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Codiaeum Variegatum Hydro Alcoholic Leaf Extracts and Their Fractions Inhibit Pro-Inflammatory Mediators *in Vitro*

Sylvain Pechangou Nsangou[1], Charifa Ngbetkom Mandou[1], Carine Matsigang Fondjou[1], Vigny Sayal Ngohoba[1], Edie Brice Enang II[1], Ibrahim Njingou[1], Emmanuel Mfotie Njoya[1,2], Frederic Nico Njayou[1*], Paul Moundipa Fewou[1]

[1]Laboratory of Pharmacology and Toxicology Department of Biochemistry, Faculty of Sciences, University of Yaounde1, Yaounde Cameroon
[2]Centre for Quality of Health and Living, Faculty of Health and Environmental Sciences, Central University of Technology, Bloemfontein, South Africa
Email: *njayou@yahoo.com

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Abstract

Codiaeum variegatum has been widely investigated for its biological proprieties ranging from the antiamoebic potential to the phytochemical analysis. The aim of the present study was to evaluate the anti-inflammatory potential of *C. variegatum* leaf extracts and fractions. A primary macrophage culture activated by *Saccharomyces cereviseae* (SC) was used to evaluate cell cytotoxicity and anti-inflammatory potential of the plant extracts and fractions. Macrophages were treated with different concentrations (0.1; 1; 10 and 100 µg/mL) of the extracts/fractions for the inhibition of 5-lipoxygenase activity, nitric oxide (NO) and Tumor Necrosis Factor Alpha (TNF-α) production. No significant difference was observed on cell viability in the presence of extracts and fractions at tested concentration during the incubation period. Extracts and fractions of *C. variegatum* inhibited the 5-lipoxygenase activity, NO and TNF-α production by viable primary mouse macrophages in a concentration-dependent manner. The fractionation process increased anti-inflammatory activity. Among fractions, HEF2, HEF3, HEF5, EEF1, EEF3 and EEF5 exhibited the best anti-inflammatory potential. *C. variegatum* extracts and fractions exhibited a greater anti-inflammatory potential throughout the inhibition of pro-inflammatory mediators such as NO, 5-Lox and TNF-α.

Keywords

C. variegatum, Anti-Inflammatory, 5-Lipoxygenase, NO, TNF-α

1. Introduction

For centuries plants have been and still remain the basis of many traditional medicine systems [1]. Plants are the most abundant suppliers of safe and successful remedies from time immemorial to present either to humans or to other animals. It is estimated that more than 90% of traditional medicine recipes comprise medicinal plants [2] which are used to treat a wide array of acute and chronic diseases ranging from common cold to complex cancerous phases throughout the world [3]. According to the World Health Organization, the use of traditional and complementary medicine is increasing rapidly in most of the countries [4]. Plants are an important source of biologically active natural products and are considered as a promising avenue for the discovery of new drugs due to easy access and relatively low cost, since they are naturally found in relative abundance. *Codiaeum variegatum* L. (garden croton) is belonging to family Euphorbiaceae. It is a native to southern India, Sri Lanka, Indonesia, Malaysia, and the western Pacific Ocean islands [5]. In Philippines, India, Indonesia and Cameroon it is used in traditional medicine to treat enteric diseases and related symptoms [6] [7] [8] [9] [10]. The leaf extracts and fractions of *C. variegatum* were reported to exhibit various pharmacological activities such as antiamoebic [11]; antigiardial [12], antimicrobial [13] [14] [15] [16] [17], antioxidant [18] [19] [20] [21], anticonvulsant [22], and antiviral activities [23]. *C. variegatum* has been proven to exhibit various secondary metabolites such as alkaloids, flavonoids phenolic compounds, terpenoids and essential oils [24] [25]. Some flavonoids compounds with anti-inflammatory activities were found to be present in methanolic extract of this plant [26] [27]. In our continuous finding on *C. variegatum*, we undertook the investigation of its hydro alcoholic leaf extracts and derived fractions for their anti-inflammatory activity.

