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Assessment of the Anti-inflammatory and Anti-oxidant Activities of Flavonoid-rich Fraction of *Pleiocarpa mutica* Leaves

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Abstract: *Pleiocarpa mutica* is a medicinal plant that is native to large areas of tropical Africa and belongs to the family of *Apocynaceae*. It is a medicinal herb used in traditional Nigerian medicine to treat a variety of diseases, including inflammatory disorders. The present study investigated the anti-inflammatory and anti-oxidant effects of flavonoid-rich fraction of *Pleiocarpa mutica* leaves (FRFPML) using both *in-vivo* and *in-vitro* based assays. A total of thirty (30) adult male albino rats (110-150g) were divided into five (5) groups of six (6) rats each for the *in-vivo* anti-inflammatory assay. Group 1 untreated; Group 2 was given 10 mg/kg body weight of Indomethacin and Groups 3, 4 and 5 were given 200, 400 and 600 mg/kg body weight of FRFPML respectively. Tannins, phenols, flavonoids, alkaloids, steroids, terpenoids and saponins were found in varied amounts in the plant secondary metabolites according to quantitative analysis. The extract demonstrated no harm in an acute toxicity assay when dosed up to 5000 mg/kg b.w. Scalar dosages of the FRFPML significantly ($p<0.05$) suppressed the development of paw oedema induced by egg albumin in the systemic rat paw oedema model. This compares favorably to the standard anti–inflammatory drug indomethacin (10 mg/kg b.w) which at 24 hours inhibited egg albumin-induced rat paw oedema (11.82%). Varying doses of the extract significantly ($p<0.05$) inhibited egg albumin denaturation, hypotonicity-induced membrane stabilization, protease inhibition and phospholipase A_2 in a concentration-dependent manner, causing inhibition comparable to that of the standard anti-inflammatory drug used. The extract at different concentrations (0.2-0.8 mg/ml), significantly ($p<0.05$) exhibited DPPH (1-1diphenyl-2-picrylhydrazyl) and H_2O_2 (hydrogen peroxide) radical scavenging activities in a concentration-dependent manner similar to the standard antioxidant ascorbic acid used. Results from the study revealed that the FRFPML exhibited remarkable anti-inflammatory and anti-oxidant activity.

Keywords: *Pleiocarpa mutica*, Anti-inflammation, Paw Odema, Anti-oxidant

1. Introduction

A medicinal plant can be defined as a species of plant with one or more bioactive molecule, used in folk medicine for treating diseases in human and animals [1]. Medicinal plants have been used since ancient civilization to treat various diseases [2]. These plants were used in ancient times to treat various ailments by trial and error method, and by this method, plants with therapeutic value was distinguished from non-medicinal plants [3]. The healing property of medicinal plants can be attributed to the presence of various bioactive molecules. Isolation and characterization of these bioactive molecules from medicinal plants has paved the way for the discovery of new therapeutics, thereby improving the pharmaceutical and health sector [4]. Various *in-vitro* and *in-vivo* assay-based studies over the decade have led to the identification of different medicinal plant species with proven anti-inflammatory activity [5, 6].

Inflammation is a complex biological response of vascular tissues generated as a result of a harmful stimulus. This response is regarded as a defense mechanism used by the body to eliminate harmful stimuli which can be damaged cells, pathogens, or irritants [7]. This non-specific defense mechanism is hastened by recruitment of immune cells and release of molecular mediators from injured cells or tissues at site of injury [8, 9]. Molecular mediators such as histamine, prostaglandins, serotonin and kinin function in a collective manner to increase permeability of capillaries and vasodilation [10-12]. Inflammation can be classified into two types: acute inflammation and chronic inflammation. Acute inflammation is an early response lasting from several minutes to a few days. Acute inflammation is marked by migration of leukocytes and leakage of plasma proteins or fluids from the blood to site of injury [13, 14]. Persistent inflammation as result of prolonged exposure to harmful stimuli can result to chronic inflammation, in which there is dense infiltration of immune cells to site of injury [15]. Various causes of inflammation have been documented which include: Protein denaturation, lysis of Red Blood Cell (RBC) membrane, production of Reactive Oxygen Species (ROS) and degradation of phospholipase A2. During early phase of inflammation, leukocytes and mast cells are recruited to site of injury accompanied by production of ROS [16]. Oxidative stress occurs when the body's endogenous antioxidant compounds are unable to mop up overproduced ROS [17]. Oxidative stress results in various degenerative diseases and inflammatory illnesses [17].

