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Original Article

Crude Extracts of *Codiaeum Variegatum* Stem Exhibit Potent Antioxidant and Anti-inflammatory Activities *in Vitro*

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Abstract

Background and objectives: *Codiaeum variegatum* (*C. variegatum*), which is commonly known as garden croton, is a medicinal plant used for the treatment of amoebiasis in Cameroon and some Asian countries. The present study aims to evaluate the antioxidant and anti-inflammatory activities of the stem crude extracts of *C. variegatum*.

Methods: Aqueous, hydroethanolic 70/30 (v/v) and ethanolic extracts were tested for antioxidant activity using DPPH radical scavenging, ferric iron-reducing antioxidant power (FRAP), and lipid peroxidation inhibitory assays. The anti-inflammatory activity was determined based on the inhibition of nitric oxide production on isolated mouse macrophages activated by *Saccharomyces cerevisiae*. Furthermore, the inhibitory effect of these extracts on 5-lipoxygenase activity and bovine serum albumin (BSA) denaturation was determined, and the activation of two lysosomal enzymes involved in phagocytosis was performed. The phytochemical screening of the plant extracts was performed using standard methods.

Results: The results revealed that the ethanolic extract (EE) exhibited the highest antioxidant activity, in terms of DPPH-free radical scavenging activity, FRAP, and its potential to inhibit lipid peroxidation (IC_{50} = 77.04 µg extract/mol of DPPH; EC_{50} = 543.6 µg/mL and IC_{50} = 21.52 µg/mL, respectively). However, this activity remained significantly lower than that of ascorbic acid (p < 0,05). Furthermore, the hydroethanolic extract (HE) had the highest anti-inflammatory activity on isolated mouse macrophages, in terms of inhibitory activity on NO production, BSA denaturation, and 5-lipoxygenase activity (IC_{50} = 8.80 µg/mL, IC_{50} = 205.9 µg/mL, IC_{50} = 0.08 µg/mL, respectively). However, there was no significant difference in the inhibitory activity of baicalin. Moreover, the activity of acid phosphatase and alkaline phosphatase increased in the presence of the HE (EC_{50} = 10.03 µg/mL and EC_{50} = 0.274 µg/mL, respectively). The phytochemical analysis of these extracts indicates the presence of phenolic compounds, and these may be responsible for the observed activities.

Conclusions: Overall, these results demonstrate that the hydroethanolic and ethanolic stem extracts of *C. variegatum* have good antioxidant and anti-inflammatory potential.

Keywords: *Codiaeum variegatum*; Antioxidant; Anti-inflammatory; Phytochemicals; Amoebiasis.

Abbreviations: AE, aqueous extract; BSA, bovine serum albumin; DPPH, 2,2-diphenyl-1-picryl hydrazyl; EC_{50}, effective concentration 50; EE, ethanolic extract; FRAP, ferric iron-reducing antioxidant power; HE, hydroethanolic extract; IC_{50}, inhibitory concentration 50; IL, interleukin; LOX, lipoxigenase; LPS, lipopolysaccharides; LTB4, leucotriènes B4; NO, nitric oxide; OD, optical density; SC, *Saccharomycces cerevisiae*; TBA, thiobarbituric acid; TCA, trichloroacetic acid; TGF-β, tumor growth factor-beta; TLR, toll-like receptor; TNF-α, tumor necrosis factor-alpha.

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Introduction

The immune system consists of a complex set of individualized organs and tissues, between which cells of both innate and adaptive immunity are constantly circulating.[1] Organisms use these types of immunity to maintain its physiological balance. Innate immune responses include several mechanisms, such as inflammatory reactions, which is the direct response of vascularized living tissues to aggression induced by numerous non-specific immune cells, of which macrophages and neutrophils are the most representative.[2] Following the recognition of a pathogen's lipopolysaccharide (LPS) pattern by Toll like receptor-2 (TLR-2) on the macrophage membrane, a cascade of reactions can be observed. This allows for the activation of cyclooxygenase and lipoxygenase pathways, and the production of pro-inflammatory mediators (interleukin [IL]1β, IL6, IL8, IL12, tumor necrosis factor-alpha [TNF-α] and nitric oxide [NO]), which eliminate this antigen.[3] The over-secretion of pro-inflammatory mediators for a long period of time may lead to a pathological state (Alzheimer's disease, rheumatoid arthritis, Crohn's disease, etc.), which is generally characterized as oxidation of the organism's structural macromolecules, such as membrane lipids.[4] In addition, cells in the immune system induce the production of antioxidant enzymes to protect themselves.[5]

Despite the body's efforts, there is sometimes a need for external help to overcome these dysfunctions. This help can be provided through diet, as a preventive measure, and drugs (steroidal and non-steroidal anti-inflammatory drugs), as a curative measure. However, these do not combine both antioxidant and anti-inflammatory effects, and some drugs are likely to have undesirable side effects (such as diarrhea, vomiting and nausea) in individuals.[6] There is an increasing emphasis on the use of herbal medicines as an alternative treatment. Plants are known to have moderate secondary effects, and those used in traditional medicine have various secondary metabolites that are known to have beneficial effects (including antioxidants, cancer prevention, and anti-platelet aggregation).[7] The World Health Organization (WHO) has established a strategy to enhance the use of medicinal plants.[8] Therefore, in developing countries, more attention is given on medicinal plants, as an alternative therapeutic strategy. *Codiaeum variegatum* (*C. variegatum*), which is commonly known as garden croton, is a plant of the Euphorbiaceae family found in tropical areas (Asia and the Pacific islands). The leaves and barks of this plant can be used for the treatment of intestinal infections.[9] Furthermore, this plant has larvicidal activity against the Aedes aegypti mosquito that carries dengue, chikungunya and zika.[10] A number of studies on various leaf extracts of *C. variegatum* have demonstrated numerous properties, such as antioxidant,[11] antilithiasis,[12] anti-amoebic,[13] anti-influenza[14] and anticonvulsant activities,[15] as well as the lack of sub-chronic toxicity of up to 200 mg/kg.[16] Furthermore, the phytochemical screening of its leaves revealed the presence of several secondary metabolites,[17] which include phenolic compounds that are well-known for its antioxidant, immunomodulatory and anti-inflammatory properties.[18] *C. variegatum* has been widely investigated, and has very useful properties for a medicinal plant. That is, this plant has multiple medicinal properties. However, the activities of several parts of a plant may differ due to the irregular distribution of secondary metabolites and the variability of extracted compounds, depending on the plant part and solvent used. Therefore, the present study aimed to evaluate the antioxidant and anti-inflammatory properties of *C. variegatum* stem extracts.

