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Inhibition of phospholipase A_2 and prostaglandin synthase activities as possible mechanistic insight into the anti-inflammatory activity of *Brenania brieyi* methanol and chloroform fractions

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ABSTRACT

Objectives: The pharmacological basis of anti-inflammatory agents is geared toward maintaining body homeostasis. This study investigated the scientific underpinning and mechanisms of anti-inflammatory activity of root bark of *Brenania brieyi.* **Material and Methods:** The plant material was extracted with a mixture of methanol and chloroform (2:1) and fractionated with 0.2 volume water. Egg albumin-induced edema and cotton pellet-induced granuloma models were used to investigate anti-inflammatory activity while the possible mechanisms of actions were evaluated using standard methods. **Results:** Significant ($P < 0.05$) reduction in edema and granuloma tissue formation were recorded in fractions treated groups compared with the normal control. There was a significant ($P < 0.05$) inhibition of membrane hemolysis and phospholipase A2 (PLA2) activity by the fractions compared with the standard drugs, indomethacin, and prednisolone, respectively. Both fractions significantly ($P < 0.05$) inhibited prostaglandin (PG) synthase and nitric oxide radical activities compared with the control. However, varying doses of both fractions did not inhibit leukocyte migration compared with rats in indomethacin and control groups. The methanol fraction was found to be more potent in most of the tests. **Conclusion:** These results show that *B. brieyi* root bark has anti-inflammatory activity, and the potential underlying mechanisms of its action are inhibition of PLA2 and PG involved in inflammatory responses.

Keywords: Anti-inflammatory activity, *Brenania brieyi,* cotton pellet, egg albumin edema, phospholipase A2, prostaglandin synthase

INTRODUCTION

Medicinal plants have proven to be beneficial, and a greater percentage (about 80%) of people now use herbal remedies for the management and treatment of numerous diseases/disorders including those mediated by an excessive or unregulated inflammatory response.[1] The pharmaceutical basis for their usage is due to the presence of phytochemicals such as flavonoids, phenols, tannins, saponins, and others.[2] *Brenania brieyi* is among the commonly used herbal remedies. *B. brieyi* is a perennial flowering plant that belongs to Rubiaceae family. It is distributed in Nigeria, Gabon, Cameroon, and Central African Republic. It has four synonyms: *Anthocleista brieyi* De Wild., *Brenania spathulifolia, Randia spathulifolia,* and *Randia walkeri Pellegr.* The ethnomedicinal applications of the plant include treatment of fever, pain, infection, swelling,

and impotency[3] which are all inflammation-related disorders.

The inflammatory cascade is one of the defensive mechanisms used in getting rid of injurious physical, chemical, infective, and immunological agents.[4] The body in a bid to eliminate these stimuli through inflammatory reaction releases mediators such as nitric oxide (NO), thromboxane A2, cytokines, and prostaglandin (PG).[5] PG is the end product of phospholipase lipase A2 (EC 3.1.1.4) catalyzed membrane phospholipid cleavage.[4] These mediators are either derived from plasma components such as clothing factor, fibrinolytic, kinin, and complement system or cells including platelet-activating factor, vasoactive amines (serotonin and histamine), reactive oxygen species, NO, leukocytes, and vascular endothelial cells.[5]

Paradoxically, inflammatory response turns out to be the key player in the etiology and pathogenesis of a wide range of diseases/disorders when the release of pro-inflammatory mediators and cells far outweighs the body's anti-inflammatory cascades. In clinical settings, clinicians prescribe steroidal and nonsteroidal anti-inflammatory agents[6] such as indomethacin and ibuprofen for the management and treatment of inflammatory conditions.[7,8]

The intensive search for natural anti-inflammatory agents was borne out of the increasing side effects that set in with the prolonged usage of these synthetic drugs.[7] Their usage is associated with unprecedented side effects,[9] especially on the cardiovascular system and gastrointestinal tract,[8] as well as mediators of respiratory depression and immune suppression.[10]

Considering the documented high antioxidant potency of *B. brieyi*[3,11] and various trado-medical claims of its efficacy in the management of inflammatory disorders/diseases, providing the scientific backings and mechanisms of action of the plant which serves as therapeutic targets will be a vital tool in drug discovery. This study, therefore, investigated the phytochemicals, anti-inflammatory activity, as well as pharmaceutical mechanisms of anti-inflammatory actions of methanol and chloroform fractions of root bark of *B. brieyi*.

MATERIALS AND METHODS

Materials

Plant materials

The root bark of *B. brieyi* collected was identified by a taxonomist, Mr. Felix Nwafor. Voucher specimens with an identification number PCG/UNN/0327 were deposited at the taxonomist herbarium at the Department of Pharmacognosy and Environmental Medicine, University of Nigeria, Nsukka, Nigeria.

Blood samples

Blood samples (5 ml each) were collected by a phlebotomist through venipuncture from three healthy male students who gave consent and were not on any medication for at least 2 weeks before blood sample collection. The blood samples were used for membrane stabilization experiment and phospholipase A2 (PLA2) activity assay.

Animals

The Wistar albino rats with a weight range of 110–132 g used for the study were gotten from the Department of Zoology Animal House. The animals were kept in a well-ventilated stainless cage, housed under a 12 h light and dark cycle, 25 ± 1°C temperature, fed growers mesh, and given clean drinking water. They were housed for at least a minimum of 2 weeks for acclimatization before use.