2. Material and Methods

2.1. 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.

2.2. Biological Material

The biological animal material used consisted of primary macrophages prepared from mice and maintained on DMEM medium at the Laboratory of Pharmacology and Toxicology of the University of Yaounde 1.

2.3. Plant Material

Leaves of *C. variegatum* (var. mollucanum) were collected in 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 and dried at laboratory temperature, then crushed in a blender to obtain the powder and preserved. The resulting powders were packaged and stored at 4˚C for later use.

2.4. Preparation of the Plant Extracts

Powdered plant material (400 g) was macerated in 4 l of ethanol/water in the ratio (70:30 v/v) or ethanol (95% v/v) for 48 hours at room temperature. The resulting extract was filtered through a whatman N˚1 filter paper and then dried using a rotary evaporator at 65˚C. The residues which constitute the crude extract was kept at 4˚C until further use.

2.5. Fractionation of the Leaf Extracts of *Codiaeum variegatum*

Fractionation was done by flash chromatography using several solvents or mixture of solvents in the following order: methylene chloride, methylene chloride/methanol (95:5 v/v), methylene chloride/methanol (90:10 v/v), methylene chloride/methanol (50:50 v/v) and methanol. The solvent change was made when the filtrate appeared clear. At the end of the procedure, all filtrates obtained with the same solvent were mixed and then concentrated in a rotary evaporator. The final fraction obtained was kept in a clean bottle and stored at 4˚C. Each fractionation yield (FY) was calculated according to the formula below.

$$\text{Fractionation yield} = (\text{masse of fraction}/\text{masse of extract}) \times 100.$$

2.6. Determination of the Anti-Inflammatory Potential of *C. variegatum* Extracts and Fractions

Primary macrophages preparation

Macrophages were isolated and maintained according to the method described previously [28]. Mice were elicited by an intra-peritoneal injection of 0.5 mL of a 2% starch solution (inflammatory agent). Four days later, animals were sacrificed by cervical dislocation. Then peritoneal primary macrophages obtained using previously described method [29], were suspended in 1ml DMEM culture medium and 25 µl 2.3×10^7 cells/mL of the suspension were used for trypan blue viability assay [28]. Counted cells were distributed in 96-well micro-plates at a concentration of 10^4 cells/mL. In the test and positive control wells, 150 µL of cells were introduced with 50 µL of *Saccharomyces cerevisiae* (250 µg/mL). In the blank wells 150 µL of cells were introduced with 50 µL of DMEM. The micro-plate was incubated for 1h at 37˚C (5% CO_2), then 50 µL of extract at different concentrations (0.1, 1, 10 and 100 µg/mL) were added to the test wells and 50 µL of DMEM were added to the positive control wells and finally 50 µL of baicalin to the standard. The micro-plate was again incubated for 3 h at 37˚C (5% CO_2). The supernatants were used for the nitric oxide assays, while the pellets were used for the 5-lipoxygenase activity assays and for MTT cytotoxicity.

2.6.1. Cell Cytotoxicity Assay on Primary Mouse Macrophages

The pellets from different incubations were taken up in 100 µL of MTT (3(4,5-dimethylthiazol-2-yl)-2,3diphenyletrazolium) solution (0.5 mg/mL in PBS) and the mixture was incubated at 37˚C for 1 h 30 min. The supernatant was then removed and 100 µL of acidified isopropanol were added to each tube to dissolve the formazan crystals formed. Finally, the absorbance of the blue-violet solution was read at 550 nm against the acidified isopropanol solution. The percentage of cell viability was calculated using the following formula:

$$\%\text{ of cell viability} = \left(\text{sample OD}/\text{control OD}\right)\times 100$$

2.6.2. Evaluation of the Effect of *C. variegatum* Extracts and Fractions on Nitric Oxide (NO) Production by Viable Macrophages

