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Analysis of *in vitro* antioxidant activity of *Caryota urens* L. leaves: A traditional natural remedy

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ABSTRACT

Objective: To estimate the antioxidant potentiality of crude ethanol extract (CEE), chloroform fraction (CLF), ethyl acetate fraction (EAF) and methanol fraction (MNF) of *Caryota urens* L. (*C. urens*) leaves.

Methods: Antioxidant activity of *C. urens* leaves was determined by total antioxidant activity, 2,2-diphenyl-2-picrylhydrazyl radical scavenging assay and hydroxyl radical scavenging assay. Total phenolic content and total flavonoid content were determined for phytochemical constituent's characterization.

Results: The CEE of the *C. urens* leaves showed the highest total antioxidant activity compared to CLF, EAF and MNF. In the 2,2-diphenyl-1-picrylhydrazyl scavenging assay and the hydroxyl radicalscavenging assay, CEE showed the highest scavenging activity (42.36% and 53.36%) having IC_{50} of 472.14 and 374.81 μg/mL respectively with respect to other fractions.The ranking order of CEE, MNF, EAF and CLF for total phenolic content was 155.74 > 106.8 > 83.95 > 71.49 mg gallic acid equivalent/g of dried extract, respectively. Total flavonoid content was found to be the highest in the CEE (101.35 mg of quercetin equivalent/g of dried extract) rather than other fractions.

Conclusions: This study recommended that CEE of *C. urens* leaves can be considered as a potential source of natural antioxidant and can be used against diseases linked with free radical mediated oxidative stress.

1. Introduction

To lead a normal life, oxidation is essential owing to carry out normal physiological and biochemical functions that lead to the generation of reactive oxygen species (ROS) plus reactive nitrogen species (RNS)[1]. ROS and RNS such as hydrogen peroxide radicals, hydroxyl radicals, nitric oxide radical, peroxyl radical, superoxide radicals and singlet oxygen are highly reactive molecules and can damage biomolecules including carbohydrates, lipids, proteins, nucleic acids (DNA and RNA) and alter their functions[2-7]. They act in normal physiological cell processes, at low to moderate concentrations, but they produce adverse alterations to cell components, at high concentrations[8-12].Imbalance between the pro-oxidant/antioxidant homeostasis creates oxidative stress that is responsible in the pathogenesis of lifestyle-related diseases for example atherosclerosis, heart disease, arthritis, gastritis, diabetes, cancer, preeclampsia, HIV/AIDS, inflammation, ischemia and neurodegenerative diseases especially Alzheimer's, Huntington, Parkinson, amyotrophic lateral sclerosis and celiac diseases[13,14].

Antioxidants shield the body from attack of free radical mediated oxidative stress. Antioxidants that are available in nature include retinoids, tocopherols, ascorbic acid (ACA), bioflavonoids (citrin) and polyphenols (hydroxytyrosol)[15,16]. These naturally occurring antioxidants are effective in the prevention of some life threatening diseases include cancer, heart disease, ageing and immune deficiency diseases[17]. Today, the safety of synthetic antioxidants is questionable for many researchers and health specialists. Artificial antioxidants, including butylated hydroxyanisole, butylated hydroxytoluene and propyl gallate are presumed to be oncogenic[18].

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Until more studies are conducted, it is best to obtain antioxidants from medicinal plants and foods.

As stated by the World Health Organization, 80% of the world's population utilize traditional medicine for their medical care[19,20]. The folkloristic concepts of medicinal plants play an active role in the treatment of various medical conditions[21]. Day by day, the use of medicinal plant and plant products are increasing to treat acute and chronic diseases due to fewer side effects with respect to synthetic drugs[22]. In case of developing countries, almost 3.5 billion people principally rely on medicinal plants and herbal medicine around for their healthcare need[23]. Complementary and alternative medicines, especially Ayurvedic, Unani and Siddha provide healthcare facility for more than 70% of people living in the rural areas[24]. The extensive antiquity of human interactions with the environment is represented by the use of complementary and alternative medicine in Asia[25]. Medicinal plants as folk medicines are extensively practiced in the rural zones of several developing countries[26,27]. Folk medicine practitioners claim that their medicine is inexpensive and more active than modern medicine[28]. The pharmacological actions of medicinal plant are due to the presence of multiple bioactive compounds. The utmost important bioactive phytocompounds are alkaloids, phenolic compounds flavonoids and tannins[29].

