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Elucidation of the Phytochemicals, Safety Profile, and Preclinical Anti-Inflammatory Activity of Ethanol Extract of *Combretum paniculatum* Leaves

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ABSTRACT

Inflammatory aberrations constitute the major pathophysiological basis for most chronic diseases. This study investigated the phytochemicals, safety profile, and anti-inflammatory activity of ethanol extract of *Combretum paniculatum* leaves (EECP). Phytochemical screening and the acute toxicity of EECP were determined using standard methods. Egg albumin (0.1 mL) was used as a phlogistic agent for induction of inflammation in the right hind paw of rats one hour after treatment with diclofenac (100 mg/kg) and EECP (100 and 200 mg/kg). Paw volume was measured before induction and hourly for 5 hours using the plethysmometer. Further, membrane stabilization tests and nitric oxide scavenging activity were used to decipher the inflammatory mechanisms. EECP is rich in phenols, flavonoids, alkaloids, and reducing sugars. Tannins and terpenoids were present in moderate amounts, while low contents of steroids and glycosides were recorded in EECP. There was no mortality and visible signs of toxicity up to a dose of 5000 mg/kg after 24 h. Moreso, EECP significantly ($p < 0.05$) suppressed edema formation, with 200 mg/kg of EECP having a higher effect than diclofenac after 1, 2, and 3 h post-induction. Similarly, EECP inhibited membrane hemolysis and nitric oxide production with IC_{50} values of 115.3 and 6.547 µg/mL, respectively, against 74.56 and 4.667 µg/mL recorded for standard drugs, diclofenac and ascorbic acid, respectively. Taken together, EECP has strong anti-inflammatory activity, and the possible mechanism of its anti-inflammatory action could be by stabilizing the membrane, thereby mitigating the release of inflammatory mediators such as nitric oxide to the inflamed region.

***Keywords*:** Acute toxicity, Anti-inflammatory, *Combretum paniculatum*, Egg albumin-induced edema, Nitric oxide activity, and Membrane stabilization.

Introduction

Inflammation is an immune response characterized by cellular, vascular, and biochemical changes vital for the elimination of aggressors through the action of mediators and cytokines.[1] However, these mediators can interact with various cellular and subcellular components in different cell types, thereby amplifying the inflammatory response.[2] Unregulated inflammatory responses have become a major public health threat globally. [2-3] Research findings have shown that aberrations in inflammatory reactions are associated with the etiology and pathogenesis of several diseases, including cancer, type 2 diabetes, periodontal diseases[4], and the COVID-19 cytokine storm.[5-6] For instance, myriad stages of cancer development, including proliferation, angiogenesis, and metastasis, have been linked to chronic inflammation.[7] Moreso, in diabetes, pancreatic inflammation and beta cell loss or damage set a pro-inflammatory scene, which leads to both microvascular and macrovascular complications.[8]

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From the viewpoint of periodontal disease, unresolved inflammation could lead to tooth loss and gradual destruction of tooth-supporting tissues like alveolar bone, gingiva, cementum, and periodontal ligament.[9-10] The hyperinflammatory response in COVID-19 patients caused by excessive tumor necrosis factor-alpha (TNF-α), chemokines, and interleukins (IL-1 and IL-6) results in viral sepsis, coagulopathy, pneumatic shock, severe respiratory distress syndrome, loss of organ functions, and death.[5-6] Undoubtedly, inflammation not only regulates the pathobiology of these diseases but also underlies their complications. Currently, many pharmaceutical interventions are employed to address inflammatory challenges, ranging from steroidal to non-steroidal anti-inflammatory drugs (NSAIDs).[2,4] Steroidal drugs decrease cell-mediated immunity, leukocyte number, and activity as well as the synthesis of interleukins and bioactive lipids.[1] On the other hand, NSAIDs such as cyclooxygenase (COX)-2 selective inhibitors (coxibs) and non-selective NSAIDs (indomethacin, ibuprofen, aspirin, and diclofenac), on the other hand, regulate inflammation by inhibiting COX-2, the enzyme that catalyzes the biosynthesis of prostaglandins, thromboxanes, and prostacyclins.[2] Although both agents are effective against inflammation, their use in chronic inflammation is insufficient and has serious side effects.[11] Long-term use of corticosteroids can cause high blood sugar, high blood pressure, a stop in growth, and osteoporosis. NSAIDs, on the other hand, can harm the kidneys, heart, and intestines.[3] So, to avoid these risks, the WHO suggested making medicines from plants instead of dangerous synthetic drugs.[3] Recently, the focal point of much scientific investigation has been centered on the investigation of the pharmaceutical potential and possible mechanisms of action of plant-derived molecules, which could be employed as lead compounds in drug discovery for the treatment of diseases including inflammation.[12] Pro-inflammatory cytokines,