The supernatants previously obtained were transferred in new 96-well microplates. In each well, 100 µL of supernatant were mixed with 100 µL of Griess reagent (1% sulphanylamide, 0.1% naphtyl ethylene diamine dihydrochloride in 2.5% v/v phosphoric acid). The mixture was incubated at room temperature for 10 min and the absorbance measured at 550 nm. The amount of nitrite was determined by reference to the standard calibration curve of the sodium nitrate [30]. The percentage inhibition of NO production was calculated according to the following formula.

$$\%\text{ inhibition} = \left(\left(\text{OD of control} - \text{OD of assay}\right)/\text{OD of control}\right)\times 100$$

2.6.3. Evaluation of the Effect of *C. variegatum* Extracts and Fractions on 5-Lipoxygenase Activity

The evaluation of the effect of *S. rhombofolia* extracts was performed in test tubes as previously described [30]. After isolating and recovering the mouse macrophages from the culture medium, 950 µL of cells were introduced into each tube (100,000 cells per tube). Then 300 µL of *Saccharomyces cerevisiae* suspension (250 µg/mL) were added to each of the tubes, except the control in which culture medium was added followed by one hour of initial incubation at 37˚C (5% CO_2). Subsequently, 50 µL of extract was introduced in each test tubes, 50 µL of ascorbic acid, acetylsalicylic acid and baicalin in the standard tubes and finally 50 µL of medium in control tubes followed by three hours of second incubation. Each tube was centrifuged at 2000 rpm for 10 minutes at 4˚C and the supernatant was discarded. In pellet containing cells, 50 µL of Triton X-100 were added and the tubes were shaken for 2 minutes then 1000 µL of linoleic acid (125 µM) were added and the mixture was incubated once more for 30 minutes. Absorbance was read at 234 nm. The inhibition percentage of enzyme activity was calculated according to the following formula.

$$\%\text{ Inhibition} = \left(\left(\text{OD of control} - \text{OD of test}\right)/\text{OD of control}\right)\times 100$$

2.6.4. Evaluation of the Effect of *C. variegatum* Extracts and Fractions on Tumor Necrosis Alpha (TNF-α) Production

All the fractions presenting higher inhibitory activity against NO production or

5-Lox compared to Baicalin, were used for the determination of their effect on TNF-α production. The levels of TNF-α was determined using commercial ELISA kits as described by the manufacturer method. First, 100 µL supernatant and standard was added in the microplates at various dilutions in triplicate, blocked and incubated in the dark at 4˚C for 1 h 30 min. The plates were washed 3 times with buffer solution while allowing the buffer solution to stand for 1 to 2 min and 100 µl antibodies was then added to the wells (biotin-labeled detection antibody). Plates were incubated at room temperature for 1 h, washed 3 times with buffer solution, and 100 µl of streptavidin-HRP was added. Plates were incubated at room temperature (37˚C) for 45 min, removed and washed 5 times with buffer solution. Then 100 µl TMB substrate (color reagent) was added in the plates and incubated in the dark at 37˚C for 30 min. The enzyme reaction was stopped with 100 µl stop solution and absorbance was measured at 450 nm. Values were expressed in pg/ml.

2.6.5. Data Analysis

Data analyses were performed using 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 $p < 0.05$.

3. Results

3.1. Cytotoxicity of Extracts on Primary Mouse Macrophage

C. variegatum extracts and fractions were evaluated for their cytotoxic effect on mouse primary macrophages maintained on DMEM culture medium for six hours.at different concentrations ranged from 0.1 µg/mL to 100 µg/mL using MTT assay. Results (**Figure 1**) showed that no significant difference was observed on cell viability in the presence of extracts and fractions at tested concentration during the incubation period.

