



Comparative In Vitro Anti-Inflammatory Activity of Aqueous and Ethanol Root Extracts of *Croton Gratissimus*

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Abstract

This study examines the root extracts of Croton gratissimus, a plant that is frequently used in traditional medicine, for their in vitro anti-inflammatory properties. Aqueous and ethanol solvents were used in two extraction techniques to assess the plant's phytochemical makeup and anti-inflammatory properties. The study looked at whether both extracts contained bioactive substances such as quinones, terpenoids, flavonoids, and saponins. The quantitative and quantitative study showed that the aqueous extract contained flavonoids (0.76%), terpenoids (2.62%), carbohydrate (23.8%) and saponins (10.74%), while the ethanol extracts contained flavonoids (0.24%), terpenoids (8.98%), carbohydrate (28.22%) and saponins (3.94%). Membrane stabilization, anti-lipoxygenase, anti-proteinase, and albumin denaturation assays were used to evaluate the anti-inflammatory activity. Despite being less effective than common medications like Diclofenac and Indomethacin, both extracts showed strong anti-inflammatory potential. The aqueous extract was consistently more effective at preventing enzyme activity and stabilizing red blood cells. The findings suggest that C. gratissimus root extracts could serve as a natural alternative for managing inflammation, with solvent choice playing a critical role in optimizing bioactive compound extraction.

Keywords: Anti-inflammatory, membrane stabilization, anti-lipoxygenase, albumin denaturation

Citation

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Introduction

Inflammation is a complex biological response triggered by harmful stimuli such as pathogens, physical injury, or chemical irritants. It is an essential component of the body's immune defense and tissue repair mechanisms (Medzhitov, 2008). However, when the inflammatory response becomes excessive or chronic, it contributes to the development of several pathological conditions including arthritis, cardiovascular diseases, and neurodegenerative disorders (Chen et al., 2018). Pain, redness, warmth, and swelling are the four primary indicators of inflammation. Arterioles in the affected tissue grow when a bodily part is harmed. As more blood

flows through the area, this makes it redder (Burke et al., 2005). Acute and chronic inflammations are recognized types of inflammation. Perhaps the body's initial reaction to potentially dangerous stimuli is acute inflammation. Chronic inflammation in the body is detrimental to the body since the inflammatory process is uncontrollable. (Medzhitov, 2008).

The principal enzyme that is engaged in the production of prostaglandins, prostacyclins, as well as thromboxanes that are associated with inflammation, pain, and platelet aggregation is known as cyclooxygenase (COX) (Pilotto et al., 2010).

Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly prescribed to manage inflammatory conditions. While effective, prolonged use of these synthetic drugs is associated with significant side effects such as gastrointestinal irritation, renal impairment, and cardiovascular risks (Alsenani, et al., 2023). These limitations have prompted growing interest in plant-based anti-inflammatory agents, which are generally considered safer and more sustainable alternatives.

Croton gratissimus (Euphorbiaceae), commonly known as “scent leaf,” is a medicinal plant used in various African ethnomedical systems to treat inflammation, infections, and gastrointestinal disturbances (Alves, et al., 2015). Its pharmacological potential has been attributed to bioactive phytochemicals such as flavonoids, terpenoids, saponins, and phenolic compounds, many of which are known for their anti-inflammatory and antioxidant activities (Cai et al., 2016).

A critical factor in the phytochemical analysis and bioactivity of plant extracts is the choice of extraction solvent. Solvent polarity greatly influences the efficiency of bioactive compound extraction. Water, a polar solvent, is effective in extracting hydrophilic compounds like flavonoid glycosides and saponins. In contrast, ethanol, being moderately polar, can dissolve a broader range of both polar and non-polar constituents such as terpenoids, steroids, and phenolics (Edeoga et al., 2005; Makkar et al., 2007). Comparing these solvents allows researchers to better understand how solvent selection affects both the phytochemical yield and the biological activity of the extract.

Therefore, the present study aims to comparatively evaluate the *in vitro* anti-inflammatory activity of aqueous and ethanol root extracts of *Croton gratissimus* using membrane stabilization, anti-lipoxygenase, anti-proteinase, and albumin denaturation assays. The results will provide insight into the optimal extraction method for maximizing the plant’s therapeutic potential and support the ongoing search for natural, safe anti-inflammatory agents.