interleukins, and prostaglandins are released by signaling pathways that can be turned off with the help of bioactive compounds from medicinal plants.[12-13]Among these bioactive compounds, phytochemicals such as flavonoids, steroids, alkaloids, phenols, glycosides, tannins, and triterpenoids have demonstrated interesting anti-inflammatory effects.[3,14] *Combretum paniculatum* Vent, also called forest frame, is an ascadent shrub with trailing branches.[15-18] It is a tropical African endemic plant belonging to the *Combretaceae* family of flowering plants. *C. paniculatum* is extensively used in ethnomedicine for the treatment of chronic dysentery, diarrhea, enlarged liver, flatulence, colic, vomiting, as an analgesic, anesthetics,[15,17] stomach pain, pain in the joints, wounds, ringworm,[16] gonorrhea, stomatitis,[18] eye diseases, and leprosy.[19] The pharmacological potency of the leaves includes antiulcer, anti-diarrhea,[17] antioxidant,[15] strong anti-viral activity against HIV-2,[19], and anti-cancer activity.[18] Due to the wide application of *C. paniculatum* in treating inflammatory-related anomalies as well as a paucity of information on the anti-inflammatory activity of the plant. This study was undertaken to elucidate the phytochemicals, safety profile, anti-inflammatory activity, and possible mechanism of action.

Materials and Methods

Plant materials

Combretum paniculatum leaves were collected from Nsukka Local Government Area of Enugu State, Nigeria, between November and December 2021. Taxonomic identification of the plant with reference number INTERCEDD/1909 was done by Mr. Alfred Ozioko, a staff of the Bioresources Development and Conservation Programme (BDCP) Research Centre, Nsukka, Enugu State, Nigeria.

Management of experimental animals

Eighteen Swiss mice (28–32 g) and twenty (20) Wistar rats (119–135 g) used as test animals were acclimatized for two weeks, fed rodent meal and water ad labium. All tests using experimental animals (mice and rats), human blood, and eggs were carried out as specified by the revised National Institute of Health Guide for Laboratory Animal Care and Use (Pub No. 85-23, revised 1985) and the ethical procedure of the Ethics and Biosafety Committee of the Faculty of Biological Sciences, University of Nigeria, Nigeria (approval number: UNN/FBS/EC/1082).

Chemicals/standard drugs

All the chemicals used were of analytical grade and procured from Sigma-Aldrich Inc., UK, and British Drug Houses, England. Diclofenac, sourced from Evans Pharmaceutical, England, was used as a standard drug in the egg albumin-induced edema model and membrane stabilization experiment, while ascorbic acid (produced by Qualitex, India) was employed as the standard drug for the nitric oxide radical scavenging activity test.

Plant preparation and extraction

The fresh leaves of *Combretum paniculatum* were dried, pulverized, and extracted with 70 % ethanol for 72 hrs. Subsequently, filtration was done first with muslin cloth and then with Whatman No. 1 filter paper. A rotatory vacuum evaporator was used to evaporate the filtrate at 45 °C to obtain the ethanol extract of *Combretum paniculatum* designated as EECP. EECP was stored in the refrigerator until needed.

