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Antioxidant and nephroprotection activities of *Combretum micranthum*: A phytochemical, in-vitro and ex-vivo studies

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Abstract

Management of chronic renal failure is exceedingly expensive. Despite of encouraging experimental outcomes, there is a lack of potent nephroprotective drugable molecules in a clinics or market. To develop a nephroprotective phytomedicine, the present study was designed to do a literature survey on reported phytochemical and biological analysis of *Combretum micranthum* and to carry out chemoprofiling, in-vitro antioxidant and ex-vivo nephroprotective capacity of the title plant. The phytochemical and biological activity survey of *C. micranthum* has reveals the presence of many bioactive compounds such as flavonoids, terpenoids, steroids and alkaloids with many biological activities. Phytochemical investigation re-confirmed the presence of these compounds. Hydroalcoholic extract of *C. micranthum* (CM extract) showed a strong antioxidant activity by scavenging AAPH, DPPH, nitric oxide, hydrogen

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peroxide and chelating metal ions. CM extract exhibited significant ($P < 0.001$) dose dependent inhibition of ferric chloride-ascorbic acid induced lipid peroxidation. Diabetic nephropathy is a serious and common complication leading to end stage renal disease. Therefore, in the present study, glucose-induced toxicity was also studied in human embryonic kidney cells (HEK-293) as an in vitro model for diabetic nephropathy. The results showed that exposure of cells to high glucose (100 mM) for 72 h significantly reduced the cell viability resulting in morphological changes such as cell shrinkage, rounded cell shape and cytoplasmic vacuolation. Treatment with CM extract at 10 and 25 μg/mL resulted in significant improvement in cell viability from 10 to 23% compared to the high glucose control. This study demonstrated the potential antioxidant and nephroprotective properties of *C. micranthum*, justifying its traditional use in the treatment of various diseases.

Keywords: Stem cell research, systems biology, Physiology, Developmental biology, Cell biology

1. Introduction

The kidneys are in charge for many essential physiological functions such as filtration and removal of metabolites and toxic wastes from the body, regulation of the internal fluid environment to sustain proper fluid volume and tonicity, pH balance, electrolyte composition and essential endocrine functions [1, 2, 3]. As main emunctory, the kidney is potentially exposed to many aggressions [4]. In chronic kidney disease (CKD) there is a progressive knock down of functional units of the kidney. Moreover, multiple underlying pathophysiological mechanisms leads to chronic renal failure [4]. Several factors such as diabetes mellitus [5], hypertension [6], drugs [7], obesity [8], chronic infection [9], sickle cell anaemia [10], smoking [11], environmental toxins [12] and advanced age [13] are involved in the initiation and progression of CKD. Approximately more than three people become diabetic once every 10 seconds or almost ten million new cases reported per year [14, 15]. Diabetes is the fourth leading cause of death globally and every 1 minute 6 persons die from the complications of diabetes include nephropathy [16, 17]. Diabetic nephropathy (DN) is the leading cause of CKD and end stage chronic kidney disease (ESCKD) [18, 19]. DN is the main cause of end stage renal disease, which results in high mortality [20, 21]. It has been estimated that DN occurs in up to 40% of patients with diabetes [14, 18] and contributing to approximately 45% of new cases of ESCKD [15]. Previous report suggests that 43% of the chronic renal failure patients on dialysis have diabetic nephropathy, 60% death cases of diabetic mellitus patients are due to DN and death case of diabetic mellitus patients due to renal failure are 17 times more as compared to non-diabetic mellitus patients [22]. The prevalence rate

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of CKD is increasing and has a profound impact on human health [23]. CKD has become a serious public health problem worldwide [24]. It affects about 10% of the global adult population and more than 30% of people over 70 years of age [13]. Annual global increase of the prevalence of CKD (8%−16%) is alarming and higher than the general population growth [25]. The ESCKD has a major impact on survival, quality of life, and the cost of health [13]. Management of chronic renal failure is extremely expensive [26]. Health insurance estimated its cost in France in 2007 to be more than 4 billion euros for 61,000 treated patients, which were splinted into about 77% for haemodialysis, 5% for treatment under peritoneal dialysis and 18 % for kidney transplantation [26]. However, access to transplantation remains difficult due to transplant shortage and complications that occur after transplantation [27]. In view of these observations, only pharmacological treatment would be ideal. Despite encouraging experimental progress, evidence for the efficacy of potentially nephroprotective molecules in a clinical context is currently lacking [28]. The need to have potential molecules in nephrology is enormous and urgent [13, 28]. Preserving kidney function and improving the transition from chronic kidney disease to the terminal stage is therefore a challenge for nephrology [13]. Indeed, the pathophysiology of nephropathies is currently well characterized. It goes through oxidative stress, inflammation and apoptosis [29, 30]. Many molecules have been recently tested in order to slow and stabilize CKD without any of them being retained as a new therapeutic strategy [28, 31]. Despite recent interest in molecular modelling, combinatorial chemistry and other synthetic chemistry techniques by pharmaceutical companies, natural products and especially medicinal plants remain an important source of new medicines [32, 33]. The use of herbal medicines to prevent the genesis and complications of nephropathies offers new alternatives since synthetic molecules pose problems [34]. It is therefore essential to identify natural molecules to counter the progression of nephropathies. Nowadays, much of attention has been given to the use of phytochemicals as a protective strategy against nephrotoxicity [35]. Many researchers have reported that biomolecules, such as phenolic compounds, were efficient in inhibiting reactive oxygen species induced organ pathologies [36]. Furthermore, there is an increasing preference for natural antioxidant rather than synthetic molecules because of the safety of the natural sources [36]. Plants are known to provide a source of inspiration for novel drug compounds and this is sequel to the fact that medicines derived from plants have made large contributions to human health and well-being [37]. Many herbal medicines are known to have various types of polyphenolic compounds and may be quite safe and effective in reducing nephrotoxic effects [38, 39]. Plants may offer new alternatives to the limited therapeutic options currently available in the treatment of CKD [34]. A major turning point has been shown in particular by demonstrating the nephroprotective character of flavonoids [39]. They possess a remarkable spectrum of pharmacological activities with nephroprotective potential due to their antioxidant, anti-inflammatory and anti-apoptotic activities [39]. *Combretum micranthum* (CM) is

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widely used in traditional medicine throughout West Africa [40]. However, there has been no available literature focused on its nephroprotective effects. To develop a nephroprotective phytomedicine, the present preliminary study was undertaken to carryout the literature review on the phytopharmacological studies of CM and to evaluate its in vitro and ex-vivo antioxidant and nephroprotective activities. Further, the effect of CM in glucose-induced toxicity was studied in human embryonic kidney (HEK-229) cells as an in vitro model for diabetic nephropathy.

