

Inhibition of Digestive Enzymes, Antioxidant and Free Radical Scavenging Capacities of Stem Bark Extracts of *Coula edulis* Baill (Olacaceae)

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Abstract

Prolonged hyperglycemia enhances oxidative stress. Bioactive compounds extracted possess antioxidant, anti-free radical potentials or the ability to reduce blood sugar levels. The objective of this study was to evaluate the antioxidant properties of extracts of stem bark of *Coula edulis* Baill., their abilities to trap free radicals and glucose, as well as their ability to inhibit α -amylase and invertase activities. *In vitro* assays were used to test the trapping capacity of extracts on DPPH, ABTS, NO, and OH radicals; to evaluate the antioxidant capacity, the activity of glycosylation and the capacity of inhibition of the activities of α -amylase and invertase were conducted. Also, phenolic, flavonoid and alkaloid contents of extracts were determined. Results showed that extracts of the stem bark of *C. edulis* have anti-radical properties. The extracts chelate DPPH, hydroxyl (OH), nitrite oxide (NO), ABTS radicals, and even glucose. The IC₅₀ values varied depending on the nature of the extraction solvent. Ethanolic extract has the highest polyphenolic content (289.12 ± 30.31 µg catechin equivalent/g), flavonoids (1.12 ± 0.09 µg quercetin equivalent/g) and alkaloids (5.54 ± 0.59 µg quinin equivalent/g). The extracts also reduce invertase and α -amylase activities. *C. edulis* extracts present strong antioxidant potentials and can be used as a source of natural antioxidants for the prevention of oxidative stress and hyperglycemia.

Keywords: *Coula edulis* Baill., antioxidant, amylase, invertase

1. Introduction

Oxidation process is one of the most important means for producing free radicals in food, drugs and even living systems (Khettaf *et al.*, 2016). Free radicals and hyperglycaemia which can be induced by a lower digestive enzymes activities play a major role in many of today's diseases (Kim *et al.*, 2015; Budin *et al.*, 2018). Further, more evidences have accumulated and indicated key roles for reactive oxygen species (ROS) and other oxidants such as singlet oxygen species, superoxide, hydroxyl, peroxy, nitroxide and ferrous in causing numerous disorders (Saeed *et al.*, 2012). When, free radicals are present in higher concentration beyond the antioxidant capacity of a biological system, this gives a disturbance between the pro-oxidant/antioxidant balance in favour of the former, rise to an imbalance known as oxidative stress (Zima *et al.*, 2001). ROS are normally generated during normal metabolism and energy production in the body (Mavi *et al.*, 2004). This production helps the normal healthy tissues to perform physiological roles such as signaling molecules, regulation of signal transduction and gene expression, activation of receptor and nuclear transduction among others (Valko *et al.*, 2006). Oxidative stress status forced by free radicals may cause lipid peroxidation, protein damages, inflammation, autoimmune pathologies, DNA damage and altering cell signalling pathways and modulating gene expression. Overwhelming literature evidence has implicated the role of free radicals in the progression of heart diseases, neurodegenerative diseases, cancer, aging process (Oluyemi *et al.*, 2007) and complications in diabetes among others (Raghuveer and Tandon, 2009). However, cells have a comprehensive array of antioxidant defense mechanisms to reduce free radical formation or limit their damaging effects (Badmus *et al.*, 2011). These antioxidants mechanism protect human cells against free radical damage by enzymes such as superoxide dismutase (SOD) and catalase, or non-enzymatic compounds such as ascorbic acid, tocopherol and glutathione (Moukette *et al.*, 2016). Then, these antioxidants are used to counteract the harmful effects of free radicals. Given that, they stabilize or deactivate free radicals, often before they attack targets in biological cells (Nunes *et*

al., 2012), antioxidants can prevent or delay the oxidation of the substrate. Sometimes these protective mechanisms are disrupted by various pathological processes, and antioxidant supplements are vital to combat oxidative damage. A lower digestive enzymes activity favours the high plasma glucose level, along with metabolic diseases. Accumulating evidence indicates that obesity and many chronic diseases are associated with systemic oxidative stress, whereas consuming polyphenols attenuates these disease conditions (Zhang *et al.*, 2015).

Several studies have demonstrated a distinct correlation between the higher intake of plant foods and lower risk of mortality from diseases (Chidambaram *et al.*, 2013). Phenols are repeatedly implicated as natural antioxidants in fruits, vegetables and other plants (Yeh *et al.*, 2011). Plant derived products may contain phenols and others phytochemicals that could act as antioxidant compound. These bioactive natural products have been proved to have unique pharmacological properties (Kannan *et al.*, 2013) throughout their ability to prevent the destructive processes caused by oxidative stress (Athiperumalsami *et al.*, 2010). Also, these compounds have the ability to bind to enzymes and inhibit their activity. The presence of inhibitors of carbohydrate and glycerides hydrolysing enzymes such as invertase and α -amylase, in plant derived products helps in the control of blood glucose level in patients with type 2 diabetes and to prevent obesity (Zhang *et al.*, 2015). Hence, the growing interest to find cheap, renewable and abundant sources of antioxidants has fostered research on current consumption plants. *Coula edulis B.*, (commonly known as African walnut), grows naturally in evergreen forests from Sierra Leone to Democratic Republic of Congo (Moupela *et al.*, 2011).

Previous studies on *C. edulis B.* revealed the presence of phenolic compounds which have been shown as having antiplasmodial activity (Zofou *et al.*, 2011). Further, in the folk medicine, the bark is used in treatments of gastro-intestinal infections, anaemia, diarrhoea, anti-inflammation and anti-microbial health's care (Bukola *et al.*, 2008). Indeed, different part of *C. edulis B.* has been several using in African traditional pharmacopeia, some of these beneficial effects could be related to the antioxidant potent (Dembitsky, 2006). In fact, the exploration, characterization and application of natural antioxidants are the focus of several research teams in the Sub-Saharan Africa (Biapa *et al.*, 2011; Nyangono *et al.*, 2012; Kuate *et al.*, 2014). The main objective of this study was to investigate the *in vitro* antioxidant, free radical scavenging properties, and some digestive enzymes effects of hexane dichloromethane, ethanol and aqueous extract from stem barks of *C. edulis B.* against *in vitro* oxidative stress.

2. Materials and Methods

2.1 Instruments

The samples were weighed using an OHAUS model PA64 microbalance. An ika Rv 10 Control type rotary evaporator was used to collect the different fractions of extracts. A Vanguard brand V6500 Iohazard micro-centrifuge was used to separate the constituents of mixtures. The absorbance of solutions was measured using a Genesys 20 brand spectrophotometer.

2.2 Chemicals & Reagents

The different reagents used in this study (Hexane, Dichloromethane, Ethanol (95%), Methanol, Sodium Carbonate, Folin-Ciocalteu, Chloride acid, 1,10-phenanthroline, Naphtylenediamine chloride, 2,2-diphenyl-1-picrylhydrazyl, Gallic Acid, Aluminum Chloride, Potassium Acetate, Quercetin, Catechin, Quinin, Ascorbic Acid, Hydrochloric Acid, Iron Chloride, Sodium dihydrogen phosphate, Sulfuric Acid, Dibasic hydrogen phosphate, Potassium ferricyanide, Ammonium molybdate, Sodium Nitroprusside, Sulfanilicacid, Acetic acid and N-(1-naphthyl)-Ethylenediamine dihydrochloride) were purchased from Merck (Germany).