The plant, *Caryota urens* (*C. urens*) L. is commonly known in Bengali as Sopari belongs to the family Arecaceae. *C. urens* is naturally found in Myanmar, India, Burma, Sri Lanka and several districts of Bangladesh[30,31]. This plant possesses several illnesses curing effect according to the Ayurveda. The various parts of this plant have outstanding medicinal properties. The flowers of *C. urens* is used against gastric ulcer and migraine headache[32]. To alleviate snake bite poisoning and rheumatic swellings, the barks, roots and terminal buds of this plant are usually used[33]. The root of *C. urens* is also used for the treatment of tooth diseases as well as bark and seed are effective in the treatment of boils[34]. Several studies suggested the antioxidant and antimicrobial effect of *C. urens* fruits.

A previous study showed that methanolic leaf extract of this plant has strong antioxidant activity[35]. Therefore, the objective of this study was to evaluate the antioxidant potential of chloroform fraction (CLF), ethyl acetate fraction (EAF), methanol fraction (MNF) and crude ethanol extract (CEE) of *C. urens* leaves.

2. Materials and methods

2.1. Chemicals

Ethanol, sulphuric acid, ammonium molybdate, ACA, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2-deoxy-2-ribose, ethylenediaminetetraacetic acid, trichloro aceticacid, thiobarbituric acid, Folin-Ciocalteu reagent, gallic acid, methanol and quercetin were bought from Sigma-Aldrich, USA. Unless otherwise specified, remaining chemicals were of analytical grade and bought from Active Fine Chemicals Ltd., Bangladesh.

2.2. Collection and identification of plant materials

C. urens leaves were collected in April, 2015 from Kahba, Brahmanbaria, Bangladesh. The leaves were recognized by a skilled taxonomist of Bangladesh National Herbarium, Mirpur, Dhaka, Bangladesh. The accession number of *C. urens* was DACB-54327.

2.3. Drying and grinding of plant materials

To remove adhering dirt, the fresh leaves of plants were first cleaned properly with water. Then, the leaves were allowed to sun dried for 10 days. For smooth grinding, the sun dried leaves were again dried in an oven at a temperature at 50 ˚C. After drying, the leaves were milled into coarse powder by using a suitable grinder. The obtained milled powdered sample was kept in an airtight glass conation until extraction.

2.4. Extraction and fractionation of plant materials

Powdered sample (250 g) was placed in an amber colored glass bottle and soaked in 800 mL of 98% ethanol at 25 ˚C. The bottle with powder sample was airtight and reserved for 7 days with random agitation at room temperature. The extract was then filtered and concentrated by the help of a rotary evaporator at 50 ˚C temperature to give CEE. The CEE was fractionated, initially with chloroform, followed by ethyl acetate then finally methanol and subsequent evaporation of solvents yielded 2.78 g, 3.75 g, 2.41 g and 2.05 g fractions respectively. Concentrated extract and different fractions were stored until further use.

2.5. Antioxidant activity

The antioxidant activity of the CEEs and fractions were assessed by total antioxidant activity (TAA), DPPH radical scavenging assay and hydroxyl radical scavenging assay. In order to characterize phytochemical constituents, total phenolic content (TPC) and total flavonoid content (TFC) were measured. A stock solution of 1 mg/mL was prepared for the crude extract and fractions prior to test.

2.5.1. TAA

A modified method of Prieto *et al*., was used for the determination of triallyl cyanurate[36]. For this test, 0.5 mL stock solution of *C. urens* extract/fractions at various concentrations (100–600 μg/mL) was added to 3 mL of reaction mixture consisting of 0.6 mol/L sulphuric acid, 28 mmol/L sodium phosphate and 1% ammonium molybdate into the test tube. The test-tubes were then incubated for 15 min at 95 ˚C in a water bath. The test-tubes were allowed to cool to 25 ˚C and the absorbance was determined at 695 nm in contrast to a reagent blank by the help of a UV spectrophotometer. ACA was used as positive control for this assay.