2. Material and methods

2.1. Drugs and chemicals

2,2′- azobis (2-amidinopropane) (AAPH), 1, 1-diphenyl-2-picryl hydrazyl hydrate (DPPH), malonedialdehyde (MDA), gallic acid, ascorbic acid, rutin, quercetin, methyl-2-phenylindole, iron chloride, 2,6-di-tert-butyl 4-methylphenol, 5,5-dithiobis-2-nitrobenzoic acid, nitric oxide, sulfanilamide, naphthylethylenediamine, phosphoric acid, sodium nitrite, 1-diphenyl-2-picrylhydrazyl, Bradford reagent, bovine serum albumin (BSA), polyvinylpolypyrrolidone, Folin Ciocalteu reagent, aluminium chloride, acetate Sodium were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other unlabelled chemicals and reagents were of analytical grade and available commercially.

2.2. Plant materials and extraction

Fresh leaves of *Combretum micranthum* were collected in December 2016 from Alibi I, a locality at North West of Tchamba (Togo). Botanical authentication was confirmed at the Laboratory of Botany and Plant Ecology of the Faculty of Science, University of Lomé, Togo and the voucher specimen was deposited at the herbarium (N° TOGO151085). The leaves were washed, dried under shade and were coarsely powdered. The powder (830 g) was macerated in 5 L of ethanol-water (8:2; v/v) at room temperature for 72 h. The filtrate was evaporated under vacuum at 45 °C by a rotary evaporator (Rotavapor Buchi R100) with 12.15% w/w yield and designed as CM extract. Distilled water was used to solubilize the extract.

2.3. Animals

Male Albino Wistar rats of 6−8 weeks weighing 200−250 g were procured from the Nigerian Institute of Medical Research, Lagos, Nigeria. Rats were housed in standard polypropylene cages and maintained under standard laboratory conditions (temperature 24 ± 1 °C, relative humidity 55 ± 5%, and 12 h light/dark cycle). They were acclimatized for two weeks before the study and fed with normal pellet diet and water ad libitum. Experimental protocols were based on World Health Organization Guidelines for care and use of laboratory animals. The use of the animals

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was approved by the Ethics Committee of the University of Lomé, a branch of the National Ethics Committee for control and supervision of experiments on animals (N° SBM/UL/14/NS0004).

2.4. Reported ethnobotanical, pharmacological and phytochemical analysis of *C. micranthum*

Systematic literature search was done according to the method used previously [41]. Chemical constituents isolated and identified from *C. micranthum*, pharmacological activities exhibited by the isolated compounds and crude plant extracts were searched across Medline (National Library of Medicine), Science Direct databases, web of science, PubMed, Google Scholar. The data were updated on April 2018, using the search terms *C. micranthum*, chemical constituents, biological activities, pharmacological activities or properties of *C. micranthum* as keywords. In addition, the reference lists of all papers identified were thoroughly reviewed.

2.5. Determination of total phenolics, tannins and flavonoids compounds

Total polyphenols, tannins and flavonoids were determined by Folin−Ciocalteu procedure [42].

2.5.1. Determination of total polyphenol contents

To 500 μL of CM extracts (1 mg/mL) was added 250 μL of Folin−Ciocalteu reagent and 1.25 mL 20% aqueous sodium carbonate solution; tubes were vortexed and absorbance of blue coloured mixtures was recorded after 40 min at 725 nm against a blank containing 500 μL of extraction solvent. The amount of total polyphenols was calculated as a gallic acid equivalent from the calibration curve of gallic acid standard solutions (5 and 200 μg/mL), and expressed as mg gallic acid/100 g dry plant material. All measurements were done in triplicate.

2.5.2. Determination of tannins

Total tannin content was determined by Folin−Ciocalteu procedure as above, after removal of tannins by their adsorption on insoluble matrix (polyvinylpolypyrrolidone, PVPP). Insoluble, cross-linked PVPP (Kollidon CL, BASF, Germany; 100 mg) was weighed into test tubes and 500 μL of CM extracts dissolve in distilled water (1 mg/mL) was added. After 15 min incubation at 4 °C, tubes were vortexed and centrifuged at 4350 g for 10 min. Aliquots of supernatant (200 μL) were transferred into test tubes and non-absorbed phenolics determined as described above. Calculated values were subtracted from total polyphenol contents and total tannin contents

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were expressed as mg gallic acid/100 g dry plant material. All measurements were done in triplicate [42].

2.5.3. Determination of flavonoids

To 2 mL of CM extracts dissolve in distilled water (1 mg/mL), 5 mL of $AlCl_3$ reagent (13 mg crystalline aluminium chloride and 40 mg crystalline sodium acetate were dissolved in 10 mL of extracting solvent) was added and absorbance was recorded at 430 nm against a blank (2 mL of analyzed solution plus 5 mL of water). The amount of flavonoids was calculated as a rutin equivalent from the calibration curve of rutin standard solutions, and expressed as mg rutin/100 g of plant material.

2.6. Fourier transform infra-red spectroscopy

The dry extract of CM is mixed with anhydrous KBr (98 mg). The resulting powder was subjected to a high pressure (10000 psi) using a press to obtain a tablet. The IR spectra of the different products was recorded, using KBr pellets, with a Fourier Transform Apparatus (FTIR) type Perkin Elmer Spectrum Bx (application software: Spectra Manager).

