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PHYTOCHEMICAL AND ANTIOXIDANT INVESTIGATIONS OF A *CLAUSENA ANISATA* HOOK, A SOUTH AFRICAN MEDICINAL PLANT

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Abstract

Background: *Clausena anisata* (Willd). Hook (Rutaceae), also known as *Iperepesi* in Xhosa language is a medicinal plant widely used by herbalists for the treatment and/or management of several ailments such as chronic cough, tuberculosis and lung ulceration in Eastern Cape, South Africa. With reference to the information gathered in our previous study, we investigated the plant's phyto-constituents, as well as its inhibitory effects using aqueous and two different organic solvent of extractions in order to justify its folkloric usage.
Methods: Antioxidant activity of the plant was screened through 1,1- diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) diammonium salt, nitric oxide (NO), and ferric reducing power. Total phenols, flavonoids, flavonols, proanthocyanidins, tannins, alkaloids and saponins were investigated using spectroscopic techniques.
Results: There were no significant differences in the flavonoid and proanthocyanidins contents between the leaves and bark extracts of *C. anisata* respectively, while the total phenolic content of the bark extract of *C. anisata* was significantly higher than that of the *C. anisata* leaf. The acetone extracts of both the leaf and bark indicated strong antioxidant activities.
Conclusion: The observed activities of the plant extracts could be attributed to the high contents of the phenolics, alkaloids, flavonoids, saponins, proanthocyanidins and tannin. The acetone extracts of the plants have also exhibited strong antioxidant activities *in vitro*. It has been established scientifically that oxidative stress is linked with several degenerative conditions and diseases; the inhibitory effects of these plant extracts on the free radicals could logically justify the folkloric usage of *C. anisata* leaf and bark in the Eastern Cape for the treatment of respiratory infection diseases.

Key words: Antioxidants, *Clausena anisata* phytochemical contents, solvent extraction antiradical

Introduction

It has been scientifically proven that living cells produce free radical reactive oxygen species (ROS) through physiological and biochemical processes in the body system (Gulcin, 2012, Cetinkaya et al, 2012). The normal metabolic body processes release free radicals such as hydroxyl radicals (OH^-), Superoxide anion (O_2^-), and ($^1O_2$) singlet oxygen and peroxyl radicals (ROO^-). There is limitation to the level of free radicals a body can accommodate, however. If the free radical productions are excessive it may result into the damage of cell wall, leading to chronic diseases like cancers and cardiovascular disease; (Mandal et al., 2009, Gulcin, 2012). Traber et al (2006) reported that the intake of green tea reduces the adverse effect of free radicals because of the composition of selenium and polyphenol that act as scavengers by donating one of their own electrons in order to replace those displaced by the free radicals.

Anti-oxidants act as defence mechanism against potential oxidative stress when there is an imbalance between the ROS formation and the body's antioxidants defence. Standard antioxidant drugs, however, have been used in recent times to reduce the adverse effect of the body free radicals. For instance butylhydroxytoluene (BHT) and rutin, but have been reported as being toxic to living cells (Gocer and Gulcin, 2011, Bursal and Gulcin, 2011). Quercetin rutinoside with the trade name as rutin is a glycoside of the bioflavonoids used in many countries, including South Africa as medications for the treatment of inflammatory disorders, allergies, bacteria infections and viruses (PDR, health). On the other hand, several side effects have been reported in connection with it, for instance, skin rashes, swelling of throats, chest pain and also carcinogenic toxicity. (Ewa et al., 2009)

The antioxidant properties of polyphenol compounds rely on their capability in scavenging free radicals and its potential to reduce iron (Nihal et al., 2007). Solvent of extraction determine the amount of total polyphenol in plants and its antioxidant activities (Omoruyi et al., 2012). It has been reported that acetone extracted both polar and non-polar compounds from plants (Wintola and Afolayan, 2011), another report also confirmed that the acetone extract total phenols in olive oil when compared to other solvent extract (Brenes et al., 2000)
In India, hexane, ethyl acetate/ chloroform, alcohol, and acetone root extract of Clausena anisata have been reported for its scavenging activities and the presence of some secondary metabolite such as, alkaloid, protein- carbohydrate and amino acid (Tarnam et al., 2014). Similarly, in Ghana, Phytochemical study of ethanol extract *C. anisata* leave has been reported to contain tannin, saponin, phenolics, anthraquinone with the exception of terpenes (Agyepong et al; 2014).

Clausena anisata (Willd). Hook can be described as a shrub or small tree belonging to Rutaceae family, and remains the only species, out of the 15 species of the genus, found on the African continent (Molino, 1994). *C. anisata* investigated in this study is very important in African traditional medicine, it is locally known as *Iperepesi* in the Eastern Cape (Dold and Cock, 1999). The various morphological parts of the plant have been identified to be effective remedies against worm infections, respiratory ailments, heart disorders, hypertension, malaria fever, rheumatism, insanity, convulsion, and other inflammatory conditions (Hamza et al., 2006, Hutching et al; 1996).The stem bark and the leaves have also been identified to be effective, during an ethno-botanical survey, and used for the treatment of tuberculosis among Xhosa people in Eastern Cape Province, South Africa (Lawal et al; 2014). The presence of essential oil in the *C. anisata* has been reported to be responsible for its several pharmacological activities such as antimicrobial, anti-diabetics properties which also affirmed its folkloric usage (Ojewole, 2002; Usman et al., 2010, York et al; 2012).