Standard drugs

Indomethacin procured from Evans Pharmaceutical, England, was used as a standard drug in egg albumin edema and cotton pellet-induced models, membrane stabilization experiment, PG synthase activity assay, and *in vivo* leukocytes mobilization test. Prednisolone (purchased from Salvavidas Pharmaceutical, India) was the standard drug used in PLA2 activity assay while ascorbic acid (produced by Qualitex, India) was employed as the standard drug for NO radical scavenging activity test.

Methods

Extraction procedure

The plant material was dried under shaded, pulverized and 1793 g obtained was extracted with chloroform and methanol in the ratio of 2:1 v/v for 48 h. Filtration was done with cheesecloth followed by filter paper (Whatman No. 4). The filtrate obtained was measured and shaken vigorously with a 20% volume of distilled water to separate it into two organic layers. Then, a separating funnel was used to separate the layers into methanol fraction of *B. brieyi* root bark (MFBB, upper layer) and chloroform fraction of *B. brieyi* root bark (CFBB, lower layer). The CFBB was evaporated at 45°C with a rotary evaporator while the MFBB was lyophilized with a lyophilizer. Both fractions were stored in a refrigerator at 4°C.

Phytochemical analysis of MFBB and CFBB

Methods of Harborne[12] and Trease and Evans[13] were used in the qualitative and quantitative investigation of these phytochemicals in MFBB and CFBB: Phenols, flavonoids, tannins, saponins, terpenoids, steroids, and alkaloids.

Dose optimization study

The optimal doses for the study were determined using Winter *et al.*[14] egg albumin-induced, acute inflammatory model. The right hind paw volume of male and female adult Wistar rats denied access to food and water for 18 h divided into 13 groups with five rats per group which were measured with Vernier caliper before the onset of the experiment (t = 0). The control group (Group 1) was administered 1 ml/kg body weight (B.W.) of normal saline (NS), Groups 2–7 and 8–13 were given 50, 100, 200, 300, 400, and 500 mg/kg b. w of methanol and chloroform fractions, respectively. Undiluted egg albumin (0.1 ml) was used to induce acute inflammation 1 h later in the rats' right hind subplantar region. This was followed by the measurement of the rats' paw volume which was used to determine edema formation at 0.5, 1, 2, 3, 4, and 5 h.

Calculations

Change in paw volume = (Vt-Vo)

Where, Vt = Paw volume at time t (0.5, 1, 2, 3, 4, and 5 h). Vo = Paw volume at time zero (initial time).

Determination of the anti-inflammatory activity of MFBB and CFBB using egg albumin-induced edema acute inflammatory model

This was investigated with the egg albumin inflammatory model of Winter *et al.*[14] as described in the dose optimization study above using a total of 40 Wistar rats of both sexes. Group 1 (egg albumin control) was administered 1 ml/kg b. w of NS, Group 2 (standard control) was given 50 mg/kg b. w of indomethacin, 50, 100, and 200 mg/kg of MFBB were administered to Groups 3, 4, and 5, respectively. Same doses of CFBB were also administered to Groups 6, 7, and 8, respectively. The index used as a measure of edema formation (inflammation) was changes in paw volume (Vt-Vo) while the inhibition of inflammation (%) was calculated relative to control using this formula:

$$\text{Inhibition of inflammation } (\%) = \frac{(Vt-Vo)control-(Vt-Vo)test\ groups\times 100}{(Vt-Vo)control}$$

Determination of the anti-inflammatory activity of MFBB and CFBB using cotton pellet-induced chronic inflammatory model

Male Wistar rats (45) grouped into nine groups with five rats each were implanted with cotton pellet according to the method of Mosquera *et al.*[15] except Group 1 which served as the baseline. Group 2 was not treated, Group 3 was treated with indomethacin (10 mg/kg B.W.), and Groups 4–6 and Groups 7–9 received 50, 100, and 200 mg/kg B.W. of methanol and chloroform fractions, respectively, for 7 days. The animals were sacrificed on the 8th day by anesthetizing with chloroform. The cotton pellets were carefully removed, dried in an oven at 60°C for 24 h, and weighed.

Percentage inhibition of granuloma tissue formation was calculated with this formula:

$$\text{Percentage inhibition of granuloma tissue formation} = \frac{WC-WT}{WC}\times 100$$

Where, WC = change in pellet weight of the control.

WT = change in pellet weight of the drug-treated groups

Anti-inflammatory Mechanisms of the Action of MFBB and CFBB

The following parameters were used to ascertain the fractions mechanisms of actions:

Determination of membrane stabilization effects of MFBB and CFBB on hypotonicity-induced hemolysis of human red blood cells (HRBCs)

Membrane stabilization assay was conducted with Shinde *et al.*[16] method. The fractions were dissolved in hypotonic (distilled water) and isotonic (NS) solutions. Then, 0.1 ml of erythrocyte suspension was pipetted into test tubes arranged in a quadruplet (two sets for hypotonic and two sets for isotonic solutions) containing 5 ml of each fraction (100–800 µg/ml), the normal control contains 5 ml of distilled water while standard control had 200 and 400 µg/ml of indomethacin (standard). The test tubes were mixed gently before incubating at room temperature for 1 h and subsequently centrifuged at 1300 g for 3 min. The absorbance of the supernatant was measured at 418 nm with a Jenway spectrophotometer. Inhibition of membrane hemolysis (%) was calculated with this formula:

$$\text{Inhibition of membrane hemolysis } (\%) = 1-\frac{Ab2-Ab1}{Ab3-Ab1}\times 100$$

Ab1 and Ab2 represent the absorbance of the test sample dissolved in isotonic and hypotonic solutions, respectively.

