability and could serve as free radical inhibitors. However, the organic solvent extract from the stem bark demonstrated a more remarkable anti-radical activity with IC_{50} values lower than those of ascorbic acid and trolox. Although many reports on the DPPH radical

scavenging activity of extracts from plants belonging to different families and from various parts of the world exist in the literature, only a few authors (25) reported low IC_{50} values as was observed with these extracts. This further suggests that these extracts contain powerful free radical scavenging phytochemicals that could have the ability to inhibit a free radical upsurge as well as oxidative stress which consequently might ameliorate oxidative stress associated metabolic disorders.

Hydroxyl radicals are known to be the most reactive species, causing damage to DNA, protein and other life essential biological molecules (26). It is said to be detrimental and initiates auto-oxidation, polymerization and fragmentation of biological molecules. The identification of compounds that have excellent hydroxyl scavenging activity would be important for diseases such as type 2 diabetes where oxidative stress is important for the disease initiation and/or progression. The hydroxyl radical scavenging activity is thus measured as the percentage of inhibition of hydroxyl radicals generated in the Fenton's reaction mixture by studying the competition between deoxyribose and the extracts for hydroxyl radicals generated from the Fe^{3+}/EDTA/ascorbate/H_2O_2 system (17). In the present study, the EtOAc extract of the stem bark and aqueous extract of the root are more effective in hydroxyl radical inhibitions, considering their lower IC_{50} values. The pro-oxidative tendencies showed by the EtOH and aqueous extracts of the stem bark as well as the EtOAc extract of the root in the hydroxyl radical based anti-oxidative model further indicate the need to use a multi method approach before a definite statement can be made on the anti-oxidative effects of any plant extract or bioactive compound.

Nitric oxide is a very unstable species that has been implicated in the pathology of cancer, T2D and several other diseases (27-29). The lower IC_{50} values observed by the aqueous extracts in all the plant parts could indicate that the anti-oxidative principles extracted by the various solvents act *via* a different antioxidant mechanism. On the other hand, the non concentration dependent pattern of NO scavenging activity demonstrated by the EtOAc and aqueous extracts of the root as well as the organic solvent extracts of the leaves could be linked to a hormesis phenomenon exhibited by these extracts. Hormesis is a dose-response relationship for a single endpoint that is characterized by a reversal of response between low and high doses of chemicals, biological molecules, physical stressors, or any other initiators of a response (30) and its occurrence has been documented in numerous biological, toxicological and pharmacological investigations (31). Thus, these extracts possess optimal points for effective inhibition of NO radicals and/or antagonism may occur with other phytochemicals at certain concentrations.

Phenolics are very important constituents among other natural antioxidants because of their multiple biological effects and direct contribution to anti-oxidative activity (26). Although a correlation analysis was not performed, our data tends to suggest strong correlations between the total phenolic content and anti-oxidative capacity because most extracts with the highest phenolic content within a plant part displayed high anti-oxidative activity in most of the experimental models used and consequently, these extracts were selected for GC-MS analysis. While not discounting the possible contributions of the other detected phytochemicals, it is possible to surmise that the main anti-oxidative agents of these extracts are simple phenolics, especially resorcinol in the stem bark and root as well as phytol in the leaves. However, the differences in the anti-oxidative activities among the extracts could be due to different qualitative and quantitative composition of their phenolics as well as other phytochemicals. This is because the antioxidant actions of phenolics and their derivatives depend on the number of hydroxyl groups in the molecule (32).

In conclusion, this work reveals that the various solvent extracts of *C. singueana* parts possessed strong anti-oxidative activities that could be relevant in the management of oxidative stress related non-communicable chronic diseases, however, further experimental and clinical studies are warranted. Phenolic compounds were found to be the main phytochemicals with resorcinol being more prominent than other compounds in the stem bark and root and phytol being more prominent than other compounds in the leaves. Bioassay guided fractionation and evaluating the *in vivo* anti-oxidative effects of some of the extracts will be the subject of our future study.