2.5.2. DPPH radical scavenging assay

The method as expressed by Choi *et al*., with slight alterations was

used for the determination of DPPH radical scavenging activity[37]. For this test, methanolic DPPH solution, 3 mL (0.02%) was added to 2 mL stock solution of *C. urens* extract/fractions at various concentrations (25–400 µg/mL) followed by incubation in a dark place for 30 min. Then the absorbance was measured at 517 nm in contrast to methanol as blank by the help of a UV spectrophotometer. As a standard, ACA was used for this test. Percent scavenging of the DPPH free radical was calculated using the formula given below:

DPPH radical scavenging (%) = $[1 – (A/A_o)] \times 100$

where, A_o was the absorbance of control; A was the absorbance of sample/standard solution.

2.5.3. Hydroxyl radical scavenging assay

The method as expressed by Kunchandy and Rao, was used for the determination of hydroxyl radical scavenging activity[38]. For this test, 0.1 mL stock solution of *C. urens* extract/fractions at various concentrations (25–400 µg/mL) was added to 1 mL of reaction mixture consisting of 0.5 mL of 2.8 mmol/L 2-deoxyribose in a 50 mmol/L phosphate buffer having pH 7.4, 0.2 mL of premixed 100 µmol/L ferric chloride and 100 µmol/L ethylenediaminetetraacetic acid (1:1; v/v), 0.1 mL of 200 mmol/L hydrogen peroxide and 0.1 mL of 300 µmol/L ACA into the test tubes. The test-tubes were then incubated for 1 h at 37 ˚C followed by the addition of 0.5 mL of the reaction mixture to 1 mL of 2.8% trichloroacetic acid. Then, 1 mL of 1% thiobarbituric acid solution was added and the reaction mixture was again incubated at 90 ˚C for 15 min in order to allow the color to develop. After that, test tubes were cooled to 25 ˚C and the absorbance was determined at 532 nm against a reagent blank using a UV spectrophotometer. As a standard, ACA was used for this test. Percent scavenging of the hydroxyl free radical was calculated using the formula given below:

Hydroxyl radical scavenging (%) = $[1 – (A/A_o)] \times 100$

where, A_o was the absorbance of control; A was the absorbance of sample/standard solution.

2.5.4. TPC

The method as expressed by Singleton and Rossi with a slight modification was used for the determination of TPC[39]. For this test, 0.1 mL stock solution of *C. urens* extract/fractions at a concentration of 1 000 µg/mL was mixed with 0.75 mL of 1 000-fold diluted Folin-Ciocalteu reagent using distilled water. The test-tubes were then incubated for 5 min at 22 ˚C followed by the addition of 0.06% sodium carbonate and again incubated at 22 ˚C for 90 min to complete the reaction. After that, absorbance was determined at 725 nm in contrast to a reagent blank by the help of a UV spectrophotometer. Gallic acid standard curve was used for the estimation of TPC. The results were represented as mg of gallic acid equivalents (GAE)/g of dried sample.

2.5.5. TFC

The method as expressed by Chang *et al*., was used for the determination of TFC[40]. For this test, 0.1 mL stock solution of *C. urens* extract/fractions with a concentration of 1 000 µg/mL was mixed with 3 mL of methanol, 0.2 mL of 10% aluminum chloride, 0.2 mL of 1 mol/L potassium acetate and 5.6 mL of distilled water into the test tubes. Then for 25 min, the test tubes were incubated at 25 ˚C to complete the reaction. After that, absorbance was determined at 420 nm against a reagent blank by using UV spectrophotometer. Quercetin standard curve was used for the estimation of TFC. The results were represented as mg of quercetin equivalents/g of dried sample.

2.6. Statistical analysis

The results obtained were expressed as mean ± SD from three distinct observations. Student's *t*-test was used to find the consequence of standard and sample for IC_{50} values. Microsoft excel 2010 (Roselle, IL, USA) was used for the statistical and graphical estimations. The value of $P < 0.05$ was considered as significant.

3. Results

3.1. Determination of TAA

The TAA of *C. urens* leaves was given in Figure 1. Results displayed that the antioxidant capacity of the extracts was in the following order: ACA > CEE > MNF > EAF > CLF. The extracts had dose-dependent activity, *i.e.*, antioxidant capacity increased with increased concentration of the extracts. An increase in absorbance at 695 nm against a blank indicated the antioxidant activity of the extract.