Chemical constituents of *C. anisata* stem and root have also been investigated in Cameroon which led to the isolation and characterization into three carbazole alkaloids: girinimbine, murrayamine-A and ekeberginine; two peptide derivatives: aurantiamide acetate and N-benzoyl-L-phenylalaninyl-N-benzoyl-L-phenylalaninate; and a mixture of two phytosterols: sitosterol and stigmasterol. The structures were elucidated using nuclear magnetic resonance 1H-NMR, 13C-NMR, COSY, HSQC, HMQC, HMBC and NOESY techniques. (Songue et al.,

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2012). Even though, the antioxidant and phytochemical activities for some parts of this plant have been investigated in different countries (Songue et al., 2012 Agyepong et al., 2014, Tarnam., 2014). The use of plants for treatment and management of several different ailments are imperative in Africa, especially in South Africa because of the richness in plant biodiversity and holistic belief in medicinal plants (Hutchings et al., 1996, Grierson and Afolayan, 1999, Lawal et al; 2014).There is inadequate information on the Phytochemical and antioxidants properties of the leaves and stem bark of *C. anisata* using various solvent of extractions in the Eastern Cape, South Africa. The aim of this study was therefore to investigate the quantitative phytochemical properties of *C. anisata* leaves and bark present using different solvent extractions.

Materials and methods

Chemicals

Aluminium chloride ($AlCl_3$), Folin-Ciocalteu's phenol reagent, Sodium carbonate (Na_2CO_3) Sodium nitrite ($NaNO_2$), 1,1-Diphenyl-2-picrylhydrazyl (DPPH), quercetin, trichloracetic acid (TCA), potassium ferricyanide, rutin, vitamin C, tannic acid, Iron III chloride ($FeCl_3$) were all purchased from Merck (South Africa). All chemicals and solvents used in this experiment were of analytical grade. Identification of the collected plant specimens was done by Prof. D.S. Grierson of the Department of Botany at the University of Fort Hare, South Africa. Voucher specimens with the voucher no (Lawmed 01) were deposited in the Giffen Herbarium of the University of Fort Hare.

Plant materials and preparation of extracts

C. anisata barks (LAWMED 01) were washed and dried in an oven (Stainless steel PA Cuthbert and Co., Modderfontein) overnight at 30^0 C. The leaves were air - dried at room temperature; the dried samples were ground, and later used for preparing solvent extracts. 50g each of the bark and leaves were soaked in 500mL of distilled water and then shaken horizontally for 24 hr using linear shaker (Labotec Scientific Orbital Shaker, SA). The resulting mixture was filtered using Whatman qualitative filter paper (Sigma-Aldrich, Germany). The filtrate was then freeze dried. The organic solvent extraction of the plant was done with acetone and dichloromethane respectively. The respective solvents was shaken horizontally for 24 hr using linear shaker (Labotec Scientific Orbital Shaker, SA), the extract were filtered using a Buchner funnel and Whatman qualitative filter paper (Sigma-Aldrich, Germany) and each filtrate was concentrated to dryness under reduced pressure at 40^0C using a rotary evaporator. Each extract was re-dissolved in its respective solvent to the required concentration needed for this study.

Preliminary Phytochemical screening

Preliminary screening tests of the three extracts were performed to ascertain the presence or absence of phyto-constituents such as phenolic compounds, flavonoids, tannin, phytosteroids, saponins, and alkaloids following the standard procedures (Alex et al., 2012, Omoruyi et al; 2012).

Determination of total phenolics

Total phenolic contents were evaluated with Folin-Ciocalteu's phenol reagent (Adedapo et al; 2009, Otang et al; 2012). 5 ml of the extract solution was mixed with 5 ml Folin-Ciocalteu reagent previously diluted with water (1:9 v/v). After 5 minutes, 4 ml of 7% Na_2CO_3 solution was added with mixing. The tubes were vortexed for 5 seconds and allowed to stand for 30 min at 40°C for colour development. Absorbance was then measured at 765 NM using the Hewlett Packard UV-vis spectrophotometer. Samples of extract were evaluated at a final concentration of 0.1 mg/ml. Total phenolic content was expressed as mg/g tannic acid equivalent using the following equation based on the calibration curve: y = 0.1216x, R2 = 0.9365, where y was the absorbance x was the concentration.

Determination of total flavonoids

Colorimetric aluminum chloride method was used for flavonoid determination (Ebrahimzadeh et al., 2001, Nabavi et al; 2008, Otang et al; 2012) 0.5 ml solution of each plant extract in methanol was separately mixed with 0.5 ml of 2% aluminum chloride. After one hour at room temperature, the absorbance was measured at 420NM. A yellow colour indicated the presence of flavonoids. Extract samples were evaluated at a final concentration of 0.1 mg/ml. Total flavonoid content was calculated as quercetin equivalents (mg/g) using the following equation based on the calibration curve: y = 0.0255x, R2 = 0.9812, where y was the absorbance x was the concentration.

Estimation of total flavonols

Total flavonol content was determined by adopting the procedure described by Wintola and Afolayan, 2011, Jimoh et al.,2010 The reaction mixture consisting of 2 ml of the sample, 2 ml of $AlCl_3$ prepared in ethanol and 3 ml of (50 g/l) sodium acetate solution was allowed to incubate for 2.5 h at 20°C. Absorbance was measured at 440 NM. Total flavonol content was calculated as mg/g of the quercetin equivalent from the calibration curve using the equation: Y = 0.0255×, R2 = 0.9812 where × is the absorbance and Y is the quercetin equivalent.

Determination of total proanthocyanidins

Determination of proanthocyanidins was based on the standard procedures (Nakamura et al; 2003, Wintola and Afolayan, 2011). 0.5 ml of 1 mg/ml of extract solution was mixed with 3 ml of 4% vanillin-methanol solution (4% v/v) and 1.5 ml of hydrochloric acid was added and vortexed. The mixture was allowed to stand for 15 minutes at room temperature. The absorbance was then measured at 500NM. Extract samples were evaluated at a final concentration of 0.1 mg/ml. Total proanthocyanidin contents were expressed as catechin equivalents (mg/g) using the following equation based on the calibration curve:
y = 0.5825x, R2 = 0.9277, where y was the absorbance x was the concentration.