2.3 Plant Collection

The stem barks of *Coula edulis B.* Stem bark were collected in July 2014 in Mbalmayo, a locality in the Centre Region of Cameroon. The plant was identified and authenticated at the Cameroon National Herbarium in Yaounde and a voucher specimen has been deposited under the number 46305 HNC.

2.4 Extraction

For each extract, 500 grams of the powder were macerated at room temperature in 3 L of boiling water or ethanol/water (1:4) mixture, for 48 hours.

Stem bark of plant (2400 g) were collected and shade, dried at 50 °C indoor drying room for 48 h. The dry sample powder obtained in last step (2000 g) was mixed successively with hexane, dichloromethane, ethanol (95%) and distilled water at room temperature (1/4: w/v) reagents. After homogenization, mixture was macerated for 48 h at room temperature and filtered through Whatman #2 filter paper (Whatman International Limited, Kent, England). The filtrate was concentrated using a rotary evaporator (ika Rv 10 Control) under

reduced pressure at 40 °C to yield four samples of *C. edulis* B. extract: hexane (CE hexane 31 g), dichloromethane (CE DCM, 70 g), ethanol (CE EtOH, 96 g) and aqueous (CE Aq 53 g) extract of stem back.

Each sample were dissolved in 95% ethanol to make a concentration of 1 mg/ml and then diluted to prepare the series concentrations for antioxidant assays. Reference chemicals were used for comparison in all assays.

2.4.1 DPPH Radical Scavenging Activity Test

The free radical scavenging activity of the fractions was measured in vitro by 2, 2'-diphenyl-1-picrylhydrazyl (DPPH) assay according to the method described and modified by (Brand-Williams *et al.*, 1999; Sánchez Moreno *et al.*, 1998). The stock solution was prepared by dissolving 24 mg DPPH with 100 ml methanol and stored at 20 °C until required.

One (1) ml aliquot of this solution was mixed with 20 µl of the sample at various concentrations (10 – 160 µg/ml). The reaction mixture was shaken well and incubated in the dark for 30 min at room temperature. Then the absorbance was taken at 517 nm. The control was prepared as above without any sample. The same procedure was used for the ascorbic acid (1 mg/ml) used as standard. The scavenging activity was estimated based on the percentage of DPPH radical scavenged as the following equation:

$$\text{Scavenging effect (\%)} = [(Abs\ Control - Abs\ Sample)/Abs\ Control] \times 100$$

2.4.2 ABTS Radical Scavenging Activity Test

The 2, 2'-azinobis (3-ethylbenzthiazoline-6-sulphonic acid), commonly called ABTS cation scavenging activity was performed according to (Re *et al.*, 1999) with some modifications. Briefly, ABTS solution (7 mM) was reacted with potassium persulfate (2.45 mM) solution and kept for overnight in the dark to yield a dark coloured solution containing ABTS radical cations.

The mixture was stored in the dark at room temperature for 12 h before used. The ABTS⁺ solution was diluted (1: 10 v/v) with a solution of phosphate buffer (0.1 M, pH 7.4, NaCl 150 mM) to an absorbance of 2.27 ± 0.05 at 734 nm. After addition of 20 µl of extract sample or ascorbic acid used as standard to 1 ml of diluted ABTS⁺ solution, absorbance was measured at 734 nm after incubation at room temperature for 30 minutes. The control was prepared as above without any sample. The decrease in absorption was used for calculating scavenging effect values. The following equation was used to determine the percentage of the radical scavenging activity of each extract.

$$\text{Scavenging effect (\%)} = [(Abs\ Control - Abs\ Sample)/Abs\ Control] \times 100$$

2.4.3 Hydroxyl Radical Scavenging Activity Test

The hydroxyl radical scavenging activity was determined as earlier described by Halliwell *et al.*, (1987). The reaction mixture consisted of FeCl₃ (300 µM), EDTA (780 µM), 2-deoxyribose (2.8 mM), ascorbic acid (300 µM), H₂O₂ (4 mM) and aliquots of extracts in a final volume of 1 ml. All reagents were dissolved in potassium phosphate buffer (20 mM, pH 7.4). This was then incubated at 37 °C for 1 h. After incubation, 1 ml of TCA (2.8%) and TBA (1%) were added to the reaction mixture and incubated at 100 °C for 20 min. A control tube was prepared similarly effect except that the extract was replaced with methanol. The absorbance was read spectrophotometrically at 532 nm. All measurements were done in triplicate and the % inhibition was calculated using following formula.

$$\text{Scavenging effect (\%)} = [(Abs\ Control - Abs\ Sample)/Abs\ Control] \times 100$$

2.4.4 Nitric Oxide Radical Scavenging Activity Test

Nitric oxide scavenging activity was determined according to the Griess Illosvoy reaction (Green *et al.*, 1982). The lesser of the production of nitrite ion was measured according to (Sreejayan and Rao, 1997). Various concentrations of the extracts (0.5 ml) were added in a final volume of 3 ml. After incubation for 60 min at 37 °C, Griess reagent [N-(1-Naphthyl) ethylenediamine (0.1%) and sulphanilic acid (1%) in H₃PO₄ (5%)] was added. The pink chromophore generated during diazotization of nitrite ions with sulphanilamide and subsequent coupling with N-(1-Naphthyl) ethylenediamine was measured spectrophotometrically at 540 nm. Ascorbic acid was used as a positive control. The control was prepared as above without any sample. The scavenging effect (%) of the nitric oxide was calculated using the formula:

$$\text{Scavenging effect (\%)} = [(Abs\ Control - Abs\ Sample)/Abs\ Control] \times 100$$

2.4.5 Phosphomolybdate Assay (Total Antioxidant Capacity)

The total antioxidant activity of the extracts was evaluated by green phosphomolybdenum complex according to a method described (Prieto *et al.*, 1999). An aliquot of 10 µl of the extract solution was mixed with 1 ml of

reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) in a micro centrifuge tube.

The tubes were incubated in a dry thermal bath at 95 °C for 60 min. After cooling, the absorbance of the mixture was measured at 695 nm against a blank. A typical blank contained 1 ml of the reagent solution and the appropriate volume of the solvent and incubated under the same conditions. Ascorbic acid was used as standard. The antioxidant capacity was estimated using following formula:

$$\text{Antioxidant effect (\%)} = [(Abs\ Control - Abs\ Sample)/Abs\ Control] \times 100$$

2.5 Evaluation of Glucose Scavenging Activity

The glucose scavenging activity was determined according to the method of Bertrand described by Kebieche (2009). At first we have introduced glucose (100 mg/l; 1 ml), sample of extract (100 µl) at various concentrations (50 – 1600 µg/ml), followed by addition of picric acid (0.4 M; 1 ml). The control was prepared as above without any sample. The mixture was incubated 30 minutes at room temperature. The absorbance of the solution was read at 570 nm.

$$\text{Glucose scavenging (\%)} = [(Abs\ Control - Abs\ Sample)/Abs\ Control] \times 100$$

2.6 Evaluation of the Power of the Extracts to Inhibit the Activity of α -amylase

The α -amylase inhibitory activity was determined according to the method of Bernfield (1951). Adding 250 µl of appropriate dilutions of the vegetable extracts prepared in different concentrations (0.0375 to 1.2 mg/ml) and 500 µl of 0.02 M sodium phosphate buffer (pH 6.9 with 0.9 % NaCl) containing rodent pancreatic α -amylase (EC 3.2.1.1) (0.5 mg/ml) were pre-incubated at 25 °C for 10 minutes. Then, 250 µl of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.9 % NaCl) was added to the reacting mixture.