Materials and Methods

Collection of Plant Material

The roots of *Croton gratissimus* were freshly harvested at Ogbayo-ijoko, Ogun State, Nigeria. They were authenticated and identified at the University of Lagos with authentication no: 8233. The Fresh roots were well rinsed with water and left to air dry at room temperature (28 °C) and ground in an electric blender to fine powder, weighed and kept till extraction.

Extraction

The root of *Croton gratissimus* was extracted by soaking 100 g of the dried powdered plant materials in 1 L of distilled water and ethanol respectively and allowed to stand at room temperature over a period of 48 hours (to allow thorough extraction). After the 48 hours elapsed, the extracts were filtered successively on Whitman filter paper No. 42 (125mm). Concentration of the filtrates were done by freeze drying the extract. Dried residue (crude extract) was then kept at -4 °C. Small portions of the crude plant extract residue was weighed and dissolved in distilled water which will be used each day of the experiments.

Qualitative Analysis of Phytochemicals

The main classes of secondary metabolites, such as glycosides, phenols, anthraquinone, tannins, alkaloids, saponins, flavonoids, terpenoids and steroids were screened using standard procedures (Sofowora, 1993; Trease & Evans, 1980; Ayoola et al., 2008).

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Quantitative Analysis of Phytochemicals

The amount of some phytochemicals in the ethanol extract of *Croton gratissimus* such as steroids, tannins, glycosides, alkaloids, flavonoid and many more were determined (Farhana et al., 2023).

Invitro Anti-Inflammatory Analysis

In-vitro anti-inflammatory analysis was performed using standard procedures and was done using four different assays which are:

- Membrane stabilization assay.
- Anti-lipoxygenase assay.
- Anti-proteinase assay.
- Albumin denaturation assay.

Membrane Stabilization Assay

The membrane stabilization assay measures the ability of the extract to stabilize red blood cells (RBCs) under hypotonic conditions, which mimics the stabilization of lysosomal membranes during inflammation (Shinde et al., 1999).

Procedure:

The packed RBCs were obtained by centrifuging 5 mL of fresh whole human blood for 10 minutes at a rate of 3,000 rpm. Three separate washes of the cells were performed using the same amount of saline. RBCs were suspended in normal saline at a rate of 10% (v/v). To 1 mL of the RBC suspension, different quantities of the plant extracts (100, 200, 400, and 800 µg/mL) were applied. Two milliliters of hypotonic solution (0.25% NaCl) were added to this mixture, then saline was added to bring the volume up to five milliliters. After 30 minutes of incubation at 37°C, the mixtures were centrifuged for 10 minutes at 3,000 rpm. The supernatant's absorbance was measured with a UV-visible spectrophotometer at 560 nm. The usual medication used was diclofenac, and the percentage membrane stabilization was calculated using the following formula:

$$\% \text{ inhibition} = \frac{\text{Absorbance control} - \text{Absorbance of test}}{\text{Absorbance control}} \times 100$$

Anti-Lipoxygenase Assay

Lipoxygenase is an enzyme that plays a role in the inflammatory response by catalyzing the oxygenation of polyunsaturated fatty acids (e.g., arachidonic acid). This

assay measures the ability of the extract to inhibit lipoxygenase activity (Kim et al., 1999).

Procedure

A 1mL reaction mixture containing 0.1mL of lipoxygenase enzyme (prepared from soybean), 0.1mL of the extract at different concentrations (100, 200, 400, 800 µg/mL), and 0.8mL of phosphate buffer (0.2M, pH 7.0) was incubated at 25°C for 10 minutes. The reaction was initiated by adding 0.1 mL of linoleic acid substrate (final concentration 140 µM). The change in absorbance was monitored at 234 nm for 10 minutes. Indomethacin was used as the reference standard. The percentage inhibition of lipoxygenase was calculated as:

$$\% \text{ inhibition} = \frac{\text{Absorbance control} - \text{Absorbance of test}}{\text{Absorbance control}} \times 100$$

Anti-Proteinase Assay

Proteinase enzymes are involved in the tissue damage associated with inflammatory conditions. This assay measures the ability of the extract to inhibit proteinase activity (Oyedepo et al., 1995).