Ab3 represents the absorbance of the control sample dissolved in a hypotonic solution, respectively.

Assay of Effects of MFBB and CFBB on PLA2 Activity

Method of Vane[17] was used to assay PLA2 activity. A volume, 5 ml of blood samples was used to obtain red cells used as PLA2 substrate while cultured *Aspergillus niger* was prepared and used as an enzyme. A volume, 1 ml of phosphate-buffered saline, red cell suspension (0.2 ml), and $CaCl_2$ (0.2 ml) were added into each set of test tubes labeled blank and test before the addition of 1 ml of boiled and free enzymes to blank and test tubes, respectively. Then, 1 ml of varying concentrations of each fraction (100–800 µg/ml) and 200 and 400 µg/ml of indomethacin in phosphate-buffered saline were dispensed into the respective test tubes. The control test tube had RBC suspension, free enzyme, and $CaCl_2$. All the test tubes were incubated at room temperature for 1 h before centrifugation was done at 3000 rpm for 10 min. Absorbance was measured at a wavelength of 418 nm.

$$\text{Inhibition of enzyme activity } (\%) = \frac{Absorbance\ of\ control-absorbance\ of\ test}{Absorbance\ of\ control}\times 100$$

Assay of the Effects of MFBB and CFBB on PG Synthase Activity

PG synthase obtained from fresh sheep seminal vesicles was assayed by a slight modification of Nwodo.[18] The seminal vesicles were homogenized in a frozen box with Tris-HCl buffer (40 ml, pH 7.6) using Cole-Parmer LabGEN 125 homogenizer and centrifuged severally at 6000, 15,000, and 18,000 rpm to get crude PG synthase. The enzyme activity was assayed by adding the substrate (0.2 ml arachidonic acid), different concentrations (100, 500, and 1000 µg/ml) of indomethacin, as well as each fraction and the volume, made up to 2.5 ml with Tris-HCl buffer (0.02 M, pH 7.6). The solution was incubated for 2 min under room temperature before terminating the reaction with 0.5 ml citric acid (0.2 M). Then, 5 ml ethyl acetate was used to extract the reaction mixture twice and the extract was centrifuged for 10 min at 2500 g to obtain an organic layer which was carefully removed and evaporated to dryness in a sand bath. The residue obtained was dissolved with 2 ml methanol (60%) and mixed with 0.5 ml of KOH (3 M). The resulting solution was incubated for 15 min at room temperature and the absorbance of the test against blank was measured at

a wavelength of 278 nm. Enzyme activity was calculated as follows:

$$\text{Enzyme activity} = \frac{\Delta A_{278}^{\text{min}-1} \times 10 \times 2.5 \times 1000}{25.6 \times 9 \times \text{mg enzyme test}}$$

$$\text{Inhibition of PG synthase activity } (\%) = \frac{Absorbance\ of\ Control - Absorbance\ of\ Test}{Absorbance\ of\ Control} \times 100$$

NO Radical Scavenging Activity of MFBB and CFBB

The method of Sreejayan and Rao[19] was used in determining the extent of scavenging (NO) radicals generated *in vitro* with sodium nitroprusside. A volume, 0.5 ml of distilled water (control), different concentrations (31.5–1000 µg/ml) of each fraction, and ascorbic acid (standard) were mixed with 5 ml phosphate buffer and 10 mM sodium nitroprusside (2.0 ml) in phosphate buffer with pH 7.4. The resulting solution was incubated for 150 min at room temperature. Thereafter, 2 ml of each solution was mixed with 2 ml of Griess reagent before another incubation which lasted for 30 min at room temperature. The absorbance of the pink chromophore formed when the nitrate was diazotized with α-naphthyl-ethylenediamine dihydrochloride which was taken at 546 nm wavelength.

NO activity inhibitory effect (%) was calculated as follows:

$$\text{Inhibition of NO } (\%) = \frac{Absorbance\ of\ control - Absorbance\ of\ Test}{Absorbance\ of\ Control} \times 100$$

Determination of the Effects of MFBB and CFBB on *In vivo* Leukocyte Mobilization in Rats

Agar-induced *in vivo* leukocyte mobilization was investigated according to Ribeiro *et al.*[20] using 40 male and female Wistar rats divided into eight groups with each having five rats. A volume, 1 ml/kg b. w. of NS was given to the control group (Group 1), 50 mg/kg b. w of indomethacin to standard group (Group 2), MFBB (50, 100, and 200 mg/kg b. w.) was administered to Groups 3, 4, and 5, respectively, while same dose of CFBB was given to Groups 6, 7, and 8, respectively. After 1 h of test substance administration, 0.5 ml agar solution (3% dissolved in NS) was intraperitoneally injected into each rat. The rats were sacrificed 4 h after agar injection and 5 ml of EDTA (5% in phosphate-buffered saline) was used to wash their peritonea. The peritoneal fluid obtained was used for total and differential leukocyte counts (TLC and DLC).