Thereafter, the reaction mixture was incubated at 37 °C for 10 min and stopped with 500 µl of dinitrosalicylic acid (DNSA). The mixture was then incubated in a boiling water bath for 5 min, and cooled to room temperature. The reaction mixture was then diluted by adding of distilled water until 10 ml. The absorbance measured at 540 nm.

Then, the α -amylase inhibitory activity was calculated as percentage inhibition.

$$\text{Inhibition (\%)} = [(Abs\ Control - Abs\ Sample)/Abs\ Control] \times 100$$

2.7 Evaluation of the Power of the Extracts to Inhibit the Activity of Invertase

The invertase inhibitory activity was determined according to the method of Melius, (1971). Invertase catalysed hydrolysis of sucrose. The first step was consisted to isolate of invertase from Baker's Yeast followed by the purification of sample.

At first introduce 50 µL of extracts in different concentrations (5, 10, 20 and 40 mg/mL) into 1.0 ml of substrate solution (composed of 20 mM sucrose in 40 mM sodium acetate buffer at pH 4.80). Those mixtures were pre-incubated at an ambient temperature of 20-24°C during 5.0 minutes. At 30 second intervals for each concentration (30 and 60 s), small aliquots (0.05 ml) of each enzyme-containing fraction are quantitatively transferred and gently mixed into disposable test-tubes where the invertase reaction is allowed to proceed for a precise time of 5.0 minutes.

At the end of each 5.0 minutes' reaction period at a temperature of 37°C, the invertase activity in each tube is instantaneously stopped at 30 second intervals by denaturation with the rapid addition of 2.0 ml of alkaline DNS solution (composed of 0.20 M NaOH, 23 mM 3, 5-dinitrosalicylate, and 0.53 M sodium potassium tartrate). After each reaction is "stopped", the entire set of assay tubes is transferred to a boiling water bath for 10 minutes. Under these conditions, the D-Fructose produced in step 1 rapidly isomerizes to D-glucose which, along with the other mole of glucose produced by the enzyme reaction, reduces the brightly yellow-coloured 3, 5-dinitrosalicylate to dark orange-coloured solutions of 3-amino-5-nitrosalicylate. The entire set of assay tubes is then cooled in a beaker of tap water before each solution is diluted to a final volume of 6.1 ml with the addition of 3.0 ml of 50 mM sodium acetate buffer at pH 4.8.

The control tube was prepared exactly as one of the assay tubes in steps 1 and 2 except for the volume of extract added to the substrate solution in step 1 is replaced with water. The absorbance of the solution in each disposable test tube at 540 nm.

$$\text{Inhibition (\%)} = [(Abs\ Control - Abs\ Sample)/Abs\ Control] \times 100$$

2.8 Determination of Phenolic and Alkaloid Contents

2.8.1 Evaluation of the Total Phenolic Content

Determination of total phenolic content was determined using Folin-Ciocalteu (FC) reagent according to the method of Singleton and Rossi (1965), with a slight modification. Briefly, the plant extract (30 μ l) was mixed with 1 ml of FC reagent (previously diluted 10-fold with distilled water) and incubated for 5 min at ambient temperature, then (0.7 ml; 7%) Na_2CO_3 solution was added. After incubation at ambient temperature for 30 min into dark room, the absorbance was measured at 750 nm. The phenolic content was evaluated from a catechin standard curve.

2.8.2 Evaluation of the Total Flavonoid Content

The total flavonoid content was determined with aluminium chloride (AlCl_3) according to a known method of Bahorun *et al.*, (1996). We were using quercetin as a standard.

The plant extract 1 ml (1 mg/ml; ethanol 95 %), was added to AlCl_3 (1 ml, 10%, ethanol 95 %), followed by potassium acetate (1 ml, 1 M). After a further 30 min, the reaction mixture was allowed to stand at room temperature for 10 min. The absorbance was measured at 420 nm. All tests were performed three times. The flavonoid content was calculated from a quercetin standard curve.

2.8.3 Assessment of the Alkaloid Content

Total alkaloids contents were determined by the method of Singh *et al.*, (2003), which involves the oxidation of alkaloids by iron (III). To each 1 ml of sample (1 mg/ml) add of FeCl_3 (1 ml, 0.25 M) in HCl (0.5 M) and 1, 10-phenanthroline (1 ml, 0.05 M) in ethanol. The flasks were placed in water bath maintained at 70 °C for 30 minutes. The absorbance of red-colored complex was measured at 510 nm against a reagent blank. All tests were performed three times. The alkaloids content was calculated from a quinin standard curve. All of these tests were carried out in triplicates.

2.9 Phytochemical Screening of CEE Bark

Phytochemical screening stem bark of *C. edulis* extracts for the presence of alkaloids, cardiac glycosides, flavonoids, saponin, phlobatannins, tannins and terpenoids was carried out.

2.9.1 Detection Test for Alkaloids

One (1) ml of stem bark of *C. edulis* extracts was boiled with 1 ml of 1% HCl for 5 minutes. The mixture was warmed and filtered (Harborne, 1973). The filtrate was treated separately with few drops of potassium mercuric iodide (Mayer's reagent) Saeed *et al.*, (2012). Turbidity or precipitation with either of these reagents was taken as evidence for existence of alkaloids.

2.9.2 Saponin Detection Test

In the presence of water, saponins form emulsions after stirring (Trease *et al.*, 1989). 0.5 g of CEE extracts were added to 5 mL of distilled water. The mixture was shaken for a few minutes. The appearance and persistence of the emulsion after 20 min was used as evidence of the presence of saponins.

2.9.3 Terpenoids Test

The presence of terpenoids in the CEE extracts was carried out by mixing 2 mL of extract (1 mg/mL) of CEE with 2 mL of chloroform, then 3 mL of concentrated H_2SO_4 added. Under these conditions, the appearance of a reddish brown coloration at the interface attested to the presence of terpenoids (Harborne, 1973).

2.9.4 Cardiac Glycoside Detection Test

Two (2) ml (10 mg/ml in ethanol) of *C. edulis* extracts were mixed with 2 ml of glacial acetic acid having one drop of FeCl_3 solution. To the mixture obtained 1 ml of concentrated H_2SO_4 was added to form a layer. The presence of brown ring of the interface indicated deoxy sugar characteristic of cardiac glycosides (Trease *et al.*, 1989).

