

Anti-inflammatory Activity of the Aqueous Leaf Extract and n-Butanol Fraction of *Croton zambesicus* Muell Arg.

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ABSTRACT

Croton zambesicus Muell Arg. (Euphorbiaceae) is a plant indigenous to West Africa and has been found to be useful in management of chronic diseases including; hypertension diabetes mellitus and gastric ulcer. These diseases are deeply rooted in inflammation, the WHO has advocated for evaluation of herbal medicines with ameliorative potentials to promote the use of effective ones and discourage the use of toxic ethno medicines. Hence, the anti-inflammatory activity of aqueous extract and n-butanol fraction of *Croton zambesicus* were evaluated. The total phenolics and flavonoids, vitamin A, C and E content were determined using spectrophotometric analysis. Inflammation was achieved using carrageenan-induced hind paw edema model in rats. The total phenolic and flavonoid contents were 0.392 mg/g gallic acid equivalent and 0.149 mg/g quercetin equivalent respectively. The vitamin A, C and E concentration were found to be 0.13, 5.9 and 0.02 mg in 100 g of dried plant material, respectively. Administration of carrageenan significantly ($p < 0.05$) increased the hind paw sizes of the animals and the administration of both extracts significantly ($p < 0.05$) reduced hind paw edema in a non-dose dependent manner. At 180 minutes after the induction of inflammation, aqueous extract (100 mg/kg) and n-butanol fraction (5 mg/kg) of *Croton zambesicus* showed 70.53% and 57.89% inhibition of the hind paw edema. The result is comparable to the effect of diclofenac at 180 minutes with 73.68% inhibition. Results from this study shows the aqueous leaf extract of *Croton zambesicus* and n-butanol fraction has anti-inflammatory activity. This may be attributed to the presence of phenolic, flavonoid and antioxidant compounds present in the leaf extract.

Keywords: Anti-inflammatory, Aqueous, n-butanol, Carrageenan, *Croton zambesicus*, flavonoids Phenolics

INTRODUCTION

Inflammation is a complex biological response of vascular tissues to physical trauma, noxious chemicals or harmful agents and it is characterized by redness, swelling, pain, heat and loss of function¹. This bodily response has been found to form the basis of most painful and chronic diseases². The

conventional drugs for management include the non-steroidal (piroxicam, ibuprofen) and steroidal anti-inflammatory drugs (prednisolone, dexamethasone)³. Despite their effectiveness, they are still limited with respect to their costs and adverse effects which worsen with age including; gastrointestinal ulceration, bleeding, renal damage, liver damage, and

hyperglycemia^{4,5,6}. Hence, the search for treatment options from natural origin which would be more active, less costly, more accessible and very tolerable to patient in management of inflammatory conditions is therefore on the rise.

Medicinal plants contain different phytochemicals like polyphenols, flavonoids, terpenoids, alkaloids, anthraquinones, polysaccharides, saponins and peptides. These have been found to have considerable anti-inflammatory activities with little to no side effects⁷. Nutraceuticals such as vitamins, minerals, amino acids and fatty acids have established nutritional and physiological benefits⁸ and they have been claimed to have benefits and provide protection against cancer, cardio metabolic and neurodegenerative diseases^{9, 10, 11, 12}.

Croton zambesicus Muell Arg. (Euphorbiaceae) is an ornamental plant and a shrub widely distributed in West Africa, Nigeria, with a small tree reaching 9-15 m high and 0.9 m in girth. The leaves are approximately 15×5 cm, oblanceolate, acuminate with whitish and brownish scales beneath¹³, the common names in Nigeria include; *Kiroba maser* (Hausa) and *Ajeobale* (Yoruba)¹⁴. The plant is used as spice in food and traditionally in the treatment of bleeding gums, ulcer, hypertension, menstrual cycle disorders, epilepsy, infection and to treat fever in Benin^{15,16,17}.

The plant has been reported to have antidiabetic, anticonvulsant and considerable antiparasitic activities^{18,19,20}. The aqueous extract of the leaves also possess depressant, sedative, hypnotic, cardioprotective and nephroprotective activities^{21,22,23}. However, many of these chronic disorders are deeply rooted in inflammation and affect the protective mechanisms in the body. It is therefore worthy to evaluate the ameliorative potential of *Croton zambesicus* on inflammation.

