Ngoua-Meye-Misso et al. Clinical Phytoscience (2018) 4:15
https://doi.org/10.1186/s40816-018-0075-x

Clinical Phytoscience

ORIGINAL CONTRIBUTION Open Access

Phytochemical studies, antiangiogenic, anti-inflammatory and antioxidant activities of *Scyphocephalium ochocoa* Warb. (Myristicaceae), medicinal plant from Gabon

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Abstract

Background: The search for new anti-cancer molecules is one of the main concerns of oncology researchers. *Scyphocephalium ochocoa* is a plant of Myristicaceae family, used in traditional medicine against inflammatory diseases and several types of cancer. It is well established that free radicals, chronic inflammation and angiogenesis play an important role in initiation, tumor progression and metastasis formation. The aim of this study was to carry out a phytochemical screening, to determine the phenolic compounds content, to investigate the antiangiogenic, anti-inflammatory and antioxidant activities of water, water-ethanol and ethanol extracts of *S. ochocoa*.

Methods: Phytochemical screening and determination of phenolic compounds content were performed using standard methods. Antiangiogenic activity was assessed using chick chorioallantoic membrane (CAM) model and Drabkin test. Anti-inflammatory activity was estimated by protein denaturation and erythrocyte membrane stabilization method. Finally the antioxidant activity was appreciated by DPPH radical inhibition and phosphomolybdenum assay.

Results: The results of phytochemical studies show that extracts of bark of *S. ochocoa* are rich in polyphenols, tannins, flavonoids, proantocyanidins, saponosides, flavonols, flavanonols, sterol and triterpenes. The water extract showed good antiangiogenic activity (IC_{50} = 1.153 μg/mL). Strong anti-inflammatory activity was observed with all extracts, IC_{50} ranging from 34.775 ± 2.543 μg/mL to 74.577 ± 3.456 μg/mL for protein denaturation inhibition test and IC_{50} values ranging from 36.793 ± 0.529 μg/mL at 48.912 ± 0.957 μg/mL for antihemolytic activity. In addition, all extracts showed good antioxidant activity marked by a strong inhibition of the DPPH radical (IC_{50} ranging from 4.969 ± 0.263 μg/mL to 16.188 ± 0.336 μg/mL and AAI ranging from 3.090 ± 0.065 to 10.080 ± 0.517) and by greater total antioxidant capacity (with contents ranging from 37.654 ± 0.995 to 131.302 ± 1.102 VtCE (mg)/g dry extract).

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Conclusion: Ultimately, these results could justify the use of *S. ochocoa* extracts in traditional medicine in the treatment of diseases related to angiogenesis and cancer, inflammatory diseases and diseases due to oxidative stress. A phyto-product with such a pharmacological profile could be a good candidate for the development of anticancer.

Keywords: *Scyphocephalium ochocoa*, Anti-inflammatory, Antiangiogenesis, Antioxidants, Chick Chorioallantoic membrane (CAM), Cancer

Background

Scyphocephalium ochocoa (*S. ochocoa*) belongs to the family of Myristicaceae and widespread in the littoral forests of Niger, Cameroon and Gabon [1]. Its vernacular names in Gabon are: Soghe, Sogho (Fang), Ossoko (Myene), Musuku (Nzebi, Punu-Shira), Otsoko, Soko (Tsogo) [2]. For its therapeutic and nutritional virtues, several populations of Central Africa use this plant. In Congo, a bark decoction is used for vaginal injection against female infertility. In a steam bath, mixing its leaves with those of *Microdermis puberula*, *Costus afer*, *Macaranga barteri* and *Chorophora excelsa* is used against febrile body aches [3]. It is also used as a decoction against gonorrhea or with salt and chili pepper [2]. In Gabon, the seeds of *S. ochocoa* are eaten grilled or looted. In traditional Gabonese medicine, a bark decoction is used in the treatment of anemia. As a drink, this decoction is used against rheumatism, joint pain, body aches and disorders of ovulation. The bark, dried and kneaded with white clay, serves to stop excessive milk secretions. This plant is also used in the treatment of several cancers: breast, stomach, skin and liver cancer.

The search for new anti-cancer molecules is one of the main concerns of researchers in oncology because, despite the advancement of medical technologies on cancer, this pathology remains among the main public health problems in the world with 14 million new cases and 8.8 million deaths in 2015 [4]. In Gabon, incomplete data available due to the lack of actual registration of the cancer population indicate that the number of new patients is constantly increasing from 183 new cases in 2000 to more than 1000 new patients in 2008 constituting a real public health problem [5]. According to the World Health Organization, metastases are the leading cause of cancer-related deaths [4]. Many studies have shown that reactive oxygen species (ROS), chronic inflammation and angiogenesis play an important role in initiation, tumor progression and metastasis formation [6, 7].

ROS, in cancer, are involved in the progression and proliferation of the cell cycle, cell survival and apoptosis, energy metabolism, cell morphology, cell-cell adhesion, cell mobility, angiogenesis, maintenance of the tumor and the formation of metastases [7, 8].

Other research has shown that there is a relationship between inflammation and cancer. In 1863, Virchow hypothesized that the origin of the cancer was at sites of chronic inflammation [9]. Inflammation acts as a tumor promoter via the pro-inflammatory cytokine TNFα (tumor necrosis factor-α) which is important in the early stages of tumors, regulating a cascade of cytokines, chemokines, adhesions, matrix metalloproteinases (MMPs), and pro-biogenic [9, 10]. In addition, there is strong evidence that the use of nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin reduces the risk of colon cancer and may be preventative for the respiratory tract, esophageal cancer and stomach cancer [11, 12]. Other NSAIDs, such as flurbiprofen, may have strong antimetastatic effects because of their inhibition of platelet aggregation [13].

Angiogenesis corresponds to all the processes leading to the formation of new blood capillaries by the outgrowth or budding of preexisting vessels [14]. Via the proangiogenic factor VEGF (vascular endothelial growth factor), angiogenesis is essential for tumor growth and for the formation of metastases because it supplies cancer cells with oxygen and nutrients [15].

ROS neutralization, inhibition of inflammation and angiogenesis may therefore be one of the strategies for finding anti-cancer products. Thus, in order to search for potential phyto-anticancer substances, this preliminary work consists in carrying out a phytochemical screening, in determining the content of phenolic compounds, in researching the antiangiogenic, anti-inflammatory and antioxidant activities of water, water-ethanol and ethanol extracts from *S. ochocoa*.

