IJPSR (2022), Volume 13, Issue 2 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 24 April 2021; received in revised form, 24 June 2021; accepted, 28 June 2021; published 01 February 2022

IN-VITRO AND *IN-VIVO* ANTIOXIDANT PROPERTIES OF STEM EXTRACTS OF *CROTON MEMBRANACEUS*

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Keywords:

Croton membranaceus, Stem extracts, Flavonoid, Phenolic, antioxidant activity

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ABSTRACT: *Croton membranaceus* is a tropical plant that grows wildly in some West African countries. Though several parts of this plant are used for the treatment of diverse disease conditions, its root extracts are traditionally used mainly in Ghana for the management of prostate diseases. This present study aimed to investigate the *in-vitro* and *in-vivo* antioxidant activities of the stem extracts of *C. membranaceus* and assess their total phenolic and flavonoid contents. DPPH free radical scavenging method was employed in the preliminary screening for antioxidant activities of the direct aqueous (CMASE) and sequential fractions (SECM) of the pulverized stem of *C. membranceus*: hexane, ethanol, ethyl acetate, and aqueous. *In-vivo* antioxidant effects of CMASE on liver antioxidant enzymes/markers [Glutathione (GSH), catalase (CAT), superoxide dismutase (SOD), and malondialdehyde (MDA)] in Sprague-Dawley (S-D) rats after 90 days were also investigated. *In-vitro* investigations revealed that all SECM and CMASE had some antioxidant activities. Ethanolic and ethyl acetate fractions exhibited the highest antioxidant activities with EC_{50} values of 0.35 and 1.41 mg/ml. Phenols were present in the SECM and CMASE. Flavonoids were present in all the fractions except the CMASE and sequential aqueous fraction. CMASE induced non-significant dose-dependent elevations of mean liver SOD, GSH, and CAT levels but marginal decline in MDA levels in the treated rat groups. SECM and CMASE possess mild antioxidant activities *in-vitro* and *in-vivo*, contributing to mitigating oxidative stress-related diseases such as benign prostatic disease.

INTRODUCTION: Oxidative stress is a condition in which there is an imbalance in the rate of release or production of free radicals in the body compared to its detoxification, hence resulting in oxidative tissue damage [1].

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.13(2).921-29

This article can be accessed online on www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.13(2).921-29

The production of by-products of cell metabolism such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) leads to significant decline in antioxidants defense systems with associated damage to deoxyribonucleic acid (DNA), protein, and lipid in cells and, ultimately, cell death [2].

Lipid peroxidation results in a number of degradation products such as malondialdehyde (MDA) in biological systems that is considered to be an important cause of cell membrane destruction and cell damage [3]. Free radicals have also been implicated in the pathogenesis of many diseases,

including cardiac arrhythmia, hypertension, atherosclerosis, diabetes, and cancer [4]. Antioxidants are agents that delay or inhibit cellular damage mainly through their free radical scavenging property, through their ability to donate electron(s) to neutralize free radicals and reduce their capacity to damage cells and induce associated diseases [5]. Antioxidants can be classified as natural or synthetic. In recent times there has been growing interest in the potential health benefits of dietary plant polyphenols as antioxidants due to safety concerns like nephrotoxicity, hepatotoxicity, and carcinogenicity associated with synthetic antioxidants such as butylated hydroxyl toluene, propyl gallate and terbutylhydroquinine [7].

Thus, there has been an increased demand for the use of non-toxic, natural preservatives; many are likely to have either antioxidant or antimicrobial activities [8]. High concentrations of phytochemicals with antioxidant properties such as phenolic compounds; phenols and phenolic acids, flavonoids, carotenoids, anthocyanins, tannins, lignans, tocopherols, as well as vitamins A, C, and E may protect against free radical damage, and these accumulate in fruits and vegetables [8, 9]. Phenolic compounds and flavonoids have been associated with anti-oxidative actions in biological systems [10]. Antioxidant activity of phenolic compounds is due to their ability to scavenge free radicals, donate hydrogen atoms or electrons, or chelate metal cations [11]. Flavonoids are also known as strong metal chelators that inhibit lipid peroxidation [12].