Material and methods

Ethical statement

All procedures in this study followed the Cameroon National Veterinary Laboratory guidelines and were approved by the Animal Ethical Committee of the Laboratory of Animal Physiology of the Faculty of Sciences, University of Yaoundé I–Cameroon.

Plant material

Fresh stems of *C. variegatum* (var. mollucanum) were collected from the locality of Nomayos, in the Centre region of Cameroon. The specimen was identified under number HNC 33570 at the Cameroon National Herbarium (CNH) in Yaounde, Cameroon. The stems were washed and rinsed with distilled water, dried at laboratory temperature, crushed in a blender to a fine powder, and preserved.

Preparation of extracts

One hundred grams of stem powder was boiled in 1 L of distilled water for 30 minutes, and cooled to room temperature. Then, the ethanolic and hydroethanolic extracts were prepared by macerating 100 g of stem powder for 48 hours in ethanol (95%) and ethanol/water (70:30, v/v), respectively. Afterwards, the resulting mixtures were filtered using Whatman No. 1 paper, and dried in an oven at 65°C.

Evaluation of antioxidant property of *Codiaeum variegatum*

The antioxidant property of *C. variegatum* was evaluated by 2,2-diphenyl-1-picryl hydrazyl (DPPH) radical scavenging, lipid peroxidation inhibition, and ferric iron-reducing antioxidant power (FRAP) assays, and determining the total antioxidant capacity (TAC). For these different tests, 50 µL of extracts at concentrations of 0.1, 1.0, 10.0, 100.0, 500.0 and 1,000.0 µg/mL were used.

DPPH radical scavenging assay

This assay was carried out based on a previously described method.[19] Briefly, 50 µL of plant extracts at different concentrations were added into a series of test tubes that contained 3.1 mL of methanolic solution of DPPH (40 µg/mL). For the negative control tubes, the extract was replaced by 50 µL of solvent, while for the positive control tubes, the extract was replaced by 50 µL of ascorbic acid. Next, the mixtures were homogenized and incubated in the dark for 30 minutes at room temperature, and the absorbance was measured at 517 nm using a spectrophotometer. Then, the percentage of inhibition was calculated using the following formula:

$$\text{\% of DPPH scavenging activity} = \frac{OD_{control} - OD_{assay}}{OD_{control}} \times 100$$

where: $OD_{control}$: absorbance of the negative control tube; OD_{assay}: absorbance of the test tube.

The inhibitory concentration 50 (IC_{50}) value expressed in µg of the extract per mol of DPPH for each extract was determined using the non-linear regression curve for the DPPH scavenging activity against the concentration of extracts tested.

Evaluation of the inhibition of lipid peroxidation

Preparation of the liver homogenate

One Wistar rat was sacrificed by cervical dislocation, and the liver was excised. Then, the rest of the manipulation was performed on ice. The organ was washed in a saline solution of 0.9% NaCl, and subsequently spin-dried and weighed. After the mass was determined, the liver was cut into small pieces in a solution of 1.15% KCl, and crushed using the Teflon plunger of the Potter apparatus and Bleau fountain sand. Then, 10% homogenate in a 1.15% KCl solution was prepared, taking into account the weight of the liver. This was divided into several tubes, and centrifuged (720 g, 10 minutes, 4°C). Each supernatant was collected, and the volume was noted. Finally, the aliquots were prepared based on the number of assays performed, and stored in a freezer until use.

Lipid peroxidation inhibition assay

The thiobarbituric acid (TBA) reactive substances were determined using a previously described method.[20] In each test tube, 50 µL of plant extracts, 1 mL of 10% rat liver homogenate, 50 µL of 0.5 mM $FeCl_2$, and 50 µL of 0.5 mM H_2O_2 were successively introduced. For the blank test tube, $FeCl_2$ and H_2O_2 were replaced by 100 µL of 1.15% KCl, while for the negative control tube, the extraction solvent was used instead of the extract. Then, the mixtures were incubated (one hour, 37°C). After incubation, 1 mL of trichloroacetic acid (TCA, 15%) and 1 mL of 0.67% TBA were added to all tubes, and boiled in a water bath for 15 minutes. After cooling and centrifugation (1,620 g, five minutes, 4°C), the supernatants were collected, and the absorbance of the pink stain was read at 532 nm against the blank. The percentage of inhibition was calculated using the following formula:

$$\text{\% of lipid peroxidation inhibition} = \frac{OD_{control} - OD_{assay}}{OD_{control}} \times 100$$

where: $OD_{control}$: absorbance of the negative control tube; OD_{assay}: absorbance of the test tube.

The evolution of the percentage of inhibition according to the concentration of extract used allowed for the determination of the IC_{50} in µg/mL.

Ferric iron-reducing antioxidant power assay

FRAP assay was performed, as previously described.[21] In each test tube, 50 µL of plant extract, 1,100 µL of phosphate buffer (0.6 M, pH 6.6), and 1,000 µL of 0.25% potassium ferricyanide were introduced. For the blank test tube, 1,100 µL of distilled water was added instead of potassium ferricyanide. After incubation for 20 minutes at 50°C, 1 mL of 10% TCA was added to all tubes. Then, the whole set of test tubes was centrifuged (1,620 g, 10 minutes 4°C). Subsequently, 1 mL of distilled water and 200 µL of ferric chloride were added to 1 mL of supernatant. The whole set of test tubes was left to stand for 10 minutes to allow the samples to homogenize well. Afterwards, the absorbance was measured at 700 nm against the blank using a spectrophotometer, and the percentage of iron reduction was calculated using the following formula:

$$\text{\% reduction} = \frac{OD_{control} - OD_{assay}}{OD_{control}} \times 100$$

where: $OD_{control}$: absorbance of the negative control tube; OD_{assay}: absorbance of the test tube.

The evolution of the percentage of reduction as a function of the extract concentration allowed for the determination of the effective concentration 50 (EC_{50}).

Evaluation of total antioxidant capacity

The TAC was measured based on a previously described method.[22] In each test tube, 50 µL of plant extract, 1 mL of 0.6 M sulphuric acid, 1,050 µL of 28 mM sodium phosphate, and 1,050 µL of 4 mM ammonium molybdate were successively introduced. Then, the test tubes were capped with beads, heated for 90 minutes, and cooled in running cold water. The absorbance of the blue staining mixture was measured using a spectrophotometer at 695 nm. The antioxidant capacity of the extracts, which was expressed in g ascorbic acid/mg extract, was determined using the calibration curve drawn using different concentrations of ascorbic acid, instead of plant extracts.