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REFERENCES

1. Fang Y.Z., Yang S., Wu G.: Nutrition 18, 872 (2002).
2. Gulcin I., Oktay M.O., Rfan K.L., Ali, A.: J. Ethnopharmacol. 79, 325 (2002).
3. Halliwell B.: Lancet 355, 1179 (2000).
4. Kaur C., Kapoor, H.C.: Int. J. Food Sci. Tech. 37, 153 (2002).
5. van Wyk B.E.: J. Ethnopharmacol.119, 342 (2008).
6. Koehn F.E., Carter G.T.: Nat. Rev. Drug Disc, 4, 206 (2005).
7. Etuk E.U., Bello S.O., Isezuo S.A., Mohammed B.J.: Asian J. Exp. Biol. Sci. 1, 55 (2010).
8. Adzu B., Abbah J., Vongtau H., Gamaniel K.: J. Ethnopharmacol. 88, 261 (2003).
9. Ode O.J., Onakpa M.M.: Int. J. App. Biol. Pharm. Tech. 1, 1352 (2010).
10. Ode O.J., Oladele G.M., Asuzu O.V.: Int. J. Plant Animal Env. Sci. 1, 54 (2011).
11. Ode O.J., Asuzu O.V. Oladele, G.M.: J. Pharm. Biomed. Sci. 8, 1 (2011).
12. Madubunyi I.F., Ode O.J.: Comp. Clin. Path. 21, 1565 (2012).
13. McDonald S., Prenzier P.D., Autokiwich M., Robards K.: Food Chem. 73, 73 (2001).
14. Oyaizu M.: Japan J. Nutr. 44, 307 (1986).
15. Gulcin I., Beydemir H.A., Alici H.A., Elmastas M., Buyukokuroglu M.E.: Pharmacol. Res. 49, 59 (2004).
16. Tuba A.K., Gulcin I.: Chem. Biol. Interact. 174, 27 (2008).
17. Hinnerburg I., Damien H. J., Hiltumen R.: Food Chem. 97,122 (2006).
18. Kurian A.G., Suryanarayanan S., Raman A., Pidakalla J.: Chin. Med. 5, 1 (2010).
19. El-Hashash M.M., Abdel-Gawad M.M., El-Sayed M.M., Sabry W.A., Abdel-Hameed E.S., Abdel-Lateef E.E.: Acta Pharm. 60, 361 (2010).
20. Kouakou-Siransy G., Sahpaz S., Irie-Nguessan G., Datte Y. J., Kabian J., Gressier B., Bailleul F.: Food Chem. 118, 430 (2010).
21. Arya V., Yadav S., Kumar S., Yadav J.P.: Nat. Prod. Res. 25, 1473 (2011).
22. Huang D., Ou B., Prior R.L.: J. Agric. Food Chem. 53, 1841 (2005).
23. Arabshahi-Delouee A., Urooj A.: Food Chem. 102, 1233 (2007).
24. Prathapan A., Singh M.K., Anusree S.S., Kumar D.R.S., Sundaresan A., Raghu K.G.: J. Food Biochem. 35, 1548 (2010).
25. Wu N., Zu Y., Fu Y., Kong Y., Zhao J., Li X. et al.: J. Agric. Food Chem. 58, 4737 (2010).
26. Lee J.C., Kim H.R., Kim J., Jang, Y.S.: J. Agric. Food Chem. 50, 6490 (2002).
27. Ceriello A.: Endocr. Pract. 12, 60 (2008).
28. Ceriello A., Bortolotti N., Motz E., Crescentini A., Lizzio S., Russo A. et al.: Diabetes Care, 21, 1529 (1998).
29. Fukumura D., Kashiwagi S., Jain R.K.: Nat. Rev. Cancer 6, 521 (2006).
30. Calabrese E. J., Baldwin L. A.: Ann. Rev. Pub. Health 22, 15 (2001).
31. Kendig E.L., Le H.H., Belcher S.M.: Int. J. Toxicol. 29, 235 (2010).
32. Soobrattee M.A., Neergheen V.S., Luximon-Ramma A., Aruoma O.I., Bahorun T.: Mutation Res. 579, 200 (2005).

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