

Comparative study on the phenolic content, antioxidant properties and HPLC fingerprinting of the leaf extracts of *Clerodendrum volubile*

P. Beauv

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ABSTRACT

The present study aimed to investigate the phenolic contents and antioxidant properties of the various leaf extracts (methanol, ethanol, ethyl acetate) of *Clerodendrum volubile*. The total phenol and total flavonoid contents of the extracts were determined. *In vitro* scavenging activities of the extracts were assessed against 1, 1-diphenyl-2-picrylhydrazyl (DPPH), 2, 2-azinobis (3-ethylbenzo-thiazoline- 6-sulfonate (ABTS) radical scavenging ability. The reducing power ability and high-performance liquid chromatography-diode-array detector (HPLC-DAD) of the extracts were also determined. The results revealed that methanolic extract had the highest total phenol and total flavonoid contents in all the extracts. The methanol extract had the highest reducing power, DPPH and ABTS radicals scavenging activities, followed by ethanol and ethyl acetate extracts. The HPLC-DAD results revealed the presence of both phenolic acids (caffeic acid, gallic acid) and flavonoids (catechin, quercetin, rutin, quercitrin, kaempferol, luteolin, apigenin) as its major bioactive polyphenolics. The difference in scavenging potential of the extracts may be due to variation in the phytoconstituents extracted using various solvents. The result suggests that the methanol leaf extract of *Clerodendrum volubile* could serve as a potential source of antioxidants and can be explored as a therapeutic agent in free radical induced diseases.

INTRODUCTION

The generation of free radicals is necessary for any biochemical process and constitutes an essential part of aerobic life and metabolism (Tiwari, 2001). The continuous generation of these radicals is because of the body's normal use of oxygen such as respiration and some cell mediated immune functions. A dynamic balance exists between the amount of free radicals generated in the body and antioxidants to scavenge them and protect the body against their deleterious effects (Ramjith *et al.*, 2013). Hence an imbalance between the generation of free radicals and the scavenging abilities of the antioxidants results in state called oxidative stress. Furthermore, oxidative stress has been linked to be the major culprit in the pathology of many

diseases for example carcinogenesis, cardiovascular diseases, rheumatoid arthritis, ulcerative colitis and neurological degenerative diseases (Adesanoye *et al.*, 2012; Bhattacharyya *et al.*, 2014). Several studies have reported on how to delay or prevent the onset of these diseases. The most feasible and practical way to fight against these degenerative diseases is by boosting the body's natural antioxidant defense system or by supplementing with dietary antioxidants. This could be achieved by higher consumption of vegetables and fruits which are rich in natural antioxidants, especially phenolics and flavonoids, hence the growing interest in research on plants and new plants with antioxidants ability and fit for human consumption (Gu *et al.*, 2014; Kasote *et al.*, 2015; Molehin *et al.*, 2014). Therefore in recent years, considerable attention has been shifted to towards identification, development and utilization of more effective antioxidants from natural sources with antioxidant ability that may be used for human consumption (Chu *et al.*, 2002).

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White butterfly (*Clerodendrum volubile* P. Beauv) is a climbing shrub of 3m high that belong to the family of *Verbenaceae*. It is commonly grown in deciduous forests across Africa including Nigeria, Ghana and Seirra Leone (Burkill, 1985). In the southern part of Nigeria, which is highly dominated by the Ijaws, Urhobos and Itsekiris and Yoruba, it is well-known as a delicious green leafy vegetable that is consumed as food and in folklore medicine, the plant was found to be effective in treatment of arthritis, diabetes rheumatism, dropsy, swellings, oedema, and gout and is also used as an anti-abortifacient and sedative (Erukainure *et al.*, 2011; Fred-Jaiyesimi and Adekoya 2012). Erukainure *et al.* (2011) reported the high nutritional value of the green leafy vegetables. In another study by Fred-Jaiyesimi and Adekoya (2012), the phytochemical screening reveal the presence of alkaloids, flavonoids, saponins, anthraquinone and cardiac glycoside. The leaf extracts of *C. volubile* has been shown to possess anti-inflammatory properties (Fred-Jaiyesimi and Adekoya 2012). Moreover, there is a dearth of information on the phenolic content, antioxidant, and chromatographic fingerprinting of the green leafy vegetable. Therefore, this study was planned to investigate the phenolic contents, antioxidant properties and chromatographic fingerprinting of various leaf extracts of *C. volubile*.