Methods

Chemicals

Butylated hydroxytoluene (BHT); 2,2-diphenyl-1-picryl--hydrazyl (DPPH); sorafenib p-toluenesulfonate salt (LC Laboratories; Woburn, MA, USA); Diclofenac (Combi--Blocks, Silverton, San Diego, CA, USA); quercetin, ethanol; ferric chloride; sulfuric acid; methanol; sodium phosphate; ammonium molybdate; chloridric acid; benzene; ammonium hydroxide; sodium chloride; Folin-Ciocalteu reagent; sodium bicarbonate; gallic acid

and ascorbic acid were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Plant material

The barks of *S. ochocoa* were collected August 2015 in Mitzic, Woleu-Ntem (Northern of Gabon) (Fig. 1). They were identified at National Herbarium of IPHAMETRA, Libreville (Gabon). Voucher specimen has been deposited in the Herbarium of IPHAMETRA and at Laboratory of Biochemistry Research (LAREBIO) Department of Chemistry-Biochemistry, Faculty of Sciences of USTM in Franceville.

Preparation of plant extract

Barks were dried at ambient temperature of the Laboratory (20–30 °C) and protected from light for several days. After drying, barks were crushed using a grinder (Laboratory Blender, Torrington, CT. USA). This powder was used for extractions by maceration method. Briefly, 200 g of powder was mixed with 2000 ml of solvent (water, water-ethanol (50/50, *v*/v) and ethanol). After 72 h, the obtained extract was filtered using Whatman N°1 filter paper. Ethanol and water-ethanol extracts were concentrated under reduced pressure at rotavapor (Büchi, Labortechnik, Switzerland) at 40 °C and 60 °C, respectively. Water extract was lyophilized using a lyophilizer (Alpha 1–2 LDplus, Germany). All crude extracts obtained were stored at 4 °C until analysis.

Phytochemical screening

Each extract was then tested for the presence of flavonoids, coumarins, tannins, total phenolic, saponosids, cardiac glycosides, reducing sugar, sterols and triterpenes, oses and holosides, anthracenics, anthocyans, alkaloids and anthracenosids as described elsewhere [16, 17].

Fig. 1 Shrub and trunk of *S. ochocoa*. Photos taken at Mitzic, Woleu-Ntem (Northern of Gabon) by Ngoua-Meye-Misso with digital camera Cannon 16 M pixel made in China

Phenolic compound content

Total phenolic content

The Folin-Ciocalteu method [18] with minor modifications was used to determine the total phenolic contents of the different extracts using gallic acid as standard. The absorbance was measured at 735 nm using a Spectrophotometre (Evolution 60S, USA). Results were expressed as gallic acid equivalent per gram of lyophilized sample (average of the triplicate analysis).

Total flavonoid content

The aluminum chloride ($AlCl_3$) colorimetric assay method [19] was used to determine total flavonoid contents, using quercetin as a standard. The absorbance was measured at 415 nm and total flavonoid contents were expressed as quercetin equivalents in milligrams per gram sample (average of the triplicate analysis).

Tannins content

Tannins content was determined according the reference method of European community [20]. The absorbance was measured at 525 nm and tannic acid was used as standard.

Proanthocyanidins (PAs) content

The proanthocyanidins content was determined according the method described by Prigent [21]. The absorbances were read at 550 nm. Apple procyanidins were used as a standard. Results were expressed as apple procyanidins equivalent (APE).

Antiangiogenic activity

Antiangiogenic activity was evalued using Chick Chorioallantoic Membrane (CAM) Model acording to previously reported method [22, 23] with minors modifications. Brefly, fertilized chicken eggs were purchased from a local poultry farm, were sterilized with 70° ethanol and incubated at 37 °C in incubator (Ecocell, LSIS-B2V/EC55, Germany), with 80% relative humidity. On day 2 of post incubation, 3 mL of albumin were withdrawn to minimize adhesion of the shell membrane with CAM. A square window of 1 cm^2 was opened in the egg shell at the opposite to blunt edge and sealed with an adhesive tape. The eggs were returned for further incubation.

Drug administration

At the 8th day, the experimental groups were divided into 5 of each containing 50 numbers of eggs. Group 1, 2, 3 and 4 were treated with water extracts and sorafenib (positive control). Sterile discs (diameter: 10 mm) of Whatman N°1 soaked of 10 μL of the water extract from *S. ochocoa* and sorafenib at concentrations ranging from 60, 125, 250 to 500 μg/mL was applied to the CAM. In

parallel Group 5 treated with phosphate buffered saline (PBS) alone as negative control, a paper disc Whatman N°1 soaked of 10 μL PBS at pH 7.4 was placed on the CAM of egg. The treated CAM samples were incubated for 48 h.

Angiogenesis quantification

After 48 h of incubation at 37 °C and 80% relative humidity, a volume of 10 μL of formaldehyde at 4% was applied to the CAM. 5 min later, the CAM was cut around the disk and all (disc and CAM) was placed in a Petri. Then the photos were taken with a Nikon digital camera D5100 (made in Thailand) and the images were subsequently analyzed with the software Image J. The numbers of vessel branch points contained in a circular region (equal to the area of each filter disk) were counted manually. The percentage of vascularization (density) is measured relative to a normal control vascularization. The ability to inhibition angiogenesis was calculated by the following equation:

$$\%\mathrm{AIA} = \left[\left(\mathrm{Nbv}_{\mathrm{control}} - \mathrm{Nbv}_{\mathrm{sample}}\right)/\mathrm{Nbv}_{\mathrm{control}}\right] \times 100$$

Nbv = number of blood vessel branch points. The IC_{50} (concentration providing 50% inhibition) of extracts and standards was determinate using regression curves in the linear range of concentrations. The experiments have been repeated at least four times, and the results were reproducible.

Drabkin reagent test

The determination of hemoglobin in CAM sections was done by the Drabkin and Austin [24] assay method. The dissected CAM sections were crushed with a pestle and mortar. The pilate was mixed with 5 ml of cooled normal salt solution and then homogenized. The resulting solution was centrifuged at 2500 rpm for 30 min. 3 mL of Drabkin reagent was added to 1 mL of supernatant. The reaction was incubated at room temperature (27 ± 3 °C) for 20 min and the absorbance measured at 580 nm using a UV spectrophotometer (Evolution 60S, USA). All tests were performed in triplicate to ensure reproducibility of the result.

Toxicity of extracts

Toxicity of extracts was determined by observation of embryos statue after extracts actions on vascularization according to a previously reported methods [25].

Anti-inflammatory activity

The anti-inflammatory activity was evaluated by protein denaturation method and membrane stabilization method.