Phytochemical screening of EECP

Qualitative elucidation of phytochemicals in the extract was carried out using the experimental procedure of Harbone [20], Trease, and Evans.[21]

Acute toxicity test of EECP

The lethal dose of EECP was evaluated in two phases with the Lorke's [22]method.

Determination of the anti-inflammatory activity of EECP

Inflammation was evaluated in twenty Wistar rats using the method of Winter et al. [23] technique. The rats were divided into four experimental groups, each of which had five rats. Group 1 received normal saline, Group 2 was administered the anti-inflammatory standard drug, diclofenac (100 mg/kg), and Groups 3 and 4 received intraperitoneal dosages of EECP of 100 and 200 mg/kg body weight, respectively. Egg albumin (0.1 mL) was used to induce inflammation on the rat's paw. Measurement of rats' paw volume was done using a plethysmometer before induction (TO) and at 1, 2, 3, 4, and 5 h (designated as T1, T2, T3, T4, and T5). The extent of inflammation was determined by calculating changes in paw volume between time intervals TO and T1-T5.

Mechanisms of anti-inflammatory activity

Membrane stabilization

This was done using the procedure of Shinde et al [24]. The erythrocytes used for the study were prepared by centrifuging (4000 rpm, Abman) 5 mL of blood collected from healthy human donors and centrifuged for 10 min at 3000 rpm. Thereafter, it was washed with sodium phosphate buffer (10 mM, pH 7.4). The erythrocyte suspension was added to different test tubes containing EECP (100-800 µg/mL) and diclofenac (100-800 µg/mL) prepared separately in an isotonic and hypotonic solution. Incubation of the resulting mixture was carried out at 37 °C for 1 h. Then, the mixture was centrifuged (10 min at 2500 rpm) and absorbance was measured at 540 nm with a 6305 Jenway UV-vis spectrophotometer (Bibby Scientific Ltd., UK). All tests were done in triplicate. Inhibition of membrane hemolysis was calculated using the following:

$$\text{Inhibition of HRBC hemolysis} = 1 - \frac{Abs\ 2 - Abs\ 1}{Abs\ 3 - Abs\ 1} \times 100$$

Where: *Abs_1 denotes the* absorbance of test and control samples in an isotonic solution, Ab_2 and Abs_3 represent the absorbance of test samples and control samples in a hypotonic solution, respectively.

Nitric oxide production scavenging assay

The extent of nitric oxide scavenging activity of EECP was measured following the procedure of Sreejayan and Rao[25] using ascorbic acid as a standard drug. Before a 150-minute incubation period at room temperature, 3 ml of sodium nitroprusside (10 mM) was added to test tubes containing a serial concentration of EECP (15.63-500 µg/mL) and ascorbic acid (15.63-500 µg/mL). Then, 0.5 mL of Griess reagent was pipetted into the test tubes, which were left to incubate again for 30 min before measuring the absorbance at 546 nm. All tests were done in triplicate. The nitric oxide scavenging activity was calculated from the following equation.

$$\text{Inhibition of nitric oxide (\%)} = \frac{Absorbance\ of\ control - Absorbance\ of\ test}{Absorbance\ of\ control} \times 100$$

Statistical analyses

The data were analyzed using IBM Statistical Package for Service Solutions (SPSS) version 23 (SPSS Inc., Chicago, IL, USA). Descriptive analysis was performed using one-way ANOVA, and differences were assessed using LCD and Duncan multiple comparisons, with a p-value of 0.05 established as the least significant criterion. Version 6.05 of GraphPad Prism (GraphPad Software, Inc., California, USA) was utilized in generating the nonlinear regression sigmoidal curve used to calculate the half maximal inhibitory capacity (IC_{50}) and R square (R^2) values.

Results and Discussion

Over the last decade, plant-based products have increasingly gained scientific and public interest because of their multi-targeted efficacy, therapeutic properties, and low toxicity.[12,13,26] Plants are rich sources of phytochemicals used to prevent and treat numerous diseases.[27,28] In the present study, qualitative phytochemical screening results presented in Table 1 showed that EECP contains phenols, tannins, flavonoids, alkaloids, steroids, and terpenoids, reducing sugar and glycosides. These phytochemicals are credited with possessing a wide range of biological and pharmaceutical activities.

