IN VITRO ANTIOXIDANT ACTIVITY AND GC-MS ANALYSIS OF THE ETHANOL AND AQUEOUS EXTRACTS OF *CISSUS CORNIFOLIA* (BAKER) SPLANCH (VITACEAE) PARTS

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Abstract: The study was intended to explore the antioxidant potential and phytochemical content of the ethanol and aqueous extracts of the leaf and root samples of *Cissus cornifolia* (Baker) Splanch (Vitaceae) across a series of four *in vitro* models. The results showed that all the extracts had reducing power (Fe^{3+}- Fe^{2+}), and DPPH, hydroxyl and nitric oxide radical scavenging abilities to varying extents. However, the ethanol root extract had more potent antioxidant power in all the experimental models than other extracts and possessed a higher total phenol content of 136.1 ± 6.7 mg/g. The GC-MS analysis of the aqueous and ethanol extracts of the roots indicated the presence of the common aromatic phenolic compounds, pyrogallol, resorcinol and catechol, a fatty acid, n-hexadecanoic acid and an aldehyde, vanillin. Data from this study suggest that both the leaves and roots of *C. cornifolia* possessed anti-oxidative activities with the best anti-oxidant activity being exhibited by the ethanolic extract of the root. The antioxidant properties of the root extracts can be attributed to the phenolic compounds present in the extracts.

Keywords: antioxidant, *Cissus cornifolia, in vitro*, polyphenols

The biochemical state of the body, when there is an imbalance between the production of reactive oxygen and a biological system's ability to readily detoxify the reactive intermediates or easily repair the resulting damage, is termed oxidative stress (OST). Oxidative stress has been implicated in the pathogenesis of a number of diseases such as atherosclerosis, Parkinson's, heart failure, myocardial infarction, Alzheimer's disease, fragile X syndrome, chronic fatigue syndrome and diabetes (1).

The deleterious effects of oxidative stress in a number of metabolic chronic disorders has prompted scientists to search for antioxidative compounds that can impede the oxidation of biomolecules in a chain reaction, which could be vital in the therapy and prevention of many metabolic disorders (2). The synthetic antioxidative agents, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propylgallate (PG), and tert. butylhydroxytoluene exhibit potent free radical scavenging effects but they induce liver and kidney dysfunction and have also been reported to be carcinogenic in laboratory animals (3-5). Thus, there is a need to identify and utilize more antioxidants of natural ori-

gin, which can relieve the deleterious effects of free radicals and other biological oxidants (5).

Plants have a long history of use in medicinal applications, especially in Sub-saharan Africa where access to medical supplies, pharmaceutics and medical doctors are limited. The uses of these medicinal plants have been well documented over the years and their use, especially in the rural areas of African countries is quite popular (6). Based on this, research activities on medicinal plants, especially on the phytochemistry and bioactivity of medicinal plants have been stimulated in order to develop alternative therapies for a number of diseases (7). Thus, in recent years the screening of medicinal plants with potential antioxidant properties has received significant attention due to an increasing concern for safe and non-toxic alternative antioxidants (8).

Cissus cornifolia (Baker) Splanch (Vitaceae), commonly called the "Ivy grape" is indigenous to Zimbabwe. It is locally called ''Mudzambiringa'' and ''Idebelebe'' by the Shona and Ndebele-speaking Zimbabweans, respectively. *Cissus* is a genus of approximately 350 species. Most of the members of

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this genus have a wide array of uses in African traditional medicine (9). *C. cornifolia*, commonly found in Zimbabwe, is traditionally used by the Shona speaking people as a remedy for gonorrhea while the leaf-sap is used among the Tanganyika as a sedative in cases of mental derangement; the root-decoction is also used for malaria, septic tonsil, diabetes, cardiac problems and pharyngitis (10). Currently, there have been no phytochemical or biological activity studies on *C. cornifolia*. Phytochemical studies on other species of *Cissus* have revealed the presence of glycosides, flavonoids, saponins, steroids, terpenoids and tannins (11-14).