Pleiocarpa mutica Benth. is a medicinal plant native to vast regions of tropical Africa, belonging to the family of *Apocynaceae* [18]. The genus of *Pleiocarpa* consist of 22 different scientific plant names, only 6 specie names are accepted [19]. *P. mutica* is an evergreen shrub which has a height of about 7.5m [20]. The plant leaves have traditionally been used to treat jaundice, oedema, and roundworm infection in patients [21-23]. A decoction of root bark has also been used to treat malaria [20]. Addae-Kyereme et al. [24] and Enechi et al. [25] have scientifically reported this pharmacological activity. Although *P. mutica*'s use in folk medicine for treating oedema shows it has anti-inflammatory activity, there have been no scientific findings on its anti-inflammatory and anti-oxidant activities. Therefore, the aim of this study is to evaluate the anti-inflammatory activity of FRFPML.

2. Materials and Methods

2.1. Collection of Plant Material and Extraction Procedure

Fresh leaves of *P. mutica* were taken on May 2021 from a habitat Ugbene-Ajima in Uzo-uwani Local Government Area of Enugu State, Nigeria. Mr. Alfred Ozioko, a taxonomist of the Bioresources Development and Conservation Programme (BDCP) Research Centre, Nsukka, Enugu State, identified and authenticated the plants. A voucher specimen numbered Intercedd/301 was deposited at the herbarium for reference purposes. The leaves were washed and dried in the shade for several weeks, turning them often until crispy. A mechanical grinder was used to crush the dried leaves into a powder form. The powder (1000 g) was macerated in 3.0 L absolute ethanol and left at room temperature for 72 hours. A white muslin cloth was used to filter the mixture. Further filtration was achieved with Whatman No, 1 filter paper and the filtrate were concentrated using rotary evaporator (IKA, Germany) at an optimum temperature of 45–50°C to get a chocolate-like semi solid extract.

2.2. Experimental Animals

Swiss albino mice (28–30 g) were used for toxicity testing while adult Wistar rats (110–150 g) were used for *in-vivo* anti-inflammatory activity of both sexes obtained from the animal house of the Department of Veterinary Medicine, University of Nigeria Nsukka. Before the studies, they were confined in metal steel cages and acclimatized in the laboratory for seven days. The rats were fed regular grower's mash rat pellets and water *ad labitum* (Grand Cereals LTD, Enugu, Nigeria). The study followed the revised National Institute of Health Guide for Care and Use of Laboratory Animal (Pub No. 85-23, revised 1985) and the International Guidelines for Handling of Laboratory Animals [26].

2.3. Chemicals, Reagents and Drugs

All of the chemicals used in this research were analytical grade and were products of Sigma Aldrich in the United States, British Drug House (BDH) in England, Burgoyne in India, Harkin and Williams in England, Qualikems in India, Fluka in Germany, and May and Baker in England. The assays were carried out using commercial kits and products from Randox and Teco (TC) in the United States. The standard anti-inflammatory drug Indomethacin, Asprin and Prednisolone (10 mg) used in this study were acquired from a renowned pharmacy, Elofex Pharmaceutical and Drug Stores in Enugu, Enugu State, Nigeria.

2.4. Phytochemical Analysis

Various quantitative chemical tests were carried out to evaluate the phytochemical composition of the crude extract using procedures outlined by Harborne [27].

2.5. Preparation of Flavonoid-rich Fraction

The approach for extracting FRFPML provided by Chu et al. [28] was followed with few modifications. In a small flask, a sample of crude extract was dissolved in 20 mL of 10% H_2SO_4 and hydrolyzed by heating on a water bath for 30 minutes at 100°C. The mixture was placed on ice for 15 minutes allow the flavonoids aglycones to precipitate. The cooled solution was filtered and the filtrate (flavonoids aglycone mixture) was dissolved in 50 mL of warm 95% ethanol (50°C). The resultant solution was filtered once again into a 100 mL volumetric flask that had been filled with 95% ethanol. Using a rotary evaporator, the filtrate was concentrated to dryness.

2.6. Acute Toxicity Study

The acute toxicity of the fraction was examined, along with the estimation of the median lethal dose (LD_{50}) using method of Lorke [29] with certain changes to define the fraction's lethal dose range. The study employed eighteen (18) Swiss albino mice that were starved of food for 18 hours but granted access to water. They were divided into six (6) groups of three mice each, with each group receiving FRFPML at varied dose levels (10, 100, 1000 mg/kg for phase one and 1600, 2900 and 5000 mg/kg for phase two). For the next 24 hours, the animals were monitored for nervousness, dullness, in-coordination, death and behavioral change (toxic symptoms).