Saponin determination

The saponin content in the plant extracts was estimated as previously described (Jimoh et al., 2010, Omoruyi et al, 2012). Ten grams of the powdered sample was placed in 200 ml of 20% ethanol. The suspension was heated in a water bath at 55°C for 4 hours with continuous stirring. The mixture was filtered and the residue was re-extracted as above. The combined extracts were reduced to 40 ml over a water bath at

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90° C. The concentrate was transferred into a 250 ml separator funnel and 20 ml diethyl ether was added and shaken vigorously. The ether layer was discarded, while the purification process was repeated. 60 ml of n-butanol was added and the extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation, the sample was dried in the oven to a constant weight. The saponin content was calculated according to the equation: amount of saponin (mg/g) = weight of the residue/ weight of sample.

Alkaloids determination

Five grams of the powdered sample were weighed into 200 ml of 20% acetic acid in ethanol and allowed to stand for 4 hours. This was filtered and the extract was concentrated using a water bath at 55°C to one-quarter of the original volume. Concentrated ammonium hydroxide was added drop wise into the extract until precipitation was complete. The whole solution was allowed to settle and the precipitate collected was washed with dilute ammonium hydroxide solution and then filtered. The residue which is the crude alkaloid was weighed and calculated according to the equation: amount of alkaloid (mg/g) = weight of precipitate/weight of sample (Obadoni and Ochuko, 2001).

Tannin determination

Tannin content of the samples was determined according to the modified vanillin-HCl methanol following the standard procedures (Noha et al; 2011 Omoruyi et al; 2012). The vanillin-HCL reagent was prepared by mixing equal volume of 8% HCl and 1% vanillin in methanol. The reagent was mixed just prior to use. About 0.2 g of the ground sample was placed in a small conical flask. Then 10 ml of 1% concentrated HCL in methanol was added. The flask was capped and continuously shaken for 20 min and the content was further centrifuged at 2500 rpm for 5 min. About 1.0 ml of the supernatant was pippetted into a test tube containing 5 ml of vanillin-HCL reagent. Absorbance at 450NM was read on spectrophotometer after 20 min of incubation at 30°C. A standard curve was prepared expressing the result as catechin equivalent as follows:
Tannin (%) = C × 10 × 100/200, where: C = Concentration corresponds to the optical density; 10 = volume of the extract (ml); 200= Sample weight (mg).

Antioxidant Assays
Ferric-reducing power (FRAP) assay

The reducing power of the extract was evaluated according to the method of Hemalatha and Kumar (2011). The mixture containing 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of potassium ferricyanide (K_3Fe $(CN)^6$ (1% w/v) was added to 1mL of each of the extracts at different concentrations ranging from 0.025 – 0.5 mg/ml. The resulting mixtures were incubated at 50°C for 30 min, followed by the addition of 2.5 ml of trichloroacetic acid (10% w/v). The mixture was centrifuged at 3000 rpm for 10 min to collect the upper layer of the solution. A volume of 2.5 ml supernatant solution was mixed with distilled water (2.5 ml) and 0.5 ml of $FeCl_3$ (0.1%, w/v). The absorbance was then measured at 700NM against blank sample. Ascorbic acid and rutin were used as positive controls. The increased absorbance of the reaction mixture indicated higher reducing power of the plant extract.

Scavenging activity of 2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) radical

For DPPH radical assay Liyana-Pathiranan et al; 2005 and Jimoh et al., 2010 procedures were followed. The effect of extracts on DPPH radical was evaluated. About 0.1 ml of DPPH-methanol solution (0.135 mM) was mixed with 1.0 ml of different concentrations (0.025–0.5 mg/ml) of various extracts of *C. anisata*. The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 min. The absorbance of the mixture was measured spectrophotometrically at 517 NM. Rutin and Ascorbic acid were used as standard drugs. The percentage of free radical scavenging was calculated according to the following equation: scavenging activity (%) = $[(Abs_{control} - Abs_{sample})/Abs_{Control}] \times 100$ where $Abs_{control}$ is the absorbance of DPPH + methanol; Abs_{sample} is the absorbance of DPPH radical + sample extract / standard.

2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid (ABTS) scavenging activity

The method of Re et al., (1990) and Otang et al. (2012) was adopted for the determination of the ABTS activity of the plant extract. First the working solution was prepared by mixing two stock solutions of 7 mM ABTS solution and 2.4 mM potassium persulphate solution in equal amount and allowed to react for 12 hrs at room temperature in the dark. The solution was then diluted by mixing 1ml ABTS solution to obtain an absorbance of 0.706 ± 0.001 units at 734 NM using the spectrophotometer. Fresh $ABTS^+$ solution was prepared for each assay. Plant extracts at different concentrations ranging from 0.025–0.5 mg/ml. Plant extract (1ml) were allowed to react with 1 ml of the ABTS solution and the absorbance was taken at 734 NM after 7 min using the spectrophotometer. The ABTS scavenging capacity of the extract was compared with that of vitamin C and rutin. The percentage inhibition was calculated as ABTS radical scavenging activity (%): $Abs_{control} - Abs_{sample} / (Abs_{control})$ x100 where: $Abs_{control}$ is the absorbance of ABTS radical+methanol; Abs_{sample} is the absorbance of ABTS radical+sample /standard.