Bioactivity and isolation of various compounds have been carried out in a number of species from the genus *Cissus* and had shown impressive results. *Cissus quadrangularis* Linn. Wall. Ex which is commonly used as a food supplement in India, was evaluated for its protective effects against tissue injury (13). Jainu and Devi (15) reported that *Cissus quadrangularis* has antimicrobial, antiulcer, antioxidative and cholinergic activity as well as potent fracture healing properties and a beneficial effect on cardiovascular diseases. In addition, the same group reported cytoprotective properties of the methanolic extract of the same plant (16). Attawish et al. (17) isolated vitamin C, β-carotene, two asymmetric tetracyclic triterpenoids, β-sitosterol, α-amyrin, α-amyrone from *Cissus quadrangularis* which they accredited to the observed activities and Potu et al. (18) also reported several phytochemical constituents, such as ascorbic acid, flavonoids, and triterpenoids in *Cissus quadrangularis*.

Cissus sicyoides L, has been widely used in folk medicine against stomach ache and indigestion (19) and has been reported to treat diabetes, pain, inflammation, rheumatism, abscesses, muscle inflammation, convulsions, epilepsy, stroke and hypertension (20). Ferreira et al. (20) also reported a coumarin glycoside, coumarin sabandin, flavonoids, steroids, sitosterol and hydrolyzable tannins from the plant. *Cissus populnea* has been reported as a food supplement in Nigeria (21), however there are no reports on the phytochemistry of the plant in the literature. Ojekale et al. (22) reported *Cissus populnea* to have a myriad of uses as a medicinal agent globally.

This study was undertaken to investigate the ethnomedicinal efficacy of the leaf and root ethanol and aqueous extracts of the plant as potential sources of therapeutic agents which can be of use in ameliorating oxidative stress related parameters using various models *in vitro*. We also subjected the extracts to GC-MS analysis in order to partially explore the phytochemistry of the plant.

MATERIALS AND METHODS

Chemicals and reagent

All chemicals used were of analytical grade. Gallic acid, ascorbic acid, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), 2-deoxy-D-ribose, iron chloride, sodium carbonate, trichloroacetic acid, ethylenediaminetetraacetic acid (EDTA), H_2O_2, 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's phenol reagent were purchased from Merck Chemical Company, Durban, South Africa.

Plant material

The fresh leaf and root samples of *C. cornifolia* were collected in the month of March, 2012 from Mrewa, Mashonaland East province, Zimbabwe. The plant samples were identified and authenticated at the herbarium unit of the Harare botanical garden and herbarium, Zimbabwe and a voucher specimen number CC082 was deposited. The leaves and roots were immediately washed with distilled water, cut into small pieces and shade-dried until constant weights were attained. The dried samples were ground to a fine powder using a blender, and stored individually in air-tight polyethylene bags for transport to the University of KwaZulu-Natal, Westville campus, Durban, South Africa for further analysis.

Preparation of the plant extracts

Forty grams of the fine powdered plant parts were separately defatted with hexane. The defatted materials were sequentially extracted with ethanol and water by soaking for 48 h in 200 mL of the relevant solvent. For ethanol extracts, after filtration through Whatmann filter paper (No. 1), the ethanol was evaporated under reduced pressure using a rotary evaporator (Buchi Rotavapor II) at 40°C. Aqueous extracts were dried using a freeze dryer. The solvent extracts in each case was weighed, transferred to micro tubes and stored in a refrigerator at 4°C until required.

Estimation of total phenolic content

The total phenolic content of each extract was determined (as gallic acid equivalents) according to the method described by McDonald et al. (23), with slight modifications. Briefly, 200 μL of the extract

(240 µg/mL) was incubated with 1 ml of ten-fold diluted Folin Ciocalteau's phenol 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) using a special glass cuvette with a 10 mm optical path length. All measurements were done in triplicate.