2.7. Experimental Design

For the study, a total of thirty (30) male Wistar albino rats were used. They were divided into five (5) groups, each with six (6) rats, and were given the following treatment:

Group 1: Toxic group

Group 2: Received 10 mg/kg body weight of indomethacin (standard drug)

Group 3: Received 200 mg/kg body weight of FRFPML

Group 4: Received 400 mg/kg body weight of FRFPML

Group 5: Received 600 mg/kg body weight of FRFPML.

2.8. Determination of the Effect of FRFPML on Egg Abumin-induced Rat Paws Oedema

The effect of FRFPML on egg abumin-induced rat paw oedema was investigated using a modified method of Winter et al. [30]. Wistar albino rats were fasted for 18hrs before the experiment to ensure uniform hydration and minimize variability in oedematous response, afterwards the right hind paw size of the rats at time zero (before the induction of oedema) was measured using a Vernier caliper. The FRFPML was administered orally one hour prior to induction of acute inflammation using 0.1 ml of freshly prepared egg albumin. Egg albumin was injected into the sub-plantar of the right hind paw of rats. The increase in the right hind paw size of rats was subsequently measured at 0, 1, 2, 3, 4, and 24 hr after injection of egg albumin. The difference between the paw size of the injected paws at time zero and at different times after egg albumin injection was used to assess the formation of oedema. These values were used in the calculation of the percentage inhibition of edema for each dose of the extract and for indomethacin at the different time intervals using the relation below:

$$\text{Paw oedema}=(V_t - V_o)$$

V_o=Size of paw oedema at time zero

V_t=Size of paw oedema at time t [0, 1, 2, 3, 4, 24 hr]

$$\text{Percentage inhibition of oedema}=\frac{(Vt-Vo)\ \text{Toxic group} - (Vt-Vo)\ \text{Treated groups}}{(Vt - Vo)\ \text{Toxic group}}\times\frac{100}{1}$$

2.9. In-vitro Anti-inflammatory and Antioxidant Activities

2.9.1. The Effect of FRFPML Egg Albumin Denaturation

The protein denaturation assay was performed using inhibition of albumin denaturation technique described by Haghparast et al. [31]. A test solution containing various concentrations of samples (0.2, 0.4, 0.6, 0.8, 1.0 mg/ml) was combined with 0.2ml of 1% egg albumin from a chicken (fresh hen egg). The pH of the reaction mixture was adjusted from pH7.0 to 3.1 using tiny amount of HCL. The mixtures were incubated at 37°C for 15 minutes before being heated in a water bath to induce denaturation at 70°C for 5 minutes. A spectrophotometer was used to assess their absorbance at 660nm after cooling. Aspirin was used as standard drug. The experiment was replicated thrice. The percentage of protein denaturation inhibition was calculated using the formula:

$$\%\ \text{inhibition}=\frac{AbsorbanceControl-AbsorbanceSample}{AbsorbanceControl}\ x\ 100$$

2.9.2. The Effect of FRFPML Membrane Stabilization

The effect of FRFPML on hypotonicity-induced haemolysis was investigated using method described by Oyedepo and Femurewa [32] with some modifications. Prednisolone, an NSAID, was used as standard reference drug and anti-inflammatory activity was expressed as percentage inhibition of haemoylsis. Human red blood cell (HRBC) function in the same manner as lysosomal membrane [33]. If the plant fraction is able to stabilize the HRBC, then it stabilizes lysosomal membrane.

Preparation of red blood cell suspension:

Fresh blood (3ml) collected from healthy volunteers was placed in a clean EDTA bottle, centrifuged at 3000rpm for 10 mins, and washed three times with equal volume of normal saline. The blood volume was measured and reconstituted as a 40% (v/v) suspension with normal saline.