2.9.5 Phlobatannin Detection Test

One hundred (100) mg of CEE extracts were boiled in 1% aqueous hydrochloric acid; the deposition of a red precipitate indicated the presence of phlobatannins (Trease *et al.*, 1989).

2.9.6 Flavonoids Test

Five hundred (500) mg of CEE extracts were suspended in 5 ml of distilled water to get the filtrate. 2 ml of dilute NaOH solution was added to 2 ml of filtrate followed by few drops of concentrated hydrochloric acid. Presence

of flavonoids was confirmed by yellow colouration (Sofowara, 1993).

2.10 Statistical Analyses

The results were presented as mean \pm SEM of triplicate assays. Analyses of data was conducted using one-way analysis of variance (ANOVA) followed by Tukeys test and Dunnett's multiple test (SPSS program version 18.0 for Windows, IBM Corporation). The Log probit was used to determinate the IC_{50} were used to achieve the Pearson Correlation Analysis (PCA). The differences were considered as significant at $p < 0.05$.

3. Results and Discussions

3.1 Extraction Yield

Depending on the nature of solvent used, extraction and fractions yield was recorded differently. With 4.8 and 3.5% CE EtOH and CE Aq appear respectively to be the best solvents for fractionation giving the maximum yield

3.2 Preliminary phytochemical screening

C. edulis soluble extracts demonstrated the presence of alkaloids, cardiac glycosides, flavonoids, saponins, phlobatannins, tannins and phlobatannins. Only cardiac glycosides and phlobatannins were not present in all the extracts.

Table 1. Phytochemical constituents of *C. edulis* extracts

Compound Class	Samples			
	CE hexane	CE dichloromethane	CE ethanolic	CE aqueous
Saponins	+	+	+	+
Flavonoids	+	+	+	+
Tannins	+	+	+	+
Phlobatannins	-	+	+	+
Alkaloids	+	+	+	+
Cardiac glycosides	-	-	+	+

+ = presence of a constituent

- = absence of a constituent

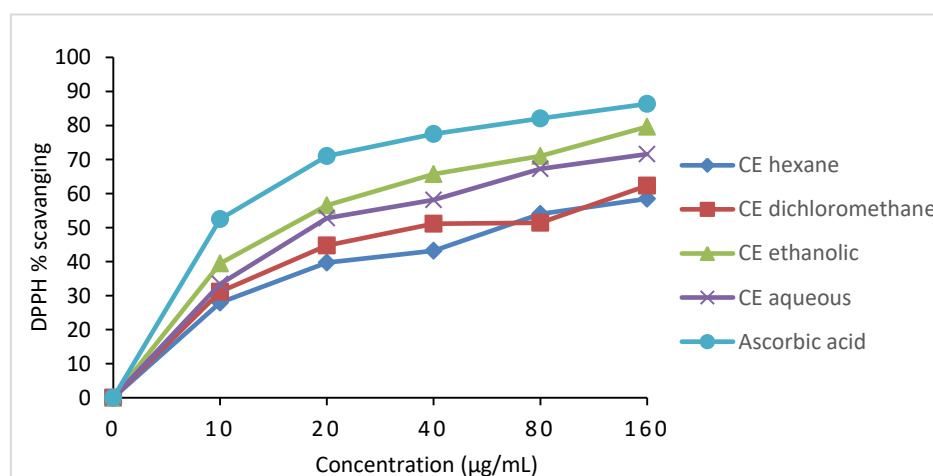


Figure 1. DPPH anti radical activity of hexane, dichloromethane, ethanolic and aqueous extracts of *C. edulis* stem bark extract

Each value represents a mean \pm SD (n = 3)

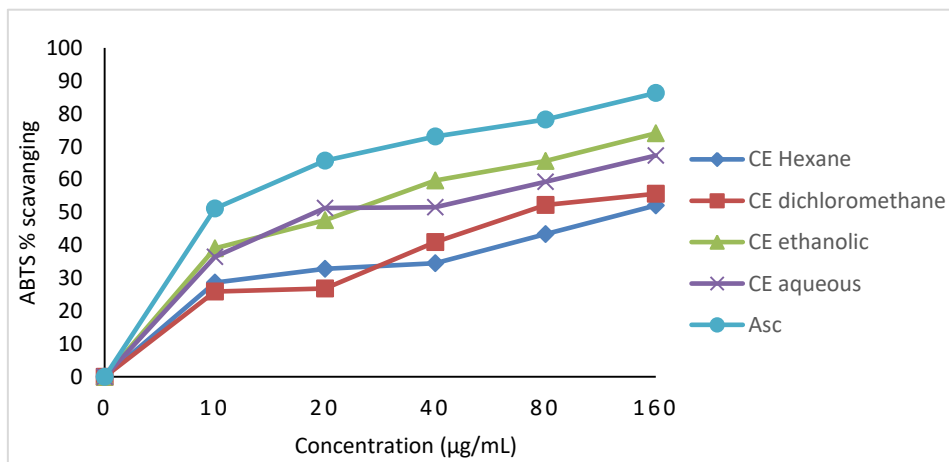


Figure 2. ABTS anti radical activity of hexane, dichloromethane, ethanolic and aqueous extracts of *C. edulis* stem bark extract

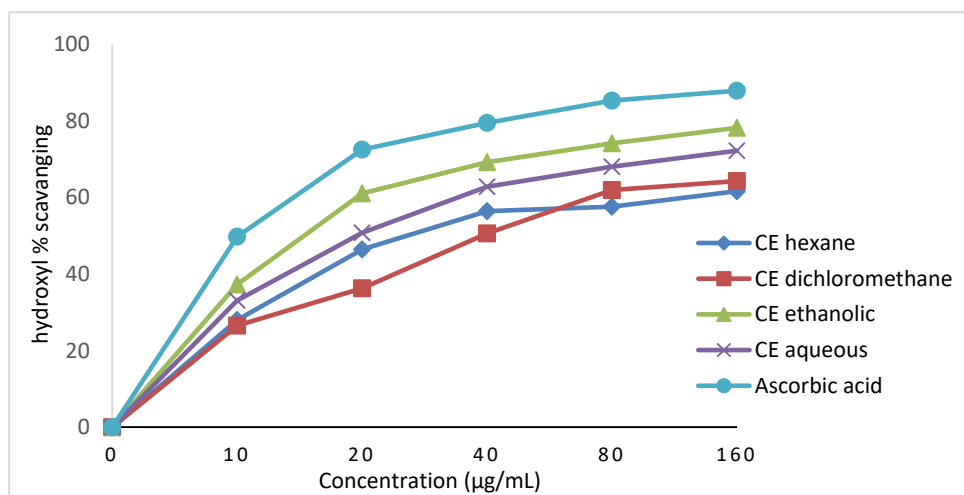


Figure 3. Hydroxyl anti radical activity of hexane, dichloromethane, ethanolic and aqueous extracts of *C. edulis* stem bark extract