Statistical Analysis

Data generated in the research work were analyzed with Statistical product and service solutions (SPSS) for Windows version 18.0 (SPSS Inc., Chicago, IL, USA). Differences between means were separated and analyzed using a one-way analysis of variance (ANOVA) alongside *post hoc* multiple comparisons. The least significant threshold employed was $P < 0.05$. All analyses were performed in triplicates.

RESULTS

Qualitative and Qualitative Phytochemical Composition of MFBB and CFBB

Results in Table 1 which shows the phytochemicals present in MFBB and CFBB revealed that there were high concentrations of alkaloids (20.83 ± 1.44; 15.83 ± 1.40 mg/100 g) and tannins (0.64 ± 0.12; 0.56 ± 0.07 g/100 g) in both fractions. High concentrations of flavonoids (62.60 ± 13.50 mg/100 g) were seen in the MFBB whereas the CFBB had high concentrations of terpenoids (44.14 ± 6.79 mg/100 g) and steroids (1.87 ± 0.04 mg/g) [Table 1].

Optimal Doses for the Study

Results of the effective doses to be used for the *in vivo* assay show that the activities of both fractions peaked at the lower concentrations (50, 100, and 200 mg/kg B.W.) at the 5th h. This is evidence by the observed decrease in paw volume signifying inhibition of edema formation. Hence, these concentrations were chosen as graded doses of the fractions to be administered in the *in vivo* experiments [Figures 1 and 2].

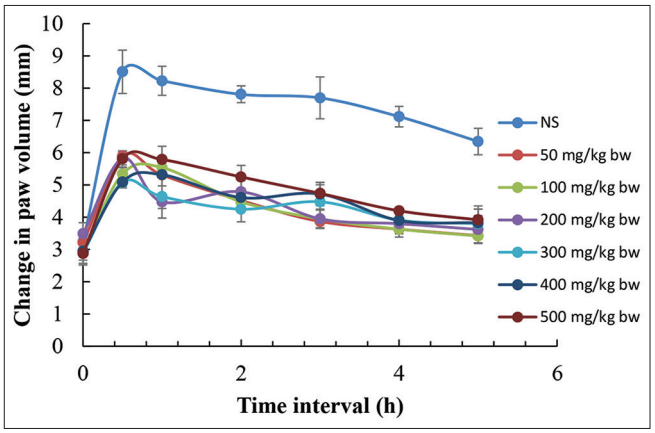

Figure 1: Change in paw volume against time using methanol fraction. The paw volumes of control (NS) and different concentrations of methanol fraction after 5 h. Data are expressed as means ± SD ($n = 5$)

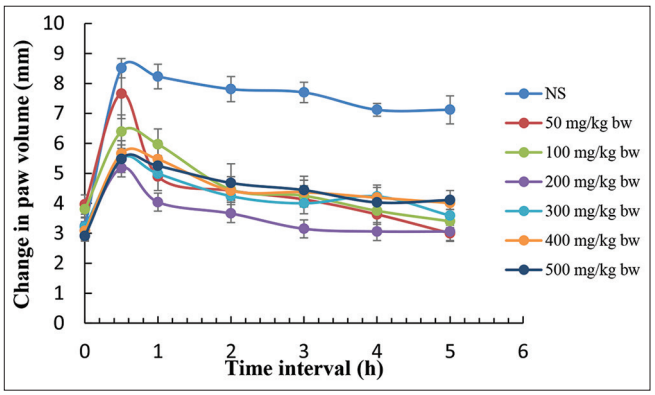

Figure 2: Change in paw volume against time using methanol fraction. The paw volumes of control (NS) and different concentrations of chloroform fraction after 5 h. Data are expressed as means ± SD ($n = 5$)

Effects of MFBB and CFBB on Egg Albumin-induced Paw Edema Model

Egg albumin administration elicited vascular changes leading to an increase in fluid exudation to the inflamed region. Groups administered doses of the fractions, as well as indomethacin, elicited a significant ($P < 0.05$) decrease in paw volume compared with control depicting a reduction of inflammation in these groups. The decrease in paw volume was time dependent with the greatest reduction seen at the 5th h in all the fractions doses except in the group administered with 50 mg/kg b. w of CFBB where edema inhibition was highest at the 4th h. No significant ($P > 0.05$) difference in paw volume was observed in groups administered the same doses of both fractions at the 1st, 2nd, 4th, and 5th h [Table 2].

Effects of MFBB and CFBB on the Cotton Pellet-induced Inflammatory Model

Implantation of cotton pellet caused granuloma tissue formation to ward off the foreign agent (cotton). The result obtained in this model in Group 1 shows a significant increase in granuloma tissue weight compared with Groups 2–8. MFBB inhibited granuloma tissue formation more than that of CFBB. However, the group treated with indomethacin (a standard anti-inflammatory drug) had higher percentage inhibition (45.26%) compared with almost all the fraction treated groups except 200 mg/kg B.W. of methanol fraction where 52.33% inhibition was recorded [Table 3].

Effects of MFBB and CFBB on Membrane Stabilization

Stabilization of membranes prevents the breakdown of membrane phospholipids. Fractions of *B. brieyi*, as well as indomethacin, stabilized HRBC. The inhibition increased with an increase in concentration in MFBB from 10.01% at 100 µg/ml to 57.97% at 800 µg/ml. Surprisingly, a reverse dose inhibition of hemolysis was observed for CFBB, which had the highest inhibitory effect of 60.86% at 100 µg/ml. Although the CFBB had higher inhibition across all the concentrations compared with MFBB, 400 µg of indomethacin inhibited HRBC hemolysis more than the same doses of CFBB [Table 4].