Hypotonicity-induced haemolysis:

Samples of the fraction and standard drug (prednisolone) were dissolved in distilled water (hypotonic solution). An

aliquot (1 ml) of graded doses of the fraction (0.2, 0.4, 0.6, 0.8, 1.0 mg/ml) were put in triplicates in a set of five test tubes. Another tube contained 1 ml of 0.6 mg/ml Prednisolone. The contents of the test tube were made up to 4.9 ml with distilled water. Control tubes contained 4.9 ml of the vehicle (distilled water) and 4.9 ml of normal saline (isotonic solution). HRBC suspension (1 ml) was added to each tube and gently mixed. The mixtures were incubated for 1 hr at 37°C, and then the tubes were centrifuged at 3000 rpm for 10 min. The absorbance (OD) of the supernatant measured at 418nm using a spectrophotometer. The percentage inhibition of haemolysis was calculated using the equation below:

$$\% \text{ Inhibition of haemolysis} = 100 \times \left(1 - \frac{OD2 - OD1}{OD3 - OD1}\right)$$

Where OD1=Absorbance of control I (isotonic solution)
OD2=Absorbance of test sample
OD3=Absorbance of control II (hypotonic solution).

2.9.3. The Effect of FRFPML Protease Inhibition

The assay was done according to method of Oyedepo and Femurewa [32] with slight modifications. The reaction mixture (2ml) contained 0.06mg trypsin, 20 mM Tris HCl buffer (pH 7.4) and 1 ml test sample of different concentrations (0.2-1.0 mg/ml). The mixture was incubated for 20 minutes at 37°C afterwards; 2ml of 70% perchloric acid was added to halt the reaction process. The solution was centrifuged at 3000 rpm for 10 mins, and the absorbance of the supernatant was read at 210nm against Tris HCl buffer as blank. Prednisolone was used as standard drug. The experiment was performed in triplicate. The percentage inhibition of protease activity was calculated as follows:

$$\% \text{ Inhibition} = \left(\frac{Abscontrol - Abssample}{Abscontrol}\right) \times 100$$

2.9.4. The Effect of FRFPML Phospholipase A_2 (PLA_2) Activity

The effect of FRFPML on PLA_2activity was evaluated using method of Vane [34] with modifications by Enechi et al. [35]. Fresh human blood samples collected from healthy individuals were centrifuged at 3000 rpm for 10 min afterwards; the supernatant (plasma) was discarded. The red cells were washed thrice with equal volume of normal saline and reconstituted as a 40% (v/v) suspension with normal saline. Fungal enzyme preparation was gotten from *Aspergillus niger*strain culture. *Aspergillus niger* was cultured using a nutrient broth for 72 h at room temperature. The culture was transferred into test tubes containing 3 ml of phosphate buffered saline and centrifuged at 3000 rpm for 10 min. The fungal cells constituted the pellet, while the supernatant was used as crude enzyme preparation. HRBC (0.2 ml), $CaCl_2$ (0.2 ml), 0.2 ml crude enzyme preparation, and varying concentration of normal saline and the fraction (0.2-1.0 mg/ml) were incubated at 37°C for 1 hr. Control tube contained HRBCs, $CaCl_2$, and crude enzyme preparation. The blanks were treated with 0.2 ml of boiled enzyme separately. The incubated reaction mixture was centrifuged at 3000 rpm for 10 min. A measured quantity of the supernatant (1.5 ml) was diluted with 10 ml of normal saline and the absorbances were the solution was taken at 418 nm. Prednisolone was used as standard drug which is an inhibitor of phospholipase A_2. The percentage maximum enzyme activity and percentage inhibition were calculated using the expression:

$$\% \text{ Maximum activity} = \frac{Absorbance of test}{Absorbance of control} \times \frac{100}{1}$$

% Inhibition=100- % maximum activity of the enzyme.

2.9.5. DPPH Radical Scavenging Assay

The scavenging ability of the flavonoid-rich fraction towards the stable radical DPPH (1, 1-diphenyl-1-picrylhydrazl) was evaluated using the method of Brand-Williams et al. [36]. Methanol solution (1.0 ml) of varying concentrations of the fraction (0.2-1.0 mg/ml) was added to 5 ml of DPPH solution and absorbance was measured at 517 nm. Ascorbic acid was used as standard drug. The percentage scavenging activity of DPPH radical was calculated using the formula:

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

2.9.6. Hydrogen Peroxide (H_2O_2) Scavenging Activity

The ability of flavonoid-rich fraction to scavenge hydrogen peroxide radical was evaluated using method of Ruch et al. [37]. Hydrogen solution was prepared in phosphate buffer (0.1 M pH 7.4). Test sample (0.2 to 1.0 mg/ml) was added to 1 ml of hydrogen peroxide. The mixture was incubated for 10 mins, and absorbance measured at 230 nm. Ascorbic acid solution served as standard control. The percentage scavenging activity of hydrogen peroxide was calculated using the equation:

$$\%\text{Scavenged } H_2O_2 = \frac{\text{Absorbance (control)} - \text{Absorbance (test)}}{\text{Absorbance (control)}} \times 100$$

2.10. Statistical Analysis

The analysis of data obtained was done using Statistical Product for Service Solution (SPSS), version 23. The results were expressed as mean±standard deviation. Significant difference of the result was established by one-way Analysis of Variance (ANOVA) and the acceptance level of significance was $p<0.05$ for the results.