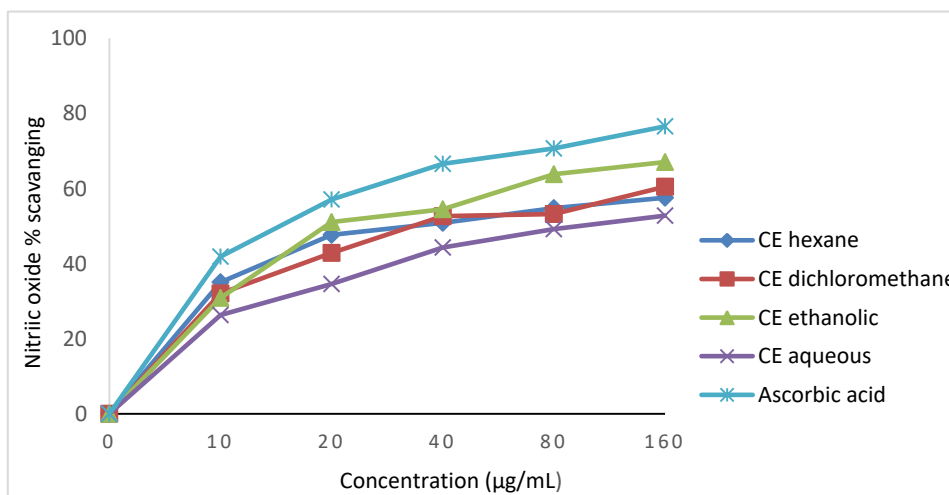


Figure 4. Nitric oxide anti radical activity of hexane, dichloromethane, ethanolic and aqueous extracts of *C. edulis* stem bark extract

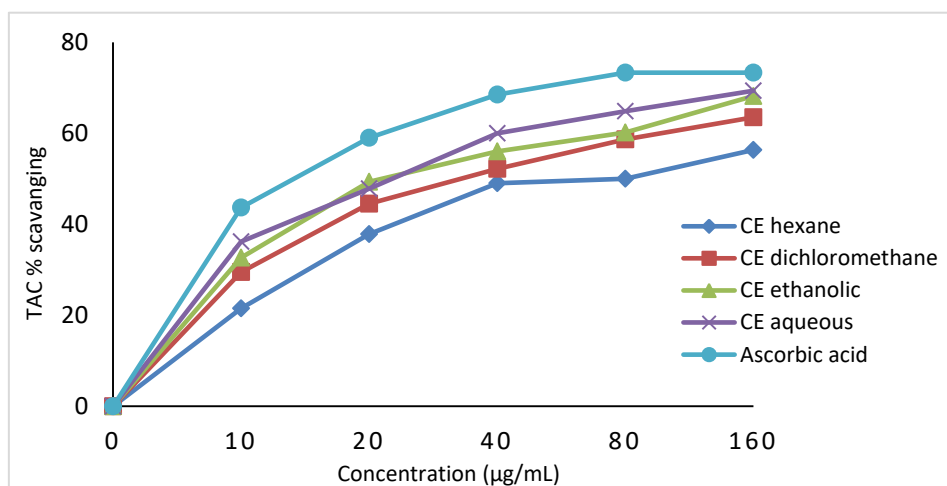


Figure 5. Total antioxidant capacity hexane, dichloromethane, ethanolic and aqueous extracts of *C. edulis* stem bark extract

Each value represents a mean \pm SD (n = 3)

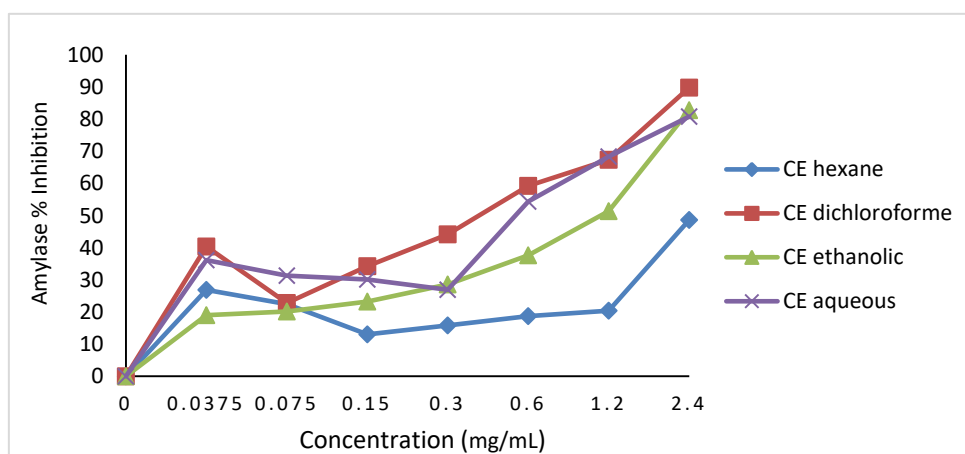


Figure 6. Alpha amylase inhibitory activity hexane, dichloromethane, ethanolic and aqueous extracts of *C. edulis* stem bark extract

Values represent mean \pm standard deviation, n = 3

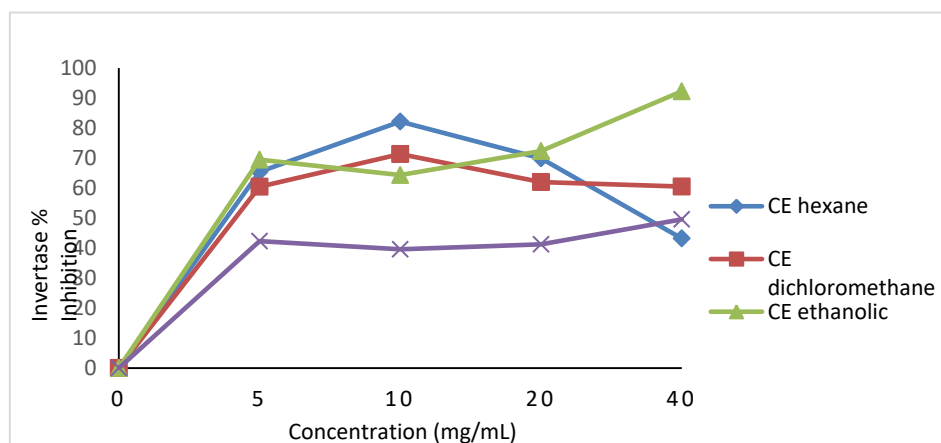


Figure 7. Invertase inhibitory activity of hexane, dichloromethane, ethanolic and aqueous extracts of *C. edulis* stem bark extract

Values represent mean \pm standard deviation, n = 3

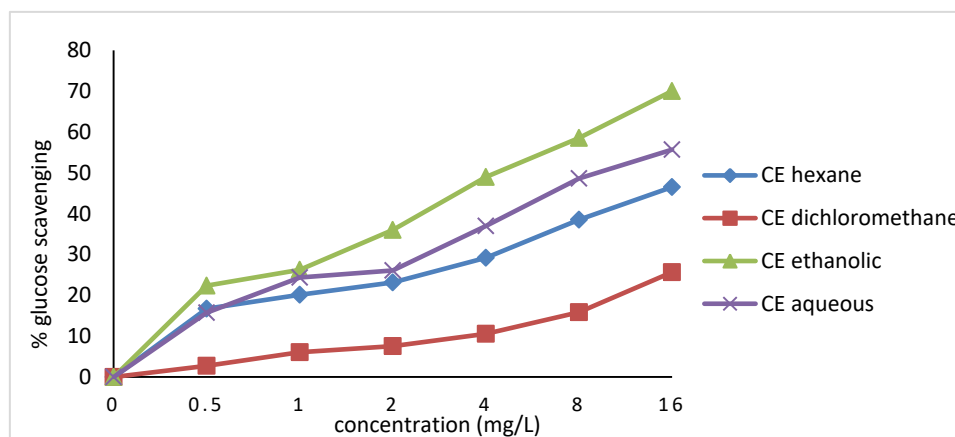


Figure 8. Glucose scavenging activity of hexane, dichloromethane, ethanolic and aqueous extracts of *C. edulis* stem bark extract