Procedure

A reaction mixture containing 0.06 mg trypsin, 1mL of 20 mM Tris-HCl buffer (pH 7.4), and 1 mL of plant extract (at concentrations of 100, 200, 400, 800 µg/mL) was incubated at 37°C for 5 minutes. To this mixture, 1mL of 0.8% casein solution was added, and the reaction was allowed to proceed for 20 minutes. The reaction was stopped by adding 2 mL of 5% trichloroacetic acid, and the mixture was centrifuged at 3,000 rpm for 10 minutes. The absorbance of the supernatant was measured at 280 nm. Diclofenac was used as the standard, and the percentage inhibition of proteinase activity was calculated using the formula:

$$\% \text{ inhibition} = \frac{\text{Absorbance control} - \text{Absorbance of test}}{\text{Absorbance control}} \times 100$$

Albumin Denaturation Assay

Denaturation of proteins, particularly albumin, is a well-documented cause of inflammation. The albumin denaturation assay assesses the ability of the extract to inhibit the heat-induced denaturation of bovine serum albumin (Sakat et al., 2010).

Procedure

A reaction mixture containing 0.2 mL of 1% bovine serum albumin and 2.8 mL of phosphate buffer (pH 6.3) was prepared. Different concentrations of the plant extract (100, 200, 400, 800 µg/mL) were added to the reaction mixture. The mixture was incubated at 37°C for 20 minutes, then heated to 70°C for 5 minutes. After cooling, the absorbance was measured at 660 nm. Diclofenac was used as the reference standard. The

percentage inhibition of protein denaturation was calculated using the following formula:

$$\% \text{ inhibition} = \frac{\text{Absorbance control} - \text{Absorbance of test}}{\text{Absorbance control}} \times 100 \quad 4$$

Results and Discussion

Table 1 shows the profile of phytochemical screening carried out qualitatively on the aqueous and ethanol extracts from *Croton Gratissimus* root to access the presence and absence of their bioactive chemical compound. The Phytochemicals present were indicated as positive (+) while the Phytochemicals absent were indicated as negative (-).

Table 1: Qualitative phytoconstituents of the aqueous and ethanol root extract of *Croton Gratissimus*

Phytochemical	Aqueous Extract	Ethanoic Extract
Carbohydrate	+	+
Tannins	-	-
Saponin	+	-
Alkaloids	-	-
Flavonoids	+	+
Glycosides	-	-
Quinones	+	+
Phenols	-	-
Terpenoids	+	+
Cardiac Glycosides	-	+
Nihydrin Test	-	-
Coumarins	+	+
Anthroquinones	-	-
Steroids	-	+
Phlobatannins	-	+
Anthrocyanine	-	-

+ = present; - = absent

Phytochemical screening offers a great understanding of the possible therapeutic value of herbal extracts. The

chemical analysis of aqueous and ethanol extracts showed some similarities and differences in the

phytochemical composition that may result in diverse effects on their biological activity. Both aqueous and ethanol extracts contained carbohydrates, flavonoids, quinones, terpenoids and coumarins. These are compounds which have wide pharmacological activities. An example of such compounds is flavonoids, which are strong antioxidants, possess anti-inflammatory, antimicrobial, and anticancer effects (Panche et al., 2016). Terpenoids and quinones too have antimicrobial and anticancer properties and are useful in the process of developing drugs (Thoppil & Bishayee, 2011). Coumarins were connected with anticoagulant and antimicrobial properties (Venugopala et al., 2013).

Saponins were only found in the aqueous extract and have been known to reduce cholesterol levels, improve immune response and have antitumor activity (Man et al., 2010). In contrast, the ethanol extract had cardiac glycosides, steroids and phlobatannins which were not found in the aqueous extract. Cardiac glycosides Democratic presidential nomination, 2008 have had wide application in the management of heart failure as they enhance the force of heart contractions (Salahdeen & Yemitan, 2006). Steroids also have anti-inflammatory

effects as well as hormone regulatory effects thus helpful in the treatment of autoimmune diseases and endocrine diseases (Evans, 2004). Phlobatannins are condensed tannins which have shown antimicrobial and antioxidant activity (Okuda, 2005).