High production levels of ROS could result in significant decrease in cell antioxidant defense enzymes such as; catalase (CAT), glutathione S-transferase (GST), glutathione peroxidase (GPx), superoxide dismutase (SOD) and glutathione reductase (GR), thus inducing oxidative stress with subsequent damage to protein, lipid, DNA, disruption of cellular functions and cell death [13, 14]. The stem and root parts of *C. membranaceus* have been of great medicinal interest in Ghana principally for the management of benign prostatic hyperplasia (BPH) and related cancers. *C. membranaceus* bears a characteristic pleasant odour in all parts. Recent pharmacological and toxicological studies on the aqueous root extract of *C. membranaceus* revealed its safety in both acute and subchronic toxicity studies. Furthermore, *C. membranaceus* possesses antimicrobial, anticancer, anti-diabetic, anti-antherogenic, and anti-ischaemic potentials as well as prostate organ targeting activity [15, 16]. A study on the ethanolic root extract of *C. membranaceus* revealed its antioxidant properties [17], however, there is no related studies on the stem extracts. Thus, this study sought to evaluate the antioxidant potentials of the stem extracts of *C. membranaceus*, as this may be associated with its mechanism of action in prostate shrinkage.

MATERIALS AND METHODS:

Plant Collection and Identification: Whole stems of *C. membranaceus* were collected in the morning (9 am 10 am) from Mampong- Akwapem. It was authenticated and a voucher specimen of the plant (UCCG-DCOP-007) was deposited at the Herbarium of the Plant and Environmental Biology Department of the University of Ghana.

Plant Extraction:

Preparation of Aqueous Stem Extract of *C. membranaceus* (CMASE): The choice of the aqueous stem extraction of *C. membranaceus* (CMASE) was based on folkloric preparation. The modified validated procedure [16] was used to prepare the extract (CMASE). Briefly, the stem parts of *C. membranaceus* were thoroughly washed, air-dried for three weeks, machine-crushed into powder, packed into labeled zip lock bags and stored at room temperature (25 ± 2 °C). Subsequently, 1 kg of pulverized stem of *C. membranaceus* was macerated for 24 h with four litres (4L) of distilled water, and heated for 1 h at 100 ºC on a water bath.

The extract was then filtered using sterile gauze and refrigerated. Three litres (3L) of distilled water was again added to the sediments, macerated for another 24 h and the previous process was repeated to obtain the second aqueous extract and refrigerated. The latter procedure was repeated to obtain the third extract. The extracts were pooled, freeze dried, total dry crude extract determined and stored in a labeled and sealed container in a refrigerator (2-8 °C) until used. This extract obtained directly from the pulverized stem of *C. membranaceus* in this study was labelled direct aqueous extract of *C. membranaceus* (CMASE).

Sequential Extraction for Hexane, Ethyl Acetate, Ethanol and Aqueous Fractions From Pulverized Stem of *C. Membranaceus* (SECM): The pulverized stem of *C. membranaceus* was cold macerated and sequentially extracted using hexane, ethyl acetate, ethanol and lastly using hot water extraction for the last residue as described by Appiah-Opong [18]. A hundred grams (100 g) of pulverized stem of *C. membranaceus* was weighed on an analytical balance (Meettler Toledo × P105, USA) and transferred into an Erlenmeyer flask. Five hundred milliliters (500 ml) of hexane was then added to it and the content was placed on an electric shaker (Yamato Shaker, SA-31, Japan) for 24 h at 300 rpm. Subsequently, the content was filtered using Whatman Filter paper, Grade 91, diam. 150 mm.

The procedure was repeated twice (and the residue air-dried for further extraction). The filtrate of each solvent obtained was pooled, subjected to a rotary evaporator (BuchiRotavapor, pump VAC V-500, Germany) to obtain hexane extract. Ethyl acetate and ethanolic extracts were sequentially obtained using the air-dried residue of *C. membranaceus* from previous procedure. Aqueous extraction was carried out using the hot extraction method. Thus, five hundred milliliters (500 ml) of distilled water was added to the air-dried residue obtained after the ethanolic extraction. The content was then heated in UGO Basile Julabo GmbH Water Bath, Germany, at 80 °C for 1 h and filtered using Whatman filter paper, Grade 91, diam. 150 mm. The procedure was repeated and filtrates obtained were pooled, freeze-dried using LTE scientific 18 kg ice capacity Lyotrap freeze dryer (United Kingdom) to obtain the lyophilized sequential aqueous stem extract of *C. membranaceus* (SAQ).