Values represent mean \pm standard deviation, n = 3

Table 2. Different values of IC₅₀ of *C. edulis* extracts on the different radicals tested

Plant extracts/ Chemical	IC ₅₀ values (μ g/mL) of radical scavenged					
	DPPH radical	ABTS radical	Hydroxyl radical	Nitric oxide radical	TAC Assay	Glucose scavenging
CE hexane	106.50 \pm 7.66 _b	210.30 \pm 6.11 _e	72.78 \pm 1.32 _d	79.21 \pm 1.36 ^c	115.00 \pm 13.18 _d	5737.0 \pm 263.4 ^c
CE dichloromethane	91.43 \pm 5.33 ^d	135.10 \pm 6.59 _d	44.98 \pm 1.38 _b	85.78 \pm 3.28 ^c	77.57 \pm 4.96 ^c	88992.0 \pm 370.4 _d
CE ethanolic	46.30 \pm 3.35 ^a	56.62 \pm 0.19 ^b	43.13 \pm 2.29 _b	63.33 \pm 1.75 ^b	60.75 \pm 2.42 ^b	887.9 \pm 21.23 ^a
CE aqueous	56.14 \pm 1.29 ^a	68.19 \pm 1.06 ^b _c	54.5 \pm 2.98 ^c	134.10 \pm 17.39 _d	58.09 \pm 0.58 ^b	1864.0 \pm 102.1 ^b
Ascorbic acid	39.86 \pm 4.55 ^a	34.75 \pm 0.51 ^a	31.06 \pm 0.83 _a	44.98 \pm 1.38 ^a	41.95 \pm 0.83 ^a	ND

Each value in the table is represented as mean \pm SD (n = 3). Values with different superscripts down the column are statistically different p < 0.05.

Table 3. Total phenolic, flavonoid and alkaloids contents *C. edulis* extracts

	Total phenolic (μ g catechin equivalent/g)	Total flavonoid (μ g quercetin equivalent/g)	Alkaloids (μ g quinin equivalent/g)
CE hexane	2.20 \pm 0.17 ^a	0.38 \pm 0.02 ^a	1.97 \pm 0.30 ^a
CE dichloromethane	3.95 \pm 0.90 ^a	0.45 \pm 0.02 ^a	1.74 \pm 0.12 ^a
CE ethanolic	289.11 \pm 30.31 ^c	1.12 \pm 0.09 ^b	5.54 \pm 0.59 ^b
CE aqueous	87.70 \pm 0.86 ^b	0.51 \pm 0.03 ^a	3.14 \pm 1.37 ^a

Each value in the table is represented as mean \pm SD (n = 3). Values with different superscripts down the column are statistically different p < 0.05.

Table 4. Different values of IC₅₀ of alpha - amylase and invertase activity of *C. edulis* extracts

IC ₅₀ values (mg/mL) of enzyme inhibition		
Plant extracts	alpha - amylase	invertase
CE hexane	634.00 ± 21.42 ^c	41.18 ± 0.49 ^c
CE dichloromethane	0.71 ± 0.01 ^a	14.72 ± 0.39 ^b
CE ethanolic	3.17 ± 0.03 ^b	13.39 ± 0.43 ^{ab}
CE aqueous	1.06 ± 0.02 ^a	13.03 ± 0.24 ^a

Each value in the table is represented as mean ± SD (n = 3). Values with different superscripts down the column are statistically different p < 0.05.

Table 5. Results of the Pearson correlation of the different *in vitro* antioxidant assays

Tests	ABTS	DPPH	OH	NO	TAC	Polyphenols	Flavonoids	Alkaloids
ABTS	1							
DPPH	0.943*	1						
OH	0.593	0.413	1					
NO	0.063	0.033	0.014	1				
TAC	0.958*	0.817*	0.673	0.115	1			
Polyphenols	0.571	0.728	0.305	0.111	0.386	1		
Flavonoids	0.477	0.599	0.367	0.229	0.319	0.966*	1	
Alkaloids	0.568	0.745	0.249	0.082	0.376	0.993*	0.934*	1

*: significant values $p = 0.05$ (bilateral test). ABTS: ABTS radical scavenging test; DPPH: DPPH radical scavenging test; OH: OH radical scavenging test; NO: NO radical scavenging test; Total antioxidant activity: Phosphomolybdenum test; Polyphenol: Polyphenol assay; Flavonoids: Flavonoid assay; Alkaloids: Alkaloid assay.

Table 6. Correlations between the IC₅₀ values of antioxidant activities and phenolic, flavonoids and alkaloids content of *C. edulis*

Assays	Correlation R		
	Polyphenols	Flavonoids	Alkaloids
IC ₅₀ of glucose scavenging ability	0.239	0.131	0.308
IC ₅₀ of alpha amylase inhibition	0.211	0.204	0.183
IC ₅₀ of invertase inhibition	0.238	0.223	0.212

Each value in the table is represented as mean ± SD (n = 3).

3.3 Scavenging Potential of Different Radicals

3.3.1 Anti-radical Activity on DPPH

The scavenging effects of samples on DPPH radical (Figure 1). All the extracts demonstrated scavenging activities which varied depending on the solvent used. The scavenging effects were in the following order: CE EtOH > CE Aq > CE DCM > CE hexane. Then, the extract CE EtOH and CE Aq showed a higher inhibitory potential while they exhibited the lowest IC₅₀ values of scavenging DPPH radicals for the CE EtOH and CE Aq were 46.30 ± 3.35 and 56.14 ± 1.29 µg/ml, respectively (Table 2). Though the antioxidant potential of extracts was found to be low ($p < 0.05$) than those of ascorbic acid with the IC₅₀ in order of 46.30 to 106.50 µg/ml.