Protein anti-denaturation test

Protein denaturation methods have been used [26, 27] with slight modifications mentioned by Ngoua-Meye-Misso et al. [28]. 0.1 mL of fresh chicken egg albumin was mixed with 1.9 mL of phosphate buffered saline (PBS, pH 6.4) and 1 mL of varying concentration of extract (6.25, 12.5, 25, 50 and 100 μg/mL). A similar volume of distilled water served as a negative control. Then the mixtures were incubated at 37 °C in an incubator (Ecocell, LSIS-B2V/EC55, Germany) for 20 min and then heated at 70 °C for 5 min. After cooling, the absorbances were measured at 660 nm on the spectrophotometer (Evolution 60S, USA). Sodium diclofenac in the final concentrations of 31.25, 62.5, 125, 250 and 500 μg/mL, was used as a reference drug and similarly treated for the determination of absorbance.

$$\text{Inhibition } (\%) = \left[\left(\mathrm{Abs}_{\mathrm{sample}} - \mathrm{Abs}_{\mathrm{control}}\right)/\mathrm{Abs}_{\mathrm{control}}\right] \times 100$$

Abs = absorbance. The concentration of the extract for 50% inhibition (IC_{50}) was determined by the dose-response curve.

Membrane stabilization test

The membrane stabilization test was evaluated by the method of hemolysis of red blood cells. This hemolysis was induced on the one hand by heat on the other hand by distilled water [29] with some modifications.

Preparation of the suspension of erythrocytes

Fresh whole blood (3 mL) collected from healthy volunteers in EDTA tubes was centrifuged at 2500 rpm for 10 min at 4 °C. A volume of normal saline equivalent to that of supernatant was used to dissolve the red blood cells. The volume of dissolved red blood cells obtained was measured and reconstituted in the form of a 40% *v*/v suspension with isotonic buffer solution (10 mM sodium phosphate buffer, pH 7.4). The buffer solution contained 0.2 g of NaH_2PO_4, 1.15 g of Na_2HPO_4 and 9 g of NaCl in 1 L of distilled water. Reconstituted red blood cells (supernatant resuspended) were used as such. The study protocol was performed according to the Helsinki declaration and approved by the Ethics Committee of Gabon (N° 009 March 2013).

Hemolysis induced by heat

Samples of used extracts were dissolved in isotonic phosphate buffer solution. A set of 5 centrifugation tubes containing respectively 2 mL of extracts at increasing concentrations (15.625, 31.25, 62.5, 125 and 250 μg/mL). Sodium diclofenac, with the same concentration range was used as the reference medicine. The negative control contained 2 mL of distilled water. A suspension of 0.1 mL of red blood cells was added to each of the tubes and

mixed gently. The tubes were incubated at 54 °C for 20 min in a water bath. After incubation, tubes were centrifuged at 2500 rpm for 10 min at 4 °C and the hemoglobin content of the supernatant was estimated using the spectrophotometer (Evolution 60S, USA) at 540 nm. The percentage of inhibition by the extract was calculated as follows:

$$\% \text{ Inhibition of hemolysis} = \left(1 - OD_{sample}/DO_{control}\right) \times 100$$

Where OD_{sample} = absorbance of the sample; $DO_{control}$ = absorbance of the control. The concentration of the extract for 50% inhibition (IC_{50}) was determined by the dose-response curve.

Hemolysis induced by hypotonicity

The extract samples were dissolved in distilled water (hypotonic solution) at different concentrations obtained by double dilution (15.625, 31.25, 62.5, 125 and 250 μg/mL). Sodium diclofenac, at the same concentrations, was used as a reference medicine. Distilled water was used as a negative control. 2 ml of sample were mixed with 0.1 mL of a suspension of erythrocytes and then the mixtures were incubated for 1 h at 37 °C. The tubes were then centrifuged at 2500 rpm for 10 min at 4 °C. the hemoglobin content of the supernatant was estimated using the spectrophotometer (Evolution 60S, USA) at 540 nm. The percentage of hemolysis was calculated assuming hemolysis produced in the presence of distilled water as 100%. The percentage inhibition of hemolysis by the extract was calculated as follows:

$$\% \text{ Inhibition of hemolysis} = \left(1 - OD_{sample}/DO_{control}\right) \times 100$$

Where OD_{sample} = absorbance of the sample; $DO_{control}$ = absorbance of the control. The concentration of the extract for 50% inhibition (IC_{50}) was determined by the dose-response curve.

Observation of erythrocyte membranes

A blood smear was performed before and after treatment with the extracts to observe the appearance of erythrocyte membranes. 5 μL of sample was deposited and spread on the slide. After drying, fixing with methanol and stained with Giemsa, the cells were observed under an optical microscope (Motic Digital Microscope) coupled to a computer using the Motic image plus 2.0 software and the photos were taken.

Antioxidant activity

DPPH test

The method described by Scherer and Godoy [30], based on DPPH radical test, was used to determine the Antioxidant Activity Index (AAI). Briefly, DPPH solution was prepared by dissolving 10 mg of DPPH powder in 100 mL methanol. Graded concentrations of extracts ranging from 3.125 to 100 μg/mL obtained by two-fold dilutions were prepared and 1 mL of each dilution were mixed with 1 mL of the working solution of DPPH (100 μg/mL). Absorbencies were measured at 517 nm after 15 min incubation at room temperature in the dark. Ascorbic acid (vitamin C), Butylated hydroxytoluene (BHT) and quercetin were used as references. The ability to scavenge DPPH radical was calculated by the following equation:

$$\%RSA = \left(A_{control} - A_{sample}/A_{control}\right) \times 100$$

A = absorbance at 517 nm. The IC_{50} (concentration providing 50% inhibition) of extracts and standards was determinate using regression curves in the linear range of concentrations. The AAI was then calculated as follows: $AAI = [DPPH]_f\ (\mu g.mL^{-1})/IC_{50}\ (\mu g.mL^{-1})$, $[DPPH]_f$ is the final concentration of DPPH (50 μg/mL). We considered criteria of Scherer and Godoy (2009) according to which plant extracts show poor antioxidant activity when AAI < 0.5, moderate antioxidant activity when AAI between 0.5 and 1.0, strong antioxidant activity when AAI between 1.0 and 2.0, and very strong when AAI > 2.0.

Total antioxidant capacity

The assay was based on the reduction of Mo (VI) to Mo (V) and subsequent formation of a green phosphate/ Mo (V) complex in acid pH [31]. A total volume of 0.3 mL extract dissolved in methanol was added to 3 mL of reagent solution (0.6 mol/L sulphuric acid, 28 mmol/L sodium phosphate and 4 mmol/L ammonium molybdate). The mixtures were incubated at 70 °C for 90 min then cooled to room temperature. The absorbance was measured at 695 nm. The total antioxidant activity was expressed as the number of equivalence of ascorbic acid, BHT and quercetin.