2.7. In vitro steady-state antioxidant studies

2.7.1. Determination of DPPH (1, 1-diphenyl-2-picryl hydrazyl) radical scavenging activity

The free radical scavenging activity of CM was measured by DPPH, following the method of [43, 44]. Equal volume of 100 μM DPPH in methanol was added to different concentration of extract or standards (ascorbic acid and quercetin) (5−200 μg/ml) in methanol, mixed well and kept in dark for 30 min. Then the absorbance was measured at 517 nm with a spectrophotometer (UV-1601 Shimadzu, Japan). The percent DPPH scavenging effect was calculated using the following equation:

$$\text{DPPH-scavenging effect } (\%) = [(A_0 - A_t/A_0) \times 100]$$

where A_0 was the absorbance of the control reaction, and A_t was the absorbance in the presence of the standard sample or extract.

2.7.2. Reducing power assay

Different concentrations of CM (5−200 μg/mL) or standards (ascorbic acid, Quercetin and BHT) in distilled water (1 mL) was mixed with phosphate buffer (2.5 mL, 0.2 mM, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)6$] (2.5 mL, 1%) and the mixture was incubated at 50 °C for 20 minutes. A volume of 2.5 mL of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3 000 r/

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min for 10 min. The upper layer of the solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%), and the absorbance was measured at 700 nm with a spectrophotometer (UV-1601 Shimadzu, Japan) [43, 45]. Reducing power was calculated as following:

$$\text{Reducing power effect } (\%) = [(A_0 - A_t / A_0) \times 100]$$

where A_0 was the absorbance of the control reaction, and A_t was the absorbance in the presence of the standard sample or extract

2.7.3. Nitric oxide radical scavenging assay

The procedure is based on the Greiss reaction [43]. Sodium nitroprusside spontaneously generates nitric oxide at physiological pH in aqueous solution, which interacts with oxygen to produce nitrite ions that can be estimated using the Greiss reagent [45]. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitrite ions. Sodium nitroprusside (10 mM) in phosphate-buffered saline (300 μL) was mixed with 300 μL of CM (1 mg/mL) dissolved in methanol and then incubated at room temperature for 150 min. In the same way, a control reaction mixture was prepared without CM, but with an equivalent amount of methanol. After the incubation period, 500 μL of Greiss reagent (1% sulphanilamide, 2% H_3PO_4, and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) was added to the mixture. The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with naphthyl ethylenediamine was measured at 546 nm. Standard solutions of ascorbic acid, Quercetin or BHT treated in the same way as tests with Greiss reagent served as positive control. The percentage of inhibition was calculated by using the following formula:

$$\% \text{ Inhibition} = [(A_0 - A_t / A_0) \times 100]$$

where, A_0 was the absorbance of the control (without extract) and A_t was the absorbance in the presence of the extract or standard.

2.7.4. Hydrogen peroxide scavenging activity

The hydrogen peroxide (H_2O_2) scavenging assay was carried out following the procedure of [43]. A solution of hydrogen peroxide (2 mM) was prepared in phosphate buffer (pH 7.4). CM sample solutions at various concentrations (5−200 μg/mL) was added to the H_2O_2 solutions (0.6 mL) and the absorbance of H_2O_2 at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without H_2O_2. The percentage inhibition was calculated using the following formula:

$$\% \text{ Inhibition} = [(A_0 - A_t / A_0) \times 100]$$

where, A_0 was the absorbance of the control (without extract) and A_t was the absorbance in the presence of the extract or standard (ascorbic acid or quercetin).

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2.7.5. Metal chelating activity

The chelating of ferrous ions by CM was measured following the method of [43]. The reaction mixture contained different concentrations of CM (5−200 μg/mL) or standard (EDTA) and 0.05 mL of $FeCl_2$ (2 mM) solution. Then the reaction was initiated by the addition of 5 mM ferrozine (0.2 mL). The reaction mixture was shaken vigorously and allowed to stand at room temperature for 10 minutes. The absorbance of the solution was then measured at 562 nm. The percentage inhibition of ferrozine-ferrous complex formation was calculated using the following formula:

$$\text{Metal chelating activity } (\%) = [(A_0 - A_t / A_0) \times 100]$$

where, A_0 was the absorbance of the control (without extract), and A_t was the absorbance in the presence of the extract or standard.

2.8. Ex-vivo antioxidant and nephroprotection studies

2.8.1. Inhibition of AAPH induced haemolysis in rat RBCs

Rats were anaesthetised with ether and blood (5−7 ml/rat) obtained was collected from the sinus orbital into heparinized tubes. Erythrocytes were separated from plasma and the buffy coat, and were washed three times with 5 volumes of phosphate-buffered saline (PBS). During the last wash, the erythrocytes were centrifuged at 3000 rpm for 10 min to obtain a packed cell preparation. The packed erythrocytes were then suspended in 4 volumes of PBS solution. In the present study, the method described by [46] was used to determine erythrocyte haemolysis mediated by AAPH. Erythrocyte suspension (2 mL) was mixed with 2 ml of PBS solution containing varying amounts of CM or standard compounds like quercetin and ascorbic acid (5−200 μg/mL). Then 2 mL of 200 mM AAPH in PBS was added to the mixture. The reaction mixture was shaken gently while being incubated at 37 °C for 3 h. After incubation, the reaction mixture was diluted with 8 volumes of PBS and was centrifuged at 3000 rpm for 5 min. The absorbance of the supernatant fraction was recorded at 540 nm with a DU 640 spectrophotometer (Beckman Instruments, Fullerton, CA). The percentage of inhibition was calculated by the following equation:

$$\% \text{ Inhibition} = [(A_0 - A_t / A_0) \times 100]$$

where, A_0 was the absorbance of the control (without extract), and A_t was the absorbance in the presence of the extract or standard.