MATERIALS AND METHODS

Sample collection

Fresh leaves of *C. volubile* were purchased from Oja Oba Market in Akure Metropolis of Ondo State, Nigeria (Figure 1). Authentication of the plant was carried out by A.A. Sorunbge of the Department of Biology, Federal University of Technology, Akure (FUTA) Nigeria. The sample was deposited at the university herbarium with voucher no FUTA/BIO/0121.



Fig. 1: *Clerodendrum volubile*.

Chemicals and reagents

All chemicals were of analytic grade, and glass-distilled water was used. 1, 1-diphenyl-2 picrylhydrazyl (DPPH), sodium trioxocarbonate (IV), aluminum (III) chloride, potassium acetate, 2,2-azinobis (3-ethylbenzo -thiazoline-6- sulfonate),

sodiumnitroprusside, naphthylethylenediamine dihydrochloride, trichloroacetic acid, potassium acetate and potassium ferricyanide were sourced from BDH Chemicals Limited (Poole, England).

Extraction of plant material

Leaves of *C. volubile* (30 g) in powdered form were extracted with 200 mL of various organic solvents (methanol, ethanol, ethyl acetate) using shaker in 2000r/min speed for 24 h at 37°C. The extracts were filtered with Whatman No. 1 filter paper for every 3h. Then, the collected extracts were evaporated to dryness under reduced pressure. The dried extracts were reconstituted in water and stored at 4°C for further investigations.

Determination of total phenol content

The total phenol content was determined on the extracts using the method reported by Singleton *et al.* (1999). Appropriate dilutions of the extracts were oxidized with 2.5mL of 10% Folin–Ciocalteu’s reagent (v/v) and neutralized by 2.0mL of 7.5% sodium carbonate. The reaction mixture was incubated for 40 min at 45 °C and the absorbance was measured at 765nm in the spectrophotometer. The total phenol content was subsequently calculated using Gallic acid as standard.

Determination of total flavonoid content

The total flavonoid content of both extracts was determined using a slightly modified method reported by Meda *et al.* (2005). Briefly, 0.5mL of appropriately diluted sample was mixed with 0.5mL methanol, 50µL of 10% AlCl₃, 50µL of 1mol/L⁻¹ potassium acetate and 1.4mL water, and allowed to incubate at room temperature for 30 min. Thereafter, the absorbance of the reaction mixture was subsequently measured at 415 nm. The total flavonoid was calculated using quercetin as standard.

Quantification of Compounds by High Performance Liquid Chromatography–Diode-Array Detector

C. volubile leaf (methanol, ethanol and ethyl acetate) extracts at a concentration of 12mg/mL were analyzed for their phenolic constituents according to the method described by Adefegha *et al.* (2016) with slight modifications. The extracts were degassed in an ultrasonic bath. Qualitative-quantitative analyses using HPLC-diode-array detector (DAD) was performed with a Shimadzu Prominence Auto Sampler (SIL-20A) HPLC system (Shimadzu, Kyoto, Japan), equipped with Shimadzu LC-20AT reciprocating pumps connected to a DGU 20A5 degasser with a CBM 20A integrator, SPD-M20A DAD and LC solution 1.22 SP1 software. Reverse phase chromatographic analyses were carried out under gradient conditions using C₁₈ column (4.6 mm x 150 mm) packed with 5µm diameter particles; the mobile phase was water containing 2% acetic acid (A) and methanol (B), and the composition gradient was: 5% of B until 2 min and changed to obtain 25%, 40%, 50%, 60%, 70% and 100% B at 10, 20, 30, 40, 50 and 80 min, respectively according to the method described by