Statistical analysis

The data were expressed as the mean ± standard deviation (SD) of three independent experiments and analyzed using one-way analysis of variance, Student's t-test and XLSTAT 2015.4.01 software. *P*-values of < 0.05 were considered to be statistically significant.

Results

Phytochemical screening

The results of the phytochemical screening are shown in Table 1 and show that the extracts of *Scyphocephalium ochocoa* bark are rich in bioactive compounds. All extracts are rich in phenolic compounds (total polyphenols, tannins, total flavonoids and proantocyanidins). The water extract has an abundance of saponosides,

Table 1 Results of the preliminary phytochemical screening

Chemical groups		Wat	Wat-eth	Eth
Saponosids		+++	++	–
polyphenols		+++	+++	+++
Sterols and triterpenes		++	++	+++
Oses and holosides		+	++	+++
Tannins	Gallics	+++	+++	+++
	Catechics	+++	+++	+++
Alkaloids		+++	+	+
Cyanidins	Flavons	+++	–	+
	Flavanons	–	–	–
	Flavonols	–	+++	–
	Flavanonols	–	+++	+++
Total flavonoids		+++	+++	+++
Anthocyans		–	–	–
Proanthocyanidins		+++	+++	+++
Anthracenics		+++	+++	–
Coumarins		–	–	–
Cardiac glycosides	Digitoxins	–	+++	–
	Digitoxigenins	–	–	–
	Gitoxins	–	–	++
	Gitoxigenins	+++	–	–
Reducing sugar		–	–	–

+++ = Very abundant; ++ = Abundant; + = Not abundant; – = Not detected; Wat = Water; Eth = Ethanol; Wat-Eth = Water-ethanol

alkaloids, anthracenics and digitoxigenins while the water-ethanol extract has a strong presence of flavonols, flavanonols and digitoxins. In particular, the ethanol extract has an abundance of sterol, triterpenes, oses and holosides.

Phenolic compound content

The Table 2 presents the contents of total phenolic, total flavonoids, total tannins and total proanthocyanidins of extracts from *Scyphocephalium ochocoa.*

Total phenolic

The contents of total phenolic in terms of gallic acid equivalent (standard curve equation: $Y = 0.0012X - 0.0004$, $R^2 = 0.990$) ranged from 1885.611 ± 31.356 to 6284.667 ± 3.333 mgGAE/ 100 g of drug and were abundants in water extracts.

Total flavonoid

Total flavonoids (standard curve equation: $Y = 0.0032X + 0.0077$, $R^2 = 1$) ranged from 159.365 ± 2.228 to 480.406 ± 51.498 mgEQ/ 100 g and were abundants in water extracts.

Total tannins

Levels of tannins were expressed in terms of tannic acid equivalent (TAE). The equation of the right-hand side of the proportioning of the total tannins by the reference method of European Community gave $Y = 0.0009X + 0.2088$ with $R^2 = 1$. Total tannins are ranged from 194.667 ± 7.286 to 1898.000 ± 62.568 mg EQ/ 100 g and were abundant in water extracts. There were abundant in water extracts than water-ethanol and ethanol extracts.

Total proanthocyanidins contents

Levels of proanthocyanidins were expressed in terms of apple proanthocyanidins equivalent (APE). The equation of the right-hand side of the proportioning of the proanthocyanidins by the HCl-Butanol method gave $Y = 0.0006X + 0.0024$ with $R^2 = 0.986$. Proanthocyanidin contents had ranged between 94.944 ± 2.158 to 1234.111 ± 39.457 mg APE/ 100 g of drug and are very abundant in all extracts of *S. ochocoa*.

Antiangiogenic activity

The antiangiogenic potential of the extracts was evaluated in vivo with the chicken chorioallantoic membrane (CAM) the eighth embryonic day. The fertilized eggs were treated with aqueous extracts (60, 125, 250 and 500 μg/mL). The degree of vessel branches formation on CAM was scored 48 h later. The vessel density is the percentage of blood supply to the analysis area. It is inversely proportional to the degree of inhibition. In the presence of phosphate buffered saline (PBS) used as a negative control, the target area has a vascularization percentage of 100%, corresponding to a normal vasculature with a number of vessels branches equal to 15. The inhibitory potential of extracts was expressed through their inhibitory concentration fifty (IC_{50}). In the

Table 2 Results of phenolic compounds dosage

Phenolic compounds	Water	Water-ethanol	Ethanol
TPC[a] (mgGAE/ 100 g of extract)	6284.667 ± 3.333	1885.611 ± 31.356	3123.444 ± 1.156
TFC (mgEQ/ 100 g of extract)	480.406 ± 51.498	159.365 ± 2.228	364.781 ± 0.722
TTC (mgTAE/ 100 g of extract)	1898.000 ± 62.568	194.667 ± 7.286	392.815 ± 1.301
TPC (mgAPE/ 100 g of extract)	1234.111 ± 39.457	94.944 ± 2.158	254.944 ± 0.893

TPC[a] = Total phenolic content; TFC = Total flavonoid content; TTC = Total tannins content; TPC = Total proanthocyanidins content

presence of sorafenib (positive control), the number of blood vessels branches is reduced to 6, 2, 1 and 0, respectively to a concentration of 60, 125, 250 and 500 μg/mL and respectively an ability to inhibition angiogenesis to 60%, 86.667%, 93.333% and 100% with IC_{50} = 0.197 μg/mL. In the presence of the water extract of *S. ochocoa*, the number of blood vessels branches is reduced to 8, 6, 4 and 0, respectively to a concentration of 60, 125, 250 and 500 μg/mL and respectively an ability to inhibition angiogenesis to 60%, 86.667%, 93.333% and 100% with IC_{50} = 1.153 μg/mL. All results are reported in Table 3 and Fig. 2. Compared to the reference molecule, the water extract of *S. ochocoa* has good antiangiogenic activity although this activity is lower than that of the reference.

Drabkin reagent test

Drabkin's reagent test determined the hemoglobin content of the dissected CAM sections. The results are summarized in Fig. 3. Considering that the hemoglobin content is 100% for the sections of CAM treated with the PBS (negative control), it is shown that in the presence of the variable concentrations of the drug of 60, 125, 250 and 500 μg/mL, the hemoglobin content is 55%, 35%, 20% and 2%, respectively for water extract. This content is 40%, 14%, 8% and 0%, respectively for sorafenib. These data indicate that the hemoglobin content decreases with the number of branches of the blood vessels and is dependent on the concentration of the drug. This test therefore confirms the antiangiogenic activity observed.