2.8.2. $FeCl_2$-ascorbic acid stimulated lipid peroxidation in kidney homogenate

The anti-lipid peroxidation effect of CM was studied following the method [46]. The kidney tissues were quickly removed from sacrificed rats. A 2 g of kidney tissue was

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sliced and then homogenized with 10 ml of 150 mM KCl Tris-HCl buffer (pH 7.4). The reaction mixture was composed of 250 μL of kidney homogenate, 100 μL of Tris-HCl buffer (pH 7.4), 50 μL of 0.1 mM ascorbic acid, 50 μL of 4 mM $FeCl_2$ and 50 μL of various concentrations of CM or standard. The mixture was incubated at 37 °C for 1 h in capped tubes. Malonedialdehyde (MDA) concentration was estimated as previously described [47, 48]. MDA levels, as marker of lipid peroxidation, was analyzed by a colorimetric assay based on the reaction of MDA with a chromogenic reagent to yield a stable chromophore with maximal absorbance at 586 nm. Briefly, 650 μL of 10.3 mM 1-methyl-2-phenyl-indole in acetonitrile diluted with methanol containing 32 μM $FeCl_3$ (3:1) was added to 250 μL of each sample and the mixture was vortexed. After adding 150 μL of 37% (v/v) HCl, samples were mixed well, closed with a tight stopper and incubated at 45 °C for one hour. The samples were then cooled, centrifuged at 4000 g for 10 min, and the absorbance was measured spectrophotometrically at 586 nm. A standard curve comprised of 1,1,3,3-tetra-methoxypropane was also run for the quantitation of MDA.

2.8.3. Determination of total protein content in renal tissue

The protein content of the experimental samples was measured by the method of Bradford using crystalline serum bovine albumin (BSA) as a standard [49]. To 15 μL of the homogenate or BSA (1 mg/mL), 750 μL of the Bradford reagent was added. The absorbance was read 5 min later at 595 nm.

2.9. *Combretum micranthum* extract's effect on glucose induced cytotoxicity in human embryonic kidney (HEK-293) cells

HEK-293 embryonic kidney epithelial cells were cultivated in DMEM supplemented with 10% heat-inactivated fetal bovine serum in a CO_2 incubator (5% CO_2 in air) at 37 °C. The cells with 70−80% confluency were trypsinized and sufficient media added to inactivate the trypsin activity. The cells were centrifuged at 1200 rpm for 5 min, supernatant was discarded and resuspended the pellet in media prior to counting on a haemocytometer by Trypan blue exclusion method. For cell growth studies, 5,000 cells/well seeded in a 96-well plate. Post 24 h of cells seeding, cells were exposed to normal (25 mM) or high glucose (100 mM) or high glucose and CM extract (5, 10, 25, 50, 100 and 200 μg/mL) for 72 h. Post 72 h drug treatment, cell viability and cell morphological analysis were executed [50].

2.9.1. Cell viability assay

Cell viability was measured using 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) bioassay, which provides a sensitive measurement of the normal metabolic status of cells, particularly that of mitochondria, which reflects

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the early cellular redox changes. Post-extract treatment (after 72 h), MTT solution (5 mg/ml) was added and incubated for 3 h. The dark-blue formazan crystals formed in the wells dissolved in DMSO and the absorbance was measured at 570 nm with a microtiter plate reader (Varioskan® Flash, Thermo Scientific).

2.9.2. Cell morphological assessment

Morphological changes in HEK-293 cells were assessed post-exposure to cells exposed to normal glucose (25 mM) or high glucose (100 mM) or high glucose and CM extract (5−200 μg/mL) for 72 h) at 37 °C. Cells were observed under a compound microscope.

2.10. Statistical analysis

All the values are expressed as mean ± SEM (n = 3 replicates). Statistical analysis was performed with Graph Pad Prism 7 software (San Diego, CA, USA) using one-way analysis of variance (ANOVA) followed by Tukey's and Dunnett's test as a post hoc analysis. The value of $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Systematic literature search report about ethnobotanical, pharmacological and phytochemical analysis of *C. micranthum*

The phytochemicals analysis of *C. micranthum* reveals many compounds with varying structural frames. These compounds are classified into terpenoids, flavonoids, alkaloids and steroids (Table 1). Further, the literature survey of ethnobotanical/biological activities of *C. micranthum,* has revealed many biological activities tabulated in Table 2.

3.2. Determination of total phenolic, tannins and flavonoids compounds

The amount of total phenolic, tannins and flavonoids present in the CM extract was respectively 154.27 ± 3.31 mg, 21.88 ± 0.01 mg, 333.23 ± 5.02 mg expressed as gallic acid or rutin equivalents per 100 g of extract respectively (Table 3).

3.3. Infra-red analysis

The analysis of the vibration frequencies of the bonds obtained from the infra-red spectra of the extract showed that the plant extract has a phenolic nucleus (3500-3300 cm^{-1} and 1300-1000 cm^{-1}), carbonyl functions (1715−1690 cm^{-1}), C-O

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Table 1. Reported phytochemical compounds of *Combretum micranthum*.

Compound name	Classes	Reference
Gallic acid		[62, 70]
Combretine		[69]
Rutin trihydrate		[70]
Benzoic acid		[70]
(+)-Catechin		[62, 70]
Vitexin		[62, 80]
Isovitexin		[62, 80]
Orientin	**Flavonoids**	[62, 80]
Homoorientin		[62, 80]
Myricetin-3-O-glycoside		[62]
(-)-Epigallocatechin:		[62]
(-)-Epicatechin		[62]
(-)-3′,4′,5′,5,7-pentahydroxyf lavan		[62]
(-)-3′,4′,5,7-tetrahydroxyf lavan		[62]
2″-O-galloylvitexin		[62]
2″-O-galloylisovitexin		[62]
2″-O-galloylorientin		[62]
2″-O-galloylhomoorientin		[62]
α-Tocopherol		[69, 81]
α -Tocopherol derivative		[69, 81]
Monoterpene		[69]
Lupeol		[69]
α -amyrin		[69, 81]
Palmitic acid	**Acid Gras**	[69]
Oleic acid		[81]
Linolenic acid		[81]
β-Sitosterol		[81]
Sorbitol		[81]
Inositol	**Sterols**	[81]
β-sitosterol		[81]
Choline		[81]
Kinkéloid A1		[81]
Kinkéloid A2		[81]
Kinkéloid B1		[81]
Kinkéloid B2		[81]
Kinkéloid C1		[81]
Kinkéloid C2	**Alkaloids**	[81]
Kinkéloid D1		[81]

(*continued on next page*)

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Table 1. (*Continued*)

Compound name	Classes	Reference
Kinkéloid D2		[81]
Botulin		[69]
Stachydrine		[81]
4-Hydroxyproline betaine		[81, 82]
Betaine		[81]

Table 2. Reported biological activities of *Combretum micranthum*.