Adefegha *et al.* (2014). The presence of nine antioxidants compounds was investigated, namely, gallic acid, caffeic acid, catechin, quercetin, quercitrin, rutin, kaempferol, luteolin and apigenin. Identification of these compounds was performed by comparing their retention time and UV absorption spectrum with those of the commercial standards. The flow rate was 0.6 mLmin⁻¹, injection volume 50 µl and the wavelength were 270 nm for gallic acid, 281 nm for catechin, 327 nm for caffeic acid, 365 nm for quercetin, quercitrin, kaempferol, rutin, apigenin and luteolin. The samples and mobile phase were filtered through 0.45 µm membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. Stock solutions of standards references were prepared in the HPLC mobile phase at a concentration range of 0.030 – 0.250 mg/ml for catechin, quercetin, quercitrin, rutin, kaempferol, apigenin and luteolin; and 0.050 – 0.300 mg/ml for caffeic and gallic acids. The chromatography peaks were confirmed by comparing its retention time with those of reference standards and by DAD spectra (200 to 500 nm). All chromatography operations were carried out at ambient temperature and in triplicate. The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation of the responses and the slope using three independent analytical curves, as defined by Barbosa *et al.* (2014) LOD and LOQ were calculated as 3.3 and 10 σ/S, respectively, where σ is the standard deviation of the response and S is the slope of the calibration curve

DPPH free radical scavenging ability

The free radical scavenging ability of the extracts against DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical was evaluated as described by Gyamfi *et al.* (1999). Briefly, an appropriate dilution of the extracts (1mL) was mixed with 1mL of 0.4 mmolL⁻¹ methanolic solution containing DPPH radicals. The mixture was left in the dark for 30min and the absorbance was measured at 516 nm. The DPPH free radical scavenging ability was subsequently calculated with respect to the reference (which contains all the reagents without the test sample).

2, 2- azinobis (3-ethylbenzo-thiazoline- 6-sulfonate (ABTS) radical scavenging ability

The ABTS[•] scavenging ability of the extracts was determined according to the method described by Re *et al.* (1999). ABTS[•] was generated by reacting an ABTS aqueous solution (7 mmol L⁻¹) with K₂S₂O₈ (2.45 mmol L⁻¹, final concentration) in the dark for 16 h and adjusting the Abs 734nm to 0.700 with ethanol. 0.2mL of appropriate dilution of the extract was added to 2.0mL ABTS* solution and the absorbance were measured at 734nm after 15 min. The trolox equivalent antioxidant capacity was subsequently calculated.

Determination of reducing property

The reducing property of the extracts was determined by assessing the ability of the extract to reduce FeCl₃ solution as described by Oyaizu (Oyaizu, 1986). A 2.5mL aliquot was mixed

with 2.5mL of 200mmol L⁻¹ sodium phosphate buffer (pH 6.6) and 2.5mL of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20min and then 2.5mL of 10% trichloroacetic acid was added. This mixture was centrifuged at 650 rpm for 10 min. 5mL of the supernatant was mixed with an equal volume of water and 1mL of 0.1% ferric chloride. The absorbance was measured at 700 nm. The ferric reducing antioxidant property was subsequently calculated using ascorbic acid as standard.

Statistical analysis

Results of triplicate experiments were pooled and expressed as mean ± SD. Means were compared using one-way analysis of variance and the least significance difference test. A statistically significant difference was accepted at P < 0.05 (Zar, 1984).

RESULTS AND DISCUSSION

It is well established that plants contain many phenolic compounds which possess a hydroxyl group moiety on their aromatic ring. These phenolic compounds break chain oxidation reactions by donating a hydrogen atom or chelating metals thus acting as reducing agents and antioxidants (Eghdami and Sadegh 2010; Pavithra and Vadivukkarasi 2015). Table 1 shows the total phenol and total flavonoid contents of all the extracts of *C. volubile* methanol, ethanol and ethyl acetate respectively. It was observed that methanol [4.92 ± 0.2 mgGAE/g gallic acid equivalent (GAE)/g] had significantly (P < 0.05) higher total phenol contents than ethanol (3.48 ± 0.1 mg GAE/g) and ethyl acetate (2.63 ± 0.2 mg GAE/g) extracts. Similarly, the total flavonoid content of methanol (2.25± 0.3mg QE/g mg quercetin equivalent (QE)/g) was higher than those of ethanol (2.15 ± 0.2 mg QE/g) and ethyl acetate (2.04 ± 0.1mg QE/g) extracts but not significantly (P >0.05) different. The observed trend of results on total phenol and total flavonoid contents as shown in table 1 agrees with several reports on tropical vegetables with similar trends between the total phenol and flavonoid contents (Adefegha and Oboh, 2011; Eghdami and Sadegh, 2010; Kasote *et al.*, 2015; Molehin and Adefegha, 2014; Pavithra and Vadivukkarasi, 2015).