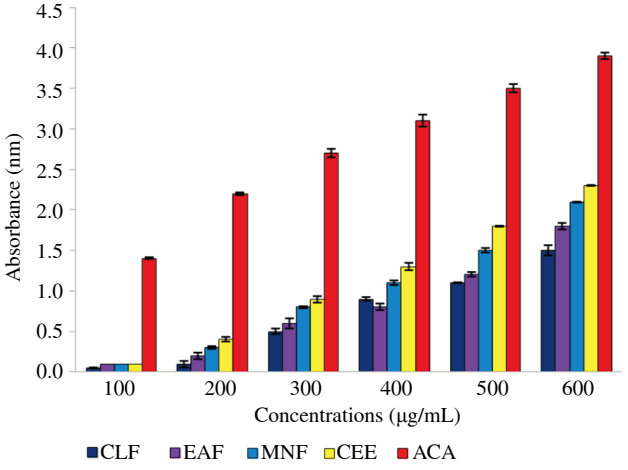

Figure 1. TAA of *C. urens* leaf extract and fractions at different concentrations.
Values were expressed as mean ± SD ($n = 3$).

3.2. Determination of DPPH radical scavenging activity

The DPPH radical scavenging activity of the extracts was given in Figure 2 in which radical scavenging activity was in the following order: ACA > CEE > MNF > EAF > CLF. It was established that the DPPH radical scavenging activity was increased by increasing the concentration of the extracts. The IC_{50} values of ACA, CEE, MNF, EAF and CLF were 18.38 µg/mL, 472.14 µg/mL, 506.59 µg/mL, 600.78 µg/mL and 605.33 µg/mL

specified in Table 1. Results suggested that the IC_{50} values of CEE were statistically significant ($P < 0.01$) with respect to ACA.

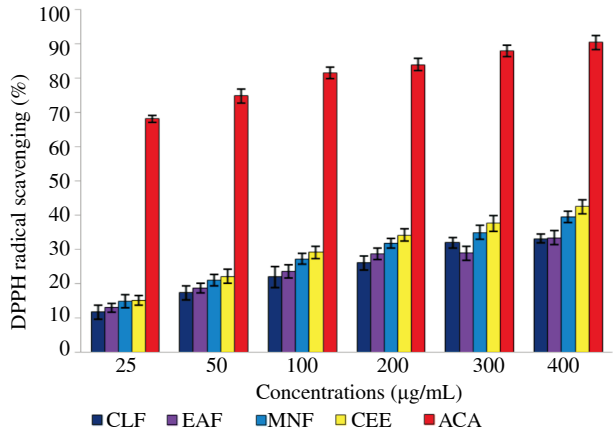

Figure 2. DPPH radical scavenging activity of *C. urens* leaf extract and fractions at different concentrations.
Values were expressed as mean ± SD ($n = 3$).

Table 1
IC_{50} values of *C. urens* leaf extract and fractions for DPPH and hydroxyl radical scavenging activity (µg/mL).

Extracts/standard	IC_{50} values for DPPH radical	IC_{50} values for hydroxyl radical
CLF	605.33 ± 1.24	450.86 ± 3.41
EAF	600.78 ± 4.89	420.87 ± 2.65
MNF	506.59 ± 2.47	401.36 ± 4.62
CEE	472.14 ± 1.68**	374.81 ± 3.87*
ACA	18.38 ± 3.68	37.66 ± 2.40

Values were expressed as mean ± SD ($n = 3$). *: $P < 0.05$; **: $P < 0.01$, significant difference from the standard.

3.3. Determination of hydroxyl radical scavenging activity

Figure 3 shows that the hydroxyl scavenging activity of extracts was in the following order: ACA > CEE > MNF > EAF > CLF. The results revealed that the CEE possessed hydroxyl radical scavenging activity with respect to standard ACA and the activity gradually increased through increasing the concentration of the extracts. IC_{50} values of CLF, EAF, MNF, CEE and ACA were 450.86 µg/mL, 420.87 µg/mL, 401.36 µg/mL, 374.81 µg/mL and 37.66 µg/mL respectively as shown in Table 1. With respect to ACA, IC_{50} value of CEE was statistically significant ($P < 0.05$).