Nitric oxide radical scavenging activity

Ebrahimzadeh et al; (2010) procedures was followed for the scavenging radical of nitric oxide assay. A volume of 2 ml of 10 mM sodium nitroprusside dissolved in 0.5 ml phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of each plant extract or BHT or rutin at various concentrations (0.025-0.5 mg/ml). The mixture was incubated at 25°C for 150 min. An aliquot of 0.5 ml of the solution was withdrawn and mixed with 0.5 mL of Griess reagents (1.0 ml sulfanilic acid reagent (0.33% in 20% glacial acetic acid at room temperature for 5 min with 1 ml of naphthylethylenediamine dichloride (0.1% w/v). The reaction mixture was incubated at room temperature for 30 min, after which absorbance was measured at 540NM. The amount of nitric oxide radical was calculated using the equation: % inhibition of NO= A0−A1/A0 × 100, where A0 is the absorbance before reaction and A1 is the absorbance after reaction has taken place.

Statistical analysis

The experimental results were expressed as mean ± standard deviation (SD) of three replicates. Where applicable, the data were subjected to one way analysis of variance (ANOVA) and significant differences between the extract used were determined by Duncan's Multiple Range test using the Minitab program (version 12 for windows).

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Table 1: Phytochemical screening results from *C. anisata*

	Leaf			Bark		
Phytochemical	**Water extract**	**DCM extract**	**Acetone extract**	**Water extract**	**DCM extract**	**Acetone extract**
Phytosteroids	-	-	+	-	-	+
Saponins	-	+	+	-	+	+
Alkaloids	+	+	+	-	+	+
Tannins	-	-	+	+	-	-
Phenolic	+	+	+	+	+	+
Flavonoids	+	+	+	+	+	+

Key: - : Absent, +: present

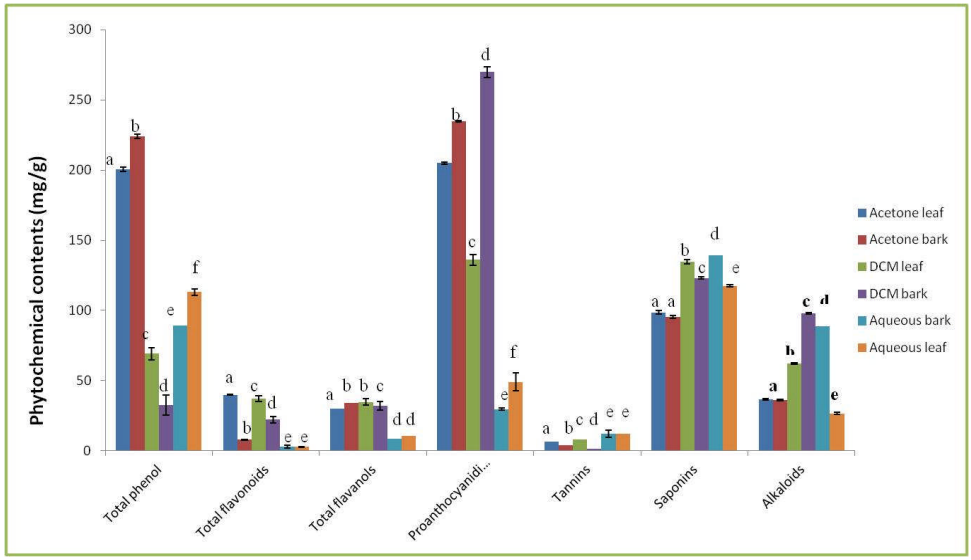

Figure 1: Phytochemical constituents identified in the various extracts of *C. anisata* (Leaf and bark), a-f are means ± SEM bars with different letters are significantly differents (P< 0.005), Vit C = Vitamin C, Rutin (standards)

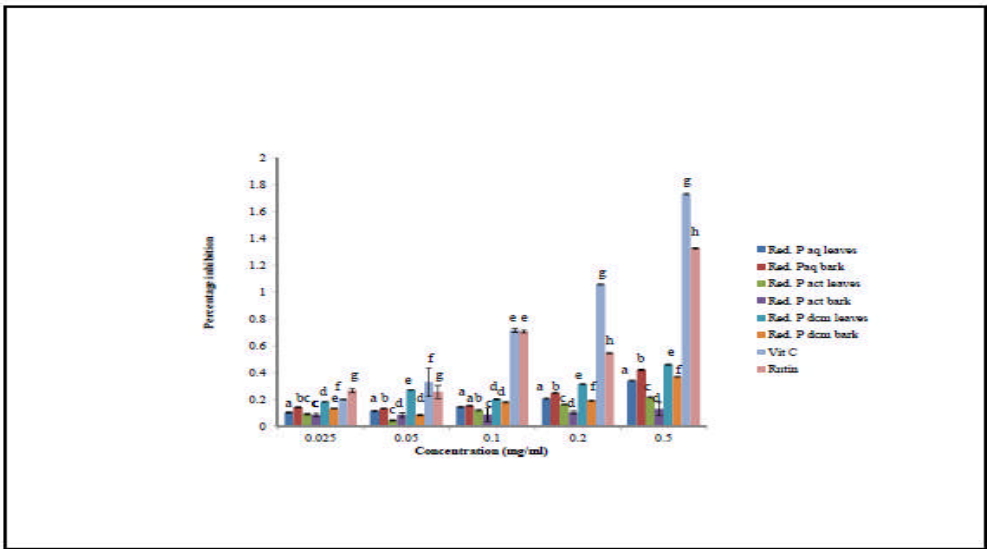

Figure 2: Reducing power of the various extract of *C. anisata* leaf and bark in comparison to rutin and vitamin C. Results are means of triplicates a-g are means ± SEM bars with different letters are significantly differents (P< 0.005), Vit C = Vitamin C, Rutin (standards)