3. Results

3.1. Quantitative Phytochemical Evaluation of Ethanol Extract of Pleiocarpa mutica Leaves

Phytochemical analysis of ethanol extract of *Pleiocarpa mutica* leaves showed high concentrations of tannins, phenols, flavonoids, alkaloids, terpenoids, glycosides, and low concentrations of steroids and saponins (Table 1).

Table 1. *Quantitative Phytochemical composition of ethanol extract of Pleiocarpa mutica leaves.*

Phytochemical Constituents	Concentration (mg/100g)
Tannins	1204.82±26.49
Phenols	1183.56±7.90
Flavonoids	1531.69±36.98
Alkaloids	528.71±13.86
Steroids	0.14±0.01
Terpenoids	398.74±3.57
Saponins	0.54±0.01
Glycosides	276.97±2.37

Results are expressed in Means±SD (n=3).

3.2. Acute Toxicity Studies

Table 2 shows that there was neither death nor any sign of toxicity and behavioral change in the groups of rats administered 10, 100 and 1000 mg/kg body weight of the FRFPML. Similarly, no record of death or any sign of toxicity and behavioral change was recorded in the groups administered 1900, 2600 and 5000 mg/kg body weight of the FRFPML.

Table 2. *Results of Phase I and II of the acute toxicity test of the FRFPML.*

Groups	Dose of extract (mg/kg b.w.)	Mortality	Behavioral changes
Phase I			
Group 1	10	0/3	Nil
Group 2	100	0/3	Nil
Group 3	1000	0/3	Nil
Phase II			
Group 1	1600	0/3	Nil
Group 2	2900	0/3	Nil
Group 3	5000	0/3	Nil

3.3. Effect of FRFPML on Egg Albumin-induced Rat Paws Oedema

Table 3 shows the effect of FRFPML on egg albumin-induced rat paw edema. The result has it that mean paw oedema and percentage inhibition of egg albumin-induced oedema in the rat paw which was sustained over a period of 24 hours. A significant ($p<0.05$) reduction in the mean paw oedema was observed for all the treatment groups from 1 hour to 24 hours when compared to the toxic group. There were no significant ($p>0.05$) reductions in the mean paw oedema of rats in the toxic group at the different time intervals. The paw size of animals treated with increasing doses of the fraction and indomethacin significantly decreased with time.

Table 3. *Effect of FRFPML on Egg Albumin- Induced Rat Paw Oedema.*

Mean Paw Size (cm) and %Inhibition			
TreatmentGroup	**0 Hour**	**1 Hour**	**2 Hours**
Toxic group	$2.50±0.32^{b}$	$4.70±0.68^{a}$ (46.41%)	$7.90±1.97^{b}$ (67.05%)
Indomethacin (10mg/kgb.w)	$1.80±0.25^{a}$	$7.09±01.27^{bc}$ (73.88%)	$4.86±0.71^{a}$ (61.91%)
FRFPML (200mg/kgb.w)	$2.05±0.16^{a}$	$5.98±0.29^{ab}$ (65.67%)	$5.43±0.19^{a}$ (62.21%)
FRFPML (400mg/kgb.w)	$2.50±0.27^{b}$	$7.71±1.36^{c}$ (67.19%)	$6.02±0.59^{a}$ (58.50%)
FRFPML (600mg/kgb.w)	$2.68±0.12^{b}$	$7.62±0.55^{c}$ (64.68%)	$6.34±0.31^{a}$ (57.68%)