Both extracts did not show the presence of Tannins, alkaloids, phenols, glycosides, anthraquinones, amino acids (Ninhydrin test), and anthocyanins. The lack of alkaloids, even though they have established pharmacological significance (antimalarial, analgesic, and anticancer activities), implies minimal prospects in those particular fields unless they occur in trace quantities undetected by current methods (Cushnie et al., 2014).

The variation witnessed in the aqueous and ethanol extracts can be discussed in terms of polarity and solubility of the phytochemicals. Since ethanol is less polar than water, it is more effective in extracting non-polar compounds such as steroids and cardiac glycosides whereas water is more effective in extracting polar compounds such as saponins (Edeoga et al., 2005)..

Table 2: Quantitative phytochemical screening of the aqueous and ethanol root extract of *Croton Gratissimus*

Phytochemical Content (%)	Aqueous Extract	Ethanol Extract
Flavonoid	0.76	0.24
Total terpenoids	2.62	8.98
Carbohydrate (CHO)	23.8	28.22
Saponin	10.74	3.94

The Table 2 provides the quantitative phytochemical screening results of both aqueous and ethanol extracts of *Croton gratissimus* root. The key phytochemical components evaluated include flavonoids, total terpenoids, carbohydrates (CHO), and saponins.

Flavonoid content was higher in the aqueous extract (0.76%) compared to the ethanol extract (0.24%). This is consistent with previous findings suggesting that

many flavonoid glycosides are more water-soluble due to their polar nature (Kumar & Pandey, 2013). On the other hand, the ethanol extract had a substantially higher total terpenoid concentration (8.98%) than the aqueous extract (2.62%), indicating that terpenoids are lipophilic and have a higher solubility in organic solvents like ethanol (Zhao et al., 2005).

The presence of carbohydrates was also high in both extracts with a relative higher percentage (28.22%) in the ethanol extract than in the aqueous extract (23.80%). This could be assigned to the existence of polar and moderately polar carbohydrates which are partly soluble in ethanol (Sasidharan et al., 2011). Surprisingly, the saponins were much more in aqueous extract (10.74%)

as compared to ethanol extract (3.94). This finding concurs with reported high polarity of saponins that increases its water solubility (Makkar et al., 2007). These variations in phytochemical distribution validate the impact of solvent polarity on the effect of solvent polarity on the extraction yield of different bioactive compounds.

Table 3: Percentage Inhibition for Membrane Stabilization of the aqueous and ethanol root extract of *Croton Gratissimus*

Concentration (µg/mL)	Aqueous Extract	Ethanol Extract	Diclofenac (Standard)
100	32.10 ± 1.02	28.60 ± 1.18	55.60 ± 0.90
200	40.70 ± 0.85	37.20 ± 0.94	68.90 ± 1.12
400	52.30 ± 1.10	45.80 ± 0.80	77.30 ± 0.78
800	61.50 ± 0.78	56.90 ± 0.95	85.10 ± 0.65

The in vitro anti-inflammatory activity of *Croton gratissimus* root extracts were assessed through membrane stabilization, anti-lipoxygenase, and anti-proteinase assays. These methods simulate key mechanisms involved in inflammation and allow for comparative evaluation of the efficacy of aqueous and ethanol extracts. Table 3 shows the percentage inhibition for membrane Stabilization of the aqueous and ethanol root extract of *Croton Gratissimus*. The membrane stabilization assay reflects the capacity of an extract to prevent the lysis of red blood cells under hypotonic conditions, mimicking the stabilization of lysosomal membranes during inflammation. Stabilizing lysosomal membranes inhibits the release of

inflammatory mediators such as proteases and histamines (Shinde et al., 1999). Results showed that both extracts exhibited a concentration-dependent inhibition of hemolysis. At the highest concentration (800 µg/mL), the aqueous extract achieved 61.50% inhibition, outperforming the ethanol extract (56.90%). Although diclofenac (the standard drug) exhibited a higher inhibition (85.10%), the results suggest that *C. gratissimus* root extracts, particularly the aqueous form, possess membrane-stabilizing properties. This may be attributed to the presence of saponins and flavonoids, which are well known for their membrane-stabilizing and anti-inflammatory properties (Man et al., 2010; Panche et al., 2016).