***In-vitro* Antioxidants Assay:**

1, 1-Diphenyl-2-Picryl-Hydrazyl (Dpph) Assay: Free radical scavenging activities of SECM and CMASE were determined by the DPPH method [19]. DPPH is a stable nitrogen-centered free radical, has a deep violet colour in methanol solution, which turns colourless or pale yellow (diphenylpicrylhydrazine) upon reduction by either the donation of a hydrogen atom or an electron (when neutralized) by an antioxidant. Briefly, 20 mg of the various fractions (hexane, ethyl acetate, ethanolic, sequential aqueous (SAQ), direct aqueous (CMASE) were dissolved in 1.0 ml of methanol to obtain a stock solution of 20 mg/ml. Two-fold serial dilutions of the stock were made to obtain concentrations of 10, 5, 2.5, 1.25, 0.625, 0.3125 and 0.015625 mg/ml. Two-fold serial dilutions of the positive control, butylated hydroxyl toluene (BHT) [1 mg/ml in absolute methanol] (St. Louis, MO, USA), were made to obtain concentrations of 0.5, 0.25, 0.125, 0.0625, 0.03125, and 0.015625 mg/ml.

A volume of hundred (100) microliters for each of the fractions and BHT dilutions were pipetted separately in triplicates into 96-well plates. Another volume of 100 μL of 0.5 mM DPPH solution (Steinheim, Germany) was added to each of the wells to obtain a total volume of 200 μL. Methanol was used as blank. The 96-well plate was gently shaken to mix the content. The plates were then covered with aluminium foil and incubated in the dark at room temperature (27 °C) for 20 min. The absorbance was read at a wavelength of 517 nm using a Tecan Infinite M200 (Austria) microplate reader. The percentage inhibition for each extract and BHT was calculated from the following formula:

$$\% \text{ Antioxidant activity [DPPH Scavenging]} = [(A_0 - A_1)/A_0 \times 100]$$

The scavenging activity was expressed as a percentage of the ratio of the decrease in absorbance of the test solution to that of DPPH solution void of the extracts. A0 is the absorbance of control (methanol), and A1 is the absorbance of test sample with DPPH. Butylated hydroxytoluene (BHT) was used as standard control. Triplicate experiments were performed for each sample/ fraction. The EC_{50} values of the reference standard and fractions, which is the concentration of the extracts that can cause 50% free radical scavenging activity, were determined from non-linear regression curves using the Graph Pad Prism version 6.0 software (San Diego CA, USA).

Total Phenolic Assay (TPC): Total phenolic content of CMASE and SECM were determined using the Folin-Ciocalteu assay as described with slight modification [19]. Stock solutions of the fractions (hexane, ethyl acetate, ethanol, SAQ and CMASE) were prepared by dissolving 5 mg of each sample in 1.0 ml of their respective solvents in an

Eppendorf tube (ethanol for hexane, ethyl acetate and ethanolic fraction, whilst distilled water was used for the aqueous fractions). Two-fold serial dilutions of this stock were made to obtain concentrations of 5.0, 2.5, and 1.25 mg/ml for each fraction. The standard (1 mg/ml) was prepared by dissolving 1.0 mg of gallic acid in 100 μL of absolute ethanol and topped up to 1000 μL with 900 μL of distilled water. Two-fold serial dilutions were made to obtain concentrations of 0.5, 0.25, 0.125, 0.0625, 0.03125 and 0.015625 mg/ml. Ten microliters (10 μL) of each of the sample dilutions (concentrations) and the standard was pipetted separately in triplicates into 24-well plates already containing 790 μL distilled water.

A volume of 50 μL of Folin-Ciocalteau reagent (Buchs, Switzerland) was then added to each well and incubated for 8 min. A volume of 150 μL of sodium bicarbonate solution (0.2 g/ml) was then added (to obtain a total volume of 1000 μL). The plates were incubated at room temperature (27 °C) for 120 min, and the absorbance read at 750 nm using a microplate reader (Tecan Infinite M200, Austria). The phenolic concentration of the extracts was evaluated from a gallic acid calibration curve. Total phenolic content in the fractions of the extract was expressed as Gallic Acid Equivalent (GAE) in grams per 100 g dry weight of plant sample (C. membranaceus).