MATERIALS AND METHODS

Plant Collection

Leaf of *C. zambesicus* was identified and authenticated at the University of Jos. A voucher specimen was deposited and number given (UJ/PGPH/HSP/0801). Fresh leaves of *C. zambesicus* were collected from Onigambari Plantation Reserve, Ibadan, Nigeria, on 28th February 2018. The leaves were cleaned, dried at room temperature and size-reduced using a milling machine to ensure better extraction process.

Preparation of Aqueous Extract and n-butanol Fraction of *C. zambesicus*

Two hundred grams (200 g) of the dried plant was weighed and soaked in distilled water (2 L) for 72 hours with intermittent agitation. After 72 hours, the supernatant was decanted, allowed to settle, and filtered with a Whatman paper (No 1). The filtrate was evaporated to dryness on a water bath at a temperature of 45°C²⁴. The concentrated extract was stored in an airtight bottle at 4°C throughout and use for this experiment. A preliminary partitioning of the aqueous extract in n-butanol, ethyl ether and hexane revealed the extract had highest solubility using n-butanol. This justifies the use of n-butanol for partitioning during this study. Twenty grams (20 g) of the aqueous extract was weighed and dissolved in 200 mL deionized water. The solution was transferred into a separating funnel with an equal volume of n-butanol (200 mL). The separating funnel was shaken gently and left to stand for a few minutes, n-butanol fraction was collected and the procedure was repeated three times to achieve complete separation. The n-butanol fraction was then concentrated over a water bath at 45 °C and the concentrate was stored inside a clean container at 4°C throughout and used for this experiment²⁴.

Experimental Animals

Forty male Wistar rats (120-130 g) were obtained from the animal house of University of Ilorin, Ilorin. Rats were allowed to adapt to the new environment for one week and maintained under ambient conditions with free access to food and water *ad libitum* at Department of Pharmacology and Toxicology, University of Ilorin, Ilorin. The experiment was performed in accordance with the principles of laboratory animal care laid down by the University of Ilorin Ethics Committee for care and use of laboratory animals. Ethical clearance was obtained with the approval number UERC/ASN/2019/1863, from the Ethical Review Committee of the University of Ilorin.

Evaluation of Nutraceutical content and Quantitative Phytochemicals

Retinol (Vitamin A)

A 0.5 g of plant material was weighed, homogenized, and saponified with 2.5 mL of 12% alcoholic potassium hydroxide in a water bath at 60 °C for 30 minutes. The saponified extract was transferred into a separating funnel which contained 10 to 15 mL of petroleum ether and mixed thoroughly, the lower layer (aqueous) was further transferred to another separating funnel and the upper layer (petroleum ether) containing the carotenoids, was collected. The extraction was repeated until the lower layer became colourless. To remove excess moisture, small amount of anhydrous sodium sulphate was added to the petroleum ether extract. The final volume of the petroleum ether extract was recorded and the absorbance was read using a UV/VIS spectrophotometer (Mettler Toledo) at 460 nm²⁶.

Ascorbic Acid (Vitamin C)

Ascorbate was extracted from 1 g of the plant sample using 4 % TCA, the sample was taken and 2 mL of distilled water was added, mixed thoroughly and allowed to stand for 30 minutes at room temperature. The tubes were centrifuged at 3000 rpm for 10 minutes. The clear supernatant was taken and absorbance was measured at 700 nm against blank²⁷.

Tocopherol (Vitamin E)

Two grams (2 g) of plant material was dissolved in 5 mL of distilled water, 1.5 mL of xylene was added to the solution and then centrifuged. From xylene layer, 1 mL was transferred to a tube and 1 mL of dipyrindyl was added. Absorbance of the mixture was measured at 460 nm. To this mixture, 0.3 ml FeCl₃ solution was added and the absorbance was measured at 520 nm²⁸.

Total Phenolic Content

The Total phenolics content was estimated by adding 1 mL of distilled water to the aliquot of the extract taken. This was followed by addition of 0.5 mL of Folin-ciocalteu reagent and 2.5 mL of sodium carbonate solution, the tubes were placed in the dark for 40 minutes and the absorbance was then measured at 725 nm. Gallic acid monohydrate was used to prepare the standard curve and the linearity obtained was in the range of 1-10 µg/mL. The standard curve was used to calculate the total phenolic content and expressed as Gallic acid equivalent in mg/g of the extract²⁹.