Evaluation of anti-inflammatory activity

Isolation of macrophages

The isolation of primary macrophages obtained from the mouse began with its elicitation through the intraperitoneal injection of 0.5 mL of 2% starch solution (inflammatory agent).[23] At four days after the injection, the animal was sacrificed by cervical dislocation. Then, 5 mL of PBS buffer (0.1 M, pH 7.4) was injected into the mice peritoneal cavity using a syringe for macrophage collection. After massaging the abdominal cavity of the animal, the injected buffer was slowly aspirated through a small incision on the abdomen. Afterwards, the resulting solution that contained the macrophages was introduced in 15 mL Falcon tubes, and kept on ice.

Next, the resulting fluid was centrifuged (1,620 g, 4°C, 10 minutes), and the supernatant was removed. Then, the red blood cells were removed by osmotic shock[24] through suspending the cells in 1 mL of hypotonic 0.05 M NaCl solution for one minute. Afterwards, the isotonicity was restored by adding 1 mL of 0.25 M NaCl. Subsequently, the mixture was centrifuged again (1,620 g, 4°C, 10 minutes), and the resulting pellet that mostly contained macrophages was suspended in 2 mL of DMEM culture medium, and kept on ice. The cell viability was determined using the trypan blue exclusion method.[24]

Treatment of isolated macrophages with the tested samples

On a 96-well plate, 150 µL of cell suspension (10^4 cells/well) were distributed in different wells. For the test and positive control wells, 50 µL of *Saccharomyces cerevisiae* (SC, 250 µg/ml) was added to stimulate the macrophages to produce pro-inflammatory cytokines, while 50 µL of DMEM was added in the blank well. Then, the microplate was incubated for one hour at 37°C (5% CO_2). Afterwards, 50 µL of plant extract or baicalin was added to the test wells at different concentrations (0.1, 1.0, 10.0, 50.0 and 100.0 µg/mL), and 50 µL of DMEM was added to the blank and positive control wells. After three hours of incubation at 37°C (5% CO_2), the cell supernatant was used for the NO quantification, and the pellets were used for the activity of lysosomal enzymes, 5-lipoxygenase and cytotoxicity.

Cytotoxicity assay

The cytotoxicity of the plant extracts was determined using 2-(4-iodophenylyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium (INT), as previously described,[25] with slight modifications. The cell pellet obtained from the different incubations was taken up in 100 µL of INT solution (0.2 mg/mL in PBS), and the mixture was incubated for one hour at 37°C for 30 minutes. Then, the supernatant was removed, and 100 µl of acidified isopropanol was added to each tube to dissolve the formazan crystals that formed. Finally, the absorbance of the pink solution was read at 490 nm against the acidified isopropanol solution. The percentage of cell viability was calculated using the following formula:

$$\text{\% viability} = \frac{OD_{assay}}{OD_{conttrol}} \times 100.$$

Quantification of nitric oxide production by stimulated macrophages

The assay was performed according to the reaction of NO with the Griess reagent.[26] One hundred microliters of the previously obtained cell supernatants were mixed with 100 µL of Griess reagent (1% sulphanylamide, 0.1% naphthyl ethylene diamine dihydrochloride in 2.5% v/v phosphoric acid). Then, the mixture was incubated at room temperature for 10 minutes, and the absorbance was measured using a plate reader at 550 nm. The amount of nitrite was measured against the standard sodium nitrate curve.

The percentage inhibition of NO production was calculated according to the following formula:

$$\% \text{ inhibition} = \frac{OD_{control} - OD_{assay}}{OD_{control}} \times 100.$$

Evaluation phosphatase activity in stimulated macrophages

The effect on acid phosphatase was assayed based on a previously described method.[27] The cell pellets obtained in the methodology were used. The obtained pellets were solubilized with 25 µL of Triton X-100, followed by the addition of 50 µL of para-nitrophenyl phosphate (10 mM) and 50 µL of citrate buffer (0.1 M, pH 5.0). All solutions were incubated for 30 minutes at 37°C. The reaction was stopped by adding 100 µL of borate buffer (0.2 M, pH 9.8). Then, the absorbance was measured at 405 nm.

Next, the effect on alkaline phosphatase was assayed based on a previously described method.[27] The cell pellets obtained in the methodology were used. The obtained pellets were solubilized with 25 µL of Triton X-100, followed by the addition of 50 µL of p-nitrophenylphosphate (10 mM) and 50 µL of glycine buffer (0.1 M, pH 9.0). All solutions were incubated for 30 minutes at 37°C. The reaction was stopped by the addition of 100 µL of NaOH buffer (0.2 M, pH 12). Then, the absorbance was measured at 405 nm.

For those two phosphatases, the percentage of change in enzyme activity was calculated based on the control tubes, and according to the following formula:

$$\% \text{ of activity} = \frac{OD_{essay} - OD_{control}}{OD_{control}} \times 100.$$

Evaluation of the effect of* Codiaeum variegatum *on bovine serum albumin (BSA) denaturation

The inhibition of BSA denaturation was assayed according to a previously described method.[28] For this assay, 450 µL of BSA were added with 50 µL of extract or sodium diclofenac for the test tubes and standard tubes, respectively, while for the control tubes, 50 µL of extract or diclofenac was added to 450 µL of distilled water. After incubating for 20 minutes at 37°C and 30 minutes at 57°C, 2,500 µL of 0.1 M phosphate buffer (pH 7.4) was added to each tube. Then, the absorbance was measured using a spectrophotometer at 660 nm.

The percentage inhibition of protein denaturation was calculated, as follows:

$$\text{Inhibition percentage} = \frac{OD_{test} - OD_{control\ test}}{OD_{control\ product}} \times 100.$$

The control represents 100% of the denatured proteins, and the results were compared with sodium diclofenac.

Evaluation of the effect of plant extracts on 5-lipoxygenase activity

The test was performed in test tubes, according to a previously described method.[29] After isolating the mouse macrophages and recovering these from the culture medium, 950 µL of cells were introduced into each tube (1×10^5 cells per tube). Then, 300 µL of SC suspension (250 µg/mL) was added to each tube, except for the negative control, in which the culture medium was added instead. This was followed by an initial incubation of one hour at 37°C (5% CO_2). Subsequently, 50 µL of extract was introduced into the test tubes, and 50 µL of medium was introduced for the controls, followed by a second incubation for three hours. Afterwards, each tube was centrifuged at 2,000 rpm for 10 minutes at 4°C, and the supernatant was discarded. Next, the pellet that contained the cells was recovered in 50 µL of Triton X-100, and the tubes were shaken for two minutes. Finally, 1,000 µL of linoleic acid (125 µM) was added, and incubated for 30 minutes. Afterwards, the optical density (OD) of the supernatant was read at 234 nm.