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

The FRAP method of Oyaizu (24) with slight modifications was used to measure the total 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°C for 30 min. After 30 min incubation, the reaction mixture was acidified with 1 mL of 10 % trichloroacetic acid (pH 0.211). 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 using above-mentioned cuvette and spectrophotometer. Increased absorbance of the reaction mixture indicates higher reduction capacity of the extracts. Results were expressed as a percentage of absorbance of the sample to the absorbance of gallic acid:

$$\text{Ferric reducing antioxidant power (\%)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of gallic acid}} \times 100$$

Free radical scavenging activity (DPPH assay)

The free radical scavenging activity of the extracts was determined and compared to that of ascorbic and gallic acids using a method described by Tuba and Gulcin (25) 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 temperature. The absorbance was measured at 517 nm against a blank lacking the scavenger.

Deoxyribose assay/hydroxyl radical scavenging (HRS) 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 Hinneburg et al. (26). 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% TCA and 1 mL of 1.0% 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 reduce the production of NO. 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:

$$\text{Scavening activity (\%)} = (\frac{Ac - As}{Ac}) \times 100$$

where: Ac is absorbance of control and As absorbance of the sample or standard.

Gas chromatography-mass spectrometric (GC-MS) analysis

The aqueous and ethanol extracts of the leaf and root samples of the plant were subjected to GC-MS analysis. The analysis was conducted with an Agilent Technologies 6890 Series gas chromatograph coupled with (an Agilent) 5973 Mass Selective detector and driven by Agilent chemstation software. A eHP-5MS capillary column was used (30 m × 0.25 mm internal diameter, 0.25 µm film thickness). The carrier gas was ultra-pure helium at a flow rate of 1.0 mL/min and a linear veloc-

ity 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 4 min at each increment. Injections of 2 μL were made in the split-less 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, quadruple temperature 150°C, solvent delay 4 min and scan range 50–700 amu. The compounds were identified by direct comparison of the retention times and mass spectral data and fragmentation pattern with those in the National Institute of Standards and Technology (NIST) library.

Statistical analysis

Data are presented as the mean ± SD of triplicate determinations. Data were analyzed by SPSS statistical software (version 19) using Tukey's multiple range *post-hoc* test. Values were considered significantly different at $p < 0.05$.