Table 1: Qualitative phytochemical screening of the ethanol extract of *C. paniculatum l*eaves

S/N	Phytochemicals	Tests	Observations	Results
1	Phenols	Folin Ciocalteau's test	Green coloration	+
2	Tannins	Ferric chloride test	Greenish-brown ppt	+
3	Flavonoids	Lead acetate test	Yellow ppt	+
4	Alkaloids	Dragendorff 's test	Reddish brown ppt	+
5	Steroids	Extract+aceticacid $+H_2SO_4$	Red coloration	+
6	Terpenoids	Salkowski test	Reddish-brown coloration	+
7	Reducing sugar	Fehling's test	Brick red ppt	+
8	Glycosides			+

Remarkably, phenolic compounds, which are rich in EECP, have health-promoting properties, especially as antioxidants and anti-inflammatory agents.[29] They work as antioxidants by getting rid of free radicals, reducing metal ions, and chelating metal ions.[30] Their anti-inflammatory potential is mainly achieved by inhibiting the expression of pro-inflammatory genes, which offers protection against inflammatory-related diseases.[30] Hence, the high abundance of phenols in EECP brings it to the limelight in the management and treatment of diseases. Flavonoids present in EECP are extensively studied due to their high therapeutic and health benefits. Flavonoids regulate abnormal hyperplasia and persistent inflammation by down-regulating expression of STAT, PI3K/Akt, Nrf2, MAPK, and NF-*κ*B signaling pathways Also, flavonoids inhibit inflammation by suppressing the release of arachidonic acid, which leads to a decrease in prostaglandins, a key mediator of the inflammatory response.[31] Alkaloids, terpenoids and tannins are effective as antioxidants, analgesics, anti-diabetic, and anti-inflammatory agents.[27,32] The fact that these bioactive compounds are found in EECP is impressive given that they are said to have health and drug benefits, especially when it comes to inflammation. The safety assessment of medicinal plants provides the basis for placing a plant-based product as an alternative, safe therapy against the already documented risk of synthetic drugs. EECP showed no toxic effect up to a dose of 5000 mg/kg **(**Table 2**).** There was no lethality, signs of toxicity, and behavioral changes such as lachrymation, increased motor activity, salvation, weakness, sedation, and muscle spasm after administration of the extract. This raises the safety profile of the extract as an alternative plant-based product. The anti-inflammatory activity of EECP was investigated with an egg albumin-induced model. The change in paw volume of the groups treated with diclofenac (100 mg/kg b. w) and EECP (100 and 200 mg/kg b. w) was significantly ($p < 0.05$) lower than that of the group administered normal saline in the entire time interval measured (1–5h). Interestingly, the group administered 200 mg/kg b. w of EECP significantly ($p < 0.05$) suppressed paw volume after 1, 2, and 3 h more than the diclofenac-treated group. However, a non-significant ($p > 0.05$) difference was recorded between the extract and diclofenac groups after 4 and 5 h **(**Figure 1). It is worth noting that in the egg albumin model, lower paw volume connotes lower edema formation in the rat's hind paw, which suggests inhibition of inflammation. Edema formation is one of the cardinal signs of inflammation that develops in the biphasic stages. The first phase is due to the release of serotonin, histamine, and platelet-activating factors. The release of prostaglandins and various cytokines such as TNF-, IL-, IL-6, and IL-10 promotes edema progression in the second phase. Considering that the egg albumin edema model is extensively used to investigate the anti-inflammatory action of test substances,[34] inhibition of edema in the test animals shows that EECP possesses anti-inflammatory effects. The capacity of the extract to suppress rat-paw edema might be through mitigation of the release, migration, and action of inflammatory stressors to the inflamed area. Research findings reveal that supplementation with phenolic compounds in experimental mice down-regulates NF-κB and activates the Nrf2 pathway, thereby