Table 3. *Continued.*

Mean Paw Size (cm) and %Inhibition			
TreatmentGroup	**3 Hours**	**4 Hours**	**24 Hours**
Toxic group	$7.21±1.88^{c}$ (63.90%)	$3.75±0.37^{c}$ (33.45%)	$3.61±0.30^{c}$ (30.96%)
Indomethacin (10mg/kgb.w)	$3.31±0.25^{a}$ (45.43%)	$2.29±0.26^{a}$ (21.51%)	$2.35±0.34^{a}$ (23.01%)
FRFPML (200mg/kgb.w)	$4.28±0.32^{ab}$ (52.01%)	$3.19±0.49^{b}$ (34.60%)	$2.71±0.25^{ab}$ (24.34%)
FRFPML (400mg/kgb.w)	$4.90±0.57^{b}$ (48.22%)	$3.55±0.21^{bc}$ (29.44%)	$3.00±0.25^{b}$ (16.68%)
FRFPML (600mg/kgb.w)	$5.28±0.69^{b}$ (48.53%)	$3.48±0.28^{b}$ (22.88%)	$3.04±0.23^{b}$ (11.82%)

Results expressed as Mean±Standard Deviation (n=6). Mean values with different letters as superscripts across the column are considered significant at $p<0.05$.

3.4. Effect of FRFPML on Albumin Denaturation

Table 4 shows the effect of FRFPML on albumin denaturation. The FRFPML exhibited a concentration dependent of inhibition of protein (albumin) denaturation. Different concentrations of the fraction exhibited significant ($P<0.05$) percentage inhibition. The highest percentage of inhibition (53.88%) was observed at the highest concentration of the FRFPML. These results were comparable to the standard drug (aspirin) used as it also exhibited concentration dependent inhibition of albumin denaturation.

3.5. Effect of FRFPML on Hypotonicity-induced Haemolysis of HRBCs

Table 5 shows the effect of FRFPML on membrane stabilization. The fraction showed a significant ($p<0.05$) percentage inhibition of hyponicity-induced lysis of HRBC at 1.0 mg/ml compared to other concentrations. The percentage inhibition increases with increase in concentrations of the plant fraction. This is comparable to the standard drug (prednisolone) used at concentrations of 1.0 mg/ml.

Table 4. *Effect of FRFPML and standard drug (aspirin) on albumin denaturation.*

Groups	Concentration (mg/ml)	Absorbance±SD$_{660nm}$	Percentage (%) Inhibition
Control	-	0.89±0.00	-
FRFPML	0.2	0.59±0.00^{e}	33.41±0.45^{a}
	0.4	0.52±0.01^{d}	41.39±1.19^{b}
	0.6	0.48±0.01^{c}	46.12±1.03^{c}
	0.8	0.45±0.00^{b}	49.79±0.36^{d}
	1.0	0.41±0.01^{a}	53.88±1.07^{e}
Aspirin	0.8	0.42±0.00^{b}	52.01±0.17^{d}
	1.0	0.35±0.00^{a}	60.48±0.39^{e}

Results are expressed in Means±SD (n=3). Mean values with different letters as superscripts across the column are considered significant at $p<0.05$.

Table 5. *Effect of FRFPML on hypotonicity-induced haemolysis of HRBCs.*

Group	Conc. (mg/ml)	Hypotonic solution	Isotonic solution	% inhibition of haemolysis
Control	-	0.62±0.00^{e}	0.40±0.01^{f}	-
Fraction	0.2	0.62±0.01^{e}	0.37±0.01^{e}	33.30±1.15^{a}
	0.4	0.56±0.01^{c}	0.33±0.01^{d}	39.83±0.55^{b}
	0.6	0.55±0.01^{c}	0.30±0.00^{d}	43.30±1.04^{c}
	0.8	0.36±0.01^{d}	0.25±0.01^{c}	60.92±1.27^{d}
	1.0	0.24±0.01^{b}	0.22±0.01^{c}	74.21±0.98^{e}
Prednisolone	0.8	0.15±0.01^{a}	0.19±0.02^{b}	71.47±0.72^{f}
	1.0	0.21±0.01^{b}	0.15±0.05^{a}	74.43±0.43^{e}

Results are expressed in Means±SD (n=3). Mean values with different letters as superscripts across the column are considered significant at $p<0.05$.

3.6. Effect of FRFPML on Protease Activity

Table 6 shows the effect of FRFML on Protease activity. The concentration of the fraction showed a significant ($p<0.05$) increase in the percentage inhibition on the Protease activity (1.0mg/ml). When compared to the other concentrations, the maximum enzyme activity was observed at 1.0mg/ml with a corresponding percentage inhibition of 58.93%. As the concentration increases from 0.2mg/ml to 1.0mg/ml, the percentage enzyme activity increases with a comparatively increase of percentage inhibition. The standard drug used (prednisolone) followed a similar trend when compared to the fraction.