3.2. Inhibitory Effect of *C. variegatum* Extracts and Fractions on Nitric Oxide (NO) Production by Viable Macrophages

The induction of primary macrophages into an inflammatory state by treatment with *Saccharomyces cereviseae* (SC) caused significant increase in NO. Extracts and fractions of *C. variegatum* inhibited the nitric oxide production by viable primary mouse macrophages in a concentration-dependent manner (**Figure 2**). However no inhibitory effect was observed with methylene chloride/methanol (50:50 v/v) fraction of hydro-ethanolic leaf extract (HEF4). Inhibitory concentration fifty (IC_{50}) were determined (**Table 1**). Inhibitory effect of extracts and other fractions ranged from 0.27 µg/mL to 30.42 µg/mL. Although fractionation process enhanced the activity of *C. variegatum*, there was no significant difference between activity of ethanolic extract as compared to hydro-ethanolic extract. The activities of fractions HEF2, HEF3, HEF5, EEF1, EEF2, EEF3 and EEF5 were significantly higher compared to Baicalin. Among fractions, methylene chloride/

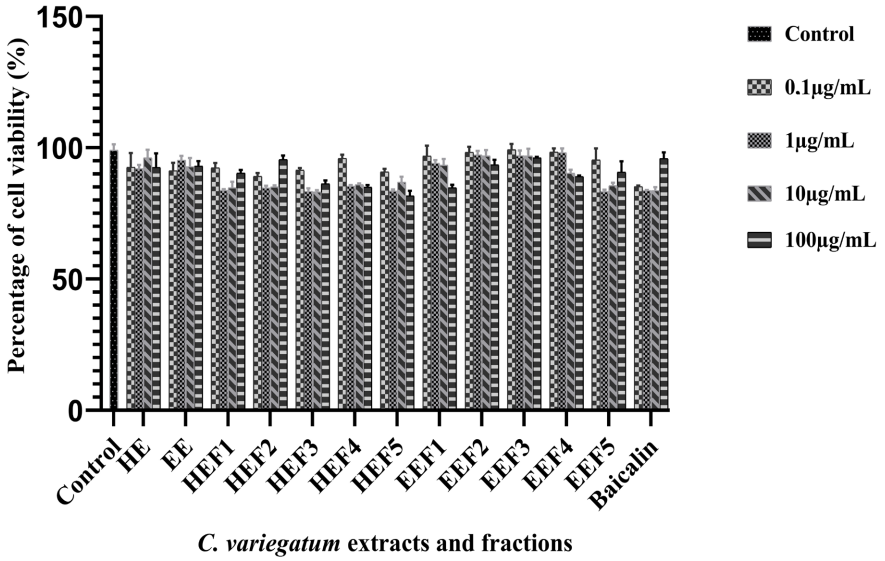

Figure 1. Cytotoxicity of *C. variegatum* leaf extracts and fractions.

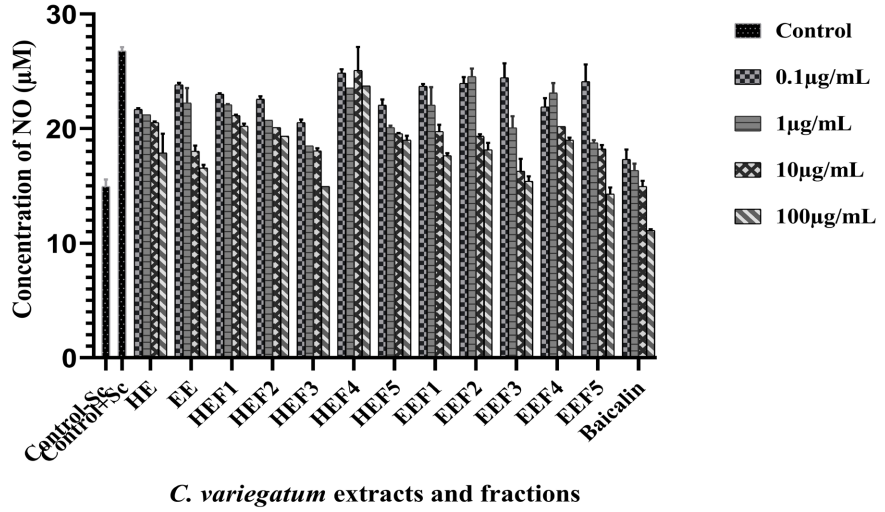

Figure 2. Inhibitory effect of *C. variegatum* extracts and fractions nitric oxide (NO) production by SC induced macrophages.