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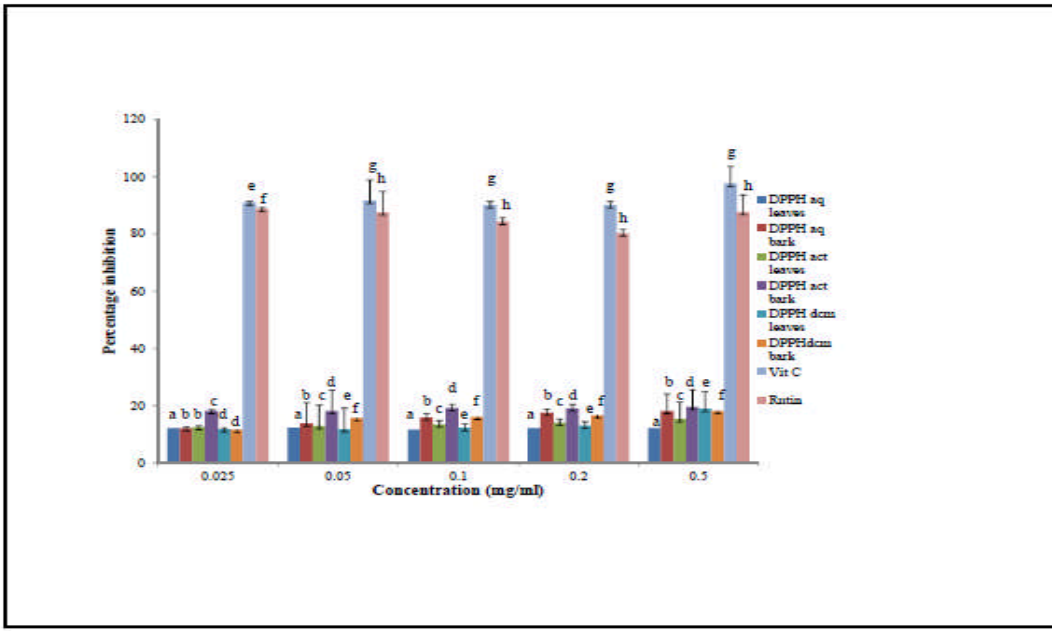

Figure 3: DPPH scavenging activity of the various extract of *C. anisata* leaf and bark in comparison to rutin and vitamin C. Results are means of triplicates, a-f are means ± SEM bars with different letters are significantly differents (P< 0.005), Vit C = Vitamin C, Rutin (standards)

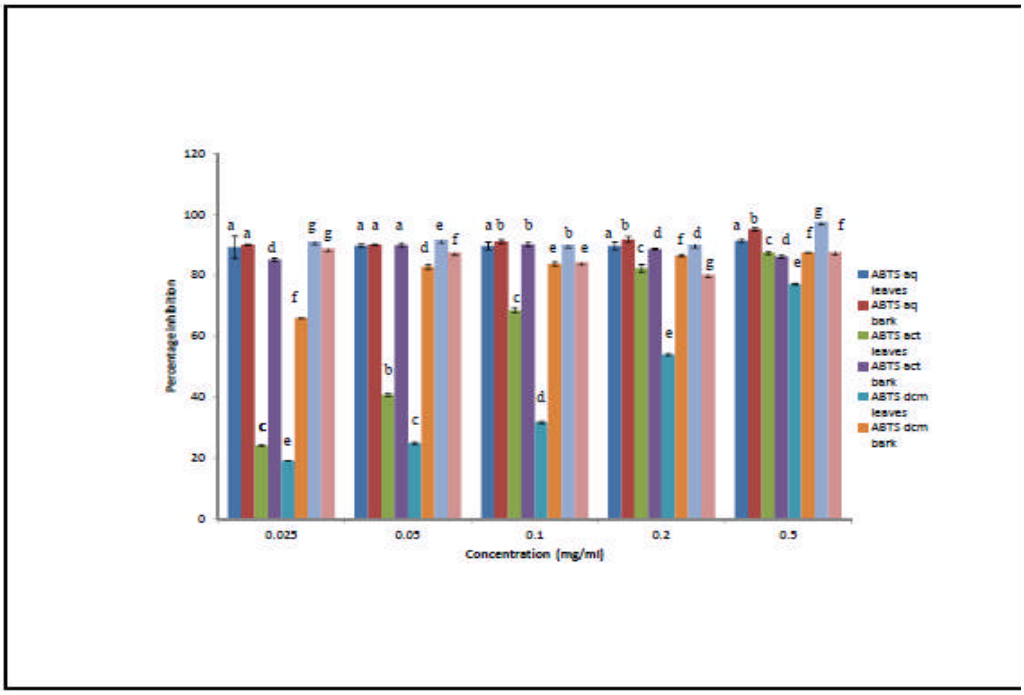

Figure 4: ABTS scavenging activity of the various extracts of *C. anisata* leaf and bark in comparison to rutin and vitamin C. Results are means of triplicates a-g are means ± SEM bars with different letters are significantly differents (P< 0.005), Vit C = Vitamin C, Rutin (standards)