Phenolic compounds are secondary metabolites found in plants and have been linked to several health benefits including antioxidant, antiglycemic, antibacterial, anti-inflammatory antiviral, vasodilator and anticarcinogenic properties (Adefegha *et al.*, 2016; Duthie *et al.*, 2000; Oboh *et al.*, 2015; Rice-Evans and Miller 1997; Pereira *et al.*, 2009; Sultan and Anwar 2009). Using the HPLC-DAD analytical assay, it was possible detect and confirm the presence of the some phenolic compounds in the extracts of *C. volubile* leaf. The HPLC-DAD chromatograms of the extracts are presented in Figures 3–5. As shown in Table 2, the result revealed the presence of both phenolic acids (caffeic acid, gallic acid) and flavonoids (catechin, quercetin, rutin, quercitrin, kaempferol, luteolin, apigenin).The extracts (methanol, ethanol and ethyl acetate) contained high amounts of quercetin,(15.34 ± 0.01 mg/g, 15.02 ± 0.01mg/g and 13.27 ± 0.01) quercitrin

(8.72±0.02mg/g, 9.14 ± 0.03mg/g and 5.01 ± 0.03mg/g)] respectively as its major phenolic compounds. Ethanolic extract had a higher significant ($P<0.05$) difference when compared with the methanolic extract (8.59 ± 0.01mg/g) and ethyl acetate extract (9.02±0.01mg/g).

Furthermore, the leaf extracts also had a high amounts of quercetin luteolin, apigenin and kaempferol (Table 2) Presence of quercetin observed in the leaves extracts of *C. volubile* (table 2) may be partly linked to the antioxidant effects of the leaf extracts (Mattila and Hellstrom 2006). Quercetin, which was also identified in all the leaf extracts has been shown to possess the antiviral, antibacterial, anticarcinogenic and anti-inflammatory effects (Aline *et al.*, 2013; Hemalatha *et al.*, 2016; Sultan and Anwar 2009; Wagner *et al.*, 2006). It is also a strong antioxidant because of its ability to scavenge free radicals and bind transition metal ions (Aline *et al.*, 2013; Scalbert *et al.*, 2005).

Quercitrin are found in many medicinal species of the plant e.g *Melissa officinalis*, *Cymbopogon citratus* (Pereira *et al.*, 2009; Wagner *et al.*, 2006). The phenolic compounds found in the leaf extracts has been reported to be powerful antioxidants present in vegetables and fruit (Guyot *et al.*, 2009; Ogunwa *et al.*, 2016; Wong-Paz *et al.*, 2015) and known as take part of the mechanism of defense environmental in vegetables, fruit and herbs (Moure *et al.*, 2001). Then, it was presumed that the antioxidant activity in *C. volubile* leaf extracts could be due to those typical phenolics. *C. volubile* may represent a good source of important phenolic phytochemicals which may enhance good health and protect against diseases in humans and animals.

Several *in vitro* model systems have been used for determining the scavenging activity in various leaf extracts of *C. volubile*. DPPH is a stable nitrogen-centered free radical commonly used for testing radical scavenging activity of the compound or plant extracts. When the stable DPPH radical accepts an electron from the antioxidant compound, the violet color of the DPPH radical was reduced to yellow colored diphenylpicryl

hydrazine radical which was measured colorimetrically. Substances which are able to perform this reaction can be considered as antioxidants and therefore radical scavengers (Adefegha *et al.*, 2016). DPPH radical scavenging activity of various leaf extracts of *C. volubile* was presented in Figure 2. All the extracts scavenged DPPH radicals in the range of the concentration (0.42–1.67 mg/ml). All the extracts scavenged DPPH radicals in the range of the concentration (0.42–1.67 mg/ml) and the EC₅₀ value of methanol, ethanol and ethyl acetate was found to be 1.7, 2.0 and 2.3 mg/mL respectively. Methanol exhibited strongest DPPH radical scavenging activity when compared to other extracts. The extracts radical scavenging activity were effective in the order methanol > ethanol > ethyl acetate. Antioxidant activity in the extracts was also investigated using the well-known ABTS method. The ABTS assay as shown in table 1 revealed that all the solvent extracts quenched ABTS radical and there was significant ($P<0.05$) difference between the solvent extracts in terms of ABTS free radical-scavenging. The extracts radical scavenging activity were effective in the order methanol > ethanol > ethyl acetate. The actions of the extracts against ABTS and DPPH radicals indicate that the vegetable species are good scavengers of radicals. The pattern of the extracts scavenging ability in ABTS assay is similar to that observed in DPPH assay. Furthermore, reducing powers of extracts were assessed based on their ability to reduce Fe³⁺ to Fe²⁺ and the results are presented in table 1 as ascorbic acid equivalent. It is noteworthy that the antioxidant capacity of these vegetable species as typified by their free radical-scavenging and reductive abilities in relation to their phenolic composition may indicate their potential roles in disease prevention and health promotion. This study agrees with the assertion that plants that are rich in phenolics play crucial roles in scavenging free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Dehpour *et al.*, 2009; Meir *et al.*, 1995; Ogunwa *et al.*, 2016; Re *et al.*, 1999; Salas *et al.*, 2010).