Table 1. Inhibitory concentration 50 (IC_{50}) of *C. variegatum* extracts and fractions.

Extracts and fractions	IC50 mean ± standard deviation (µg/mL)		
	NO	5-Lox	TNF-α
HE	1.59 ± 0.14*	18.11 ± 1.85	//
EE	1.94 ± 0.41*	31.42 ± 10.29	//
HEF1	30.42 ± 3.72	44.42 ± 3.34	//
HEF2	3.05 ± 0.24*	6.47 ± 1.05*	13.83 ± 1.39
HEF3	0.89 ± 0.07*	9.48 ± 1.48**	21.2 ± 3.41
HEF4	//	45.31 ± 5.87	//
HEF5	1.09 ± 0.05*	2.78 ± 0.17*	2.99 ± 0.18*

Continued

EEF1	3.57 ± 2.12*	23.08 ± 3.34	1.18 ± 0.37*
EEF2	3.20 ± 1.88*	>500	//
EEF3	2.88 ± 0.10*	12.21 ± 3.47**	0.19 ± 0.02*
EEF4	0.27 ± 0.13[a]	51.55 ± 11.85	//
EEF5	10.20 ± 2.38	23.33 ± 5.64	5.36 ± 2.66**
Baicalin	6.77 ± 2.07	11.20 ± 2.53	6.68 ± 1.18

*values of IC_{50} of significantly lower compared to the value of Baicalin; **values of IC_{50} of significantly non different compared to the value of Baicalin.

methanol (90:10 v/v) fraction of hydro-ethanolic leaf extract (HEF3), and methanol fraction of ethanolic leaf extract exhibited the highest inhibitory effect on NO production. HEF1 and EEF4 exhibited the lowest activity on NO production.

3.3. Effect of *C. variegatum* Extracts and Fractions on 5-Lipoxygenase Activity

The inhibitory activity of the extracts and fractions on the 5-lipoxygenase of 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 extract and fractions (Figure 3). IC_{50} obtained (Table 1) revealed that the inhibitory activity of hydro-ethanolic extract (18.11 ± 1.85 µg/mL) was significantly higher than that of ethanolic extract (31.42 ± 10.29 µg/mL). Among fractions, HEF2 and HEF5 (IC_{50}: 6.47 ± 1.05 and 2.78 ± 0.17 µg/mL respectively) exhibited significantly higher inhibitory effects on 5-Lox than Baicalin used as standard in this assay (11.20 ± 2.53 µg/mL). The inhibitory effects of HEF3 (9.48 ± 1.48 µg/mL) and EEF (12.21 ± 3.47 µg/mL) on 5-Lox were significantly non different from that of Baicalin.

3.4. Inhibitory Effect of *C. variegatum* Extracts and Fractions on Tumor Necrosis Alpha (TNFα) Production

Six fractions (HEF2, HEF3, HEF5, EEF1, EEF3 and EEF5) were selected based on their NO and 5-Lox inhibitory activity, for the evaluation of their inhibitory effect on TNF-α production. Activation of primary mouse macrophages with *Saccharomyces cereviseae* (SC) caused significant increase in TNF-α production. Fractions of *C. variegatum* showed a concentration-related inhibition of TNF-α production in which significant inhibition was still evident (Figure 4). Following the bIC_{50} determination (Table 1), fractions HEF5, EEF1 and EEF3 (2.99 ± 0.18; 1.18 ± 0.37, 0.19 ± 0.02 µg/mL respectively) presented significantly higher inhibitory effect on TNF-α production than Baicalin. The inhibitory activity of EEF5 (5.36 ± 2.66 µg/mL) was significantly non different from that of Baicalin. Fractions HEF2 and HEF3 (13.83 ± 1.39; 21.2 ± 3.41 µg/mL respectively) exhibited moderate anti-TNF-α activity.