Biological activity	Reference
Antimalarial	[83, 84, 85, 86]
Antiviral	[87, 88, 89, 90]
anti-inflammatory	[81, 91]
wound healing	[92]
Antioxidant	[70, 81, 93, 94, 95]
antibacterial	[90, 93, 96, 97, 98]
Neuroprotective	[99, 100]
Antihyperglycaemic	[81, 101]
Anti-diabetic	[62, 95, 102, 103]
Antiradical	[70]
Antifungal	[90]
Anti-trypanosomal	[104]
Anti-Ebola [108]	[105]
Anti-Obesity [109] hypotensive [40, 110]	[40, 106, 107]

Table 3. Total phenols, flavonoids and tannins content of *Combretum micranthum* extract.

Tests	Amount in extract
Total phenols (mg gallic acid/100 g of extract)	154.27 ± 3.31
Flavonoids (mg Rutin/100 g of extract)	333.23 ± 5.02
Tannins (mg gallic acid/100 g of extract)	21.88 ± 0.01

function of ethers (1150-1020 cm^{-1}), alcohol functions (OH) and aromatic unsaturations C=C (1500−1450 cm^{-1}) (Fig. 1). This IR spectrum showed that CM extract contains significant amounts of phenolic compounds that can be quantified by standard methods.

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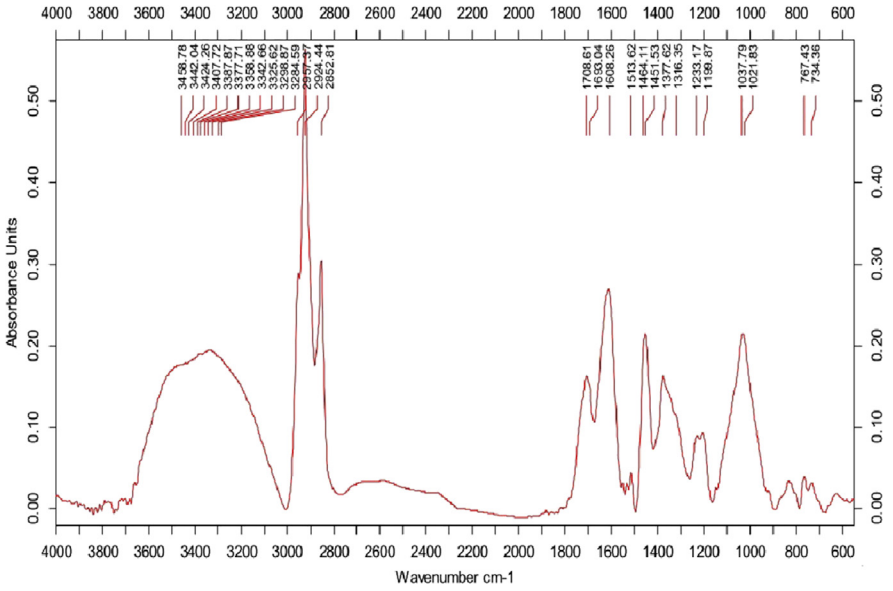

Fig. 1. FT-IR spectra of *Combretum micranthum* extract (CM).

3.4. In-vitro steady-state antioxidant activity

3.4.1. Determination of DPPH (1, 1-diphenyl-2-picryl hydrazyl) radical scavenging activity

In this present study, quercetin and ascorbic acid were used as standard radical scavengers. The percent of DPPH scavenging effect were found to be 87.16 ± 0.58% (CM extract), 78.55 ± 0.78% (Quercetin) and 96.33 ± 0.53% (Ascorbic acid) at the concentration of 25 μg/mL. The IC_{50} values of the extract, quercetin, and ascorbic acid were calculated using the equation obtained from linear regression analysis and were 2.49 ± 0.53, 5.80 ± 0.42 and 0.02 ± 0.33 μg/mL, respectively (Table 4).

Table 4. In vitro study-state antioxidant activities of *Combretum micranthum* extract (CM) against DPPH, H_2O_2, NO, AAPH radicals, Metal chelating and reducing power.

Descriptions (5−200 μg/mL)	**IC_{50} (μg/mL)**					
	DPPH radical	**Metal chelating activity**	**Reducing power**	**H_2O_2 scavenging**	**NO scavenging**	**AAPH scavenging**
Extract (CM)	2.49 ± 0.53	10.60 ± 0.22	2.50 ± 0.47	10.80 ± 0.42	36.48 ± 0.33	57.00 ± 0.13
Quercetin	5.80 ± 0.42	–	2.60 ± 0.38	10.00 ± 0.63	9.80 ± 0.41	25.30 ± 0.18
Ascorbic Acid	0.02 ± 0.33	–	2.40 ± 0.54	44.20 ± 0.13	10.41 ± 0.23	50.70 ± 0.26
EDTA	–	10.00 ± 0.34	–	–	–	–
BHT	–	–		–	2.50 ± 0.16	–

IC_{50} (μg/mL) represented as mean ± SEM, n = 3.

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3.4.2. Reducing power assay

The reducing power of CM extract compared to quercetin, ascorbic acid, and BHT is shown in (Table 4).

3.4.3. Assay of nitric oxide scavenging activity

The scavenging of nitric oxide by CM extract was evaluated by observing the reduction of linear time-dependent nitrite production in the sodium nitroprusside-PBS system. The results showed the scavenging of nitric oxide by CM extract and the standard compounds quercetin and ascorbic acid. The inhibition of nitric oxide by CM extract, quercetin and ascorbic acid was calculated as 60.59 ± 0.17%, 96.26 ± 0.73% and 76.24 ± 0.08% at a concentration of 50 μg/mL, respectively. The IC_{50} values of the extract, Quercetin, and Ascorbic acid were calculated using the equation obtained from linear regression analysis and were found to be 36.48 ± 0.33; 9.80 ± 0.41 and 10.41 ± 0.23 μg/mL, respectively (Table 4).