Table 4: Percentage Inhibition for Anti-lipoxygenase of the aqueous and ethanol root extract of *Croton Gratissimus*

Concentration (µg/mL)	Aqueous Extract	Ethanol Extract	Indomethacin (Standard)
100	25.30 ± 1.12	22.50 ± 0.95	52.20 ± 1.08
200	37.90 ± 1.05	33.80 ± 1.02	62.70 ± 0.89

400	49.50 ± 0.94	44.10 ± 0.85	72.90 ± 0.78
800	58.80 ± 0.88	53.90 ± 0.78	83.60 ± 0.65

Table 4 shows Percentage Inhibition for Anti-lipoxygenase of the aqueous and ethanol root extract of *Croton Gratissimus*. Lipoxygenase is a key enzyme in the synthesis of leukotrienes from arachidonic acid, which are potent mediators of inflammation. Inhibiting this pathway can significantly reduce inflammatory responses (Kim et al., 1999).

In the current study, both extracts demonstrated moderate inhibition of lipoxygenase activity in a dose-dependent manner. At 800 µg/mL, the aqueous extract

recorded 58.80% inhibition, while the ethanol extract showed 53.90%. Indomethacin, used as a standard, showed a much higher inhibition of 83.60%. The higher performance of the aqueous extract may again be associated with a higher content of water-soluble bioactive compounds such as flavonoids and saponins. These compounds are recognized for their ability to modulate enzyme activity and scavenge reactive oxygen species that propagate inflammation (Panche et al., 2016; Kumar & Pandey, 2013).

Table 5: Percentage Inhibition for Anti-proteinase of the aqueous and ethanol root extract of *Croton Gratissimus*

Concentration (µg/mL)	Aqueous Extract	Ethanol Extract	Diclofenac (Standard)
100	18.50 ± 1.25	15.80 ± 1.12	48.90 ± 1.04
200	30.80 ± 1.10	26.50 ± 1.05	61.40 ± 0.92
400	44.10 ± 0.98	39.20 ± 0.92	71.20 ± 0.78
800	55.90 ± 0.87	50.60 ± 0.85	82.10 ± 0.68

Table 5 shows the Percentage Inhibition for Anti-proteinase of the aqueous and ethanol root extract of *Croton Gratissimus*. Proteolytic enzymes like proteinases contribute to inflammation by degrading extracellular matrix components and promoting tissue injury. Their inhibition is a valuable strategy in managing chronic inflammation (Oyedepo & Femurewa, 1995).

Both extracts exhibited inhibitory effects on proteinase activity, with a trend of increasing inhibition across higher concentrations. At 800 µg/mL, the aqueous extract showed a greater inhibition (55.90%) compared

to the ethanol extract (50.60%). Diclofenac again showed superior inhibition at 82.10%. The relatively higher activity of the aqueous extract suggests that polar phytochemicals, which were more prevalent in the aqueous extract, contribute significantly to this activity. This is consistent with the observed phytochemical composition, where the aqueous extract contained more saponins and flavonoids than the ethanol extract.

Across all three assays, the aqueous extract consistently outperformed the ethanol extract. This suggests that solvent polarity plays a crucial role in extracting anti-inflammatory constituents from *C. gratissimus* roots.

Water, being a polar solvent, appears more effective at extracting flavonoids and saponins—compounds associated with anti-inflammatory and antioxidant effects (Kumar & Pandey, 2013; Edeoga et al., 2005). While neither extract achieved the level of inhibition

observed with standard synthetic drugs, the findings provide scientific validation for the traditional use of *C. gratissimus* and highlight its potential as a natural source of anti-inflammatory agents

Table 6: Percentage Inhibition for Albumin Denaturation of the aqueous and ethanol root extract of *Croton Gratissimus*