Table 1: Qualitative and quantitative phytochemicals of MFBB and CFBB

Phytochemicals	Relative abundance		Amount (mg/100 g)	
	MFBB	CFBB	MFBB	CFBB
Flavonoids (mg/100 g)	+++	++	62.60±13.50^{b}	8.29±2.61^{a}
Tannins (g/100 g)	+++	+++	64.00±0.12^{b}	56.12±0.07^{a}
Saponins (mg/100 g)	++	+++	26.32±4.02^{a}	53. 45±5. 62^{b}
Terpenoids (mg/100 g)	++	+++	22.63±3.13^{a}	44.14±6.79^{b}
Steroids (mg/g)	++	+++	0.07±0.01^{a}	1.87±0.04^{b}
Alkaloids (mg/100 g)	+++	++	20.83±1.44^{b}	15.83±1.40^{a}
Phenols µg/100 g GAE	+++	++	547.67±5.51^{a}	522.00±13.32^{a}
Glycosides (mg/g)	+++	+	9.60±0.14^{b}	3.54±0.70^{a}

+, ++, and +++ represent present in low, moderate, and high amounts, respectively while GAE stands for gallic acid equivalent. Values represent means±SD of triplicate samples. Different letters of the alphabet were used across the row to illustrate significant ($P<0.05$) difference but the same letters portray not significantly ($P>0.05$) different

Table 2: Effect of MFBB and CFBB on egg albumin-induced edema model

Treatment (mg/kg)		Doses Δ in paw edema (ml) and (%) inhibition of edema					
		0.5 h	1 h	2 h	3 h	4 h	5 h
Control	-	4.24±0.39^{c}	4.28±0.35^{b}	3.58±0.74^{c}	3.38±0.40^{c}	3.39±0.66^{b}	3.09±0.55^{b}
MFBB	50	3.19±0.35^{ab} (24.76)	2.69±0.29^{a} (37.15)	2.05±0.19^{ab} (42.73)	1.66±0.34^{ab} (50.89)	1.23±0.24^{a} (63.72)	0.86±0.11^{a} (72.16)
	100	3.21±0.23^{b} (24.29)	2.56±0.23^{a} (40.19)	1.88±0.19^{ab} (47.49)	1.68±0.32^{b} (50.30)	1.14±0.14^{a} (66.37)	0.57±0.16^{a} (81.55)
	200	2.77±0.30^{ab} (34.67)	2.08±0.32^{a} (51.40)	1.60±0.28^{ab} (55.31)	1.48±0.15^{ab} (56.21)	1.84±0.27^{a} (75.22)	0.49±0.08^{a} (84.14)
CFBB	50	2.76±0.49^{ab} (34.90)	2.02±0.35^{a} (52.80)	1.49±0.28^{a} (58.38)	1.31±0.29^{ab} (61.24)	0.81±0.16^{a} (76.11)	0.79±0.06^{a} (74.43)
	100	2.56±0.15^{a} (39.62)	1.91±0.17^{a} (55.37)	1.59±0.05^{ab} (56.15)	1.18±0.16^{a} (65.09)	0.83±0.14^{a} (75.52)	0.60±0.10^{a} (80.58)
	200	2.78±0.31^{ab} (34.43)	2.14±0.25^{a} (50.00)	1.48±0.17^{ab} (58.66)	1.30±0.17^{a} (61.24)	0.79±0.13^{a} (76.70)	0.55±0.05^{a} (82.20)
Indomethacin	50	3.00±0.26^{ab} (29.25)	2.08±0.11^{a} (51.40)	2.07±0.10^{b} (42.18)	1.64±0.06^{b} (51.48)	1.22±0.18^{a} (64.01)	0.72±0.06^{a} (76.70)

Values represent means±SD of triplicate samples. Different letters of the alphabet were used across the row to illustrate significant ($P<0.05$) difference but the same letters portray not significantly ($P>0.05$) difference

Table 3: Inhibition of granuloma tissue formation in rats by root barks fractions of *Brenania brieyi*

Groups	Treatments	Doses (mg/kg b. w)	Granuloma tissue weight (mg)	Inhibition (%)
1	Control (N. S)	-	381.8±8.9^{f}	0
2	Indomethacin	10	209.0±6.7^{b}	45.26
3	Methanol fraction	50	237.4±4.9^{d}	37.82
4		100	225.4±4.8^{c}	40.96
5		200	182.0±3.2^{a}	52.33
6	Chloroform fraction	50	249.0±4.7^{c}	34.78
7		100	243.0±4.3^{c}	36.35
8		200	257.6±2.3^{e}	32.53

N.S: Normal saline. Results are presented as mean±SD (n=5). Granuloma tissue weight with mean values having the same letter of the alphabet is not significant (P>0.05) while granuloma tissue weight with mean values having different letters is significant (P<0.05). Key: Group 1: Normal rats not implanted with cotton pellets (baseline). Group 2: Rats treated with 1 ml/kg body weight of normal saline after cotton pellets implantation (negative control). Group 3: Rats treated with 10 mg/kg body weight of indomethacin after cotton pellet implantation (standard). Groups 4, 5, and 6 rats were treated with varying doses of methanol fraction (50, 100, and 200 mg/kg body weight, respectively) after cotton pellets implantation. Groups 7, 8, and 9 rats were treated with varying doses of a chloroform fraction (50, 100, and 200 mg/kg body weight, respectively) after cotton pellets implantation