3.3.2 Anti-radical Activity for ABTS

Extracts of *C. edulis* have all shown a significant ability to trap the ABTS radical. However, this anti-radical power varied depending on the nature of the solvent and the concentration of extract (10 - 160 µg/mL) (Figure 1b). According to these results presented in Figure 2, the extracts can be classified according to their capacity to capture the ABTS radicals in the following order: CE Ethanol > CE Aqueous > CE DICHLOROMETHANE > CE hexane. These results also showed that the IC₅₀ of all the extracts varied from 56.62 to 210.30 µg/mL depending on the type of solvent used. However, these values remain significantly higher than that of ascorbic acid, the IC₅₀ value of which was 34.75 ± 0.51 µg/mL

3.3.3 Anti-radical Activity for Hydroxyl Radical

Hydroxyl radical scavenging activity was quantified by measuring the inhibition of the degradation of 2-deoxyribose by the free radicals generated by the Fenton reaction. The hydroxyl radical scavenging activity of *C. edulis* extract and its derived fractions can be ranked as CE EtOH > CE DCM > CE Aq > CE hexane (Table 2). All the extracts showed antioxidant activity in dose dependent manner at concentration 10 - 160 µg/ml (Figure 3). In the present investigation, the IC₅₀ value of hydroxyl radical scavenging activity for the CE EtOH and CE dichloromethane was 43.13 ± 2.29 and 44.98 ± 1.38 µg/ml while for ascorbic acid was 31.06 ± 0.83 µg/ml (Table 3). The markedly strong (p < 0.05) antioxidant response of CE EtOH in comparison with ascorbic acid.

3.4 Anti-radical Activity of Nitric Oxide

The results nitric oxide scavenging potential of the different extract samples represented in the Figure 4 showed that the inhibitory potential of the different samples increased with the concentration. CE EtOH extract showed the lower potential from the lower to the higher concentration. CE EtOH and CE hexane extracts exhibited the highest inhibitory potential (Figure 4) with the respective IC₅₀ value equal to 63.33 ± 1.75 and 79.21 ± 1.36 µg/ml. Nevertheless, the values of extracts are significant higher to ascorbic acid that IC₅₀ value was 44.98 ± 1.38 µg/ml.

3.5 Antioxidant Activity of Extracts by the TAC Method

The phosphomolybdate method or the total antioxidant capacity (TAC) of the different plant extracts are shown in the Figure 5. We noted that CE Aq extract had the higher antioxidant capacity using TAC method compared to the other extracts. The antioxidant capacity of various solvent fractions of *C. edulis* was found to decrease in this order: CE Aq > CE EtOH > CE DCM > CE hexane extracts. However, the phosphomolybdate antioxidant capacity, the IC₅₀ of CE Aq (58.09 ± 0.58 µg/ml) was higher compared to that of ascorbic acid (41.95 ± 0.83 µg/ml).

3.6 Protective Property of Extract Samples Against α-amylase and Invertase Activities

Figure 6 and Figure 7 showed the results of the effect of the extracts on digestive enzymatic activities. From Figure 6, we can observe that CE dichloromethane presented the α-amylase % inhibition higher than the other extracts with an IC₅₀ of 0.71 ± 0.01 µg/mL, followed by CE aqueous with an IC₅₀ of 1.06 ± 0.02 µg/mL. Figure 7 showed CE aqueous had the better invertase % inhibition effects. *C. edulis* effect on this enzyme decreased in this order: CE aqueous > CE ethanolic > CE dichloromethane > CE hexane. CE aqueous have IC₅₀ of 13.03 ± 0.24 µg/mL.

3.7 Protective Property of Extract Samples on Scavenging of Glucose

Figure 8 showed that CEE have an *in vitro* glucophage activity. All the extracts showed glucophage activity in dose dependent manner at concentration 50 - 1600 µg/ml. In the present study, the effect of these extracts are ranged in this order: CE EtOH > CE Aq > CE hexane > CE DCM. However, CE EtOH and CE Aq had presented the best effect on glucose scavenging activity with the IC₅₀ of 891.75 ± 31.06 µg/ml.

3.8 Polyphenols, Flavonoids and Alkaloids Contents

Table 3 summarizes the levels of polyphenols, flavonoids and alkaloids compounds in CEE ranging from the solvent. The obtained results showed that all the CEE content polyphenols with a level dependent of solvent. The results had expressed as micrograms of catechin equivalents by g dried extract for total polyphenol and as micrograms of quercetin equivalents by g dried extract for flavonoids.

Also, the CE EtOH (289.10 + 30.32) [µg catechin equivalent/g dried extract] and CE Aq (87.70 + 0.85 [µg catechine equivalent/g dried extract] have the highest polyphenol contents compared to other extracts. The highest concentration of flavonoid (1.12 + 0.09 µg quercetin equivalent/g dried ext.) was identified in the CE EtOH meanwhile the CE Aq had the second highest concentration of flavonoid with (0.51 + 0.03 µg quercetin equivalent/g dried extract). Further, CE EtOH presented the best levels of alkaloids with (1.12 + 0.09) µg quinine equivalent/g dried ext.).

The positive correlation between TAC, flavonoids contents and ABTS, OH, NO was noted (Table: 4). Further, a better correlation was observed between alkaloids and glucose scavenging, on the other hand between polyphenols and alpha amylase, invertase inhibition (Table 6).

4. Discussion

Free radicals are known to play a definite role in a wide variety of pathological manifestations. Antioxidants

fight against free radicals and protect us from various diseases. They prevent or delay the oxidation of the substrate (Proestos *et al.*, 2013). They exert their action either by scavenging the reactive oxygen species or protecting the antioxidant defence mechanisms (Umamaheswari and Chatterjee, 2008). Further, many synthetic drugs protect against oxidative damage but they have adverse side effects. An alternative solution to the problem is to consume natural antioxidants from food supplements and traditional medicines (Yazdanparast and Ardestani, 2007). Several studies demonstrated that plant derived antioxidants may be useful in preventing the deleterious consequences of oxidative stress (Matsinkou *et al.*, 2012; Nyangono *et al.*, 2013). This is a reason of natural antioxidant stimulates the research interest on the investigation of protective effects of natural antioxidants. This study demonstrated the free radical scavenging and antioxidant effects of *C. edulis* a Cameroonian non-timber forest product which the walnuts are used like foods and stem bark are used in local traditional plates. The phytochemical screenings of *C. edulis* different parts of plant have also been determined by Bukola *et al.*, (2008).

Stable DPPH radicals are widely used to evaluate the antioxidant activities of proton-donating substances according to hydrogen donating ability. DPPH· radicals accept electrons or hydrogen radicals to form stable diamagnetic molecules. The effect of extracts on DPPH· radical results for their hydrogen donating ability (Al-Temimi and Choudhary, 2013). This is associated with the decrease in absorbance of DPPH· radical along with a discoloration from purple to yellow.

The results showed an overall DPPH scavenging activity of all the tested extracts. Among them, CE EtOH and CE Aq extracts showed a higher DPPH scavenging potential. These results prove that *C. edulis* stem bark extracts act as proton donors. High scavenging ability in ethanolic extract may attribute to high level of antioxidant components in the extract, which react rapidly with DPPH radicals and reduce most DPPH radical molecules (Yeh *et al.*, 2013). Several studies stated that the polyphenols from plants extracts are responsible in a large part for the DPPH scavenging potential (Omoregie and Osagie, 2012)

ABTS assay measures the relative ability of antioxidant to scavenge the ABTS^{•+} generated in aqueous and organic solvent systems. In the presence of hydrogen-donating antioxidants, pre-formed radical cation reduced it to ABTS in a concentration-dependent manner. Our results showed that all the tested samples demonstrated a scavenging potential of the ABTS with varying inhibitory potential with the CE EtOH extract demonstrating the relative highest ABTS inhibitory power. This result confirmed that the presence of higher flavonoids level (Biskup *et al.*, 2013), could be explained by the structure, number and arrangement of their phenolic hydroxyl group responsible for these activities (Matsinkou *et al.*, 2012).