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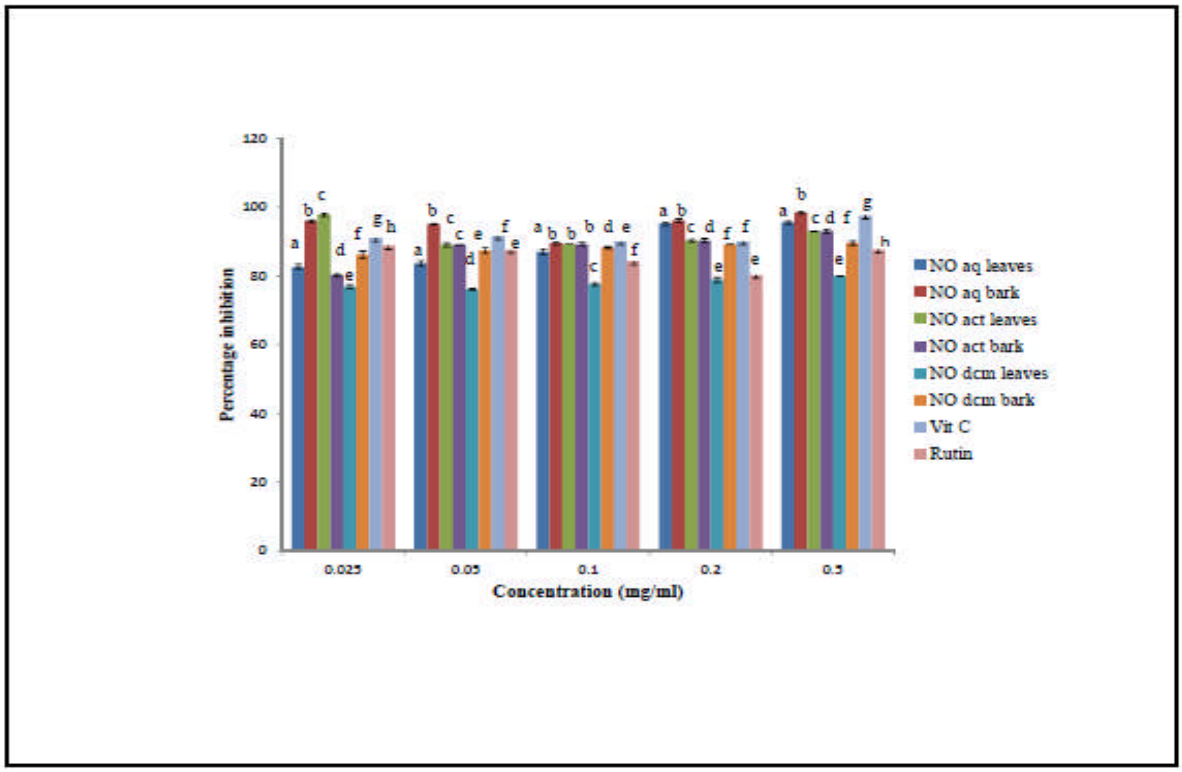

Figure 5: Nitric oxide scavenging activity of the various extracts of *C. anisata* leaf and bark in comparison with rutin and vitamin C. Results are means of triplicates, a-g are means ± SEM bars with different letters are significantly differents (P< 0.005), Vit C = Vitamin C, Rutin (standards)

Table 2: Scavenging activities of three solvent extracts of *C. anisata*

Sample/ Extracts of *C.anisata* Leaves and Bark	DPPH		Nitric oxide		Reducing power		ABTS	
	Ic50 [a]	R^2 [b]	Ic50 [a]	R^2 [b]	Ic50 [a]	R^2 [b]	Ic50 [a]	R^2 [b]
Act leaf	0.06	94.7	-	-	0.18	80.8	0.13	61.7
Act bark	0.18	67.2	0.07	49.5	0.26	97	0.26	99.3
Dcm leaf	0.29	96.6	0.24	84.1	0.24	89.4	0.23	94.6
Dcm bark	0.08	54.1	0.26	99.3	0.24	93.3	-	-
Aqueous leaf	0.31	50.3	0.18	71	0.25	99.5	0.26	89.1
Aqueous bark	0.13	66.7	0.35	29.6	0.26	98.5	0.26	99.3
Vitamin C	-	-	-	-	0.22	94.4	-	-
Rutin	-	-	-	-	0.25	88.6	-	-

a: IC50 is described as the concentration (mg/ml) sufficient to obtain 50% of a maximum scavenging capacity.
b: coefficient of determination; values obtained from the regression lines with 95% confidence level.- : Values not determined

Results and Discussion

Qualitative Phytochemical screening

The Phytochemical analysis of the dichloromethane (DCM) and acetone (ACT) leaves and bark extracts of *C. anisata* was carried out (Table 1). The Phytochemical contents of the leaves and bark of *C. anisata* was investigated qualitatively; it revealed the presence of phenolic and flavonoids in all the solvents of extraction of the leaf and bark of *C. anisata.* On the other hand, phytosteroid was only present in the ACT leaf extract and ACT bark respectively. Alkaloids were present in leaf aqueous, DCM and ACT, as well as in the DCM and ACT extract of the bark but show no indication in the aqueous bark extract of *C. anisata*. Saponins was present in the organic solvent of extraction of both leaf and bark, but was not observed in aqueous extract of the leaf and bark. However, tannin was absent in all organic extracts of the leaf and bark (*C. anisata*) but present in the aqueous extracts (Table 1)

Quantitative Phytochemical contents

This study considered using aqueous, acetone and dichloromethane according to their polarity in order to extract different chemical compound present in the leaves and bark of *C. anisata.* It was observed that the choice of different solvent of extraction contributed in identifying various chemical constituents quantitatively which complements the qualitative analysis as described in Table 1. The quantification of phytochemical contents using various solvents, the high contents of proanthocyanidine, total phenol, Saponins, alkaloid, flavonoids, flavonols and

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tannin are presented in Figure 1. The values were analysed using one way ANOVA test to verify, if the phytochemical contents in the three solvents extracts were significantly different from each other at 95% confidence interval, the values were represented in a bar chart.