Toxicity of extracts

The status of embryos after extracts action on vascularization inform on toxicity (Table 3). No embryo death was recorded in the concentration range tested indicating that the observed antiangiogenic activity is not due to the toxicity of the extracts.

Anti-inflammatory activity

The anti-inflammatory activity was assessed on the one hand by protein denaturation method [26, 27] and on the other hand by membrane stabilization method [29].

Protein anti-denaturation test

The results of protein anti-denaturation test are shown in Table 4. These results indicate that *S. ochocoa* extracts have good anti-denaturation activities with IC_{50} proteins ranging from 34.775 ± 2.543 μg/mL to 74.577 ± 3.456 μg/mL. Inhibition of protein denaturation is stronger with water-ethanol extract (IC_{50} = 34.775 ± 2.543 μg/mL). This anti-denaturation activity is similar to that of the reference drug, sodium diclofenac (IC_{50} = 35.746 ± 2.374 μg/mL) (p value = 0.582). In addition this activity is dependent on the concentration. Thus for extract concentrations of 6.25, 12.5, 25, 50 and 100 μg/mL, the percentages of inhibitions are 17.588 ± 1.553%, 33.040 ± 2.442%, 38.317 ± 1.797%, 46.357 ± 1.599% and 65.201 ± 1.178%, respectively for water-ethanol extract.

Membrane stabilization test

The anti-inflammatory activity of *S. ochocoa* extract observed by inhibition of albumin denaturation is confirmed by membrane stabilization test. The results are summarized in Table 5. The extracts showed good inhibition of hemolysis of red blood cells.

For heat-induced hemolysis, all the extracts showed a very strong anti-hemolytic activity (IC_{50} ranging from 36.793 ± 0.529 μg/mL to 48.912 ± 0.9573 μg/mL) compared to the reference drug, sodium diclofenac (IC_{50} = 180.911 ± 2.205 μg/mL) (p value = 0.001). The inhibition percentages of water extract are 46.850 ± 0.059%, 57.261 ± 0.102%, 56.985 ± 0.120%, 33.639 ± 0.137% and 7.710 ± 0.120% at respective concentrations of 250, 125, 62.5, 31.25 and 15.625 μg/mL.

For hemolysis induced by a hypotonic solution, only the water extract was more active (IC_{50} = 56.713 ± 0.492 μg/mL) than the other two extracts.

Table 3 Antiangiogenic effect of *S. ochocao* and Sorafenib

Drugs	Dose μg/mL per œuf	Tested eggs (n)	Embryos status after 48 h	Branches vessels nombers	%AIA	IC_{50} (μg/mL)	P value
PBS (negative control)	–	5	living	15 ± 0.954	–	–	0.002
Sorafenib (positive control)	62.5	5	living	6 ± 0.753	60 ± 1.056	0.197 ± 0.062	
	125	5	living	2 ± 0.954	86.667 ± 1.045		
	250	5	living	1 ± 0.976	93.333 ± 0.965		
	500	5	living	0	100		
Water extract of *S. ochocao*	62.5	5	living	8 ± 0.834	26.667 ± 1.634	1.153 ± 0.089	
	125	5	living	5 ± 0.850	60 ± 0.507		
	250	5	living	3 ± 0.500	73.333 ± 0.910		
	500	5	living	0	100		

A significant difference is observed between the antiangiogenic effect of sorafenib and that of water extract of *S. ochocoa* (p value = 0.002)

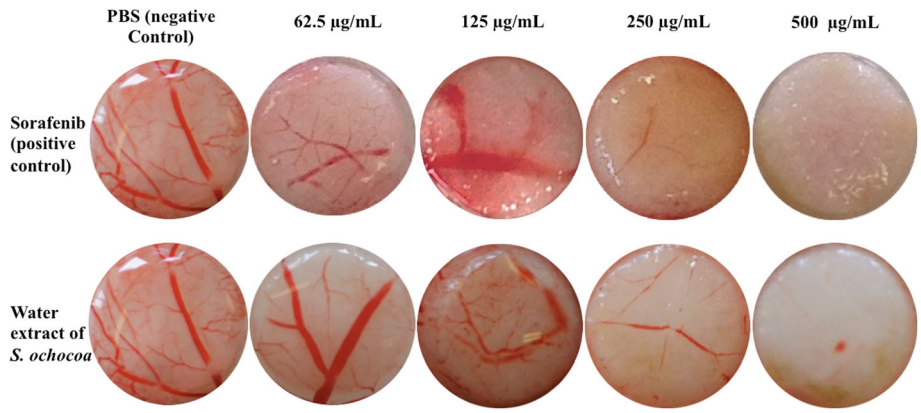

Fig. 2 Inhibitory effects of water extracts of plants on angiogenesis. The CAM of a 8 days old chick embryo was separately exposed to PBS (control). Extracts were introduced on top of the CAMs. After 48 h of incubation, the CAM tissue directly beneath each filter disk was resected, and digital images of the CAM sections were captured

Anti-hemolytic activity exhibited by the water extract is similar to that of sodium diclofenac (58.590 ± 1.021 µg/mL) (p value = 0.471).

The observation of the blood smears performed after the various treatments shows that red blood cells have a normal form in presence of an isotonic solution, in presence of water extract (250 µg/mL) and in presence of sodium diclofenac (250 µg/mL). In presence of a hypotonic solution, the haemolysis is complete and the limbs are disorganized in presence of low concentration of water extract (15.625 µg/mL) and sodium diclofenac (15.625 µg/mL) (Fig. 4).

Antioxidant activity

The antioxidant activity was evaluated by DPPH radical inhibition test and phosphomolybdenum method. The results are shown in Table 6. Compared with the other crude extracts, water-ethanol extract has a greater antioxidant activity marked by a stronger inhibition of DPPH radical (IC_{50} = 4.969 ± 0.263 µg/mL, AAI = 10.080 ± 0.517) and by a greater total antioxidant capacity (87.766 ± 0.852 EQ (mg)/g, 131.302 ± 1.102 VtCE (mg)/g and 570.364 ± 5.307 BHTE (mg)/g dry extract). Comparing this antioxidant activity with those of the reference molecules, it appears that water-ethanol extract has an antioxidant activity three times stronger than that of BHT (IC_{50} = 13.759 ± 0.029 µg/mL, AAI = 3.634 ± 0.008), twice as strong as that of quercetin (IC_{50} = 11.224 ± 0.611 µg/mL, IAA = 4.463 ± 0.245) and similar to that of vitamin C (IC_{50} = 4.597 ± 0.095 µg/mL, AAI = 10.880 ± 0.228). Other extracts also show good antioxidant activity with IC_{50} ranging from 8.373 ± 0.242 µg/mL to 16.188 ± 0.336 µg/mL and AAI ranging from 3.090 ± 0.065 to 5.975 ± 0.172.