Table 6. *Effect of FRFPML on protease Activity.*

Group	Concentration (mg/ml)	Absorbance±SD$_{210nm}$	Percentage (%) Inhibition
Control	-	0.44±0.01^{e}	-
FRFPML	0.2	0.26±0.00^{d}	42.50±1.24^{a}
	0.4	0.25±0.00^{cd}	44.00±0.57^{ab}
	0.6	0.24±0.00^{c}	45.42±0.34^{b}
	0.8	0.24±0.00^{b}	47.07±0.98^{c}
	1.0	0.18±0.00^{a}	58.93±0.91^{d}
Prednisolone	0.8	0.15±0.01^{b}	66.67±1.03^{d}
	1.0	0.12±0.00^{a}	72.75±1.03^{e}

Results are expressed in Means±SD (n=3). Mean values with different letters as superscripts across the column are considered significant at $p<0.05$.

Table 7. *Effect of FRFPML on Phospholipase A_2 Activity.*

Group	Concentration (mg/ml)	Absorbance±SD$_{418nm}$	Percentage (%) Inhibition
FRFPML	0.2	0.23±0.00^{e}	47.58±0.68^{a}
	0.4	0.21±0.01^{d}	52.95±1.35^{b}
	0.6	0.19±0.00^{c}	56.97±0.93^{c}
	0.8	0.18±0.00^{b}	59.66±1.01^{d}
	1.0	0.16±0.01^{a}	63.61±1.27^{e}
Prednisolone	0.8	0.25±0.01^{b}	71.47±0.72^{d}
	1.0	0.23±0.00^{a}	74.43±0.43^{e}

Results are expressed in Means±SD (n=3). Mean values with different letters as superscripts across the column are considered significant at $p<0.05$.

Table 8. *DPPH radical scavenging activity of the FRFPML.*

Group	Concentration (mg/ml)	Absorbance±SD$_{517nm}$	Percentage (%) Inhibition
Control	-	0.29±0.01^{e}	-
FRFPML	0.2	0.18±0.01^{e}	35.26±2.50^{a}
	0.4	0.16±0.01^{d}	39.00±2.53^{b}
	0.6	0.13±0.00^{c}	48.65±0.77^{c}
	0.8	0.11±0.00^{b}	55.34±0.80^{d}
	1.0	0.10±0.00^{a}	59.59±1.82^{e}
Ascorbic acid	0.6	0.15±0.01^{c}	78.38±0.77^{f}

Results are expressed in Means±SD (n=3). Mean values with different letters as superscripts across the column are considered significant at $p<0.05$.

Table 9. *H_2O_2 radical scavenging activity of the FRFPML.*

Group	Concentration (mg/ml)	Absorbance±SD$_{230nm}$	Percentage (%) Inhibition
Control	-	0.46±0.01^{e}	-
FRFPML	0.2	0.29±0.00^{e}	35.75±0.83^{a}
	0.4	0.23±0.00^{d}	48.50±0.67^{b}
	0.6	0.21±0.00^{c}	53.11±0.46^{c}
	0.8	0.19±0.00^{b}	59.12±0.58^{d}
	1.0	0.17±0.00^{a}	61.90±0.71^{e}
Ascorbic acid	0.6	0.15±0.01^{b}	63.97±0.44^{e}

Results are expressed in Means±SD (n=3). Mean values with different letters as superscripts across the column are considered significant at $p<0.05$.

3.7. Effect of FRFPML on Phospholipase A_2 Activity

Table 7 shows the effect of FRFPML on phospholipase A_2 activity. The FRFPML significantly ($p<0.05$) inhibited the PLA_2 at varying concentrations. The highest percentage of inhibition of the fraction, 63.61%, was observed at the highest concentration 1.0 mg/ml. The standard drug, prednisolone followed a similar trend as the plant fraction.

3.8. DPPH Radical Scavenging Activity

The result shows that the ability of the FRFPML to scavenge DPPH radical increased significantly with increase in concentration with % inhibition of 59.59±1.82 at 1.0 mg/ml. This result is comparable to the standard ascorbic acid used which also showed a higher percentage inhibition at 1.0 mg/ml.

3.9. H_2O_2 Radical Scavenging Activity

Data from table 9 shows that the fraction significantly ($p<0.05$) inhibited H_2O_2 radicals when compared to control. The fraction exhibited a concentration dependent inhibition of H_2O_2 radicals. This result is comparable with the standard drug ascorbic acid, which showed a percentage inhibition 63.97%.