Phytochemical contents in plants vary due to the genetic composition of plant and their biodiversity in nature. Evaluation of plant constituents, however, cannot be attributed to any medium of extraction (Ordonez et al., 2006). Hence, Plants composed of several bioactive compounds which are generally classified as primary and secondary metabolites; this plant has demonstrated potency for the management or cure of different ailment traditionally. Hence, this metabolite is responsible for the defence mechanism of human against pathogens Anjali Soni and Sheetal Sosa, 2013). The results obtained from this study indicated that different solvents according to their polarity have the ability to extract more secondary metabolites in plants (Fig. 1). The quantification of total phenol in the three solvent extracts is in the following order: acetone bark> acetone leaf > aqueous leaf > aqueous bark > dichloroethane leaf > dichloromethane bark. Acetone bark extract of C. anisata was significantly higher than that of other solvent of extractions (P> 0.05). The indication of the high phenolic contents in acetone leaf and bark extract corroborated with the study of (Nagavani et al., 2010, Otang et al., (2012).

Flavonoids comprise a diverse range of biological substances that inhibited diseases and serve as a defence mechanism in the human body, including antimicrobial (Harbone and Williams, 2000), anti-oxidants (Ghasemzadeh et al., 2011), cytotoxicity activity (Murakami et al., 2004). The contents of flavonoids in all the three solvent extracts, acetone leaf extracts have the highest contents as compared with other solvent of extraction (P> 0.05). However, there was no significant difference between aqueous extracts of leaf and bark respectively. This study is in agreement with the study of other researchers that reported the quantitity of flavonoid in plants and functions (Olorunisola et al., 2011, Bhumi et al., 2014).

The highest content of flavonols was recorded in the dichloromethane leaf extract of *C. anisata*, followed by acetone bark extract (Fig.1). The polarity of the solvents could be attributed to the variation in the extracted compounds, which are responsible for the scavenging of free radicals and also for its antimicrobial activity as reported by Bhumi et al., 2014. This study can also justify its traditional use in the treatment of bacterial related diseases like tuberculosis as reported from our previous study (Lawal et al., 2014). Again, this study is in agreement with previous studies of Omoruyi et al., 2012 on using different solvents for phytochemical content determination. Although, the aqueous leaf and bark extract were not significantly different.

The proanthocyanidin content was observed in solvent of extraction in the following order dichloromethane bark > acetone bark > acetone leaf > dichloromethane leaf >aqueous leaf > aqueous bark extract. The dichloromethane bark extract of *C. anisata* is significantly higher than the leaf extracts of other solvents. With an indication of the high content of proanthocyanidin in this study, this plant could possibly be a good source in the neutraceuticals for standardization and drug control, antioxidants, immune booster. (Hong and Wrolstad, 1990, Okwu and Emenike, 2006, Zora and Fawzia, 2011).

A significant amount of tannin was observed in the aqueous leaf and bark extract of *C. anisata* respectively but they are not significantly different to each other. On the other hand, they are significantly different to the organic solvent of extraction in the study (Fig. 1). Some of the functions of the tannin have been previously reported to have the capacity to inhibit the growth of bacteria, fungi and viruses (Bhumi et al., 2014). These are in support of this study and also corroborate with the report that tannin is used as antibiotics against some pathogenic strains, this could provide a rational justification for the use of the plant in the Eastern Cape for treating bacteria associated infection like tuberculosis

Saponins are characterized by its foaming nature and is traditionally used as pesticide, detergent and fish poisoning- agent and among other uses attributable to human health benefits (Bhumi et al., 2014). The Phytochemical contents were significantly higher in the aqueous bark extract of *C. anisata* (P>0.05) compared to other solvent of extraction of both the leaf and bark. However, the saponin contents of the acetone leaf and bark are not significantly different. The saponin contents in this plant is important because of its health attributed benefits such as protecting the immune system against bacteria and viruses and also because of its non-sugar part, it is considered as a good antioxidant; in view, of these it may therefore be justified in its traditional use/s in the treatment of bacteria and virus-dependent infections (Gulcin et al., 2004., Akinjogunla et al., 2010,). This study also revealed the presence of alkaloid contents in the leaf and bark within the three solvents as: dichloromethane bark extract > acetone leaf extract > aqueous leaf extract > aqueous bark extract > dichloromethane leaf extract >dichloromethane bark extract. Dichloromethane bark extract is significantly different with other extracts with 95% confidence of interval. Alkaloids are crucial secondary metabolite with several functions in medicinal plant and its attributed capacity for oxidative stress and anti-inflammatory properties (Okwu and Emenike, 2006). Hence, this may probably ascertain its medicinal value for the management of oxidative stress induced disorder. Quantitative Phytochemical contents of the leaf and bark extract of *C. anisata* revealed that the plant bioactivity dependent on the solvent of extraction.

Reducing power

The electron donating capability of an antioxidant activity can be quantified through reducing power assay. Reducing power activity of a plant extract probably indicates the confirmation of its antioxidant activity by changing the fe^{3+} /ferricyanide complex to the ferrous form. (Yildirim et al., 2001, Jayanthi and Lalitha, 2011). The herbal plant extract with the antioxidant potency need further research to combat several conditions and ailment associated with oxidative stress. The antioxidant activity of the leaves and bark extracts of *C. anisata* was measured through the conversion of Fe^{3+} /ferricyanide complex to the ferrous form. In view of the fact that, ferricyanide complex has been transformed through redox reaction by donating its hydroxyl ion, it can deduce that the extracts are capable of inhibiting radical ion fe^{3+} /ferricyanide

This could serve as a potent antioxidant. The reducing capability of all the extracts of the leaf and bark of *C. anisata* were observed to be potent given the increase in absorbance being directly proportional to the increased, reducing power; it was as well observed that reducing ability is concentration - dependent of all the solvent extracts and the standard drug (Fig. 2). This study was in agreement with what was reported on the antioxidant activity of the root of *C. anisata* (Tarnam et al., 2014). The reducing ability of the plant extracts could also attribute to the different solvents of extractions which are in support of the previous report of Tiwari et al; (2011). It can as well be assumed that polyphenolic compound present in the plant extracts could enhance its reducing power ability (Park and Jhon 2010). The reducing power activity of *C. anisata* are in the following order vitamin C > rutin > dichloromethane leaf > aqueous bark > aqueous leaf > dichloromethane bark > acetone leaf > acetone bark. However, it was observed that leaf extract was significantly higher than the bark extract which was comparably low when compared with rutin and Vitamin C standard. This study identifies reducing power, potency of the bark and leaf extracts of *C. anisata*. Further research is required to ascertain the mechanism of action and the compound responsible for its reducing capabilities.