RESULTS

A higher yield of aqueous extract was recorded in the two parts of the plant. The highest total phenolic content was recorded in the ethanol extract of the roots (136.1 ± 10.6 mg/g GAE) (Table 1). All the extracts of *C. cornifolia* showed an ability to donate electrons to convert $Fe^{3+} \rightarrow Fe^{2+}$ as indicated by the concentration dependent increase in the percentage
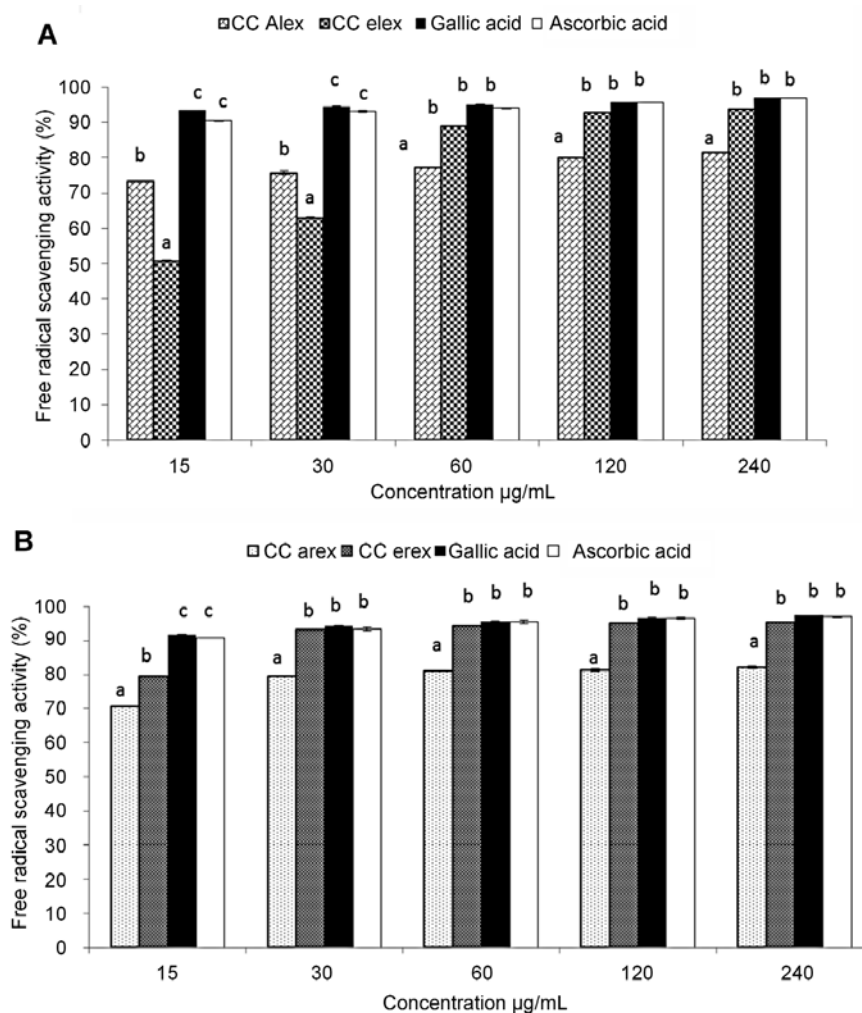

Figure 1. DPPH radical scavenging activity of ethanol and aqueous extracts of the leaves (A) and roots (B) of *C. cornifolia*. Data are presented as mean ± SD of triplicate determinations.
[a-c]Values with different letters over the bars for a given concentration of each extract are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $p < 0.05$). Arex = aqueous root extract and Erex = ethanol root extract; Alex = aqueous leaf extract and Elex = ethanol leaf extract
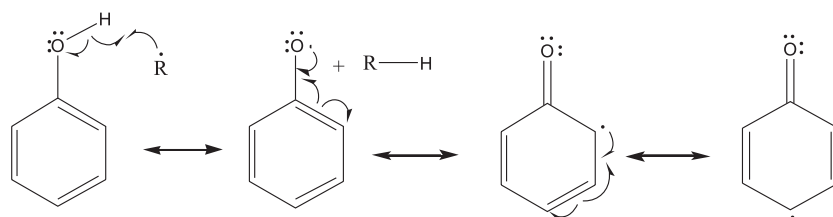
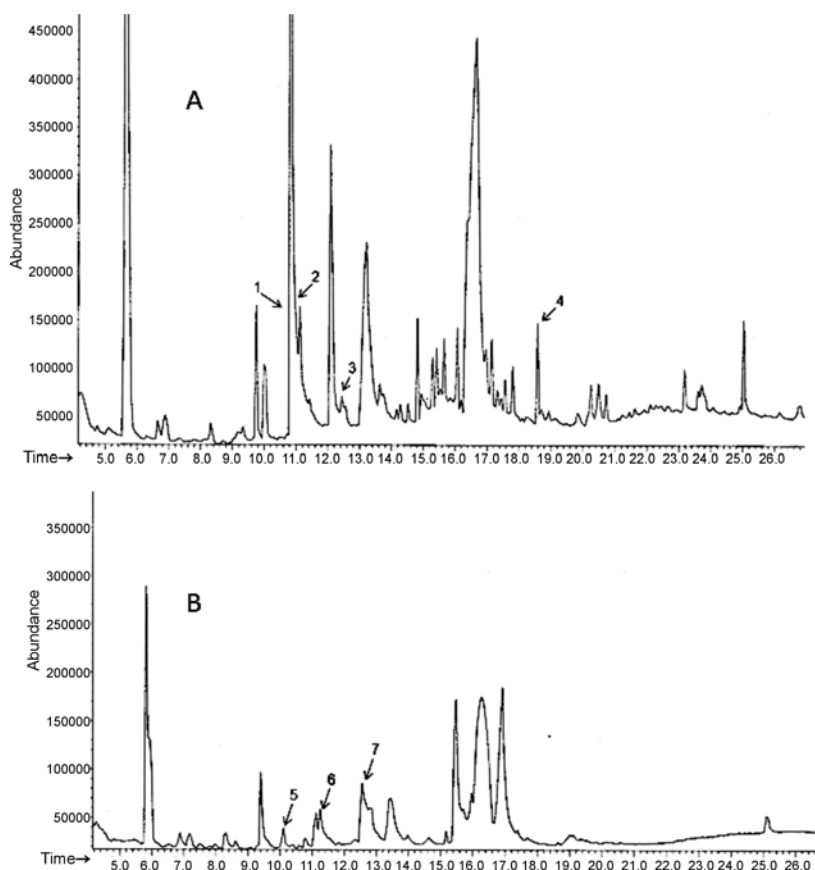Figure 2. Hydrogen radical donation to a reactive radical species and stabilization of the resultant radical