Total Flavonoids Content (TFC): Total flavonoid assays of SECM and CMASE were determined as described with slight modification [20]. Five milligrams (5 mg) of the fractions (hexane, ethyl acetate, ethanolic, SAQ, CMASE) were dissolved in 1.0 ml of their respective solvents to obtain stock solution of 5 mg/ml. Two-fold serial dilutions of the various fractions of the extract (5, 2.5, and 1.25 mg/ml) were prepared from their stock concentrations of 5 mg/ml. Using Quercetin (Buchs, Switzerland) as standard (1 mg/ml methanolic stock solution) serial dilutions were prepared to obtain 0.1, 0.05, 0.025, 0.0125, 0.00625, 0.003125, 0.0015625, 0.000781, 0.000391 and 0.000195 mg/ml for calibration. Then 100 µl of each of the sample dilutions and the standard were pipetted separately into a 96-well plate. Furthermore, 100 µl of 2% aluminium chloride solution (2 mg/100 ml methanol) was added to each concentration in the 96-well plate and incubated for 20 min at room temperature (25 ± 2 °C), and absorbance was determined at 415 nm using a microplate reader (Tecan Infinite M200, Austria). The experiment was carried out in triplicates. The calibration curve was plotted from the various concentrations of quercetin against absorbance. The quercetin equivalence in each of the fractions was extrapolated from the curve. Total flavonoid content (TFC) was expressed as milligrams of quercetin equivalent (RE) per 100 grams of the dry pulverized stem of *C. membranaceus* (mg Quercetin/ 100 g sample).

Experimental Animals: Male Sprague-Dawley (S-D) rats weighing between 100-200 g were purchased from Noguchi Memorial Institute for Medical Research (NMIMR) animal experimentation unit. The animals were acclimatized to laboratory conditions for 7 days prior to the experiment and were housed in metabolic cages. The rats were maintained at a room temperature of 25-27 °C, with a 12 h light/dark cycle. During acclimatization, the rats were fed with standard pellet formulation from AGRICARE, Ghana and water ad libitum. All animal experimentations were conducted in line with the requirements and approval of the University of Ghana Institutional Animal Care and Use Committee at the NMIMR, Legon, Accra. The ethical clearance number issued was UG-IACUC 003/18-19.

***In-vivo* Antioxidants Assays on Liver:**

Sub Chronic Effects of CMASE on Liver Antioxidant Enzymes: The sub-chronic study was done following the protocol of OECD guideline 408 for testing chemicals [21]. Twenty male Sprague-Dawley rats were randomly assigned into four groups of five rats each: control, low (30 mg/kg), median (150 mg/kg), and high (300 mg/kg) dose groups. Freshly prepared aqueous stem extract of *C. membranaceus* (CMASE) was administered orally by gavage daily for 90 days at single doses of 30, 150 and 300 mg/kg, whilst the control group received only distilled water. The rats were weighed weekly, and visual observations for mortality, behavioral patterns, changes in physical appearance, injury, pain, and signs of illness were conducted once daily during that period. At the end of the experiment (91st day), all animals were anesthetized with ether. Blood samples were

collected via cardiac puncture, rats were sacrificed, and half of the liver organs from each rat in every group were incised, placed in a labelled plastic envelope, and frozen at -80 °C until analyzed for antioxidant enzyme levels.

Preparation of Tissue Homogenates for Oxidative Stress Markers Determination: A volume 5 ml TNG buffer (50 mM Tris pH 7.4, 0.1M NaCl, 10% glycerol) was added to each frozen tissue in a mortar (on ice pack) and homogenized. The homogenized tissue was centrifuged at 4000 × g for 20 min in a refrigerated centrifuge. The supernatant (sample) was pipetted into an Eppendorf tube, labeled, and preserved (on ice packs) for various antioxidant enzyme assays. This procedure was done for liver tissue obtained from each rat in all four groups.