3.4.4. Hydrogen peroxide scavenging activity

The ability of CM extract at different concentrations (5−200 μg/mL) to scavenge H_2O_2 was evaluated. CM showed good H_2O_2 scavenging ability when compared to the standard compound quercetin, and ascorbic acid. The percentage inhibition of nitric oxide by CM extract and standard compound quercetin and ascorbic acid was calculated as 74.03 ± 0.12%, 75.92 ± 0.31% and 56.21 ± 0.28% at a concentration of 25 μg/mL, respectively. The IC_{50} values of the extract, Quercetin, and Ascorbic acid were calculated using the equation obtained from linear regression analysis and were found to be 10.80 ± 0.42; 10.00 ± 0.63 and 44.20 ± 0.13 μg/mL, respectively (Table 4).

3.4.5. Metal chelating activity

The formation of ferrous complex with ferrozine reagent was interfered by both CM extract and the standard compound (EDTA). The results illustrated the chelating activity of CM and EDTA in terms of percentage of metal chelating activity with increasing concentration (5−200 μg/mL). From these results, it was evident that the metal chelating activity of CM extract was concentration-dependent. The IC_{50} values calculated using the equation obtained from linear regression analysis and were found to be 10.60 ± 0.22 μg/mL for the extract (CM) and 10.00 ± 0.34 μg/mL for EDTA (Table 4).

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3.5. Ex-vivo antioxidant and nephroprotection studies

3.5.1. Inhibition of AAPH induced haemolysis in rat red blood cells (RBCs)

The influence of the CM extract on erythrocyte haemolysis was examined by incubating rat erythrocytes in the presence of 200 mM AAPH as an initiator of oxidation. In the absence of AAPH, RBCs were stable and the haemolysis was negligible. When aqueous suspension of RBCs was incubated with AAPH, about 80 % of haemolysis was observed. The CM extract provided a strong inhibitory effect against erythrocyte haemolysis (Table 4). All the tested compounds inhibited AAPH-induced RBCs haemolysis in a dose-dependent manner (5−200 μg/mL). The percent of haemolysis in RBCs incubated with CM (5−200 μg/mL) in the absence of AAPH was almost identical to that of control sample indicating CM itself could not induce haemolysis. The IC_{50} value of CM extract, quercetin, and ascorbic acid was 57.00 ± 0.13; 25.30 ± 0.18 and 50.70 ± 0.26 μg/mL, respectively (Table 4).

3.5.2. Combretum micranthum extract's effect on $FeCl_2$-ascorbic acid-induced lipid peroxidation on kidney homogenate

Incubation of rats' Kidney homogenate with $FeCl_2$-ascobic acid resulted in a significant ($P < 0.001$) increase in kidney MDA content as represented in Fig. 2. However, the CM and quercetin were able to significantly ($P < 0.001$) lower the kidney MDA content in a dose-dependent manner in the concentration range of 5−200 μg/mL

3.6. *Combretum micranthum* extract's effect on glucose induced cytotoxicity in human embryonic kidney (HEK-293) cells

The effect of CM extract on high glucose induced cytotoxicity was examined using the MTT assay. After the initial 24-h attachment period, cells were treated with

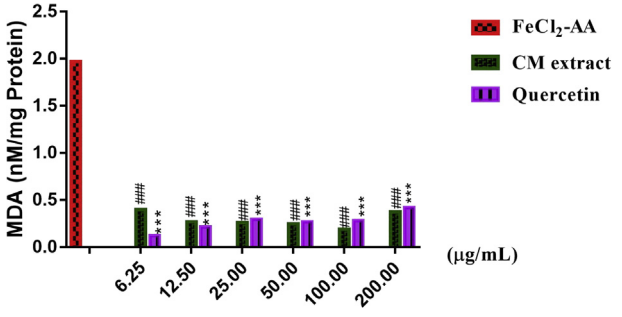

Fig. 2. Effects of *Combretum micranthum* extract (CM) on $FeCl_2$-ascorbic acid-induced lipid peroxidation on kidney homogenate. Data are expressed as mean ± SEM (n = 5). one-way ANOVA followed by Tukey's multiple comparisons test. Compared to $FeCl_2$-ascorbic acid group: ***P < 0.001; Compared to Quercetin group: $^{###}$P < 0.001.

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various concentrations of CM extract (5, 10, 25, 50, 100 and 200 μg/mL) for 72 h in the presence of normal (25 mM) or high glucose (100 mM). No change in cell viability was observed in the cells treated with CM alone (Fig. 3). Exposure of cells to high glucose (100 mM) for 72 h significantly reduced the cell viability resulting in morphological changes such as cell shrinkage, rounded cell shape and cytoplasmic vacuolation (Fig. 4). Treatment with CM extract at concentrations of 10 and 25 μg/mL resulted in significant improvement in cell viability from 10 to 23% compared to the high glucose control. However, the cytoprotective effect of CM extract was not retained at higher concentrations (>50 μg/mL) in the presence of high glucose. The EC_{50} of CM was found to be 140.7 μg/mL (Table 5).

4. Discussion

The search for natural medicines from plants with antioxidant activities has become a concern in nephrology [51, 52]. The nephroprotective activities of molecules isolated from natural products represent an exciting advance in the search for efficient nephroprotective, because of the urgent need for new and innovative molecules [53, 54]. A major turning point was, in particular, the demonstration of the nephroprotective character of polyphenols including flavonoids [39, 54]. Flavonoids as nephrotropic molecules can be explored to obtain a real potential source as drug candidates against kidney diseases. They have a remarkable spectrum of pharmacological activities, including antioxidant properties [55, 56, 57], anti-inflammatories [55, 56] and anti-apoptotic [58] explaining their nephroprotective potential. The phytochemical study of CM extract as performed by the FT-IR spectra has given many characteristic functional groups with various structural skeletons such as: amino acids, hydroxyl compounds, ethers, carbonyl compounds, carboxylic acid, aldehyde, ketone, amide [59, 60]. These characteristic functional groups correspond

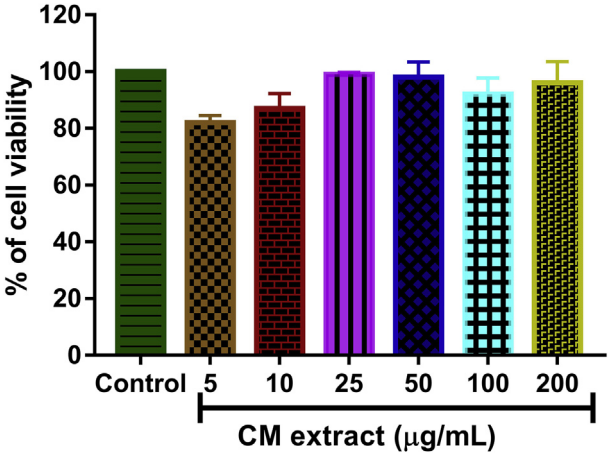

Fig. 3. Effect of various concentration of *Combretum micranthum* extract (CM) on cell viability in HEK-293 cells.