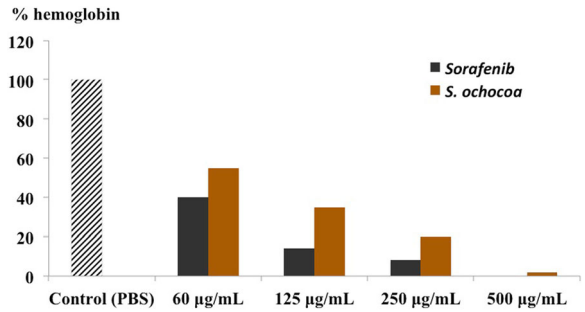

Fig. 3 Haemoglobin content of CAM sections detected by Drabkin's assay. The hemoglobin content in the CAM sections was determined by Drabkin method. As in the case of macroscopic observation of CAM that showed that the number of blood vessel branches was concentration dependent, so the present figure shows a hemoglobin content dependent on the concentration of *S. Ochocoa* water extract and sorafenib

Discussion

Cancer correspond to a rapid proliferation of abnormal cells that, beyond their usual boundaries, can invade adjacent parts of the body and then form metastases, which according to WHO are the leading cause of cancer deaths [4]. It is clearly established that reactive oxygen species (ROS), chronic inflammation and angiogenesis play an important role in initiation, tumor progression and metastasis formation [6, 7]. In order to contribute to the search for potential phyto-anticancer drugs, this preliminary study aimed to perform a phytochemical screening, to determine the content of phenolic compounds, to evaluate the antiangiogenic, anti-inflammatory and antioxidant activities of ethanol, water-ethanol and water extracts of *S. ochocoa* barks. This African medicinal plant is used in the treatment of several diseases such as female infertility, febrile body aches, gonorrhea, anemia, rheumatism, joint pain,

Table 4 Effect of *S. ochocoa* extracts and diclofenac sodium against protein denaturation

% Inhibition of *S. ochocoa* extracts				Sodium diclofenac	
Conc. (μg/mL)	Water	Water-Ethanol	Ethanol	Conc. (μg/mL)	% Inhibition
100	66.332 ± 1.777	65.201 ± 1.178	56.281 ± 1.264	500	129.481 ± 1.508
50	31.281 ± 1.244	46.357 ± 1.599	39.573 ± 1.954	250	103.015 ± 2.970
25	17.714 ± 1.178	38.317 ± 1.797	32.161 ± 2.132	125	96.566 ± 2.609
12.5	13.693 ± 1.178	33.040 ± 2.442	24.497 ± 2.152	62.5	71.106 ± 3.048
6.25	4.899 ± 1.533	17.588 ± 1.553	10.678 ± 1.244	31.25	43.300 ± 1.002
IC_{50} (μg/mL)	74.577 ± 3.456	34.775 ± 2.543	40.345 ± 3.567		35.746 ± 2.374
P value	0.014	0.582			

There is no significant difference between the IC_{50} of the water-ethanol, ethanol extract and diclofenac (p value = 0.582). On the other hand the difference is made with the water extract (p value = 0.014)

ovulation disorders, breast cancer, stomach cancer, skin cancer and liver cancer.

The results of the phytochemical screening revealed that the extracts of *S. ochocoa* are rich in total polyphenols, tannins, total flavonoids and proantocyanidins, saponosides, alkaloids, anthracenics and digitoxigenins, flavonols, flavanonols, digitoxins, sterol, triterpenes, oses and holosides. High levels of phenolic compounds (total polyphenol) were recorded. The polyphenol content of *S. ochocoa* is higher than that recorded in *Oncoba welwitschii* plants (with contents ranging from 232.050 ± 2.200 to 735.150 ± 23.650 mgGAE/g) and *Tetrorchidium oppositifolium* (with quantities ranging from 126.400 ± 0.750 to 718.100). ± 0.100) [32]. Several researchers have shown that polyphenols are highly active compounds against cancer, inflammatory diseases, cardiovascular, neuro-degenerative (Parkinson's and Alzheimer's disease), are powerful antioxidants and antivirals agents [33].

The results also indicate that water extract has a strong anti-angiogenic activity marked by the inhibition of the formation of new blood vessels in the CAM. This activity is confirmed by Drabkin test which makes it possible to determine the hemoglobin level in the sections of CAM after treatment with the extracts. The hemoglobin level is proportional to the number of blood vessels. By comparison of IC_{50}, the antiangiogenic activity of *S ochocoa* (IC_{50} = 1.153 ± 0.089 μg/mL) is lower than that of the reference drug, sorafenib (IC_{50} = 0.197 ± 0.062 μg/mL). However, this activity is greater than that of *Oncoba welwitschii* and *Tetrorchidium oppositifolium* which at 500 μg/mL exhibit an antiangiogenic activity of 83.334% [32] while at this same concentration, *S.*

Table 5 Effect of *S. ochocoa* extracts and diclofenac sodium against membrane stabilization

	Conc. (μg/mL)	% Inhibition of extracts from *S. ochocoa*			Sodium diclofenac
		Water	Water-ethanol	Ethanol	% Inhibition
Hemolysis induced by hypotonicity	250	66.144 ± 1.663	56.867 ± 1.948	76.348 ± 0.017	100.000 ± 0.000
	125	56.532 ± 0.744	50.626 ± 0.452	51.937 ± 0.296	100.000 ± 0.000
	62.5	53.436 ± 0.603	43.873 ± 0.152	30.573 ± 0.163	99.583 ± 1.078
	31.25	32.633 ± 0.452	21.256 ± 1.285	14.720 ± 0.311	99.132 ± 1.068
	15.625	10.874 ± 0.149	10.539 ± 1.095	7.672 ± 1.976	0.885 ± 0.121
	IC_{50} (μg/mL)	56.713 ± 0.492	71.200 ± 1.833	133.375 ± 0.661	58.590 ± 1.021
	P value	0.471		0.016	0.471
Hemolysis induced by heat	250	46.850 ± 0.059	56.867 ± 2.948	69.822 ± 2.791	71.039 ± 0.112
	125	57.261 ± 0.102	41.033 ± 1.826	56.118 ± 3.369	32.971 ± 0.640
	62.5	56.985 ± 0.120	33.077 ± 1.442	35.542 ± 2.841	13.788 ± 3.527
	31.25	33,639 ± 0.137	20.704 ± 2.170	12.777 ± 1.437	6.430 ± 1.453
	15.625	7.710 ± 0.120	1.667 ± 0.995	7.553 ± 1.878	2.911 ± 0.147
	IC_{50} (μg/mL)	36.793 ± 0.529	39.526 ± 0.710	48.912 ± 0.9573	180.911 ± 2.205
	P value	0.273			0.001