Total Flavonoid

The total flavonoid was estimated using Aluminium chloride colorimetric assay, to 1 mL of extract, 4 mL of distilled water was added and this was followed by addition of

0.3 mL of 5 % sodium nitrite and after 5 minutes, 0.3 mL of 10 % AlCl_3 was added. A 2 mL quantity of 1 M NaOH was added after six minutes and made up to 10 mL with distilled water. Absorbance was measured at 510 nm. The total flavonoid content was expressed as percentage of Quercetin equivalent per 100 mg of fresh mass³⁰.

Evaluation of Anti-Inflammatory Activity of *Croton zambesicus*

This was carried out using carrageenan-induced hind paw edema model in rats as described by Winter *et al.*, (1962) with slight modification. The rats were grouped into eight (8) of five (5) animals each as below;
Group I (Normal control/NC): Received 1 mL/kg of distilled water per oral
Group II (Positive control/DC): Received 10 mg /kg of Diclofenac per oral
Group III-V (Aqueous extract/AQ1-3): Received 50, 100 and 200 mg /kg of extract per oral
Group VI-VIII (n-Butanol fraction/NB1-3): Received 5, 10 and 20 mg /kg of extract per oral.

One hour after treatment, rats were injected subcutaneously with 0.1ml of carrageenan solution (1 % w/v) into the sub planter region of the left hind paw. Paw sizes were measured using a Digital Vernier Caliper 30 minutes before administration of carrageenan

and at 30 minute intervals for a period of 3 hours³¹.

The anti-inflammatory activity was calculated using the formula below.

$$\% \text{ antiinflammatory activity} = \frac{(Vt - Vo)_{\text{control}} - (Vt - Vo)_{\text{treated}}}{(Vt - Vo)_{\text{control}}} \times 100$$

Where Vt is paw edema at different time point and Vo is paw edema at time 0 minute

Statistical Analysis

Data were presented as means $\pm$ SEM and analyzed using GraphPad Prism (version 8.03, Graph pad Software Inc.) for window. Statistical analysis was carried out using one-way analysis of variance (ANOVA) test, this was followed by Dunnett's test for multiple comparisons between groups and statistical significance was taken at $P < 0.05$.

RESULTS

Concentrations of selected phytochemical in crude extract of *C. zambesicus*

The crude extract of *C. zambesicus* was found to contain vitamin C (5.91 mg), vitamin A (0.13 mg) and vitamin E (0.02 mg) as shown in Table 1.

Table 1: Vitamin content of *Croton zambesicus* Crude extract

Nutraceuticals	Amount (mg/100g)
Vitamin A	0.13 ± 0.00
Vitamin C	5.91 ± 0.11
Vitamin E	0.02 ± 0.01

Data are presented as mean ± S.E.M, n=3

Phenolic and Total Flavonoid Content of the Crude Extract of *C. zambesicus*

The crude extract of *C. zambesicus* has a total phenolic and flavonoid content of 0.392 and 0.149 mg/g respectively.

Table 2: Phenolic and Total Flavonoid Content of *C. zambesicus* Crude extract

Phytochemicals	Amount (mg/g)
Total phenolic (GAE)	0.392± 0.025
Total flavonoids (QAE)	0.149 ± 0.001

Data are presented as mean ± S.E.M, n=3

Effect of Aqueous and n-Butanol fraction of *C. zambesicus* on Hind Paw Edema

Induction of inflammation with 0.1mL of carrageenan solution (1 % w/v) into the sub-plantar region of the left hind paw produced significant increases ($p<0.05$) in the hind paws of the animals, as evident from the changes in paw sizes (Table 3). Thirty minutes after the induction of the inflammation, pre-treatment with AQ1, NB1, NB2, and NB3 produced a significant reduction in inflammation by 40.00, 40.00, 33.33, and 30.67 %, the result of which is comparable to the effect observed with the positive control (DC) with 40.00% anti-inflammatory activity (Figure 1a).