Reduced Glutathione (GSH) Estimation: The glutathione levels were estimated using the method described by Alam [22] with slight changes. The prepared liver samples (50 µl) were aliquoted into 96 well plates. Sodium phosphate (0.1M, 50 µl) was then added to each of the samples, followed by the addition of ortho-phthalaldehyde (OPA) (10 mg/ml, 10 µl) to start the reaction. The mixture was incubated for 15 min at room temperature. Afterward, the fluorescence absorbance was measured at 460 nm (emission) and 340 (excitation) against blank using a microplate reader (Tecan M200 Infinite Pro). The absorbance values were compared with a standard curve generated from known GSH values.

Catalase (CAT) Estimation: The catalase activity was determined using the method by Hadwan [23] with slight modifications. Briefly, the various samples (50 µl) were transferred into 1.5 ml eppendorf tubes (each sample/tube) and its control. Freshly prepared hydrogen peroxide (65 mM, 500 µl) was then added. Distilled water was added to the sample controls in place of hydrogen peroxide. The mixture was vortexed and incubated for 3 min at 37 °C. After wards, dichromate and acetic acid solution (50 µl of 5% aqueous solution of potassium dichromate with 150 µl of glacial acetic acid, 1 ml) was added and incubated at 100 °C for 10 min. After cooling with tap water, they were centrifuged to remove precipitated protein (2500 g for 5 min), the changes in absorbance were recorded at 570 nm against the reagent blank and catalase activity was determined.

Lipid Peroxidation: The lipid peroxidation assay was conducted using the method described by Ohkawa [24] with slight changes in volumes of reagents and samples. Briefly, the samples (20 µl) were transferred into Eppendorf tubes. The following reagents were added: 20 µl of 8.1% (w/v) sodium dodecyl sulphate, 150 µl of 20% acetic acid and 150 µl of 8% (w/v) Tris butyrate acetate. Distilled water was added to make a total volume of 400 µl. The mixture was incubated in a water bath at 95 °C for 60 min. After cooling with water, the volume was topped up to 500 µl with distilled water. Five hundred micro-liters (500 µl) of the butanol: pyridine mixture (15:1) were added and vortexed thoroughly. The mixture was centrifuge 3000 rpm for 10 min. The upper layer was aliquoted, and the absorbance read at 532 nm against the appropriate blank (without sample). The levels of lipid peroxide were expressed in moles (n) of thiobarbituric acid reactive substances (TBARS)/mg protein with an extinction coefficient of $1.56 \times 105\ MLcm^{-1}$.

Superoxide Dismutase (SOD) Estimation: The SOD activity was determined using a method described by Alam [22] with slight modifications. Samples (70 µl) were aliquoted into 96 well plates. Tris-HCl buffer (75 mM, 200 µl, pH 8.2 containing 30 mM EDTA) was then added. After which pyrogallol (2 mM, 30 µl) was added. The absorbance was measured at time intervals of 0, 3, and 5 min at 420 nm. The enzyme activity is 50% inhibition of the rate of auto-oxidation of pyrogallol as determined by change in absorbance/min at 420 nm. The activity of SOD was expressed in units/mg protein.

Statistical Analysis: All values are expressed as mean ± SEM. Comparisons between groups were performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests using Graph Pad 6 prism software. A p-value of < 0.05 was considered significant.

RESULTS AND DISCUSSION:

Antioxidant Activity of SECM and CMASE: The DPPH radical scavenging activities of the

SECM and CMASE assay revealed that all extracts possessed antioxidant activity. Based on effective concentration (EC_{50}) values obtained, the order of increasing antioxidant activities of the various fractions in scavenging 50% of the DPPH free radicals was; hexane, CMASE, SAQ, ethyl acetate, and ethanolic **Table 1.** The ethanolic fraction of the stem of *C. membranaceus* possessed the highest antioxidant activity (EC_{50} = 0.35 ± 0.001 mg/ml) when compared to the rest of the fractions. There were significant ($p<0.001$) differences in the EC_{50} values obtained between all the various fractions except between SAQ and CMASE.