Table 2: Acute toxicity study (LD_{50}) of ethanol extract of ***C. paniculatum*** leaves

Phases	Doses of EECP (mg/kg)	Mortality
Phase 1	10	nil
	100	nil
	1000	nil
Phase 2	1600	nil
	2900	nil
	5000	nil

N = 3

suppressing the transcription of cytokines and COX-2 which are pro-inflammatory mediators.[12] Hence, the high abundance of phenols in EECP proves beneficial based on this premise. Flavonoids, also present in EECP, inhibit inflammation by suppressing the release of arachidonic acid, which leads to a decrease in prostaglandins, a key mediator of the inflammatory response, thereby protecting tissue injury.[31] So, the fact that the EECP has a lot of bioactive compounds could explain why it has a unique anti-inflammatory effect and make it possible to use the plant to treat problems like joint pain and stomatitis that are caused by inflammation.[18] Membrane stabilization regulates the exudation of fluids out of the membrane in an activated inflammatory response. Undoubtedly, lysis of the lysosomal membrane, which contains key enzymes required for regulation of mediators of systemic inflammation, increases inflammation progression and cellular oxidative damage.[35] Consequently, membrane stabilization is one of the key tests used to investigate the mechanism of anti-inflammatory activity. Herein, EECP inhibited hemolysis of the HRBC membrane just like diclofenac. At 800 µg/mL, EECP inhibited RBC hemolysis by 66.75%, whereas diclofenac had a maximal inhibition of 89.46% at the same concentration **(**Table 3**)**. Overall, the inhibitory effects of diclofenac were greater, as evidenced by its IC_{50} values of 74.56 versus the 115.3 µg/mL recorded in EECP. However, the inhibitory capacities of both were positively correlated with concentration, as recorded in their R^2 values of 0.8326 and 0.8685, respectively (Figure 2). Stabilization of the membrane prevents cell rupture and discharge of lytic enzymes into the tissue as well as the release of phospholipases, which catalyze the conversion of membrane phospholipids into arachidonic acid, a precursor of prostaglandins.[4] Several studies have shown that stabilizing the erythrocyte membrane, which is similar in structure to the lysosomal membrane, could reduce inflammation in the host's body.[34-35] The membrane-stabilizing effect of EECP could be anchored to the interaction of its rich phytoconstituents with membrane proteins. Such interactions hinder the release of pro-inflammatory mediators including proteases, histamines, and activated neutrophils into the inflamed region.

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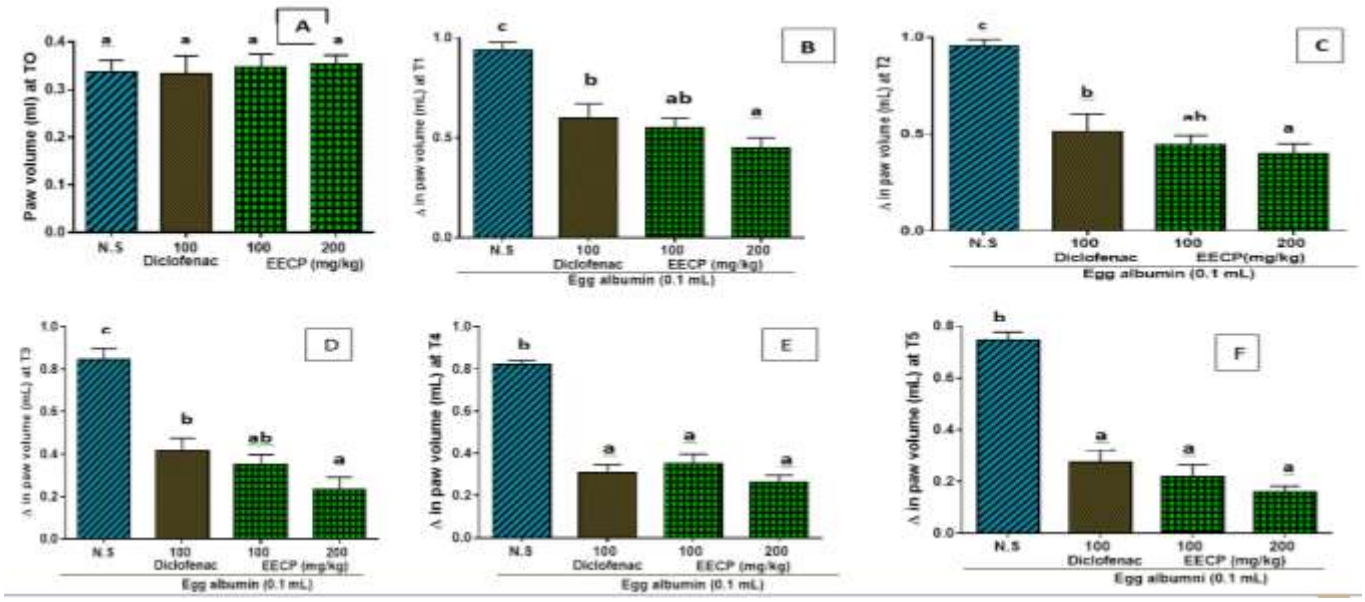