DPPH radical scavenging assay

1, 1-diphenyl-2-picrylhydrazyl (DPPH), referred to as a stable organic radical (Aparadh et al., 2012). The capability of an extract to scavenge DPPH radical can be measured as the strength for its antioxidant. Total antioxidant capacity of the aqueous, acetone and dichloromethane extract of *Clausena anisata* (Willd.) Hook was investigated by the DPPH method. The assessment of the extracts and standard

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drug were carried out based on the free radical scavenging effect of the stable DPPH free radical activity (Braca, et al., 2002). The amount of the concentration used to obtain 50% of maximum scavenging activity (IC50). The IC50 was determined from the results of several concentrations examined. The IC_{50} is inversely proportional to scavenging activity of an extracts; that is the lower the IC_{50} the higher the scavenging activity (Songue et al., 2014) (Table 2). The observation in the changes of the free radicals scavenging capability of the three solvent of the leaf and bark extracts of *C. anisata* was expressed as percentage inhibition (Fig. 2). The results of the DPPH assay also indicated that the acetone leaf extract with IC_{50}, 0.06 has a coefficient of determination of 94.7 when compared to the standard drug (Table 2). This significant antiradical activity probably may an be important content in the total phenol of the plant extract, given that a strong negative correlation existing between total phenol content and the IC_{50} of various scavenging systems as shown in table 2. This study is in total agreement with report of Songue et al., 2014. Based on the IC_{50} result, it can be deduced that the acetone leaf extract has the strongest scavenging activity than that of the bark extract at all concentration. However, the IC_{50} values of the acetone leaf extract are significantly higher than that of Vitamin C and rutin this could be attributed to the stronger phenolic contents in the plants when compared to the synthetic drug; this contrasts with the report of Otang et al, 2012, but in agreement with the report of Dai and Mumper (2010). It is also logical to assume that scavenging activity not only depended on plant type, but also on the solvent of extraction which was demonstrated in this study, as corroborating with the findings of Anjali and Sheetal (2013). Therefore, the use of *C. anisata* traditionally in treating different ailments associated with bacteria and oxidative stress like, tuberculosis, diabetes and inflammatory conditions (Ojewole, 2002, Hamza et al, 2006, Lawal et al., 2014) can hereby be justified. This study also revealed that the acetone leaf extract could be a good source of potent antioxidants and still requires further research.

ABTS radical - scavenging activity

The result of this study revealed that the percentage inhibition of ABTS radical scavenging activity showed a concentration dependent as well as the increase in the reaction mixture for all the extracts, standard drug inclusive (Fig. 4). At the concentration 0.05 there is no significant different between the aqueous extract and the standard (P> 0.05). The IC50 value of the acetone leaf extract (0.13, 61.7%) is significantly higher than that of standard (vitamin C 0.30, 74.3%) the result is in agreement with Omoruyi et al (2012), who reported that IC50 values of methanol plant extract is significantly higher than that of BHT and Rutin. It was also revealed that the leaf extracts contain antioxidant properties than the bark extracts as shown in Table 2; this implies that most bioactive compound are stored on the trichomes which might probably be on the leaves as reported by Afolayan and Meyer (1997). This result shed more light on the scavenging potency of *C. anisata* and medicinal properties of this genus in South Africa which is in agreement with the report of York et al. (2012).

Nitric oxide scavenging activity

The result of the nitric oxide scavenging activity was presented in Fig. 5. The acetone extracts showed the highest significant differences out of the three extracts investigated. The acetone extract of *C. anisata* showed a concentration dependent decrease in NO antiradical activity. It attained a minimum concentration of 0.2mg/ml and increased afterwards (Fig. 5). However, there is no significance difference in the percentage inhibition of vitamin C and the acetone extract. The minimum inhibitory concentration obtained to reduce the nitric oxide radicals by 50% of the extracts of the leaves and bark of *C. anisata* is in the following order: acetone bark > dichloromethane bark > aqueous leaf > dichloromethane leaf > aqueous bark, it can be deduced that organic solvent processes strong antioxidant activity, this study is in agreement with other researchers investigating scavenging activity of nitric oxide (Omoruyi et al., 2012, Otang et al., 2012). This will contribute to scientific knowledge and could be a lead to potent herbal drug to the ailments and diseases associated to oxidative stress.

Conclusion

The presence of the phytochemical contents such as phenolic, alkaloid, flavonoid, saponins, proanthocyanidin and tannin in *C. anisata* (leaf and bark) contributing to scientific advancement in knowledge to the acclaimed health significance in the treatment and management of diseases. The acetone extracts of the plants have also exhibited strong antioxidant activities *in vitro*. It has been scientifically established that oxidative stress are directly connected with arrays of degenerative conditions and diseases, the inhibitory effects of these plants extract on the free radicals could be logically justified for its folkloric usage of *C. anisata* (leaf) and (bark) within the Eastern Cape for the treatment and management of diseases.

Competing interests: We declare no competing interest

Authors' Contributions: I.O was responsible for the collection of plant materials within the study areas, carried out all the experiments, performed data analysis and drafted the manuscript. AJ designed the study, coordinated plant material storage, supervised the laboratory experiments and made substantial contributions to revise the manuscript critically. DS edited the manuscript. We all read and approved the final manuscript.

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