Phytochemical screening and determination of phenolic compounds and total flavonoids

Phytochemical screening

In order to detect the group of secondary metabolites present in the different plant extracts, qualitative and specific colorimetric tests were performed. For each of these tests, the extracts were prepared at 1 mg/mL

Phenol test: Reaction with 1% $FeCl_3$

Two milliliters of extract was added to 2 mL of 5% $FeCl_3$ aqueous solution. After shaking, the appearance of a blue coloration indicated the presence of phenols.[30]

Flavonoid test: Reaction with 20% NaOH in acidic medium

Three drops of 20% NaOH aqueous solution were mixed with 2 mL of extract. After shaking, the formation of an intense yellow coloration, which disappeared after the addition of three drops of 70% HCl aqueous solution, indicated the presence of flavonoids.[30]

Coumarin test: 10% NaOH reaction

Two milliliters of the extract was added to 3 mL of 10% aqueous NaOH solution. After shaking, the appearance of a yellow coloration indicated the presence of coumarins.[31]

Tannin test: Reaction with 10% $FeCl_3$

Two milliliters of the extract was added to 1 mL of the alcoholic solution of 10% $FeCl_3$. After shaking, the appearance of a black coloration indicated the presence of tannins.[30]

Anthocyanin test: Reaction with NH_3 in acidic medium

One milliliter of extract was added to 2 mL of the aqueous 2 N HCl solution. After shaking and the addition of 1 mL of 25% aqueous ammonia solution, the appearance of a purple-red coloration that turned blue-violet indicated the presence of anthocyanins.[31]

Alkaloid test: mayer test

Two hundred microliters of 10% aqueous HCl solution was added to 2 mL of the extract. Then, 1 mL of Mayer's reagent was added to the mixture. After shaking, the appearance of a yellowish coloration indicated the presence of alkaloids.[32]

Steroid test: Libermann-Burchard test

Fifty milligrams of extract was dissolved in 2 mL of acetic acid anhydride. After shaking, the appearance of a violet coloration that turned green or blue after the addition of two drops of concentrated HCl indicated the presence of steroids.[33]

Terpenoid test: HCl precipitation reaction

Half a milliliter of chloroform was added to 1 mL of extract. After shaking and the addition of five drops of concentrated HCl, the appearance of a reddish-brown precipitate indicated the presence of terpenoids.[30]

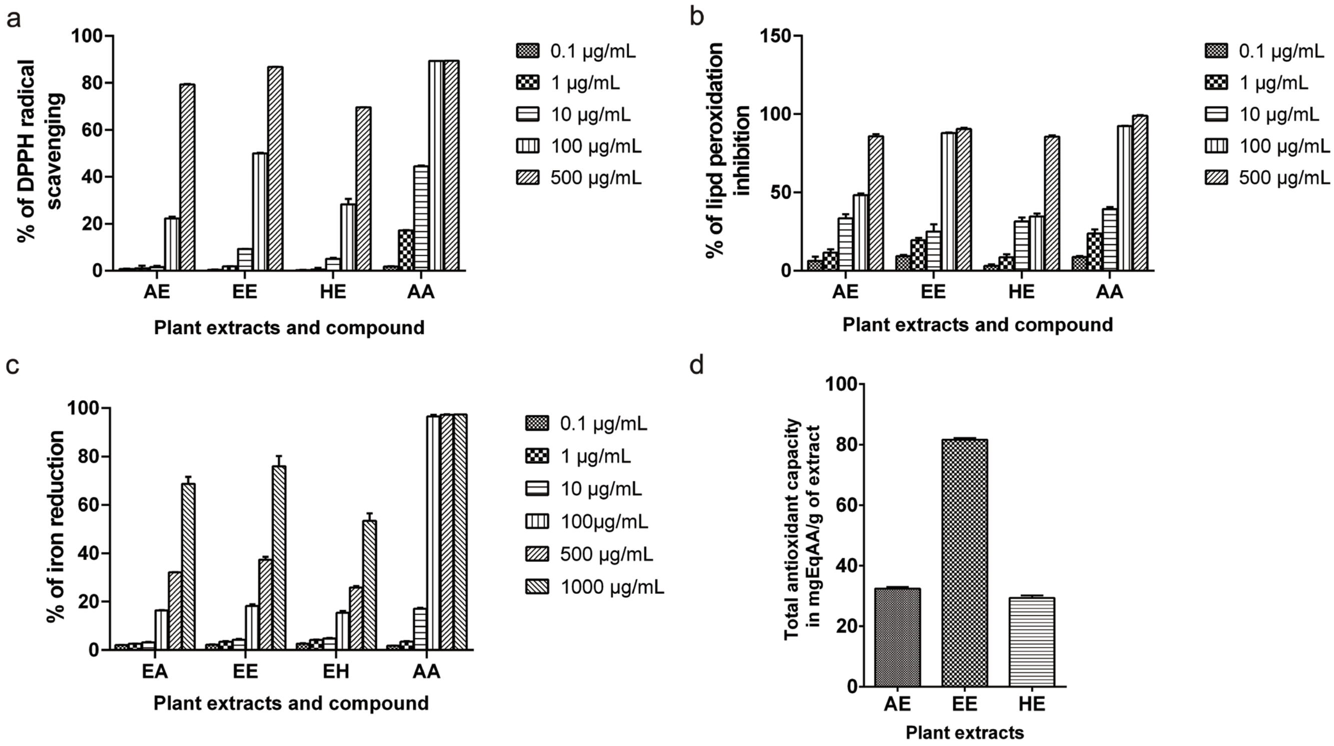

Fig. 1. Antioxidant activities of *Codiaeum variegatum* stem extracts. (a) DPPH scavenging assay; (b) Inhibition of lipid peroxidation; (c) Ferric iron-reducing antioxidant power assay; (d) Total antioxidant capacity assay. AA, ascorbic acid; AE, aqueous extract; DPPH, 2,2-diphenyl-1-picryl hydrazyl; EE, ethanolic extract; HE, hydroethanolic extract.