Figure 1: Paw volumes against treatment measured at TO, T1, T2, T3, T4, and T5 were presented in A, B, C, D, E, and F, respectively. Values are presented as mean ± SD of triplicate determinations at 95% confidence interval. The significant difference among the groups denoted by different letters at $p < 0.05$ was obtained by one-way ANOVA followed by LSD and Duncan post hoc multiple comparisons.

Table 3: Effects of ethanol extract of ***C. paniculatum*** leaves on HRBC membrane hemolysis

Test samples	Conc. (µg/ml)	Absorbance of isotonic sample	Absorbance of hypertonic sample	Inhibition (%)
Control	-	0.245 ± 0.010	0.672 ± 0.007	-
EECP	100	0.032 ± 0.009*	0.221 ± 0.003*	55.97
	200	0.045 ± 0.001*	0.233 ± 0.004*	55.74
	400	0.041 ± 0.003*	0.222 ± 0.044*	63.70
	600	0.056 ± 0.023*	0.211 ± 0.011*	57.61
	800	0.041 ± 0.019*	0.183 ± 0.014*	66.75
Diclofenac	100	0.092 ± 0.023*	0.281 ± 0.009*	55.74
	200	0.031 ± 0.010*	0.192 ± 0.009*	68.62
	400	0.045 ± 0.001*	0.179 ± 0.003*	63.93
	600	0.069 ± 0.004*	0.224 ± 0.015*	62.30
	800	0.289 ± 0.022*	0.334 ± 0.012*	89.46

Values are presented as mean ± SD of triplicate determinations at 95% confidence interval. Inhibition (%) of HRBC hemolysis of EECP and diclofenac were calculated relative to the control. Significant difference denoted by * at $p < 0.05$ compared with normal saline (control) was obtained with one-way ANOVA followed by LSD and Duncan post hoc multiple comparisons.

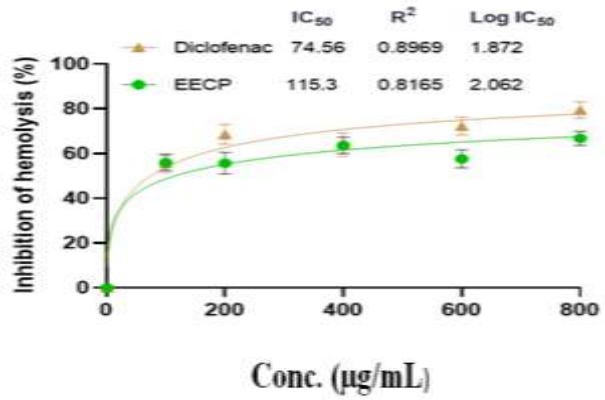

Figure 2: Non-linear regression curve for generation of IC_{50} and R^2 values of inhibition of HRBC membrane hemolysis by EECP.