TABLE 1: EC_{50} VALUES OF CMASE AND SECM

Test Fractions/compound	EC_{50} (mg/ml)	P-value
BHT	0.02± 0.001	
SAQ	2.57 ±0.011***	
CMASE	3.44 ±0.001***	<0.001
Ethanolic	0.35 ±0.001***a	
Ethyl acetate	1.41± 0.011***b	
Hexane	8.57 ± 0.001***	

Keys: BHT: -2, 6-di-tert-butyl-4-methylphenol (1 mg/ml). Values represent mean ± SEM from triplicate experiments. '***'p<0.001 BHT compared to fractions/CMASE from stem of *C. membranaceus* (CS). Concentrations of CS extracts were 5 mg/ml. 'a' p<0.001 compared to rest of *C. membranaceus* extracts. 'b' p<0.001 compared to rest of CS fractions except ethanolic fraction.

DPPH is used to evaluate the free radical scavenging capacity of several natural or synthetic compounds. Extracts of the stem, leaf, and essential oils obtained from some species of Croton such as *Croton hypoleucus*, *Croton lundianus*, and *Croton urucurana* have been found to possess good antioxidant properties [25 - 28].

The relatively high presence of phenolic and flavonoids in the ethanolic and ethyl acetate fractions respectively compared to the other fractions could be responsible for their high antioxidant activities observed in this study. EC_{50} value obtained for the ethanolic fraction revealed it possessed significantly higher antioxidant properties compared to the rest of the sequential fractions and CMASE. Furthermore, the SAQ possessed almost twice antioxidant activity compared to the CMASE. According to Blois [29], EC_{50} values of plants extracts lower than 50 µg/ml are considered very strong antioxidants, values of 50-100 µg/ml are considered a potent antioxidant, values between 101-150 µg/ml possess medium antioxidant activity, whilst greater than 150 µg/ml are considered weak antioxidants. Per Blois's categorization of the plant antioxidant activity levels, even the EC_{50} value obtained for the fraction with the highest antioxidant activity in this study was greater than 150 µg/ml, thus suggesting that all the fractions analyzed in this study could be considered weak antioxidants. A similar study on the ethanolic root extract of *C. membranaceus* revealed its antioxidant activity with an EC_{50} value of 0.100 mg/ml [17]. A comparison of the latter study results to findings in this study which EC_{50} of ethanolic stem fraction was 2.57 mg/ml, suggests that the ethanolic root extract possesses much higher antioxidant activity than the ethanolic stem fraction. Previously mentioned studies have reported the potential of antioxidants of plant origin in reducing the risk of arteriosclerosis, cardiovascular diseases, and some forms of cancer. Consequently, the presence of antioxidant activity in all SECM, and CMASE suggests their potential ito reducethe risks of oxidative dtress-related diseases among patrons especially the aqueous and ethanolic stem extracts of C. membranaceus,

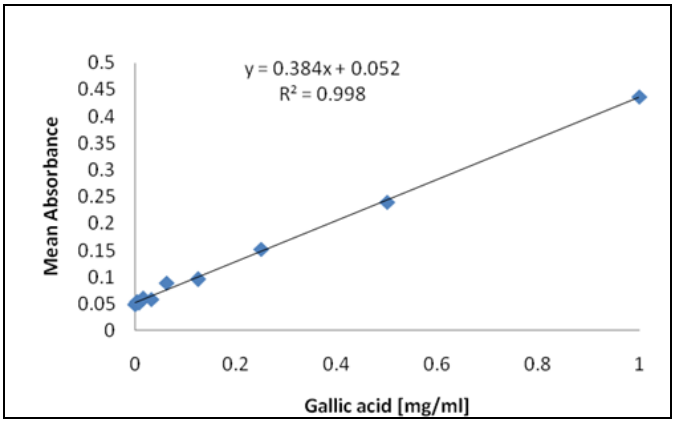

FIG. 1: GRAPH SHOWING MEAN ABSORBANCE FOR VARIOUS CONCENTRATIONS OF GALLIC ACID USED FOR PHENOLIC ASSAY.

Total Phenolic Content of the SECM and CMASE: The absorbance of series of gallic acid concentrations was plotted to yield a linear calibration curve of gallic acid, as seen in **Fig. 1.** The total phenolic content for 2.5 mg/ml concentration of the sequential fractions and CMASE of the pulverized stem of *C. membranaceus* were extrapolated from the

calibration curve for gallic acid standard (y= 0.384 × +0.052, R2 =0.998).