Glycoside test: Reaction with 20% NaOH in aqueous medium

Three drops of an aqueous 20% NaOH solution were added to 1 mL of the extract. After shaking, the appearance of a yellow coloration indicated the presence of glycosides.[32]

Saponin test: Reaction with distilled water

Two milliliters of extract was added to 6 mL of distilled water. After vigorous shaking, the formation of a persistent foam indicated the presence of saponins.[31]

Determination of total phenolic compound content

The total phenolic content of the extract was determined using the modified Folin-Ciocalteu method.[34] In each tube, 2,000 µL of distilled water, 100 µL of the extract at 100 µg/mL, and 200 µL of Folin-ciocalteu (2 N) solution were introduced. The calibration curve for gallic acid was drawn at a fixed concentration of 100 µg/ml at different volumes (0, 20, 40, 60, 80 and 100 µL), and by completing the volume of 100 µL with pure methanol, with the subsequent addition of 200 µL of Folin-ciocalteu. The mixture was left to stand for three minutes. Then, 1,000 µL of 20% sodium carbonate was added. Afterwards, the mixture was incubated for one hour in the dark at room temperature, and the OD was read at 700 nm. The total phenolic compound content was expressed in milligram equivalents of gallic acid per gram of extract (mg GAE/g extract) using the calibration line.

Determination of flavonoids content

The total flavonoids content was determined according to a previously described method.[34] In the white and test tubes, 500 µL of methanol and 500 µL of the extract solution were respectively introduced. Then, 500 µL of aluminium trichloride ($AlCl_3$) 2% (w/v) was introduced in each of the tubes. All tubes were vortexed and incubated at room temperature for one hour. Then, the OD of the yellow stain was read at 430 nm against the blank. The assay was performed in triplicate, and Quercetin was used as the standard.

Data analysis

The data analysis was performed using the GraphPad Prism 8.0.1 software. The results were expressed as mean ± standard deviation, and the different values were compared using the analysis of variance test (one-way ANOVA), followed by the multiple comparison test of Turkey, with a *p*-value of <0.05.

Results

Antioxidant activities of the studied plant extracts

DPPH free-radical scavenging activity

The antiradical activity was evaluated through the scavenging of the DPPH radical. *C. variegatum* stem bark extracts can effectively trap the DPPH radical in a concentration-dependent manner, between 0.1 and 500.0 µg/mL (Fig. 1a). The IC_{50} revealed that the ethanolic extract (EE) had the greatest antiradical power, although this was lower than that of ascorbic acid (77.04 ± 7.66 µg extract/mol of DPPH and 8.98 ± 4.11 µg extract/mol of DPPH, respectively).

Lipid peroxidation inhibitory activity

The ability of the different extracts of *C. variegatum* to inhibit

Table 1. EC_{50} and IC_{50} for the antioxidant and anti-inflammatory tests, and the total phenolic compound and flavonoids compound content in plant extracts

	Aqueous extract	Ethanolic extract	Hydroethanolic extract	Standard
DPPH (IC_{50} in µg/mol of DPPH)	173.41 ± 27.70[b]	77.04 ± 7.66[b]	186.46 ± 19.29[b]	8.98 ± 4.11
FRAP (EC_{50} µg/mL)	670,40 ± 44,10[b]	543,60 ± 42,52[b]	ND	21.34 ± 2.91
Inhibition of lipid peroxidation (IC_{50} in µg/mL)	51.57 ± 7.23[b]	21.52 ± 9.87[b]	89.06 ± 11.26[b]	10.26 ± 4.02
Total antioxidant capacity (in mgEq AA/g of extract)	32.40 ± 0.54	81.58 ± 0.55	29.32 ± 0.84	
Inhibition of NO production (IC_{50} in µg/mL)	90.94 ± 22.75[b]	95.09 ± 25.30[b]	8.80 ± 4.52	6.77 ± 3.07
Inhibition of 5-lipoxigenase (IC_{50} in µg/mL)	5.40 ± 0.15[b]	2.61 ± 0.20	0.07 ± 0.00[a]	2.55 ± 0.31
Inhibition of BSA denaturation (IC_{50} in µg/mL)	234.60 ± 19.13[b]	346.10 ± 18.43[b]	205.90 ± 20.55[b]	150.00 ± 18.63
Alkaline phosphatase (EC_{50} µg/mL)	29.68 ± 8.34[b]	2.35 ± 0.37	0.27 ± 0.03	1.16 ± 0.26
Acide phosphatase (EC_{50} µg/mL)	22.31 ± 13.15	86.65 ± 23.65[b]	10.03 ± 4,28[a]	51.08 ± 9.19
Phenolic compounds content (in mg/g of extract)	34.54 ± 0.13	44.54 ± 0.07	39.67 ± 0.23	
Flavonoids content (in mg/g of extract)	9.67 ± 0.10	7.73 ± 0.07	5.42 ± 0.17	

Notes: [a]Significantly higher than the standard, [b]Significantly lower than the standard. AE, aqueous extract; EC_{50}, effective concentration 50; EE, ethanolic extract; HE, hydroethanolic extract; IC_{50}, inhibitory concentration 50; mgEqAA/g, milligram equivalent of ascorbic acid per gram; NO, nitric oxide; SC, *Saccharomyces cerevisiae*; DS, sodium diclofenac.

membrane lipid peroxidation was effective (Fig. 1b). Furthermore, the EE exhibited the best inhibitory activity. However, the ascorbic acid activity was more pronounced, when compared to that of the extract (21.52 ± 9.87 µg/mL and 10.26 ± 4.02 µg/mL, respectively).

Iron reducing activity (FRAP) of *Codiaeum variegatum* stem extracts

The FRAP assay revealed that EE had the highest activity, with the evolution changing in a concentration-dependent manner (Fig. 1c). However, the activity of the EE was lower than that of ascorbic acid (543.60 ± 42.52 µg/mL and 21.34 ± 2.91 µg/mL, respectively; Table 1).

Total antioxidant capacity of *Codiaeum variegatum* stem extracts

The TAC was determined using the phosphomolybdenum method, and the results were expressed in milligram ascorbic acid equivalent per gram of plant extract (mgEAA/g extract). It was revealed that the EE had the greatest ability to reduce the phosphomolybdic complex (81.589 ± 0.60 mgEq AA/g extract, Fig. 1d).

Anti-inflammatory activities of Codiaeum variegatum stem extracts

Viability and cytotoxicity of extract on primary macrophage culture

Viability of primary macrophages in trypan blue

After incubating the macrophages and counting these after each hour using the trypan blue method, it was observed that the viability of the macrophages merely slightly decreased between zero and eight hours (Fig. 2a).

Cytotoxicity of trypan blue and INT extracts

The evaluation of the cytotoxicity of *C. variegatum* stem extracts on primary macrophages was carried out using trypan blue (with the plant extract at 500 µg/mL, Fig. 2a) and INT (at different extract concentrations, Fig. 2b). The results revealed that isolated primary macrophages can survive in the culture in the presence of extracts at 500 µg/mL, between zero and six hours, and that it was merely from 1,000 µg/mL that the *C. variegatum* stem bark extract started to be cytotoxic.