Thus, this suggests that EECP bioactive compounds could also stabilize cellular components of inflammation such as basophils, eosinophils, mast cells, and blood platelets that produce the inflammatory mediators involved in edema formation.

Nitric oxide is a signal transduction agent that is produced as a result of the oxidation of L-arginine by nitric oxide synthases in the cells, either constitutively or induced by various cell activators that mediate inflammatory reactions.[29,34,36] Ideally, the release of macrophages, mast cells, and leukocytes in the inflamed region increases inducible nitric oxide synthase (iNOS) expression, provoking the generation of higher NO.[37] The excess NO generated promotes vasodilation and exudation of fluids and plasma proteins via nitrosylation of the heme group of guanylate cyclase, leading to an increase in the level of cGMP.[34-35] EECP inhibited NO generation from sodium nitroprusside just like the standard drug, ascorbic acid (Table 4). The inhibitory action of EECP was positively corrected and comparable with that of ascorbic, as revealed by their IC_{50} values of 6.547 and 4.667 µg/mL, respectively (Figure 3**)**. This suggests that the extract could be very good at getting rid of NO that is made in the body by physiological and cellular processes. This would stop NO from combining with ROS to make peroxynitrite, which starts the process of protein, lipid, and

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DNA peroxidation.[32] Overwhelming evidence shows that stabilization of the membrane, which suppresses iNOS and free radical production, is one of the major therapeutic targets of standard anti-inflammatory drugs. Suppression of iNOS will mediate the degradation of the p65 subunit of the NF-kB signaling pathway, leaving the p50 subunit, which prevents tissue damage.[37] Thus, inhibition of NO production could be another possible mechanism of the anti-inflammatory action of EECP.

Table 4: Nitric oxide scavenging activity of ethanol extract of *C. paniculatum* leaves

Conc. (µg/mL)	EECP	Ascorbic acid
15.63	41.51 ± 0.65^{a}	84.54 ± 1.90^{b}
31.25	47.79 ± 3.79^{b}	85.05 ± 1.14^{b}
62.50	49.08 ± 3.83^{bc}	84.63 ± 1.78^{b}
125	54.22 ± 4.10^{cd}	82.10 ± 0.50^{b}
250	57.26 ± 4.46^{d}	77.21 ± 5.37^{b}
500	63.55 ± 1.81^{e}	60.40 ± 3.05^{a}

Values are presented as the mean ± SD of triplicate determinations at a 95% confidence interval. The significant difference within a column was denoted by different letters at $p < 0.05$ and was obtained by a one-way ANOVA followed by LSD and Duncan post hoc multiple comparisons.

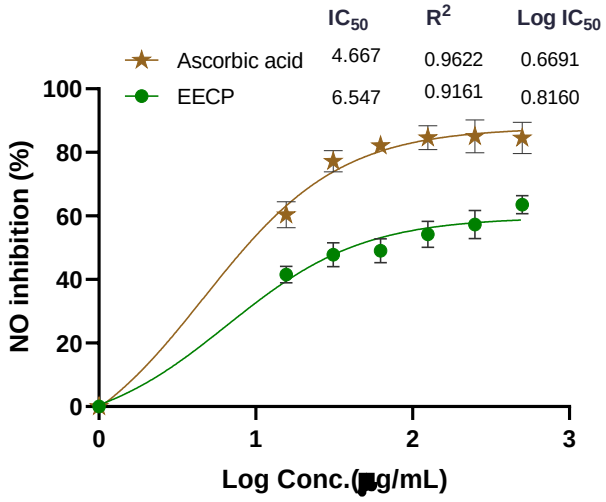

Figure 3: Non-linear regression curve for generation of IC_{50} and R^2 values of nitric oxide scavenging activity of EECP.

Conclusion

This study has shown that the ethanol extract of *C. paniculatum* possesses anti-inflammatory activity, as demonstrated by its edema inhibition. The possible mechanisms of action could be by stabilizing the membrane, which could mitigate the extravasation of neutrophils and exudate fluids to the inflamed site. This potency can be deduced from the ascribed activities of its rich phytochemicals.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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