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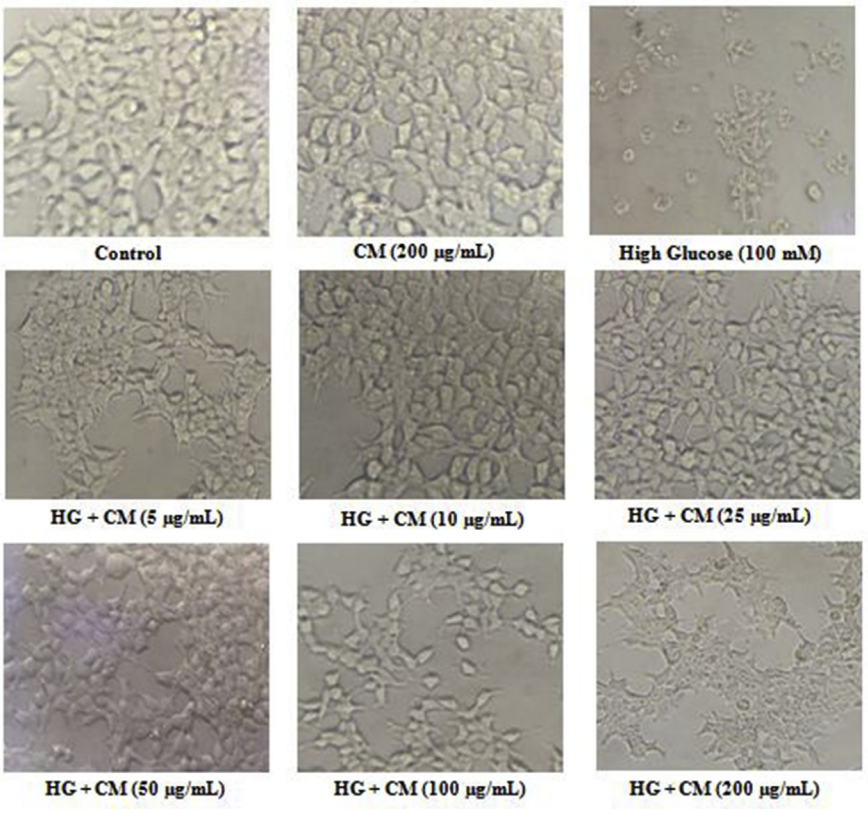

Fig. 4. Microsocpic image of HEK-293 cells treated with glucose alone or in combination of different concentration of *Combretum micranthum* extract (CM).

to polyphenols, flavonoids, tannins, saponins, terpenoids, alkaloids, polysaccharides and steroids [59, 60, 61]. More than thirteen different polyphenolic compounds were identificated in CM extract by HPLC methods [62]. Quantitative tests yield as total polyphenol contents (154.27 ± 3, 31 mg AG/100 g), flavonoids (333.23 ± 5.02 mg R/100 g) and tannins (21.88 ± 0.01 mg AG/100g), all confirming the effective presence of flavonoids, potentially nephroprotective [54] in CM extract. One of the main goals of drug discovery is to identify molecules with antioxidant activity, which is often done through in vitro evaluation before in vivo confirmation. There are currently a multitude of methods for evaluating the antioxidant activity of plant samples (approximately 19 in vitro and 10 in vivo methods) [63]. Among these in vitro models for evaluating the antioxidant potential, mention may be made in particular of DPPH, superoxide, hydrogen peroxide, AAPH, hydroxyl radicals and the nitric oxide radical. The antioxidant capacity of the CM extract was measured spectrophotometrically using these models. The CM extract clearly showed strong antioxidant activity by scavenging free radicals. In addition, erythrocytes are fragile cells and are very susceptible to free radical-induced cell membrane damage by lipid peroxidation leading to hemolysis [64]. AAPH is well known for generating peroxyl radicals by decomposition at 37 °C and with a half-life of approximately 175 h. Therefore, the

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Table 5. Effect of *Combretum micranthum* extract (CM) on Glucose induced toxicity in HEK-293 cells.

Treatment	Concentration	% Cell Viability	Improvement of % Cell Viability	EC_{50} (μg/mL)
Control Glucose (mM)	25	100 ± 0.0	–	–
Glucose (mM)	100	42 ± 2.6$^{###}$	–	–
CM Extract (μg/mL)	5	52 ± 1.5	10	140.7
with Glucose (100 μM)	10	62 ± 7.6*	20	
	25	65 ± 3.5*	23	
	50	60 ± 6.4	18	
	100	52 ± 8.7	10	
	200	40 ± 5.7	-2	

Percentage cell viability (%) represented as, Mean ± SEM, n = 3.
$^{###}P < 0.001$, One-way ANOVA followed by Dunnett's test compared with Control.
*$P < 0.05$, One-way ANOVA followed by Dunnett's test compared with cisplatin treated.