Table 4: Effects of MFBB and CFBB on hypotonicity-induced hemolysis of HRBC

Treatments	Conc. (µg/ml)	Absorbance (418 nm) solution	Isotonic hypotonic solution	% inhibition of HRBC hemolysis
Control	-	0.567±0.003	1.378±0.003	0.00
MFBB	100	0.029±0.001*	1.243±0.004*	10.01
	200	0.039±0.004*	1.030±0.003*	25.99
	400	0.054±0.004*	0.725±0.004*	49.32
	600	0.050±0.003*	0.619±0.003*	57.13
	800	0.060±0.004*	0.614±0.004*	57.97
CFBB	100	0.057±0.003*	0.574±0.005*	60.86
	200	0.061±0.005*	0.591±0.001*	59.76
	400	0.181±0.006*	0.698±0.004*	56.81
	600	0.271±0.004*	0.846±0.003*	48.06
	800	0.315±0.002*	0.756±0.002*	58.51
Indomethacin	200	0.585±0.045*	0.907±0.005*	59.39
	400	0.460±0.020*	0.596±0.002*	85.19

Results are presented as mean±SD of triplicate determination. Values with an asterisk (*) are significantly (P<0.05) different from the control

Effects of MFBB and CFBB on PLA2 Activity

Results in Table 5 reveal that the fractions of *B. brieyi* root bark as well as the standard drug prednisolone inhibited the release of hemoglobin into the medium by PLA2. This was revealed by the recorded decreases ($P < 0.05$) in absorbance in the fraction and standard test tubes. The inhibitory effect by the fractions was reverse concentration dependent. The fractions had higher ($P < 0.05$) PLA2 inhibitory activity than the standard drug, prednisolone.

Effect of MFBB and CFBB on PG Synthase Activity

The result in Table 6 shows that both MFBB and CFBB just like indomethacin inhibited PG activity which will limit the release of inflammatory mediators and cells. The inhibitory activity increased with an increase in the concentration of the fractions and indomethacin. Minimal enzyme activity of 2.20 and 1.29 with% inhibition of 81.69 and 89.27 was produced by 1000 µg/ml of MFBB and CFBB, respectively. Results in Table 6 also show that indomethacin had more propensity to inhibit the enzyme activity except at 1000 µg/ml than the MFBB but the reverse was the case with CFBB.

NO Scavenging Activity of MFBB and CFBB

As shown in Figure 3, both the MFBB and CFBB of *B. brieyi* scavenged NO *in vitro* which indicates that they have antioxidant activity. The NO scavenging activity of the fractions increased with an increase in concentration from 58. 07 ± 1.31 and 74.63 ± 4.76 at 31.3 µg/ml to 93.71 ± 1.16 and 86.58 ± 6.82 at 1000 µg/ml for MFBB and CFBB, respectively. CFBB displayed a better NO scavenging activity at lower concentrations (31.3–250 µg/ml) whereas MFBBB was found to be more effective at higher concentrations (500 and 1000 µg/ml). Furthermore, as it is evident from Figure 3, the activity of ascorbic acid was higher ($P < 0.05$) in almost all the concentrations tested.

Table 5: Effects of MFBB and CFBB on PLA2 activity

Treatment	Conc. (µg/ml)	Absorbance (418 nm)	% inhibition of PLA_2 activity
Control	-	1.588±0.181	0.00
MFBB	100	0.491±0.005*	69.08
	200	0.498±0.015*	68.64
	400	0.530±0.002*	66.62
	600	0.558±0.003*	64.86
	800	0.629±0.007*	60.39
CFBB	100	0.524±0.004*	67.00
	200	0.471±0.007*	70.34
	400	0.513±0.010*	67.70
	600	0.577±0.005*	63.66
	800	0.619±0.008*	61.02
Prednisolone	200	0.971±0.009*	38.85
	400	0.754±0.015*	53.09

Results are presented as mean±SD of triplicate determination. Values with an asterisk (*) are significantly ($P<0.05$) different from the control

Table 6: Effect of MFBB and CFBB on PG synthase activity

Treatments	Conc. (µg/ml)	Absorbance (278 nm)	Enzyme activity	% inhibition of enzyme activity
Control		0.885±0.025	12.00	0.00
MFBB	100	0.264±0.033*	3.58	70.17
	500	0.168±0.008*	2.28	81.02
	1000	0.162±0.007*	2.20	81.69
CFBB	100	0.230±0.009*	3.12	74.01
	500	0.147±0.003*	2.00	83.39
	1000	0.095±0.007*	1.29	89.27
Indomethacin	100	0.132±0.003*	1.79	85.08
	500	0.128±0.003*	1.74	85.54
	1000	0.110±0.011*	1.49	87.62

Results are presented as mean±SD of triplicate determination. Values with an asterisk (*) are significantly ($P<0.05$) different from the control