Table 1: The total phenol, total flavonoid contents, ABTS radical scavenging ability and reducing property of *C. volubile* leaves extracts.

Parameter (Unit)	methanol extract	ethanol extract	ethyl acetate extract
Total Phenol (mgGAE/g)	4.92±0.2 ^a	3.48 ± 0.1 ^b	2.63 ± 0.2 ^c
Total flavonoid (mgQUE/g)	2.25±0.3 ^a	2.15 ± 0.2 ^a	2.04 ± 0.1 ^a
ABTS ((mmol TEAC/g)	0.000082±0.003 ^a	0.000060±0.002 ^b	0.000044±0.001 ^c
Reducing property (mgAAE/g)	2.65±0.21 ^a	1.57±0.15 ^b	1.41±0.07 ^b

Values represent means ± standard deviation of triplicate readings. Values with same superscripts are not significantly different at $P > 0.05$.

ABTS, 2,2'-azino-bis(3-ethylbenzo-thiazoline-6-sulfonate) ; GAE, garlic acid equivalent; TEAC, Trolox-equivalent antioxidant capacity; QUE, quercetin equivalent

Table 2: Phenolic Composition of *Clerodendrum volubile* (methanol, ethanol, ethyl acetate) extracts.

Compounds	<i>Clerodendrum volubile</i>			LOD µg/mL	LOQ µg/mL
	Methanol (mg/g)	Ethanol (mg/g)	Ethyl acetate (mg/g)		
Gallic acid	3.49 ± 0.01 ^a	3.45 ± 0.03 ^a	2.03 ± 0.01 ^a	0.024	0.079
Catechin	1.85 ± 0.01 ^b	1.92 ± 0.02 ^b	0.61 ± 0.02 ^b	0.009	0.034
Caffeic acid	3.56 ± 0.03 ^a	1.93 ± 0.01 ^b	6.74 ± 0.02 ^c	0.015	0.050
Rutin	3.19 ± 0.01 ^a	3.57 ± 0.01 ^a	8.95 ± 0.01 ^d	0.018	0.062
Quercitrin	8.72 ± 0.02 ^c	9.14 ± 0.03 ^c	5.01 ± 0.03 ^c	0.011	0.037
Quercetin	15.34 ± 0.01 ^d	15.02 ± 0.01 ^d	13.27 ± 0.01 ^f	0.029	0.096
Kaempferol	11.62 ± 0.03 ^c	5.83 ± 0.02 ^e	8.98 ± 0.02 ^d	0.023	0.075
Luteolin	8.59 ± 0.01 ^c	11.49 ± 0.01 ^f	9.02 ± 0.01 ^d	0.014	0.046
Apigenin	8.83 ± 0.01 ^c	5.98 ± 0.03 ^e	6.67 ± 0.02 ^c	0.008	0.027

Results are expressed as mean ± standard deviations (SD) of three determinations.