At 60, 90, 120, 150 and 180 minutes after the induction of inflammation, pre-treatment with the three doses of the extracts produced significant reductions in hind paw edema; AQ1 (44.22, 44.17, 37.50, 40.90 and 34.21), AQ2 (55.55, 52.91, 62.50, 68.18 and 70.53%), AQ3 (38.89, 39.32, 45.00, 54.54 and 42.10%), NB1 (44.44, 56.31, 60.00, 61.30 and 57.89%), NB2 (34.44, 46.60, 50.00, 51.00 and 36.84%), NB3 (44.44, 44.17, 48.00, 49.10 and 55.20%) respectively. These results are comparable to the activity of the positive control/diclofenac (44.44, 61.16, 65.00, 70.45 and 73.68%) at the different time points, as shown in Table

Table 3: Effect of aqueous and n-butanol fraction of *C. zambesicus* on hind paw edema

Groups	Hind Paw Edema (mm)					
	30 min	60 min	90 min	120 min	150 min	180 min
NC	1.50 ± 0.12	1.80 ± 0.23	2.06 ± 0.17	2.00 ± 0.14	2.20 ± 0.30	1.90 ± 0.16
DC	0.90 ± 0.08 ^a	1.00 ± 0.19 ^a	0.80 ± 0.09 ^a	0.70 ± 0.04 ^a	0.65 ± 0.07 ^a	0.5 ± 0.01a
AQ1	0.90 ± 0.04 ^a	1.04 ± 0.21	1.15 ± 0.20	1.25 ± 0.16	1.30 ± 0.12	1.25 ± 0.28
AQ2	1.25 ± 0.11	0.80 ± 0.03 ^a	0.97 ± 0.06 ^a	0.75 ± 0.11 ^a	0.70 ± 0.08 ^a	0.56 ± 0.01 ^a
AQ3	1.15 ± 0.23	1.10 ± 0.28	1.25 ± 0.28	1.10 ± 0.13	1.00 ± 0.15 ^a	1.10 ± 0.13
NB1	0.90 ± 0.07 ^a	1.00 ± 0.12 ^a	0.90 ± 0.16 ^a	0.80 ± 0.11 ^a	0.85 ± 0.09 ^a	0.8 ± 0.01 ^a
NB2	1.00 ± 0.29 ^a	1.18 ± 0.09	1.10 ± 0.09 ^a	1.00 ± 0.12 ^a	1.10 ± 0.15	1.25 ± 0.27
NB3	1.04 ± 0.15	1.00 ± 0.19 ^a	1.15 ± 0.16	1.04 ± 0.21	1.12 ± 0.17	0.85 ± 0.13 ^a

Data are expressed as mean ± SEM, n=5, ^a*p* < 0.05 is significant difference in comparison with normal control. NC, DC, AQ1, AQ2, AQ3, NB1, NB2 and NB3 are; Normal control, diclofenac, aqueous extract (50 mg/kg), aqueous extract (100 mg/kg), aqueous extract (200 mg/kg), aqueous extract (200 mg/kg), n-butanol fraction (5mg/kg), n-butanol fraction (10mg/kg) and n-butanol fraction (20mg/kg) respectively.

Table 4: Inhibitory effect of aqueous and n-butanol fraction of *C. zambesicus* on hind paw edema

Groups	Inhibition of Hind Paw Edema (%)					
	30 min	60 min	90 min	120 min	150 min	180 min
DC	40.00 ± 3.42	44.44 ± 3.84	61.16 ± 4.34	65.00 ± 6.39	70.45 ± 5.27	73.68 ± 6.43
AQ1	40.00 ± 2.98	42.22 ± 2.17	44.17 ± 3.69	37.50 ± 4.13 ^b	40.9 ± 3.73 ^b	34.21 ± 3.55 ^b
AQ2	16.67 ± 2.99 ^b	55.55 ± 2.24	52.55 ± 4.98 ^b	62.5 ± 7.11	68.18 ± 6.59	70.53 ± 5.67
AQ3	23.33 ± 2.36 ^b	38.89 ± 3.04	39.32 ± 2.46 ^b	45.00 ± 3.25 ^b	54.54 ± 4.05 ^b	42.1 ± 2.13 ^b
NB1	40.00 ± 1.56	44.44 ± 2.11	56.31 ± 5.02	60.00 ± 3.97	61.3 ± 3.99	57.89 ± 3.99
NB2	33.33 ± 1.47	34.44 ± 3.64 ^b	46.6 ± 5.13	50.00 ± 5.34	51 ± 3.72	36.84 ± 2.68 ^b
NB3	30.67 ± 1.16	44.44 ± 2.67	44.17 ± 3.45 ^b	48.00 ± 5.92 ^b	49.1 ± 4.77 ^b	55.2 ± 4.02

Data are expressed as mean ± SEM, n=5, ^b*p* < 0.05 is significant difference in comparison with positive control (DC).