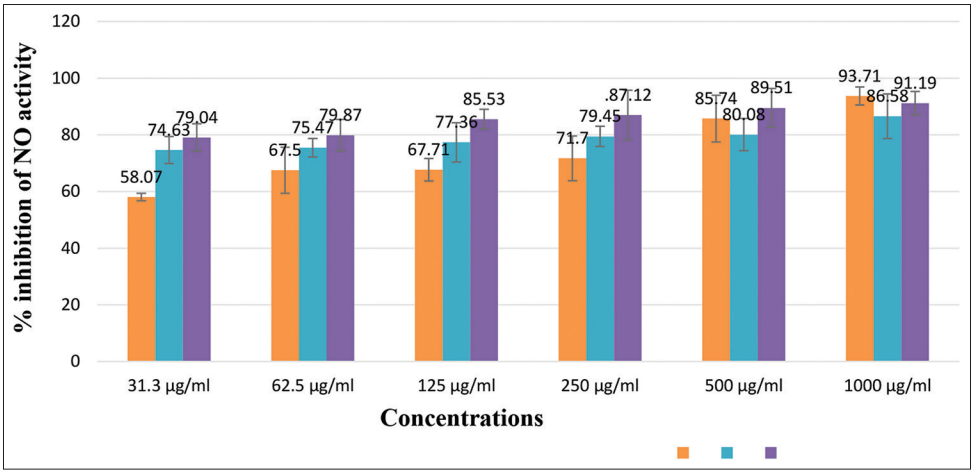

Figure 3: Nitric oxide scavenging activity of MFBB (orange) and CFBB (aqua) ascorbic acid (purple). Inhibition of NO activity increased in a concentration-dependent manner with methanol extract having the highest inhibition (93.71%) at 1000 µg/ml. Values are expressed in % ($n = 3$, triplicate)

Effects of MFBB and CFBB on *In vivo* Leukocyte Mobilization in Rats

Leukocytes are mobilized to the inflamed region to eliminate the injurious agent(s). Results in Table 7 show that the administration of different doses of MFBB and CFBB did not inhibit leukocyte mobilization compared with control and indomethacin. A remarkable increase in leukocytes predominantly neutrophil and lymphocytes was observed in groups administered both in MFBB and CFBB. Mobilization of neutrophils increased with an increase in doses with Groups 5 and 8 which received the highest doses of both fractions having the highest neutrophil but the reverse was the case with lymphocytes.

DISCUSSION

Plants' phytochemicals including flavonoids, phenols, alkaloids, tannins, and saponins are known to play an essential role in ameliorating inflammation and oxidative stress.[21] The abundance of these phytonutrients in MFBB and CFBB as shown in Table 1 makes the plant a promising agent in performing the aforementioned functions.

The fractions of *B. brieyi* at various concentrations and different time intervals had the peak activities at the lower doses (50, 100, and 200 mg/kg b. w.) as evidence in Figures 1 and 2. The fractions afforded protection against egg albumin-induced paw edema [Table 2]. An inhibition in the early phase (first few h) which is associated with the release and activation of kinins, histamine, and serotonin[3] and late phases (after 3–4 h) essentially mediated by PGs[6] was observed. This finding suggests that *B. brieyi* root bark has anti-inflammatory activity since egg albumin-induced edema inhibition is one of the models used extensively for the evaluation of compounds' anti-inflammatory effect.[6]

The cotton pellet was used to investigate the fractions efficacy in inhibiting exudation and proliferation of neutrophils, macrophages, fibroblasts, and blood cell multiplication which results in granuloma tissue formation.[22] Interestingly, groups administered *B. brieyi* fractions had significantly ($P < 0.05$) lower granuloma tissue weight compared with Group 2 (untreated). Although the exert mechanism of its action was not ascertained, it could be deduced that the antioxidant-rich bioactive constituents' presence in the fractions inhibited tissue hyperplasia.[23,24] This suggests the potency of the fractions in reducing collagen synthesis, angiogenesis, and the release of other inflammatory cytokines[25] which also provide robust evidence about the anti-inflammatory activity of the fractions.

Accumulated evidence has shown that mechanisms behind anti-inflammatory effects of test compounds could be ascertained with the investigation of its membrane stabilization, inhibition of enzymes metabolizing arachidonic acids, and release of inflammatory mediators (PLA2 and PG synthase), generation of NO, as well as inflammatory cells mobilization.[26]

Stabilization of the lysosomal membrane is important because it interferes with the release and/or action of lytic enzymes, and inflammatory mediators.[5] The result obtained from our membrane stabilization gives evidence that both fractions of *B. brieyi* offered protection against hypotonic solution-induced RBC hemolysis [Table 4]. The biochemical basis for the fractions' antihemolytic effect could be either through direct interaction of the phytoconstituents present in the fractions with membrane components or inhibiting the action of membrane hydrolyzing enzymes. Several phytonutrients such as flavonoids, saponins, terpenoids, or squalene identified in the fractions through GC–MS[3] have been reported to have a membrane-stabilizing effect. Stabilization of the RBC membrane which is equivalent to lysosomal membranes[5] proves beneficial and directly confirms one of the protective effects and anti-inflammatory mechanisms of the fractions.