Averages followed by different letters differ by Tukey test at $p < 0.05$. LOD; limit of detection LOQ: limit of quantification

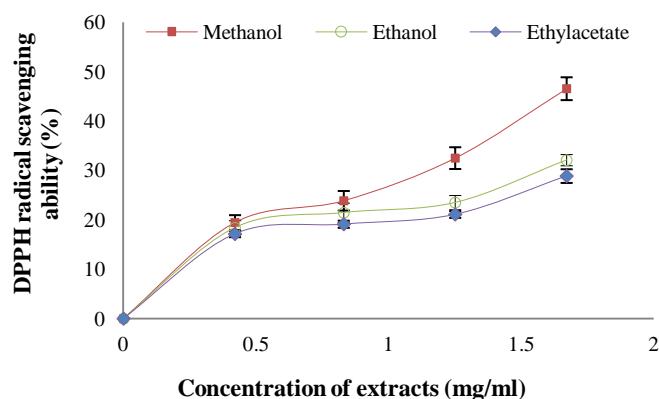


Fig. 2: 1, 1-Diphenyl-2 Picrylhydrazyl (DPPH) free radical-scavenging ability of *C. volubile* extracts. Values represent mean \pm standard deviation of triplicate experiments.

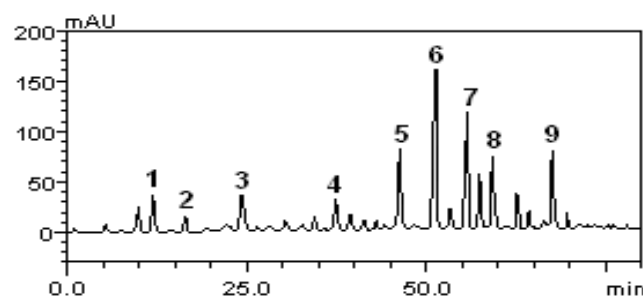


Fig. 3: Representative high performance liquid chromatography profile of methanolic extract of *C. volubile*. Gallic acid (peak 1), catechin (peak 2), caffeic acid (peak 3), rutin (peak 4), quercitrin (peak 5), quercetin (peak 6), kaempferol (peak 7), luteolin (peak 8) and apigenin (peak 9).

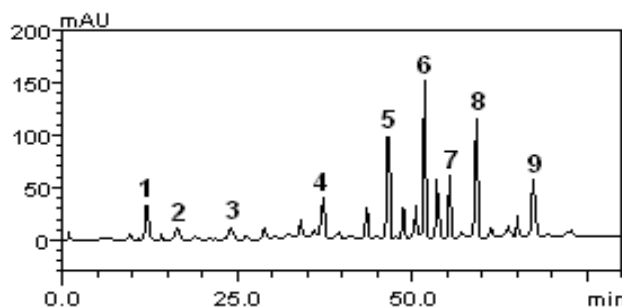


Fig. 4: Representative high performance liquid chromatography profile of ethanolic extract of *C. volubile*. Gallic acid (peak 1), catechin (peak 2), caffeic acid (peak 3), rutin (peak 4), quercitrin (peak 5), quercetin (peak 6), kaempferol (peak 7), luteolin (peak 8) and apigenin (peak 9).

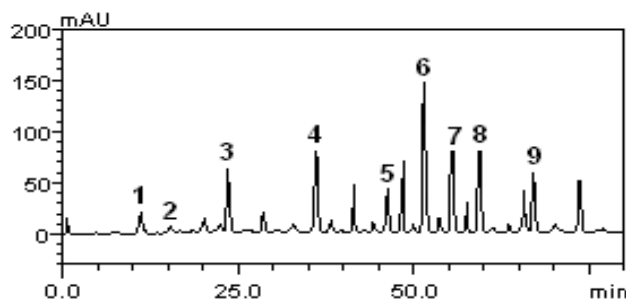


Fig. 5: Representative high performance liquid chromatography profile of ethylacetate extract of *C. volubile*. Gallic acid (peak 1), catechin (peak 2), caffeic acid (peak 3), rutin (peak 4), quercitrin (peak 5), quercetin (peak 6), kaempferol (peak 7), luteolin (peak 8) and apigenin (peak 9).

CONCLUSION

The current surge of interest in antioxidant properties of the plants today is due to their possible role as natural additives to replace synthetic ones. Results of the present study showed phenolics, flavonoids and antioxidant capacity, although with different efficiencies. Quercitrin, quercetin, kaempferol, luteolin were the major phenolic compounds in all the extracts of *C. volubile*. Methanol extract exhibited higher potency of free radical scavenging activity and phenolic contents which is highly related to the presence of hydroxyl groups in the phenolic compounds. Thus present data suggest that methanol extract can be used as a good source of natural antioxidants for health benefits and further isolation of bioactive compounds is required for identifying the unknown compounds to establish their pharmacological properties.

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