Table 2 shows the results of the presence of phenols in all the fractions obtained from the pulverized stem of *C. membranaceus*, with ethanolic and aqueous fractions possessing the highest contents. The ethanolic extract possessed a significantly higher amount of phenols ($p<0.001$) when compared to the rest of the fractions. SAQ and CMASE possessed a significantly higher amount of phenols ($p<0.01$) when compared to the ethyl acetate fraction, and also possessed a significantly ($p<0.001$) higher amount of phenols than hexane. There was no significant difference in phenolic contents between SAQ and CMASE. Similar observation was made between the ethyl acetate and hexane fractions. Phenolic compounds are known to possess antioxidant activity based on their scavenging and chelating properties, in addition to other physiological functions such as antimutagenic, antitumor activities and their ability of modifying gene expression.

The phenolic contents of SAQ and CMASE were similar, suggesting that the different aqueous extraction methods did not significantly affect the phenolic contents of extracts from the stem of *C. membranceus*. Furthermore, phenols in the SECM and CMASE fractions underpins the antioxidant activities observed in these extracts. However, a study reported that the presence of other secondary metabolites in extracts such as volatile oils, carotenoids, and vitamins could also complement or augment the antioxidant capacity of phenolic and flavonoid compounds [30].

TABLE 2: TOTAL PHENOLIC CONTENT OF SECM AND CMASE

Fractions	Total phenolic content (TPC) [g/100g Gallic acid equivalent-GAE]
SAQ	9.72 ±0.58
CMASE	9.85± 1.23
Ethanol	17.46 ± 1.91
Ethyl acetate	4.63 ± 0.74
Hexane	3.16± 0.16

Total Flavonoid Content (TFC) In SECM and CMASE: The content of flavonoids was expressed in terms of quercetin equivalents using the regression equation of the calibration curve obtained from quercetin (y = 0.034 × + 0.015, R2 = 0.990) **Fig. 2.**

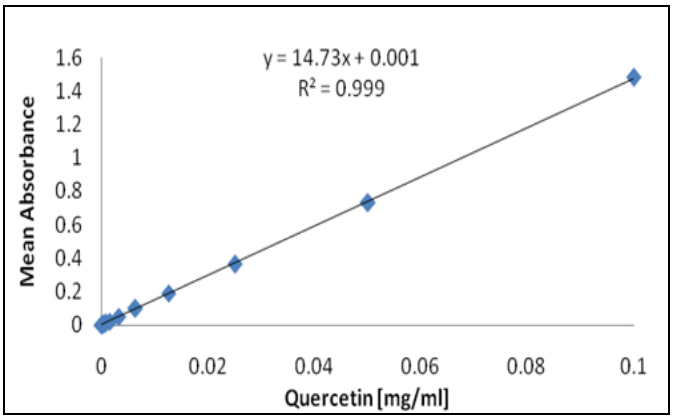

FIG. 2: SHOWS THE MEAN ABSORBANCES OF VARIOUS CONCENTRATIONS OF QUERCETIN (MG/ML)

The detailed results on the flavonoid content assay on SECM and CMASE are presented in **Table 3.** Results revealed that there were no flavonoids in SAQ and CMASE. Ethyl acetate fractions significantly possessed the highest total flavonoid content compared with ethanolic ($p<0.01$) and hexane ($p<0.001$) fractions, respectively. Furthermore, the ethanolic fraction of flavonoids possessed significantly ($p<0.01$) higher content compared to the hexane fractions.

The study revealed that flavonoids were present in all the fractions except the SAQ and CMASE. Thus, the antioxidant potentials of CMASE and SAQ could be attributed to mainly its phenolic content, as several studies have shown a linear correlation between antioxidant activity and phenolic content of plant extracts. Also, the potential antioxidant activities of the aqueous extracts may also be attributed to other secondary metabolites other than flavonoids [31]. The flavonoid content in the ethyl acetate fraction was the highest among the fractions assayed and may be the main secondary metabolite responsible for its antioxidant activity.