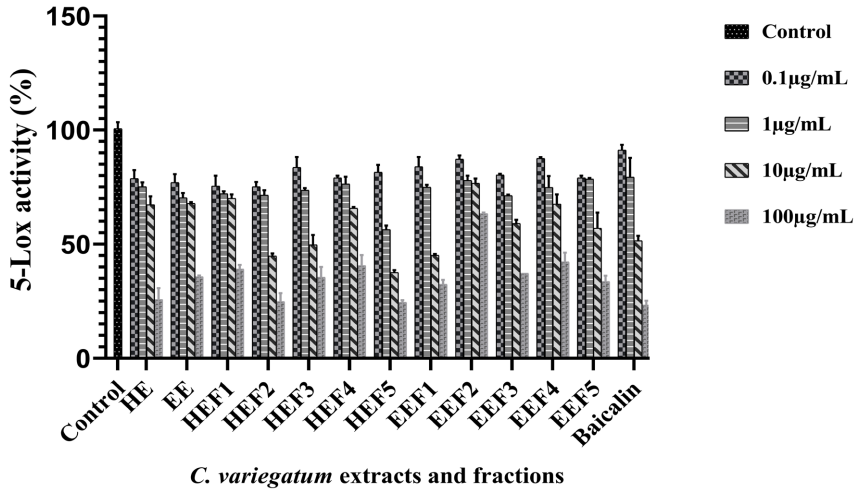

Figure 3. Inhibitory effect of *C. variegatum* extracts and fractions on 5-lipoxygenase activity of SC induced macrophages nitric oxide (NO) production by viable macrophages.

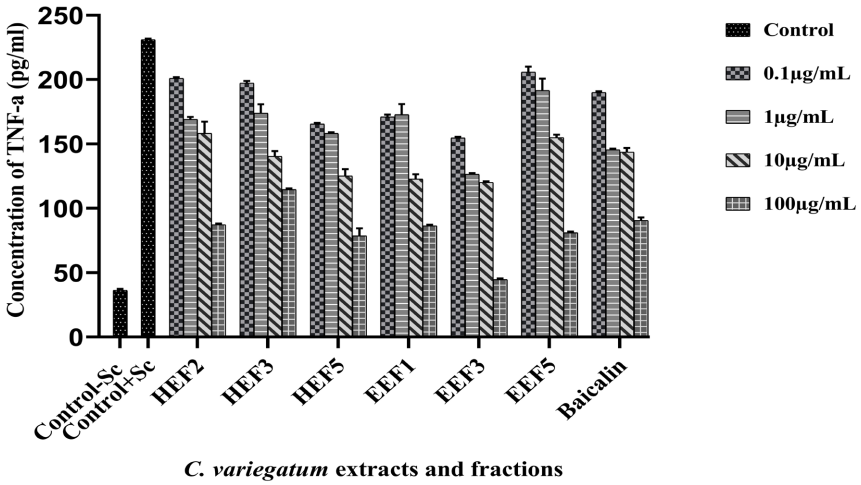

Figure 4. Inhibitory effect of *C. variegatum* extracts and fractions Tumor Necrosis Alpha (TNF*a*) production by SC induced macrophages.