AAPH/Erythrocyte model is an excellent model for antioxidant research [64]. In this study, CM extract significantly inhibited AAPH-induced oxidative hemolysis as well as quercetin and ascorbic acid and also exhibits effective scavenging activities against DPPH, superoxide, hydrogen peroxide and nitric oxide radicals. Our results showed also that CM extract has potent metal chelation activity just like the standard compound (EDTA). Thus, the CM extract affect the formation of free radicals and their damage process. This is further evident by the protection shown by the CM extract in lipid peroxidation induced by $FeCl_2$-ascorbic acid on the kidney homogenate. Most nephrotoxic chemicals, including cisplatin and gentamicin, damage the kidneys by inducing directly or indirectly lipid peroxidation. Ex vivo lipid peroxidation in the kidney homogenate can be carried out in a non-enzymatic manner. The process is induced by ascorbate in the presence of Fe^{2+}/Fe^{3+}, by generating hydroxyl radicals (ROS, eg H_2O_2, $HO^{\bullet}$, O_2) [46, 65]. According to our results, CM extract inhibits $FeCl_2$-ascorbic acid stimulated lipid peroxidation in kidney homogenate by decreasing significantly MDA formation. Kidney cells derived from human embryonic kidney cells (HEK-293) in tissue culture are an excellent model for the study of kidney cells [50]. In fact, apoptosis of the mesangial, epithelial and tubular cells of the kidneys has been demonstrated in cell cultures in the presence of high glucose concentrations [66]. In this study, the role of CM in glucose-induced cytotoxicity was studied in HEK-293 cells as an in vitro model of diabetic nephropathy. Our results showed that incubation of CM alone with HEK-293 cells did not affect cell viability. The viability of HEK-293 cells was significantly reduced after 72 hours of incubation with 100 mM glucose in the culture medium. High glucose concentration has been a major factor in the initiation and progression of renal complications of diabetes. There is evidence to explain the role of hyperglycemia in the production of free radicals responsible for oxidative stress [21, 67]. Exposure of HEK-293 cells to high glucose (100 mM) significantly decreased the viability and was associated with morphological changes. Incubation of high glucose and lower concentration

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of CM (10 and 25 μg/mL) exhibited significantly improvement in cell viability. Whereas higher concentrations of CM extract (>50 μg/mL) in presence of high glucose concentration exhibited gradual increase in cell death. This effect suggested that CM extract possess biphasic response. It is a well known fact that the pharmacological responses are concentration dependent. For example, ascorbic acid exhibited antioxidant or protection at lower concentrations in presence of inducer, whereas at higher concentrations in presence of inducers act as pro-oxidant or damager. Therefore, the optimum protection concentration of CM extract was found to be 10 μg/mL. Hyperglycemia-induced cell death involves several steps, starting with the activation of key enzymes in the polyol pathway that can be linked to glucose transporters at the cell membrane level. Glucose-mediated polyol and hexosamine pathways may be related to the production of free radicals, and certainly contribute to the overall oxidative burden [68]. It is well established that ROS and RNS can alter the mitochondrial membrane and eventually cause apoptotic cell death [67]. In addition, our experimental results demonstrate that the CM extract has antifree radical activity by inhibiting reactive species of oxygen and nitrogen. All these results confirm antioxidant activity of *C. micranthum* and its probable nephroprotective potential. In this study, the chemical constituents isolated and identified from *C. micranthum*, pharmacological activities exhibited by the isolated compounds and the crude plant extracts were searched across Medline, Science Direct databases, web of science, PubMed and Google Scholar. Many authors had proved the presence of phenols (flavonoids and tannins) [62, 69, 70]. Our results corroborate these previous findings. In West Africa, particularly in Togo several plants are used in the traditional medicine including *C. micranthum*. An ethnobotanical survey [71] reported that *C. micranthum* is also used in Burkina Faso in the treatment of renal disorders. The current study showed that CM extract exhibited a considerable lipid peroxidation inhibiting activity in kidney tissue homogenate. Although many plants have shown antioxidant potential in vitro, only a few have been confirmed in vivo. The relevance of the results of these tests in in vivo systems is sometimes uncertain. Only a few of have been confirmed in vivo because of molecule interference with physiopharmacological processes such as absorption, distribution, metabolism, storage and excretion.

During the last 15 years, several research studies have been published on the bioavailability of different classes of natural antioxidants such as phenolic compounds. The majority of them have important antioxidant potentials, mainly as scavengers of free radicals, which makes them attractive for human therapy. Many studies show that the bioavailability of most polyphenols is not very high, due to their low absorption, instability and others [72]. Their low bioavailability, does not affect their pharmacological activities [73]. By chromatographic and spectrometric methods, leaves extracts of *C. micranthum* were studied chemically. This allowed the identification of thirteen flavonoid compounds. In vitro bioassays have

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shown that the identified compounds may have synergistic effects to reduce glycemia. An additional in vivo study in C57BL/6J mice indicates that *C. micranthum* can reduce plasma glucose levels in a dose-dependent manner without significant weight loss or toxicity [62]. These in vitro and in vivo experiments support a potential new application of *C. micranthum* leaves as an antidiabetic agent. Our results on HEK-293 cells corroborate these previous finding. The bioavailability of some polyphenols and their metabolites (catechin, tannic acid, quercetin, maldivian, gallic acid, catechin, epicatechin, caffeic acid, cinnamic acid, coumaric acid, ferulic acid, epigallocatechin, epigallocatechin gallate) have been investigated [74, 75, 76, 77, 78, 79]. For medicinal plants, these tests are used to prove their antiradical activity which plays an important role in the therapeutics of pathologies related to oxidative stress such as renal damage. In vitro antioxidant potential assessment methods do not provide the exact therapeutic implications of plant antioxidants. In addition, the antioxidant potential of plants or their phytochemicals is influenced by several factors under in vivo conditions, including intestinal absorption, metabolism and bioavailability [80]. Although the bioavailability of some natural antioxidants such as polyphenols is relatively low, they have been shown to retain their biological activity at low plasma levels. It would be prudent to use several antioxidant tests to evaluate the antioxidant activities of plants and to include at least one test with biological relevance. In vitro models are useful for predicting and directing models in vivo [81, 82]. The commonly recommended strategy is to evaluate plant extracts in vitro and in vivo to confirm their therapeutic antioxidant potential [82]. Keeping this in view, in vitro evaluation of *C. micranthum* antioxidant and nephroprotective potential as the first step before confirmation in vivo is done. Work is in progress to validate the nephroprotective effect of *C. micranthum* in different in vivo nephropathy models.

Declarations

Author contribution statement

Mabozou Kpemissi, Kwashie Eklu-Gadegbeku, Veeresh P Veerapur: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

S. Vijayakumar S, Adrian-Valentin Potârniche, Thimmaiah NV: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Kodjo Adi, Siddalingesh M Banakar: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Kossi Metowogo, Kodjo Aklikokou: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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