Similarly, the root bark fractions of *B. brieyi* also inhibited PLA2 activity [Table 5]. Invariably inhibition of PLA2 activity by the fractions could either be assessed from its ability to stabilize membrane, thereby preventing the release of free phospholipids from erythrocyte membrane or through direct inhibition of PLA2 release or action. The observed inhibition of PLA2 activity by the fractions might be attributed to the bioactive compounds found in the fractions such as n-hexadecenoic acids identified through GC–MS which is a known competitive inhibitor of PLA2[27] or by terpenoids which induce lipocortin, an inhibitor of PLA2 activity.[26] Quite a several herbal plants have exhibited anti-inflammatory effects through inhibition of PLA2 activity.[26]

Table 7: Effects of MFBB and CFBB *in vivo* leukocyte mobilization

Groups	Treatment	TLC (10^3/mm)	Differentia leukocyte mobilization (%)				
			N	E	M	L	B
1	N.S 1 ml/kg	12480.0 ± 614.0^{b}	64.0^{b}	2.6^{b}	1.2^{a}	32.2^{c}	0.0
2	Indo 50 mg/kg	7120.0 ± 165.53^{a}	59.0^{a}	2.0^{b}	1.8^{abc}	37.0^{d}	0.0
3	MFBB 50 mg/kg	42860.0 ± 961.0^{e}	68.2^{bc}	1.4^{ab}	1.4^{ab}	29.0^{bc}	0.0
4	100	47520.0 ± 676.7^{f}	67.6^{bc}	1.4^{ab}	1.8^{abc}	29.2^{bc}	0.0
5	200	17720.0 ± 288.8^{c}	71.2^{c}	0.4^{a}	2.2^{abcd}	26.2^{ab}	0.0
6	CFBB 50 mg/kg	18320.0 ± 259.6^{c}	$67.2b^{c}$	1.8^{b}	3.0^{cd}	$28.0b^{c}$	0.0
7	100	27500.0 ± 702.1^{d}	$68.0b^{c}$	1.6^{ab}	$2.6b^{cd}$	$27.8b^{c}$	0.0
8	200	15840.0 ± 143.5^{c}	71.6^{c}	2.0^{b}	3.4^{d}	23.0^{a}	0.0

N.S: Normal saline, Indo: Indomethacin, TLC: Total leukocyte count, N: Neutrophils, E: Eosinophils, M: Monocytes, L: Lymphocytes, and B: Basophils. Results are presented as mean±SD of triplicate determination. Values with an asterisk (*) are significantly ($P<0.05$) different from the control

In the same vein, the result obtained from PG synthase assay, a known anti-inflammatory target of test compounds,[22] shows that both fractions of the root bark of *B. brieyi* inhibited PG synthase activity. Inhibitory effects of the fractions on PG synthase could be as a result of inhibition of PLA2 which deprived PG synthase of its substrate or direct inhibition of cyclooxygenase (COX) which is another therapeutic target.[5] This finding contributes remarkably in giving credence to the explanation that one of the mechanisms by which the therapeutic effects of the fractions are registered is through inhibition of PG synthesis. This result is in tandem with reports of several other investigators: Adebayo *et al.*,[28] Nworu, and Akah[26] who also reported the COX inhibitory effect of fractions of various plant species.

Oxidative stress products such as NO can be used as a marker of the inflammatory response.[29] Our result showed high percentage inhibition of NO [Figure 3]. In general, the radical scavenging potency of phytochemicals including polyphenols appears to depend on the OH groups found on the phenolic ring which serves as an electron/hydrogen donor.[29] The MFBB which is rich in flavonoids had the highest inhibition in this antioxidant assays, indicative of its high antioxidative potency. Furthermore, from the above premise, it is worthy to note that the fractions could prevent tissue injury, thereby subverting etiology and complications associated with a wide range of diseases including inflammation, cancer, cardiovascular, and neurological diseases.[11]

Surprisingly, the result from this present study showed that MFBB and CFBB increased the total leukocyte count of the perfusate than that of the control and indomethacin groups. It is, therefore, likely that the fractions had a stimulatory effect on leukocyte production, thereby boosting the immune system. However, the membrane-stabilizing and antioxidant activity of the fractions may offer protection against the mobilized leukocytes. This suggests that the inhibition of leukocyte mobilization may not be one of the potential mechanisms of the plant's anti-inflammatory activity.

CONCLUSIONS

Findings from this study show that the root barks of *B. brieyi* have anti-inflammatory activities. The potential mechanisms of its action could be through stabilization of lysosomal membrane, which inhibits PLA2 activities with resultant inhibition of some arachidonic acid metabolizing enzymes, PG synthase. Thus, *B. brieyi* could be used in the synthesis of cheaper, safer, and effective anti-inflammatory drugs.

AUTHORS' CONTRIBUTIONS

Conceptualization: CIF. Methodology: CIF, AVO, ELUS, and OVN. Investigation: CIF, NFN, and NCC. Formal analysis: CIF, AVO, and JE. Writing – original draft: CIF, AVO, NFN, NCC, and JE. Writing – review and editing: ELUS and OVN. All authors read and approved the manuscript before submission.

ETHICS APPROVAL

Approved international, national, and institutional guidelines on the use of human subjects for research work (Helsinki Declaration) and also the guide given by the National Institute of Health on use and care of experimental animals were strictly adhered to throughout the conduct of the research work. Approval for the study was obtained from the Faculty of Biological Sciences Ethics and Biosafety Committee (Ref no: UNN/FBS/EC/1049).

DATA AVAILABILITY

ALL data generated in this research work are embedded in the manuscript.

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