For hypotonicity-induced hemolysis, the ability to stabilize the membrane is similar for water extract, ethanol and diclofenac (p value = 0.471). A significant difference is observed with ethanol extract (p value = 0.016)

For heat-induced hemolysis, all extracts have the same effect on membrane stabilization (p value = 0.273). However, their effect is significantly different from that of diclofenac (p value = 0.001)

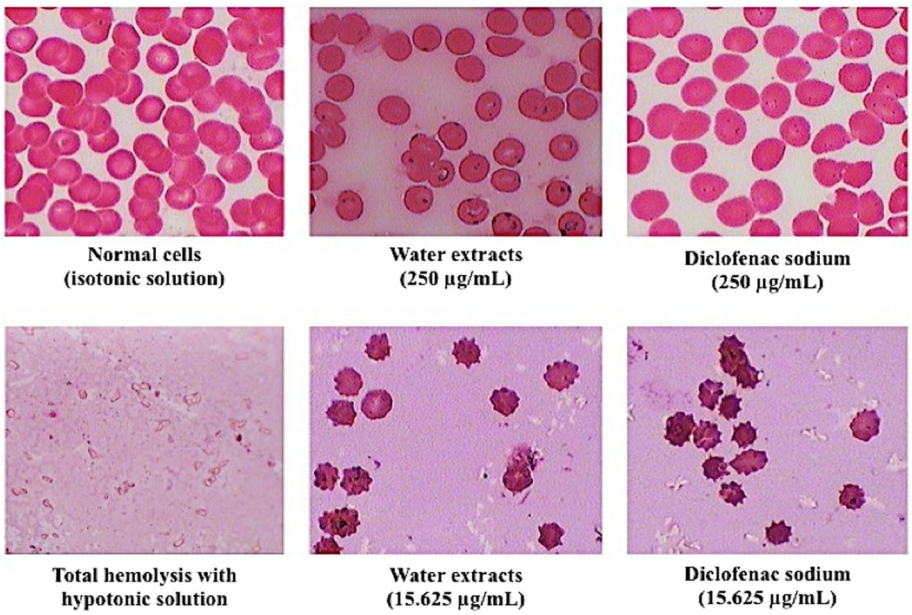

Fig. 4 Appearance of erythrocytes before and after the treatment with the extract and sodium diclofenac. The smear was performed before and after treatment with extracts and sodium diclofenac to observe the appearance of erythrocytes. Here it is the case of hemolysis induced by a hypotonic solution. It is well shown that at 250 µg/mL of drugs, erythrocytes have a normal form. However at low concentration (15.625 µg/mL), these cells have an irregular contour testifying to disorganization of the membrane but the cells are not lysed

ochocoa has a 100% activity. It is similar to the activity of *Lophira procera*, which has an inhibition of angiogenesis of 100% at 500 µg/mL [28]. From these results, it appears that water extract of *S. ochocoa* could be used in the treatment of angiogenesis-related diseases such as diabetic proliferative retinopathy, psoriasis, rheumatoid arthritis, atherosclerosis and cancers. The use of *S. ochocoa* as anticancer in traditional medicine could therefore be justified.

In addition to having antiangiogenic activity, *S. ochocoa* exhibited strong anti-inflammatory activities marked by inhibition of protein denaturation. Water-ethanol extract was more active than the other extracts and activity was similar to that of sodium diclofenac. It is well established that denaturation of tissue proteins leads to inflammatory and arthritic diseases [34]. As a result, phyto-substances that can inhibit protein denaturation may be useful for the research and development of anti-inflammatory drugs. The anti-inflammatory activity of *S. ochocoa* extracts was confirmed by erythrocyte membrane stabilization test. The results showed that at different concentrations of extracts, human erythrocyte membranes were protected against hypotonic solution-induced lysis and heat. Indeed, during the inflammatory reaction, lysosomes release their acidic lytic enzymes and pro-inflammatory mediators that will degrade most of the proteins [35]. The beneficial effect of nonsteroidal anti-inflammatory drugs (NSAIDs) is their ability to inhibit the release of lysosomal enzymes or their ability to stabilize lysosomal membranes [36]. Exposure of red blood cells to harmful substances such as hypotonic media and heat leads to membrane lysis,

Table 6 Results of antioxidant activity of *S. ochocoa* extracts

Samples	Antioxidant activity DPPH assay		Total antioxidant capacities		
	IC_{50} (µg/mL)	AAI	QE (mg)/g dry extract	VtCE (mg)/g dry extract	BHTE (mg)/g dry extract
Eth ext	8.373 ± 0.242	5.975 ± 0.172	77.207 ± 0.704	117.654 ± 0.909	504.606 ± 4.382
Wat-Eth ext	4.969 ± 0.263	10.080 ± 0.517	87.766 ± 0.852	131.302 ± 1.102	570.364 ± 5.307
Wat ext	16.188 ± 0.336	3.090 ± 0.065	15.309 ± 0.770	37.654 ± 0.995	119.152 ± 4.792
BHT	13.759 ± 0.029	3.634 ± 0.008			
Quercetin	11.224 ± 0.611	4.463 ± 0.245			
Vitamin C	4.597 ± 0.095	10.880 ± 0.228			

Wat ext. = Water extract; Eth ext. = Ethanol extract; Wat-Eth extract = Water-ethanol extract; QE = Quercetin equivalent; VtCE = Vitamin C equivalent; BHTE = BHT equivalent

followed by hemolysis and oxidation of hemoglobin [37]. Human erythrocyte membranes have been shown to be identical to lysosome membranes [36], so the antihemolytic effect of *S. ochocoa* extracts observed on erythrocytes can be transposed to lysosomes. The observation of blood smears shows that erythrocyte membranes are more stable in the presence of a concentration of 250 μg/mL of water extract and sodium diclofenac. Thus, *S. ochocoa* extracts can exert anti-inflammatory effect by membranes stabilization and prevent lytic enzymes release and pro-inflammatory active mediators.