Figure 3. GC-MS chromatogram of the ethanol (A) and aqueous (B) extracts of the root of *C. cornifolia*

reducing power (Table 2). However, the ethanol extract of the root had a consistent and significantly ($p < 0.05$) higher total reducing power than other extracts.

Figure 1A and 1B, shows the DPPH radical scavenging activities of the leaf and root extracts of *C. cornifolia*. The ability to quench DPPH free radicals was evident in all extracts as indicated by the concentration dependent increase in the percentage inhibitions. Further to this, the ethanol root extract indicated a consistently higher free radical scavenging activity than all other extracts tested.

All the extracts possess the ability to scavenge hydroxyl radicals generated by Fenton's reaction with the ethanol extracts displaying a significantly ($p < 0.05$) higher HRS ability compared to the aqueous extracts and the standards used (Table 3). The ethanol extract of the leaves demonstrated a higher ($p < 0.05$) HRS activity than the same extract in the roots at lower concentrations (15–60 µg/mL) but at

Table 1. Percentage recovery (w/w) and total phenolic content of various solvent extracts of *C.cornifolia* parts.

Samples	% Recovery (w/w)	Total polyphenol (mg/g GAE)
Leaves		
EtOH	2.70	52.9 ± 12.5[a]
Aqueous	5.91	89.9 ± 7.40[b]
Root		
EtOH	1.69	136.1 ± 10.6[d]
Aqueous	7.60	106.0 ± 23.6[c]

Data are presented as the mean ± SD values of triplicate determinations. [a-d]Different superscript letters for a given value within a column are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $p < 0.05$).

Table 2. Percentage total reducing power (GAE) of solvent extracts from various parts of *C. cornifolia*.

Extracts	Concentration (µg/mL)				
	15	30	60	120	240
Leaves					
EtOH	9.81 ± 1.30[a]	14.12 ± 2.45[a]	21.37 ± 1.33[b]	28.59 ± 1.14[a]	34.00 ± 9.07[a]
Aqueous	4.65 ± 3.39[a]	10.79 ± 4.54[a]	14.51 ± 0.88[a]	29.15 ± 5.69[a]	30.71 ± 1.00[a]
Root					
EtOH	29.75 ± 1.12[c]	45.75 ± 2.66[c]	52.80 ± 0.45[d]	75.22 ± 2.39[c]	93.09 ± 1.05[c]
Aqueous	15.95 ± 2.02[b]	30.18 ± 0.20[b]	40.69 ± 0.63[c]	45.71 ± 1.80[b]	75.51 ± 1.10[b]
Standard					
Ascorbic acid	76.83 ± 4.92[d]	80.24 ± 3.00[d]	90.26 ± 5.51[e]	91.08 ± 5.43[d]	93.60 ± 6.21[c]

Data are presented as the mean ± SD values of triplicate determinations. [a-e]Different superscript letters for a given value within a column are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $p < 0.05$).