4. Discussion

The search for the efficacy of plant extracts and the determination of their mechanism of action are major and permanent challenges for the valorization of phytotherapy [31]. In fact, Plants are the reservoir of important bioactive molecules classified as phenolics, alkaloids, carotenoids, organosulfur compounds, etc. on the basis of their chemical nature, and these molecules are reclassified as antioxidants, analgesics, cardioactive, anticancerous, immunity potentiating, detoxifying, neuropharmacological agents, etc. on the basis of their pharmacological action [32]. *Codiaeum variegatum* belongs to the genus Codiaeum A. Juss under the Euphorbiaceae family. It is one of the most popular ornamental plants which grows easily in tropical and warm regions [33]. It can be cultivated by various methods such as cuttings, grafting, by seeds and air layering [34]. Several biological activities are being demonstrated in our group previous studies potent

antiparasitic anti amoebic and anti-oxidant properties of extracts and fractions of *C. variegatum*. In the present study we aimed to highlight the potent anti-inflammatory activity against production of some pro-inflammatory mediators (NO, TNF-*α*) and inhibition of pro-inflammatory enzymes (5-Lox), by *Saccharomyces cerevisiae* induced primary macrophages. Macrophages are critical mediators of the innate immune response against foreign pathogens, including bacteria, physical stress, and injury. Therefore, these cells play a key role in the "inflammatory pathway" which in turn can lead to an array of diseases and disorders [35]. Several pathogen associated molecular patterns (PAMPs) located in the cell wall or cell surface of fungi have been identified as potential ligands. Yeast zymosan activates TLR2/TLR6 heterodimers, whereas Saccharomyces cerevisiae derived mannan seems to be detected by TLR4 [36]. *C. variegatum* extracts and fractions were nontoxic to primary macrophages up to the concentration of 100 µg/mL. The results corroborated those obtained *in vitro* with crude stem extract on primary macrophages [29]. Inhibiting NO production is a well demonstrated anti-inflammatory mechanism exhibited by many isolated compounds and extracts from various medicinal plants [37]. In mammalian cells, NO is mainly produced from the L arginine: NO metabolic pathway by the enzyme called nitric oxide synthase (NOS), which has three isoforms of NOS—eNOS (endothelial NOS), nNOS (neuronal NOS), and iNOS [38]. eNOS and nNOS produce a controlled amount of NO in endothelial cells and neurons, respectively, under the Ca^{2+}/calmodulin system [39], while iNOS produces NO only upon activation by specific cytokines (e.g., TNF and INF) or microbial products (e.g., LPS and Saccharomyces cerevisiae derived mannan). Sustained NO production enhances the formation of reactive nitrogen oxide species (RNOs), and prolonged exposure to such free radicals is harmful to healthy body cells [40] whereby iNOS must be regulated. Extracts and fractions of *C. variegatum* showed NO production inhibitory activity except HEF4. The best inhibitory activity was observed with HEF3 and EEF5. Lipoxygenases, nonheme iron-containing enzymes, catalyze the peroxidation/oxidation of the polyunsaturated fatty acids (mainly arachidonic acid) to form various reactive inflammatory mediators. These enzymes were found to significantly contribute to the pathogenesis of various diseases [41]. One important enzyme from the lipoxygenase family is 5-lipoxygenase (5-LOX) that was found to trigger the inflammatory response through the biosynthesis of leukotrienes B4 (LTB4) [42]. The inhibitory activity of hydro-ethanolic extract was significantly higher than that of ethanolic extract. These activities remained significantly lower compared to those obtained with crude extract of S. rhombifolia [43]. Among fractions, HEF2 and HEF5 exhibited significantly higher inhibitory effects on 5-Lox than Baicalin used as standard in this assay. The inhibitory effects of HEF3 (9.48 ± 1.48 µg/mL) and EEF (12.21 ± 3.47 µg/mL) on 5-Lox were significantly non different from that of Baicalin. The inhibition of 5-LOX by *C. variegatum* fractions demonstrated the great therapeutic potential of those fractions against inflammation