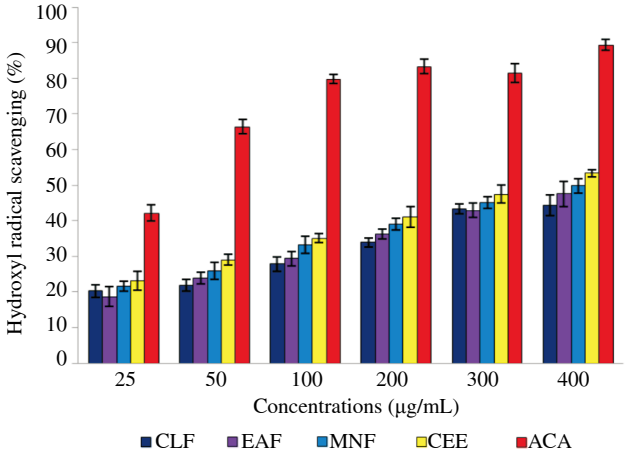

Figure 3. Hydroxyl radical scavenging activity of *C. urens* leaf extract and fractions at different concentrations.
Values were expressed as mean ± SD ($n = 3$).

3.4. Determination of TPC

TPC of *C. urens* leaf extract and fractions was calculated from the standard curve of gallic acid ($y = 0.0158x + 0.0475$; $R^2 = 0.9924$). Figure 4 shows the TPC where phenolic contents of the extracts fluctuating from (12.952 ± 0.787) to (124.355 ± 2.077) mg GAE/g. The TPC was in the following order: CEE > MNF > EAF > CLF.

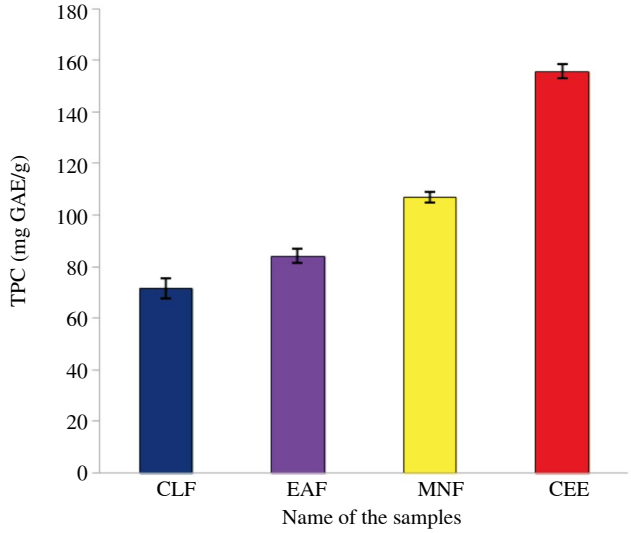

Figure 4. TPC of *C. urens* leaf extract and fractions.
Values were expressed as mean ± SD ($n = 3$).

3.5. Determination of TFC

Quercetin standard curve ($y = 0.0098x + 0.1163$; $R^2 = 0.9911$) was used for the calculation of TFC. The TFC was in the following order: CEE > MNF > EAF > CLF displayed in Figure 5. The CEE exhibited the highest flavonoid contents compared to MNF, EAF and CLF.

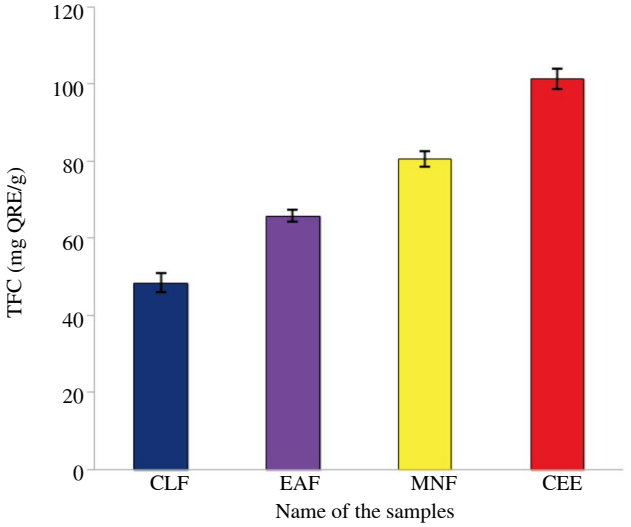

Figure 5. TFC of *C. urens* leaf extract and fractions.
Values were expressed as mean ± SD ($n = 3$).