Table 3. Percentage hydroxyl radical scavenging activity of extracts from the root and leaves of *C. cornifolia*.

Extracts	Concentration (µg/mL)				
	15	30	60	120	240
Leaves					
Ethanol	38.96 ± 1.2[c]	55.41 ± 1.3[e]	70.34 ± 0.3[d]	78.57 ± 0.1[d]	83.54 ± 0.7[d]
Aqueous	24.98 ± 1.0[b]	27.89 ± 1.2[b]	43.51 ± 4.8[c]	48.63 ± 3.3[c]	54.12 ± 2.2[b]
Root					
Ethanol	27.51 ± 1.7[b]	47.40 ± 1.3[d]	64.71 ± 0.2[d]	88.46 ± 2.0[e]	92.96 ± 2.1[e]
Aqueous	26.82 ± 1.3[b]	20.42 ± 1.6[a]	32.13 ± 2.0[b]	51.50 ± 0.3[c]	69.90 ± 5.6[c]
Standard					
Ascorbic acid	14.50 ± 2.20[a]	17.35 ± 3.10[a]	21.85 ± 0.10[a]	26.80 ± 0.70[a]	30.30 ± 1.20[a]
Gallic acid	32.00 ± 0.60[c]	35.57 ± 2.30[c]	36.75 ± 1.80[b]	38.90 ±1.10[b]	40.20 ±0.20[a]

Data are presented as the mean ± SD values of triplicate determinations. [a-e]Different superscript letters for a given value within a column are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $p < 0.05$).

Table 4. Percentage nitric oxide (NO) scavenging activities of extracts from various parts *C. cornifolia*.

Extracts	Concentration (µg/mL)				
	15	30	60	120	240
Leaves					
Ethanol	18.75 ± 2.5[a]	27.48 ± 1.9[b]	28.24 ± 0.7[b]	43.51 ± 1.4[b]	48.85 ± 0.5[b]
Aqueous	ND	11.19 ± 0.6[a]	12.91 ± 1.0[a]	16.28 ± 0.7[a]	22.95 ± 0.7[a]
Root					
Ethanol	42.23 ± 1.4[b]	44.52 ± 0.9[c]	57.58 ± 1.0[c]	83.11 ± 2.2[d]	91.18 ± 1.5[d]
Aqueous	46.56 ± 2.4[b]	50.63 ± 1.0[c]	54.7 ± 0.7[c]	63.15 ± 0.1[c]	70.48 ± 0.8[c]
Standard					
Ascorbic acid	51.44 ± 0.05[c]	56.94 ± 0.90[c]	58.37 ± 1.40[c]	62.44 ± 0.70[c]	77.22 ± 1.20[c]
Gallic acid	43.06 ± 9.20[b]	47.36 ± 0.70[c]	49.76 ± 0.70[c]	59.33 ± 3.20[c]	64.59 ± 6.20[c]

Data are presented as the mean ± SD values of triplicate determinations. [a-d]Different superscript letters for a given value within a column are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $p < 0.05$); ND = not determined.

Table 5. Compounds identified from the root of *Cissus cornifolia* by GCMS

Name of compound	Retention time (min)	Percentage match (%)	Molecular mass (g/mol)
Ethanol extract			
Hydroquinone	10.71	86	110
Resorcinol	10.83	99	110
Vanillin	12.56	92	152
n-Hexanoic acid	18.58	91	256
Aqueous extract			
Catechol	10.12	95	110
Resorcinol	11.25	97	110
Pyrogallol	12.55	72	126

higher concentrations (120–240 µg/mL), the HRS activity in the ethanol extract of the roots were higher than in the leaves.