TABLE 3: TOTAL FLAVONOIDS CONTENT (TFC) IN SECM AND CMASE

Fractions	TFC (mg/100g)
SAQ	NIL
CMASE	NIL
Ethanol	187± 25.00
Ethyl acetate	550± 64.00
Hexane	25.25± 7.68

Key: TFC was expressed as mean ± S.E.M in terms of a milligram of quercetin equivalents per 100 grams of dry weight of plant (mg TFC/100g).

Effect of Sub-Chronic Doses of CMASE on Liver Antioxidative Stress Markers: Oral administration of sub-chronic doses (30, 150, and 300 mg/kg) of CMASE to S-D male rats during 90 days did not have any significant effect on liver antioxidative stress markers; SOD, GSH, CAT, and MDA examined. However, dose-dependent elevations of mean liver values were observed in SOD, GSH and CAT, whilst there was marginal dose-dependent decline in mean MDA values. Mean SOD values obtained showed marked elevations in the low, median and high dose group values of 3.40 ± 0.90, 3.70 ± 0.47 and 4.84 ± 0.96 U/ml, respectively, compared to the control value of 2.00 ± 0.00 U/ml. Mean serum GSH values obtained also showed a consistent increase in values in the low, median and high dose groups being 2.75 ± 0.18, 2.82 ± 0.33, and 3.24 ± 0.25 × 10-7 mg/ml respectively, whilst control value was 2.21 ± 0.14x 10-7 mg/ml. The mean serum CAT values obtained in the low, median, and high dose groups were 2.32 ± 0.16, 3.22 ± 0.87, and 3.23 ± 0.48 kU/L, respectively compared to control value of 2.08 ± 0.75 kU/L. The levels of mean serum MDA of the control group at the end of this study was 0.32 ±0.07 μmol/mg, whilst values obtained in low, median and high dose group were 0.33 ± 0.17, 0.32 ± 0.12 and 0.31 ± 0.04 μ mol/mg, respectively.

High levels of ROS as a result of oxidative stress can affect antioxidant defense mechanisms and decrease SOD, CAT, and glutathione peroxidase activity levels, with subsequent disruption of cellular functions and cell death [32]. These vital antioxidant enzymes scavenge reactive oxygen species (ROS) such as superoxide, hydroxyl radicals and other reactive products produced in mammalian tissues during biochemical processes. The antioxidant potentials of CMASE observed in this study appear to confirm observations from similar antioxidant studies on other Croton species mentioned earlier. It is worth noting the related studies reported strong antioxidant potentials whilst this study revealed mild antioxidant potentials of CMASE. Additionally, the absence of flavonoids in the CMASE to complement other phenolic constituents' antioxidant activities could have contributed to the non-significant change in serum MDA, as flavonoids are known as strong metal chelators that inhibit lipid peroxidation. A study reported the association between the development, progression of BPH and prostate cancer (PCa), and response to drug therapy with oxidative stress [33]. Observations of the antioxidant potentials of CMASE from the DPPH assay and in this sub-chronic toxicity study suggest its mild antioxidant potential could complement other mechanisms in the management of BPH, PCa, and other oxidative stress-related chronic diseases.

CONCLUSION: The *in-vitro* and *in-vivo* antioxidant activities of the extracts from the stem of *C. membranaceus* observed in this study could be attributed partly to the presence of phenolics and flavonoids. However, the folkloric aqueous preparation (CMASE) antioxidant properties in vitro and *in-vivo* could be attributed to phenolic and other compounds but not flavonoids. To the best of our knowledge, this is the first study to report on the total phenolic and flavonoid content of extracts from the pulverized stem of *C. membranaceus* and their antioxidant activities. Further studies to ascertain another mechanism(s) of the CMASE in BPH and PCa management are recommended.

ACKNOWLEDGEMENT: We are grateful to the Department of Biomedical Sciences, the University of Cape Coast, for facilitating and supporting the successful completion of this study.

CONFLICTS OF INTEREST: The authors declared no conflict of interest.

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How to cite this article:
Afriyie DK, Ofori-Ameyaw E, Acheampong DO, Tuffour I and Appiah-Opong R: *In-vitro* and *in-vivo* antioxidant properties of stem extracts of croton membranaceus. Int J Pharm Sci & Res 2022; 13(2): 921-29. doi: 10.13040/IJPSR.0975-8232.13(2).921-29.