4. Discussion

Cells can not live without oxidative systems. On account of the dependence, this system leads to the genesis of free radicals, other ROS and RNS[41]. These formed free radicals, ROS and RNS are increasing evidence for interfering in a diversity of normal *in vivo*

controlling structures[1]. Once an excess amount of free radicals is produced, they can reduce endogenous defensive enzymes and consequently generate deleterious cellular effects by oxidizing biomolecules like membrane lipids, cellular proteins, enzymes, DNA and RNA, later ending cellular respiration[42]. Numerous studies have revealed that antioxidants obtained from plant may be beneficial in preventing or slowing the destructive outcome of oxidative stress[43-45]. Herein, the antioxidant activity of CEE and its resulting fractions at several concentrations were tested.

In this study, total antioxidant capacity of the plant extract were measured based on phosphomolybdate method. This method depends on reduction of molybdenum (VI) to the molybdenum (V) by the extract. The development of green phosphate color molybdenum (V) compounds indicates the antioxidant capacity of the plant extract[46]. This study confirmed that CEE displayed the highest antioxidant capacity. Several studies suggest that total antioxidant capacity of the plant is mainly due to the presence of flavonoid and related polyphenols within the plant[47,48].

The antioxidant potential of the plant extracts based on free radical scavenging is determined by DPPH test. This test measured the electron donating capability of the plant extract to reduce DPPH to DPPH-H. The conversion of purple color of DPPH solution to yellow to varying grade depending on the existence and amount of antioxidant compounds within the extract[49]. The present study inspected that all the extracts had radical scavenging activity, but the highest activity was reported by CEE extracts. In comparison with a previous report on the DPPH radical scavenging activity of methanolic extract of *C. urens* fruits, Uddin *et al*., also reported that CLF had maximum scavenging activity[33].

Hydroxyl radicals are short-lived, highly reactive source of ROS[50]. The decomposition of hydroperoxides is accountable for the generation of hydroxyl radicals[51]. In addition to this, these can infrequently be produced as a byproduct of immune action. The hydroxyl radical is considered as a potential resource of carcinogenesis, mutagenesis and cytotoxicity[52]. It is responsible for the destruction of almost all types of cellular structures such as carbohydrates, nucleic acids, lipids and amino acids[53]. Mainly it interacts with polyunsaturated fatty acid portion of cell membrane phospholipid and causes destruction to cells[54,55]. In this study, the low intensity of the red color solution indicated that the plant extracts especially CEE had hydroxyl radical scavenging capacity. Charles and Ramani reported the DPPH radical, hydrogen peroxide radical and reducing power scavenging activity of ethanolic extract of *C. urens* and showed maximum scavenging activity against hydrogen peroxide radicals[56].

The examination of antioxidant potentiality of the plant is increased since they retard oxidative degradation of biomolecules. Various secondary metabolites of plant phenolic compounds are predominant due to the presence of hydroxyl groups in phenolic compounds that exert scavenging ability[57]. Plants with phenol contents exert various pharmacological activities including antioxidant, anti-inflammatory, analgesic, antiobesity and antidiabetic actions[58]. The polyphenol of plants exerts their antioxidant activity by their redox potential and consequently plays an important role in the engrossing, counteracting, reducing and decomposing the ROS. In this study, CEE reported the highest phenol contents among others fractions. Polyphenolic compounds, especially flavonoids have a number of beneficial effects on human health[59]. Several studies revealed that flavonoids are responsible for anticancer, anti-inflammatory, anti-allergic, antibacterial, antifungal, antiviral and anti-diarrheal activities[60,61]. Flavonoids are markedly effective for controlling lipid peroxidation that lead to thrombosis, atherogenesis and carcinogenesis. It has been proved that the pharmacological effect of flavonoids is directly interconnected with their antioxidant activities[62]. In this study, the highest flavonoid contents were reported for CEE. A previous study exposed that immature fruit and fruit skin of the *C. urens* methanolic extract contained higher amount of phenolic and flavonoid contents respectively[63].

These existing data suggest that CEE of *C. urens* leaves has promising antioxidant activity.

The finding from the existing study confirmed that *C. urens* extract and fractions exhibited significant antioxidant and free radical scavenging activities and contained enormous amounts of phenolic and flavonoids compounds. CEE can be considered as a potent source of natural antioxidant, that might be beneficial in preventing and or slowing the progress of various free radicals mediated oxidative stresses related disorders. Therefore, further study is needed to perform *in vivo* antioxidant activity and identify the antioxidant compounds existing in the leaves extract of this plant.

Conflict of interest statement

We declare that we have no conflict of interest.

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