Concentration (µg/mL)	Aqueous Extract	Ethanol Extract	Diclofenac (Standard)
100	22.70 ± 1.15	20.30 ± 1.00	50.60 ± 0.95
200	33.90 ± 1.05	30.80 ± 1.02	64.50 ± 0.88
400	47.20 ± 0.98	43.10 ± 0.90	74.80 ± 0.75
800	58.90 ± 0.80	52.40 ± 0.85	83.90 ± 0.63

Table 6 shows the Percentage Inhibition for Albumin Denaturation of the aqueous and ethanol root extract of *Croton Gratissimus*. The in vitro anti-inflammatory activity of *Croton gratissimus* root extracts were assessed through membrane stabilization, anti-lipoxygenase, and anti-proteinase assays. These methods simulate key mechanisms involved in inflammation and allow for comparative evaluation of the efficacy of aqueous and ethanol extracts.

The conducted anti-inflammatory assays, namely membrane stabilization assay, anti-lipoxygenase assay, anti-proteinase assay and albumin denaturation assays indicate that the aqueous and ethanol extracts of *Croton gratissimus* have a remarkable anti-inflammatory activity and that their effectiveness rises in a dose-dependent mode. The membrane stabilization assay involves the ability of the extracts to stabilize red blood cells in hypotonic situation which is similar to the stabilization of lysosomal membranes during inflammation (Shinde et al., 1999). Under this assay, it was observed that both aqueous and ethanol extract displayed significant inhibition of red blood cell lysis with the greatest inhibition displayed at 800 µg/mL with aqueous extract (61.50%) and ethanol extract (56.90%).

The inhibitory activity of diclofenac, a standard, was found to be more (85.10%) than the extracts.

To ascertain whether the lipoxygenase enzyme, which is essential in the inflammatory process, is inhibited, an anti-lipoxygenase assay is utilized (Kim et al., 1999). At every concentration in this assay, the aqueous extract showed significantly higher inhibition than the ethanol extract. At 800 µg/mL, the aqueous extract showed 58.80% and 53.90%, respectively. The inhibition of indomethacin was higher, at 83.60 percent. The ability of extracts to suppress proteinase activity, one of the mechanisms of tissue degradation during inflammation, is the foundation of the anti-proteinase assay (Oyedepo et al., 1995). The two extracts demonstrated a strong ability to suppress proteinase activity, which is crucial for reducing the tissue damage brought on by inflammation. Compared to ethanol extract, aqueous extract showed greater inhibition (55.90%) at 800 g/ml while much higher inhibition was observed with diclofenac.

The ability of extracts to prevent heat-induced protein denaturation, one of the most frequent causes of inflammation, is assessed by the albumin denaturation

assay (Sakat et al., 2010). Significant protein denaturation inhibition was also shown by the two extracts; at 800 g/ml, the aqueous extract showed 58.90% inhibition, while the ethanol extract showed 52.40% inhibition. Again, diclofenac worked better.

Conclusion

This study demonstrated that both aqueous and ethanol root extracts of *Croton gratissimus* possess significant in vitro anti-inflammatory activity, as evidenced by their performance in membrane stabilization, anti-lipoxygenase, anti-proteinase, and albumin denaturation assays. The results showed that anti-inflammatory activity was dose-dependent across all tested models, with increased inhibition observed at higher concentrations (100–800 µg/mL). Among the two extracts, the aqueous extract consistently exhibited higher inhibitory activity compared to the ethanolic extract across all assays: Membrane stabilization: 61.50% (aqueous) vs. 56.90% (ethanolic), Lipoxygenase inhibition: 58.80% (aqueous) vs. 53.90% (ethanolic), Proteinase inhibition: 55.90% (aqueous) vs. 50.60% (ethanolic)

These findings are attributable to the higher content of polar phytochemicals such as flavonoids and saponins in the aqueous extract, which are known for their membrane-stabilizing and enzyme-inhibiting properties. Although both extracts were less potent than standard anti-inflammatory drugs like diclofenac and indomethacin, they still demonstrated remarkable natural anti-inflammatory potential.

The results not only validate the traditional use of *Croton gratissimus* root in treating inflammatory disorders but also highlight the importance of solvent selection in optimizing the extraction of bioactive compounds. Further in vivo studies and toxicity profiling are recommended to establish its therapeutic viability and possible formulation into natural anti-inflammatory remedies.

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