[44]. First identified as a serum factor causing necrosis of transplanted tumors, TNF-α is a multifunctional cytokine that participates in the regulation of immune-inflammatory reactions involved in host defense against infectious, autoimmune, and endocrine diseases and cancer [45] [46] [47] [48]. Its actions help to determine the survival or death of various cells [49]. Excessive TNF-α expression is associated with tumor promotion via a strong immune inflammatory response and angiogenesis, which can modify the risk for gastric, breast, hepatocellular, cervical, or bladder carcinoma [50] [51] [52]. In the present study, we found that Fractions of *C. variegatum* showed a concentration-related inhibition of TNF-α production in which significant inhibition was still evident. Fractions HEF5, EEF1 and EEF3 presented significantly higher inhibitory effect on TNF-α production than Baicalin. The inhibitory activity of EEF5 was significantly non different from that of Baicalin. Fractions HEF2 and HEF3 exhibited moderate anti-TNF-α activity. These activities are more pronounced than those obtained in previous publication with the crude extract of C. minima [53]. The effect of oil emulsion from B. javanica in DSS (Dextran Sodium Sulphate)-induced acute colitis mouse model (0.5, 1, and 2 g/kg) evaluated, and cytokines production was analyzed, B. javanica oil emulsion at higher concentrations (1 and 2 g/kg) has significantly ($p < 0.01$) lowered the levels of six inflammatory cytokines including TNF-α in the colon tissues when compared to positive controls (sulfasalazine and azathioprine) [54]. Costatamins A-C isolated from the leaves of Australian Angophora costata inhibited the production of NO and pro-inflammatory cytokine TNF-α in LPS-activated RAW 264.7 cells with IC50 values in the range of 20 - 30 μg/mL [55].

5. Conclusion

Our results demonstrated that *C. variegatum* extracts and fractions exhibited a greater anti-inflammatory potential throughout the inhibition of pro-inflammatory mediators such as NO, 5-Lox and TNF-α. Considering that the anti-amoebic activity of the same extracts and fractions has been previously described, our future study will focus on its *in vivo* anti-inflammatory activity with regards to bowel inflammatory diseases.

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Disclosure

The study was independently designed by the authors and the funding body had no role in Lab experiments, analysis and interpretation of the data

Conflicts of Interest

The authors have no conflicts of interest to declare.

Author Contributions

SPN, CNM, CFM, EBE, VSN, and IN, carried out all experiments reported in the manuscript. SPN, 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.

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Abbreviations

DMEM: Dulbecco's Modified Eagle Medium;
IC_{50}: Inhibitory concentration 50;
LPS: Lipopolysaccharide;
HE: Hydro-ethanolic leaf extract;
EE: Ethanolic leaf extract;
HEF1: Hydro-ethanolic leaf methylene chloride fraction;
HEF2: Hydro-ethanolic leaf methylene chloride/methanol (95:5 v/v) fraction;
HEF3: Hydro-ethanolic leaf methylene chloride/methanol (90:10 v/v) fraction;
HEF4: Hydro-ethanolic leaf methylene chloride/methanol (50:50 v/v) fraction;
HEF5: Hydro-ethanolic leaf methanol fraction;
EEF1: Ethanolic leaf methylene chloride fraction;
HEF2: Ethanolic leaf methylene chloride/methanol (95:5 v/v) fraction;
HEF3: Ethanolic leaf methylene chloride/methanol (90:10 v/v) fraction;
HEF4: Ethanolic leaf methylene chloride/methanol (50:50 v/v) fraction;
HEF5: Ethanolic leaf methanol fraction;
LTB4: Leucothrien B4;
MTT: 3(4,5-dimethylthiazol-2-yl)-2,3diphenyletrazolium;
NO: Nitric Oxide;
RNOs: Reactive nitrogen oxide species;
TLR: Toll-liked Receptor;
TNF-*α*: Tumor Necrosis Factor alpha.