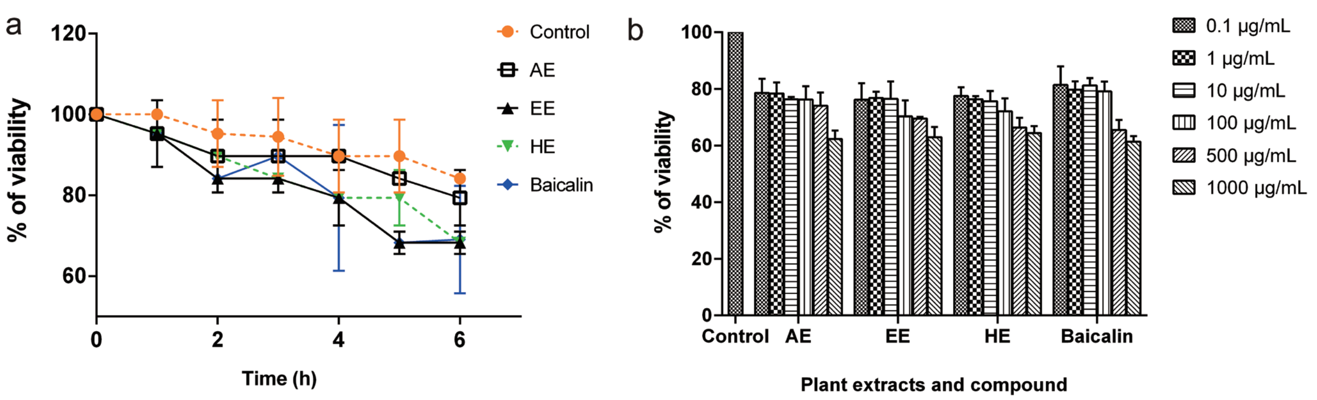

Fig. 2. Plant extract cytotoxicity. (a) Plant extract cytotoxicity on primary cell culture using trypan blue; (b) INT cytotoxicity of plant extracts on cell culture. AA, ascorbic acid; AE, aqueous extract; EE, ethanolic extract; HE, hydroethanolic extract; INT, 2-(4-iodophenylyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium.

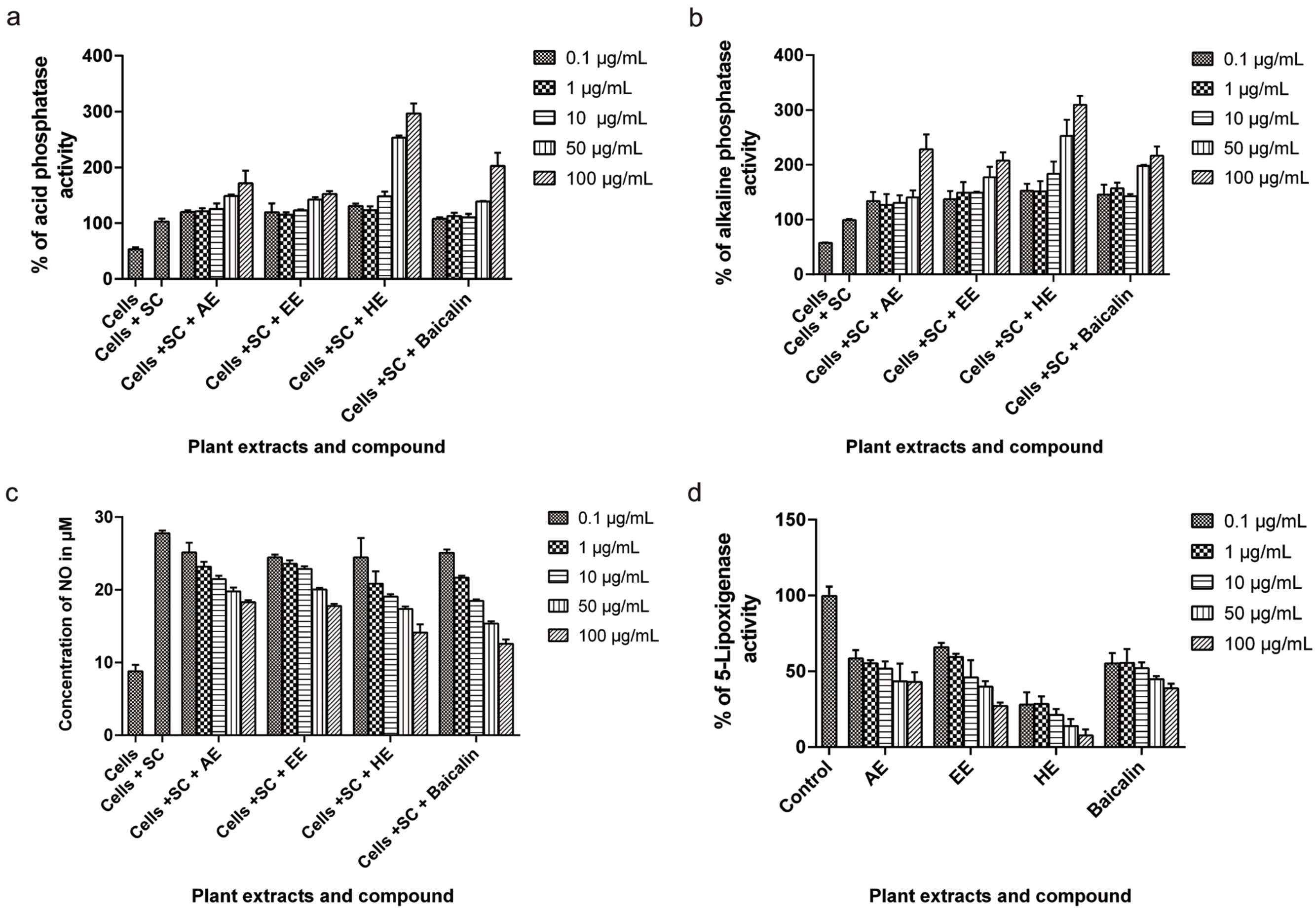

Fig. 3. Anti-inflammatory activity of *Codiaeum variegatum* stem extracts. (a) Effect of plant extracts on acid phosphatase activity; (b) Effect of plant extracts on alkaline phosphatase activity; (c) Effect of plant extracts on NO production; (d) Effect of plant extracts on 5-lipoxygenase activity. AE, aqueous extract; EE, ethanolic extract; HE, hydroethanolic extract; NO, nitric oxide; SC, *Saccharomyces cerevisiae*.