Biologically, the hydroxyl radical is widely believed to be generated when hydrogen peroxide reacts with Fe(II) (Fenton reaction). The hydroxyl radical has been described as a highly damaging species which can cause lipid peroxidation and enormous biological damage (Aurand *et al.*, 1977). When the sample is mixed with Fe(II), it may alter the activity of Fe(II) by chelation. Our IC₅₀ values indicate that the scavenging potential of the extracts increased proportional to the concentration, which is depicted by the low intensity of red colour. The extract CE EtOH exhibited a highest HO· scavenging power among all the extracts. Antioxidants in food (such as ascorbic acid) may act as pro-oxidants by reducing Fe (III) to Fe(II) and make the HO· generation catalytic. (Huang *et al.*, 2005).

It is well known that nitric oxide radical has an important role in various inflammatory processes. It is associated with several diseases (Tylor *et al.*, 1997). Oxygen reacts with nitric oxide to generate nitrite and peroxynitrite anions which act as free radicals. Under physiological conditions, peroxynitrite forms a compound with carbon dioxide dissolved in body fluid. This compound is believed to be responsible for the oxidative damage of proteins (Squadrito and Pryor, 2002). Our results showed that extracts can tightly inhibits nitrite formation by directly competing with oxygen in the reaction with nitric oxide. CE dichloromethane and CE Aq showed the lowest NO radical scavenging power whereas CE EtOH and CE hexane exhibited the highest NO· inhibitory potential. Previous research reported that the antioxidant activity of phenols against NO· radical is related to the quantity, number and position of hydroxyl groups in the molecule (Sahreen *et al.*, 2011).

Given that, the nature of the inhibition is ambiguous because there are several potential inhibition pathways, studies recommended several testing methods for the investigation of antioxidant activity of extracts from natural resources (Gordon, 1990, Huang *et al.*, 2005). The antioxidant capacity of the fractions was measured spectrophotometrically through phosphomolybdenum method, based on the reduction of Mo (VI) to Mo (V) by the test sample and the subsequent formation of green phosphate/Mo (V) compounds. The present study demonstrated that CE EtOH and CE Aq exhibited the highest antioxidant capacity for phosphomolybdate

reduction. Recent studies have shown that many flavonoid and related polyphenols contribute significantly to the phosphomolybdate scavenging activity of medicinal plants (Sharififar *et al.*, 2009, Khan *et al.*, 2012).

Polyphenols have antioxidant properties correlated to their chemical structure, the number and arrangement of their phenolic hydroxyl groups (Bravo, 1998; Pandey and Rizvi, 2009). That said, polyphenols are known to be the effective antioxidants in plants due to their hydroxyl groups. Scientists also said that the purpose of used solvents have different polarity to extract phenolic compounds from plants with a high degree of accuracy (Razali *et al.*, 2012). They were mentioned that the high polar solvents have a high effectiveness of antioxidants such as ethanol. The ethanol extract exhibited the highest total phenolic content, whereas the contents obtained with hexane extract were much smaller that is in agreement with other reports (Ao *et al.*, 2008).

Typical phenols with antioxidant activity have been characterized as phenolic acids and flavonoids. Flavonoids are one categories of phenolic compounds which are naturally occurring in plants and are thought to have positive effects on human health. Flavonoids have been shown to be highly effective scavengers of most oxidizing molecules, including singlet oxygen, and various free radicals (Bravo, 1998) implicated in several diseases. So comparable with the findings in the literature for other extracts of plant products (Sahreen *et al.*, 2011) our results suggested that phenolic acids and flavonoids may be the major contributors for the antioxidant activity as the IC₅₀ values of radical scavenging activity of various soluble fractions of *C. edulis* and the contents of polyphenols or flavonoids exhibited significant correlation.

Some alkaloids (ajmaline, ajmalicine and reserpine) has been developed in tablets of pharmaceutical formulations (Singh *et al.*, 2004). According to literature review ethanol was the better extracted solvent. Alkaloids are a group of bioactive compounds. It has been recognized that alkaloids show antioxidant property and their effects on human nutrition and health care are considerable (Kumpulainen and Salonen, 1999). Mechanism of action of alkaloids is through inhibition of peroxidation (Kessler *et al.*, 2003).

α -amylase (EC 3.2.1.1.) is an enzyme which catalyses the breakdown of starch into sugars in the mouth and small intestine. The inhibitory effects of dietary polyphenols against α -amylase have being proved by several researchers (Xiao *et al.*, 2013). CE dichloromethane and CE Aq had shown a significant ($p < 0.05$) better inhibition of α -amylase activity as a result of a low decrease of starch concentration in middle (Figure 6). Otherwise, Figure 7 showed that CE Aq and CE EtOH had the better inhibition effect on invertase activity. In fact, invertase (E.C. 3.2.1.26 - β -D-fructofuranosidase) is another digestive enzyme. It catalyses the sucrose hydrolysis producing an equimolar mixture of glucose and fructose named inverted sugar. This result is in agreement with a recent worked reported by Oboh *et al.*, (2010) where red and white ginger inhibited invertase activity *in vitro*. Polyphenols may affect glycaemia through different mechanisms, including the inhibition of glucose absorption in the gut or of its uptake by peripheral tissues (Pandey and Rizvi, 2009). Plant phytochemicals are mild inhibitors of α -amylase and strong inhibitors of invertase activity (Kwon *et al.*, 2007). A property that confers advantage over synthetic drugs such as acarbose; use by diabetics in the management of postprandial blood glucose, which strongly inhibit α -amylase.

Glycosylation is the reaction in which a carbohydrate, such as a glycosyl donor, is attached to a hydroxyl or other functional group of another molecule. Our results further show that CEE were potent *glycophagic* compounds. Nevertheless, CE EtOH had presented the better *glycophagic* effect probably due to the level of polyphenols. Many compounds such as proteins, polyphenols that have a glycosylation activity (Xiao and Kai, 2011). Thereafter, these researchers said, glycosylation of flavonoids decreases the affinities for plasma proteins by 1–3 orders of magnitude depending on the conjugation site and the class of sugar moiety, along with oxidative stress.

5. Conclusion

On the basis of the results obtained in the present study, it is concluded that, ethanolic extract of *C. edulis* stem bark, which contains large amounts of phenolic, flavonoids and alkaloids compounds, exhibits high antioxidant and free radical scavenging activities. CEE also chelate glucose and inhibited α -amylase and invertase (key enzyme linked to type-2 diabetes) activities. These *in vitro* assays indicate that this plant extract is a significant source of natural antioxidant, which might be helpful in preventing the progress of various oxidative stresses.

Author Contributions

EB and BGKA designed the study; EB performed the laboratory analysis; EB and MMA performed the statistical analysis; EB and BGKA performed the data interpretation. BGKA drafted the manuscript; JEO supervised the study. All authors read and approved the final version of the Manuscript.

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Conflicts of Interest

The authors declare there is no conflict of interest.

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