Table 4 presents the NO inhibition activities of the ethanol and aqueous extracts of *C. cornifolia* leaves and roots. All the extracts were found to exhibit NO inhibition activity to an extent, however the root extracts demonstrated a higher NO inhibition potential than the leaf extracts. Notably, the ethanol extract of the root had significantly higher NO inhibition potential compared to the other extracts and the standards used.

The crude aqueous and ethanol leaf and root extracts were analyzed by GCMS to identify compounds in the extracts by comparison with standard mass spectra in the NIST library. Three polyhydroxylated phenols, pyrogallol, catechol and resorcinol were identified in the aqueous extract of the roots, with resorcinol also being present in the ethanol extract along with hydroquinone, vanillin and a long chain fatty acid, n-hexadecanoic acid (Table 5, Figs. 2 and 3).

DISCUSSION

Cissus cornifolia has been reported to have a variety of uses among which, the root decoction is used by traditional healers and herbalists to treat and manage a vast array of non-metabolic chronic disorders and ailments such as diarrhea and other stomach ailments, back pain, sore throat, wounds, diabetes and cardiac problems in the Mrewa district in Zimbabwe (personal communication with traditional healers).

Preliminary investigations on the *in vivo* neuropharmacological activity of the methanolic leaf extract as well as its hypoglycemic activity on normoglycemic rats have been reported (7, 11). Nevertheless, according to our current knowledge, there is no report on detailed anti-oxidative activities from any part of the plant or its phytochemistry. A set of *in vitro* assays were employed to cover most possible known mechanisms by which different antioxidants operate to inhibit oxidative chain reactions.

The results from all the experimental models (total reducing power ($Fe^{3+} \rightarrow Fe^{2+}$), DPPH, hydroxyl radical and nitric oxide reducing ability) indicated that all the different parts of *C. cornifolia* extracted in ethanol and water possess antioxidant potential in a concentration dependent manner to varying extents. The ethanolic root extract has consistently shown to have exceptionally high antioxidant ability and the recorded high phenolics content (136.1 ± 10.6 mg/g GAE), which supported the observed high activity and effectiveness of the extract in the *in vitro* assays.

To identify the responsible phytochemicals, we carried out GC/MS analysis of the aqueous and ethanol extracts of the leaves and roots. Only long chain aliphatic compounds were identified in the ethanol and aqueous extract of the leaves. Palmitic acid, arachidic acid and nonacosane were identified by comparing their MS spectra to those of standard spectra from the NIST library. While no phytochemicals could be identified in the leaf extracts of the plant, the root extracts contained polyphenol compounds, known to be potent antioxidant compounds and is most likely responsible for the antioxidant activity in the root extract of the plant. Two isomers of benzenediol (hydroquinone and resorcinol) were found in the ethanol extract of the roots.

It is highly probable that the polyphenols in the roots are the reason why the roots are more active as antioxidants than the leaves. The mechanism by which these compounds act as radical scavengers is *via* the transfer of a hydrogen atom to a radical species, thereby creating a radical charge on the polyphenol, which is delocalized on the aromatic ring stabilizing the radical charge (Fig. 2). The radical benzene molecule can be quenched by reacting with other radical species.

In conclusion, the results of this study suggest that the ethanolic extracts of leaf and root have strong anti-oxidative effects which might be contributed by some major bioactive active compounds such as hydroquinone, resorcinol, vanillin and n-hexanoic acid. The anti-oxidative effects of the root

ethanolic extract were better than the leaf ethanolic extract which can be used to investigate the *in vivo* anti-oxidative and anti-diabetic effects in the animal model of type 2 diabetes. Additionally, findings of this study further support the use of the root and leaf extracts by traditional healers as an anti-diabetic medicine.

Acknowledgments

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