DISCUSSION

Inflammation has been found to underline chronic diseases like asthma, diabetes, cancer, arthritis, neurodegenerative disorders, and autoimmune diseases³². According to the World Health Organization (WHO), inflammation and its related diseases pose the greatest threat to public health, and a steady rise in the prevalence of such diseases is anticipated for the next 30 years³³. Hence, efforts have been made in

recent years to search for newer anti-inflammatory agents from natural sources because they are generally believed to be safer and more tolerable^{34,35,36}. The extraction yield and biological activity of medicinal plants are not only affected by the extraction technique but also by the polarity of the extraction solvent, temperature, and the composition of phytochemicals^{37,38}. The results from this study showed a higher extraction yield (28.6%) with the aqueous extract of *C. zambesicus* while n-butanol

fraction gave a lower yield (11.2%), indicating the presence of more polar compounds in the former^{39,40}. The solvent (n-butanol) was chosen based on the preliminary fractionation analysis, the extraction efficiency favored the highly polar solvents which is consistent with the extraction yield of *Limophila aromatic*⁴¹ and some other medicinal plants⁴².

The Carrageenan induced hind paw edema is a well-established animal model for evaluating the antiedematous effect of drugs or compounds⁴³. The formation of edema caused by carrageenan is in two phases; the first hour after carrageenan injection (first or early phase) involves the release of serotonin, histamine and bradykinin while the second or late phase (2–5 h) with increased edema formation that remains up to the fifth hour involves the release of prostaglandins⁴⁴ and COX-2 induction in the hind paw⁴⁵. The two extracts produced significant reduction of hind paw edema, depict a non-dose dependent activity and were more effective at the second phase of the inflammatory processes. The effects of both extracts suggest more inhibitory effect on prostaglandins, an effect similar to standard drug (diclofenac) used in the management of inflammatory conditions.

Plant phenolics are desirable compounds in the human diet because of their antioxidant properties and different preventive roles against diseases associated with oxidative stress, such as cancer, cardiovascular and neurodegenerative⁴⁶. The quantitative evaluation of the aqueous extract of *C. zambesicus* revealed considerable amount of flavonoids, phenolics and vitamins which might be responsible for its anti-inflammatory activity. This was corroborated in a study reported by Ayanniyi *et al.*, which evaluated the plant for its phytochemical constituents⁴⁷. This is in line with previous

studies that have reported the anti-inflammatory properties of flavonoids, phenolics and tannins^{48,49}. A study by Manthey *et al.*, also reported that a number of flavonoids act by inhibiting key enzymes that are central in the synthesis of prostaglandins⁵⁰. This may justify the effect of the extract at the late phase of inflammatory processes.

Exploration of the relationship between inflammation and oxidative stress has increased in recent years and vitamins have been reported to ameliorate chronic low-grade inflammation and overproduction of reactive oxygen and nitrogen species (ROS and RON)⁵¹. In addition, vitamins C, E, and Zinc have been reported to reduce hind paw edema induced by carrageenan either alone or in combination with diclofenac. Another study showed that administration of vitamin C reduced expected increases in hind paw inflammatory edema in a model of adjuvant-induced arthritis in rats⁵². The anti-inflammatory effect observed may be attributed to the additive effects of the vitamins (A, C, and E), phenolics and flavonoids present in the extract.

CONCLUSION

This study evaluated the anti-inflammatory activity of *C. zambesicus* aqueous leaf extract and n-butanol fraction on carrageenan-induced hind paw edema. From the results obtained, both extracts had significant activity against inflammation. The aqueous extract was found to contain phenolics, flavonoids, vitamin A, C, and E which may be responsible for its anti-inflammatory activity. Further studies need to be conducted to isolate and characterize the active constituents in the plant.

ACKNOWLEDGEMENT

Technologist in the Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, University of Ilorin for providing technical assistance during the course of this study.

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