Finally, all extracts of *S. ochocoa* showed strong antioxidant activities manifested by the inhibition of DPPH radical and by the reduction of Mo (VI) in Mo (V) leading to the formation of a complex Mo (V) of green phosphate. Water-ethanol extract showed greater antioxidant activity compared to the other extracts. It was shown that water-ethanol extract of *S. ochocoa* had an antioxidant activity half as much as that of vitamin C with the respective IC_{50} values of $IC_{50} = 0.169 \pm 0.019$ μg / ml and $IC_{50} = 0.267 \pm 0.009$ μg/mL [38]. However, the results of this study show that water-ethanol extract of *S. ochocoa* has antioxidant activity similar to that of vitamin C. This extract has an antioxidant activity slightly above that of water-acetone extract of *Englerina gabonensis* ($IC_{50} = 5.67 \pm 0.32$ μg/mL) [39]. *S. ochocoa* has a strong activity as the methanol extract of *Syzygium rowlandii* ($IC_{50} = 9.22 \pm 0.02$ μg/mL) [40] and *Eucalyptus citriodora* ($IC_{50} = 34.2 \pm 2.3$ μg/mL) [41].

It is well known that reactive oxygen species play an important role in angiogenesis, tumor progression and metastasis formation [6, 7]. Once formed, the cancer cell secretes reactive oxygen species (ROS). These ROS will activate the hypoxia-inducing factor (HIF-1α) which will lead, on the one hand, to the secretion of vascular endothelial growth factors (VEGFs) whose role is to stimulate the proliferation and migration of endothelial cells to increase the microvascular permeability. These VEGFs will interact with their receptors (VEGFR2) and induce neovascularization [8]. On the other hand, HIF-1α will induce the production of matrix metalloproteinases 2 and 9 (MMP 2 and 9), which will degrade membranes and cause tumor expansion. ROS can also interact directly with VEGF receptors and induce angiogenesis or oxidize lipids that will interact with Toll receptors and activate nuclear kappa B (NF-kB) factors that are a transcription factor involved in immune response and response in cell stress, its activation triggers the transcription of anti-apoptotic genes in the nucleus [42]. ROS can also induce the production of thioredoxin which leads to the synthesis of matrix metalloproteinases 9 [8]. As a result, a phyto-product with a high content of polyphenols and flavonoids, with good anti-angiogenic, anti-inflammatory and antioxidant activities such as extracts of *S. ochocoa* could be an ideal candidate for the development of anticancer drugs.

Conclusion

In conclusion, this study provides convincing evidence that bark extracts of *Scyphocephalium ochocoa* have beneficial health effects. Water, water-ethanol and ethanol extracts of barks showed high levels of polyphenols, flavonoids, tannins and proanthocyanidins. The phyto-constituents identified in water, water-ethanol and ethanol extracts of *S. ochocoa* were found to be biologically active by their antiangiogenic ability manifested by the inhibition of the news branches vessels formation on CAM model. Then by their powerful anti-inflammatory effects marked by the inhibition of the denaturation of the proteins and by the stabilization of the erythrocyte membranes. Finally by their strong antioxidant activities in terms of reducing power and a significant ability to trap the DPPH radicals. The water extract is richer in phenolic compounds, has a stronger anti-inflammatory activity than the other extracts and very active on angiogenesis. These results could justify the use of *S. ochocoa* extracts in traditional medicine in the treatment of cancers, other diseases related to angiogenesis (diabetic retinopathy, rheumatoid arthritis, plaque of atherosclerosis), inflammatory diseases due to pathogenicity microbial and oxidative stress caused by the overproduction of radicals.

Abbreviations

AAI: Antioxidant activity index; AIA: Ability to inhibition angiogenesis; APE: Apple procyanidins equivalent; BHT: Butylated hydroxytoluene; BHTE: BHT equivalent; CAM: Chicken chorioallantoic membrane; DPPH: 2,2-diphenyl-1-picryl-hydrazyl; EDTA: Ethylene diamine tetraacetic acid; GAE: Gallic acid equivalent; HIF-1α: Inducing factor of hypoxia; MMP: Matrix metalloproteinases; NF-kB: Kappa B nuclear factors; NSAIDs: Non-steroidal anti-inflammatory drugs; PBS: Phosphate-buffered saline; QE: Quercetin equivalent; TAE: Tannic acid equivalent; TFC: Total flavonoid content; TNFα: Tumor necrosis factor-α; TPC *: Total phenolic content; TPC: Total proanthocyanidins content; TTC: Total tannins content; VEGF: Vascular endothelial growth factor; VtCE: Vitamin C equivalent

Acknowledgements

The authors are very much thankful to Shell Gabon for the financial support of materials in Laboratory of Research in Biochemistry (LAREBIO) USTM, Franceville-Gabon. We are very much grateful to local informants and Mr. Meye Misso Paul-Edouard who shared their knowledge on the use of medicinal plants with us. We also thank Mr. Meye M'Ella Antoine, Mr. Allogho Essono Judicaël and Ms. Ada Mengome Meredith for their participation.

Funding

Laboratory of Research in Biochemistry (LAREBIO), Department of Chemistry-Biochemistry, Faculty of Sciences of USTM in Franceville has partially supported this research work. The authors have no other affiliation or financial involvement with any organization.

Availability of data and materials

All data and materials are contained and described in the manuscript. The dataset has been deposited in publicly available repositories. The barks of *S. ochocoa* were collected August 2015 in Mitzic, Woleu-Ntem (Northern of Gabon). They have been identified at the National Herbarium of IPHAMETRA, Libreville (Gabon). Voucher specimen has been deposited in the Herbarium of IPHAMETRA and Laboratory of Biochemistry Research (LAREBIO), Department of Chemistry-Biochemistry, Faculty of Sciences of USTM, Franceville.

Authors' contributions

RLNMM is the lead author, designed the study, developed the protocols, analyzed the data and drafted the manuscript. JDLCN reviewed the protocols, provided material support, and made corrections to the manuscript. CSO participated in all the experiments and the manuscript. JPO and GRNA participated in the interpretation and analysis of the data. FOA is Head of Laboratory of Biochemistry and the Mixed Unit for Biomedical Research, University of Health Sciences, Libreville, Gabon. LCOE, Head Laboratory of Research in Biochemistry, oversaw this work and made the necessary editorial corrections and gave final approval for the submission of the revised version. All authors have read and approved the final version.

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Ethics approval and consent to participate

The study protocol was performed according to the Helsinki declaration and All volunteers have completed informed consent. The study protocol was approved by the Ethics Committee of Gabon (N° 009 March 2013).

Competing interests

The authors declare that there are no competing interests.

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Received: 15 February 2018 Accepted: 21 May 2018
Published online: 08 June 2018

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