Effect of *Codiaeum variegatum* stem extracts on acid and alkaline phosphatase activities

Next, it was determined how *C. variegatum* stem bark extracts can influence the activity of two lysosomal enzymes (acid and alkaline phosphatase). It was observed that the activity of these enzymes considerably increased in the presence of the plant extracts (Figs. 3a and b). The calculation of the EC_{50} revealed that the HE boosted these enzymes better, when compared to baicalin. This was the reference anti-inflammatory compound for the present study.

Effect of *Codiaeum variegatum* stem extracts on nitric oxide production by macrophages

The ability of *C. variegatum* stem bark extracts to modulate the NO secretion through SC-activated macrophages was determined using the Griess diazotization method. It was found that the plant extracts significantly ($p < 0.05$) reduced the NO produced by the macrophages (Fig. 3c). Furthermore, the HE exhibited a more pronounced inhibitory activity, when compared to baicalin (Table 1).

Effect of *Codiaeum variegatum* stem extracts on BSA denaturation

The test allowed the investigators to perform an experiment for the model of heat denaturation of proteins in the presence of plant extracts. It was observed that the inhibitory effect on this denaturation evolved in a concentration-dependent manner (Fig. 4). The HE exhibited the best inhibitory activity, followed by the aqueous extract (AE) and EE. In addition, the activity of the standard (sodium diclofenac) was higher, when compared to that of the extracts (Table 1).

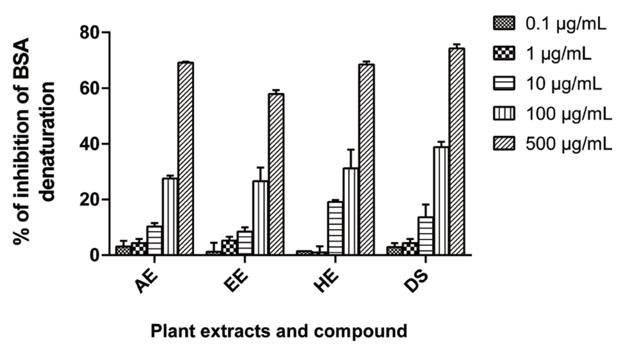

Fig. 4. Anti-inflammatory activity of *Codiaeum variegatum* stem extracts: Effect on BSA denaturation. AE, aqueous extract; BSA, bovine serum albumin; DS, sodium diclofenac; EE, ethanolic extract; HE, hydroethanolic extract; SC, *Saccharomyces cerevisiae*.

Table 2. Phytochemical screening

Tests	Aqueous extract	Ethanolic extract	Hydroethanolic extract
Phenols	+	+	+
Flavonoids	+	+	+
Alkaloids	+	+	+
Tannins	+	+	+
Saponins	+	+	+
Coumarins	+	+	+
Antocyanins	−	−	+
steroids	−	−	−
Terpenoids	−	−	−

Notes: +, present; −, absent.

Effect of *Codiaeam variegatum s*tem extracts on macrophage 5-lipoxygenase activity

The inhibitory activity of the extracts on the 5-lipoxygenase of SC-activated mouse macrophages was evaluated by hydroperoxide assay. It was observed that there was a concentration-dependent decrease in the activity of this enzyme in the presence of the plant extracts (Fig. 3d). This inhibition was more pronounced in the presence of the HE (Table 1).

Families of phytochemicals found in *Codiaeum variegatum* ***stem extracts***

Families of the compound present in *Codiaeum variegatum* extracts

The phytochemical screening revealed that *C. variegatum* stem bark extracts possess numerous biologically active secondary metabolites, such as flavonoids, alkaloids and saponins (Table 2).

Total phenolic compounds and flavonoids content of *Codiaeum variegatum* extracts

The determination of phenolic compounds with the Folin Ciocalteu reagent revealed that the EE contained significantly more phenolic compounds, when compared to the others. In addition, the determination of flavonoids through the aluminium chloride complexation method revealed that the AE contains more flavonoids, when compared to the others (Table 1).

Linear correlations between the level of phenolic compounds and the different activities

In order to establish a possible link between the different activities observed and the level of total phenolic compounds, linear correlation tests were carried out, in which the coefficients obtained served as indicators (Table 3). In general, it was found that the phenolic compounds were almost totally responsible for the different activities tested for the AE and EE. However, this was merely partially responsible for the different activities tested for the HE.

Discussion

Oxidative stress is the imbalance between the production of reactive oxygen species (ROS) and antioxidant defense activity. Excessive ROS can cause damage to various cellular components, leading to tissue damage, and is associated with aging and various chronic diseases. Natural antioxidants play remarkable roles in the inhibition of ROS production.[35,36] A plethora of evidence has validated that the antioxidant present in plant-derived products encompass various biomedical applications. The present study evaluated the effect of *C. variegatum* stem extracts on oxidative stress. It was found that the *C. variegatum* stem EE exhibits the highest DPPH radical scavenging power, with 50.01% of scavenging at 100 µg/mL, when compared to the 89.32% for ascorbic acid at the same concentration. Furthermore, the EE possesses a large amount of secondary metabolic, such as flavonoids, which can donate hydrogen and electrons to radical species, in order to stabilize these species.[37] Subsequently, it was observed that the EE had the best capacity to reduce Fe III to Fe II, but this was lower than that of the standard. This shows the extent of involvement of these extracts in iron metabolism, particularly in the reduction of Fe III provided by plant diet to Fe II, which is important for hemoglobin porphyrin synthesis.[38] Next, the inhibitory activity of these extracts on the lipid peroxidation induced by hydrogen peroxide was evaluated. It was found that the EE could moderately protect the cell's membrane lipids from peroxidation (87.93% of inhibition at 100 µg/mL). However, this activity remained significantly lower, when compared to that for ascorbic acid (92.43% of inhibition at the same concentration). Subsequently, the total antioxidant capacity of the assayed extracts was evaluated through the reduction of the phosphomolybdic complex. Again, a better capacity was observed with the stem EE. These results demonstrate that the EE exhibits the best capacity to protect against oxidative stress, since this has already been proven for leaf extracts of two other varieties of

Table 3. Correlation between the total phenolic compounds content and the different biological activities

Tests	Aqueous extract	Ethanolic extract	Hydroethanolic extract
DPPH	0.98	0.99	0.78
FRAP	0.99	0.93	0.99
Inhibition of lipid peroxydation	0.72	0.98	0.49
Inhibition of NO production	0.80	0.96	0.84
Inhibition of BSA denaturation	0.96	0.97	0.75
Inhibition of 5-lipoxygenase	0.87	0.79	0.80
Acide phosphatase	0.99	0.97	0.95
Alkaline phosphatase	0.80	0.97	0.84

Notes: a, in dependence; b, low dependence. BSA, bovine serum albumin; DPPH, 2,2-diphenyl-1-picryl hydrazyl; FRAP, ferric iron-reducing antioxidant power; NO, nitric oxide.

the same plant found in Bangladesh.[11] The inflammatory process in macrophages involves various players, such as pro- and anti-inflammatory mediators (NO, TNF-α, Il1β/Il4, Il6, Il10, and tumor growth factor-beta [TGFβ]), and enzymes, such as lipoxygenase (LOX). The catalytic activity of LOX is activated by several stimuli, including reactive oxygen and nitrogen species. All these are conducted to eliminate a pathogen.[39] Therefore, the present study evaluated the effect of *C. variegatum* stem bark extracts on the production of some of these elements.

Initially, the research of the working conditions allowed the investigators to conclude that the viability of isolated mouse macrophages during the eight-hour duration (interval time necessary for the different incubations for tests that involved macrophages) decreased in a non-significant manner. In addition, it was observed that this viability was almost unaffected in the presence of the different extracts, and that there was no significant difference, when compared to the viability of the control macrophages, indicating a still functional membrane for six hours. Furthermore, cytotoxicity was performed using INT, which is a tetrazolium salt that mainly targets the effectiveness of the respiratory process. It was merely from the concentration of 500 µg/mL that an effective cytotoxicity could be observed, especially for baicalin, which is the reference compound for the present study, and at this concentration, a viability of 72.29% was observed. However, this concentration reflected a slight decrease in mitochondrial succinate dehydrogenase activity. Based on that concentration, 100 µg/mL was set as the maximum working concentration for the subsequent assays using those cells. A significant ($p < 0.05$) decrease in the amount of NO produced by mouse activated macrophages was observed, when compared to the control, in a concentration-dependent manner, and in the presence of *C. variegatum* stem crude extracts. NO is a pro-inflammatory mediator synthesized by NO synthase from arginine, and is involved in vasodilatation during inflammatory reactions.[40] Therefore, the inhibition of its synthesis is a sign of anti-inflammatory activity. The HE had the highest inhibitory activity between extracts, which is similar to that of the two other plant extracts already assayed.[41,42] Furthermore, the activity of lysosomal enzymes (PAC and PAL) in the presence of *C. variegatum* stem bark extracts exhibited the effectiveness to boost the activity of these enzymes. The HE boosted these enzymes more efficiently, increasing the activity to 296.50% and 309.42% at 100 µg/mL for acid phosphatase and alkalin phosphatase, respectively. Since these two enzymes are involved in the phagocytosis processes, its activation would thereby reflect the capacity of *C. variegatum* stem bark extracts to regulate the inflammatory process by redirecting this towards the phagocytosis pathway.

In order to act at the level where the different pro-inflammatory mediators exert its action, the inhibition test for the denaturation of serum protein (BSA) was carried out. It was observed that *C. variegatum* stem bark extracts can effectively inhibit the heat-induced denaturation of this protein in a concentration-dependent manner, which was indeed conducted through the stabilization of the different constituent bonds of this protein. Again, the HE exhibited a better inhibitory activity, even though this was lower than that of the standard.[43] Finally, the effect of *C. variegatum* stem bark extracts on the activity of 5-lipoxygenase was evaluated. This enzyme allows for the synthesis of leukotrienes (LTB4). It was observed that the HE could effectively inhibit this enzyme, which is similar to the report of a previous study.[29]

Secondary metabolites have been shown to have various biological activities. A recent study revealed the effectiveness of the *in vitro* and *in vivo* antioxidant and anti-inflammatory activities of six flavonoids with similar structures.[37] In order to determine which group of secondary metabolites could be responsible for the biological activities reported in the literature, the phytochemical screening of the aqueous, ethanolic and hydroethanolic extracts of *C. variegatum* was performed. It was revealed that major concentrations of phenols and flavonoids were present in all the studied extracts, in addition to tannins and saponins in the AE, coumarins, tannins, alkaloids and glycosides in the EE, and saponins in the hydroethanol extract. Other secondary metabolites were also detected in trace amounts, with the absence of terpenoids and steroids. Subsequently, the determination of phenolic compounds and flavonoids was carried out due to the high anti-inflammatory activity. The results revealed that the EE contained slightly more phenolic compounds, when compared to the others. In addition, the flavonoid assay revealed that the AE and EE contained slightly more flavonoids, when compared to the HE.[12] Phenolic compounds represent a variety of pharmacologically active phytochemicals, and these have been investigated mainly due to its ability to delay or inhibit the oxidation process and inflammatory disorders, as consequences of some cellular pathological conditions.[44,45] In the present study, it was found that phenolic compounds were almost totally responsible for the different activities tested with the AE and EE. However, these phenolic compounds were partially correlated to the antioxidant and anti-inflammatory activities of the HE. These results are in some way similar to the results reported by some researchers, in which there was a very strong and positive correlation between the total phenolic compound content in *Khaya grandifoliola* crude extracts and some antioxidants activities.[46] A previous research also reported phenolic compounds as the major secondary metabolite responsible for the anti-inflammatory activity of plant biodiversity in Cuba.[47] This confirms the results, in which a phenolic compound, such as baicalin, can possess anti-inflammatory properties.[41]

Further directions

Although the present study demonstrated the antioxidant and anti-inflammatory potentials of the studied plant, further molecular and *in vivo* studies, as well as studies on the correlation between antioxidant and anti-inflammatory activities, are needed to value these findings.

Conclusions

Based on the above mentioned observations, it can be concluded that the *C. variegatum* stem EE has moderate antioxidant activity. In addition, the *C. variegatum* stem HE exhibits the best anti-inflammatory activity throughout its inhibitory effect on NO production, 5-lipoxygenase activity, the denaturation of serum protein, and the activation of the two lysosomal enzymes. Furthermore, *C. variegatum* stem extracts have numerous secondary metabolites, particularly phenolic compounds, which are partially responsible for the observed biological activities.

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None.

Conflict of interest

The authors have no conflicts of interest to declare.

Author contributions

SNP, BEE, EMN and VSN carried out all *in vitro* experiments reported in the manuscript. SNP, EMN, FNN and PFM designed the study. All authors read and approved the final manuscript.

Ethical statement

All procedures in this study followed the Cameroon National Veterinary Laboratory guidelines and were approved by the Animal Ethical Committee of the Laboratory of Animal Physiology of the Faculty of Sciences, University of Yaoundé I–Cameroon.

Data sharing statement

No additional data are available.

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