4. Discussion

In the present study *in-vivo*, *in-vitro* anti-inflammatory and *in-vitro* anti-oxidant based assays were used to find out the anti-inflammatory activities and anti-oxidant capacities of the FRFPML. The quantitative phytochemical analysis of the plant contains tannins, phenols, flavonoids, alkaloids, terpenoids, glycosides, steroids and saponins in varying amounts, as shown in table 1.

Flavonoids which were shown to be present in high amounts in the plant have been reported to possess free radical scavenging activity and are known to modify the production of cyclooxygenase (COX-1 and COX-2) involved in the prostaglandin synthesis [38].

Some medicinal plants' anti-inflammatory properties are thought to be due to these bioactive constituents [39]. Flavanoids exert their anti-inflammatory activities through various mechanisms such as inhibition of transcription factors and regulatory enzymes. These variables play a significant role in the control of mediators involved in inflammation [40]. Acute toxicity investigations have shown that oral dosages of FRFPML have high safety profile. The plant fraction was tolerated by animals up to 5000 mg/kg and no deaths were reported as shown in table 2.

The impact of FRFPML on egg albumin-induced paw oedema in rats is shown in Table 3. The extract's ability to suppress the early phase of oedema suggests that it inhibits the release of histamine and serotonin. The suppression of oedema during the second and third phases of inflammation shows that the FRFPML anti-inflammatory activity is attributable to the suppression of kinin and prostaglandin production induced by egg albumin during this period. Since these mediators cause oedema by increasing vascular permeability and vasodilatation at the site of injury, the FRFPML lowers vascular permeability and fluid exudation, most likely by preventing the contraction of endothelial cells, and therefore suppresses oedema.

Table 4 shows that the plant fraction was effective in inhibiting denaturation of egg albumin (protein). At the highest concentration (1.0 mg/ml), the FRFPML was inhibited by the highest percentage (53.88%), whereas the standard drug was inhibited by the highest percentage (60.48%). The ability of a medicinal plant extract to inhibit thermally induced protein denaturation is an indication of its anti-inflammatory activity [41]. Various nonsteroidal anti-inflammatory drugs (NSAIDs) such as prednisolone, aspirin

and diclofenac sodium exert their action through protection against protein denaturation [42].

In table 5, the FRFPML was effective in inhibiting lysis induced by hypotonic solution at doses ranging from 0.2-1.0mg/ml. When erythrocytes are exposed to harmful stimuli or external substances such as heat, hypotonic medium or formaldehyde, lysis of membranes occurs which is accompanied by haemolysis and haemoglobin oxidation [43]. Because erythrocyte membranes are similar to lysosomal membranes, inhibition of erythrocyte lysis induced by hypotonic solution can be a convenient technique to measure a plant's anti-inflammatory property [44]. Membrane stability which is an anti-inflammatory indicator is linked to protection against hypotonicity-induced haemolysis [38].

Evaluation of protease inhibitor action of the FRFPML in table 6 showed that it significantly inhibited protease activity. Protease activity was significantly ($p<0.05$) inhibited at various doses of the plant fraction. At test concentration of 1.0 mg/ml, the highest percentage of inhibition was observed at 58.93%. The lysosomal granules of neutrophils contain serine proteases [45]. Leukocyte Proteases are important in the development of tissue damage during inflammation [42].

Table 7 examined the effect of FRFPML on Phospholipase A_2 activity and found that different doses of the fraction caused significant ($p<0.05$) concentration-dependent inhibited of Phospholipase A_2 activity. Phospholipase A_2 is an enzyme that breaks down membrane phospholipids to release free fatty acids. The cyclooxygenase (COX) and lipoxygenase (LOX) enzymes react with the arachidonic acid produced by these phospholipids, resulting in the creation of lipid mediators [44]. The action of COX on arachidonic acid produces mediators such as PGE_2, PGD_2, PGI_2 and TXA_2 while the action of 5-LOX on arachidonic acid releases leukotrienes such as LTB4. PLA_2 activity was significantly ($p<0.05$) inhibited by the FRFPML at concentrations ranging from 0.2 to 1.0 mg/ml. This was demonstrated by reduced absorbance, as the inhibition prevented PLA_2 from acting on the erythrocyte membrane, resulting in reduced hemoglobin leakage which absorbs maximally at 418 nm. The plant inhibition of PLA_2 suggests that it was able to prevent the release of free fatty acids from RBC membrane phospholipids, thereby depriving COX and LOX substrates for the synthesis of inflammatory mediators, hence limiting their effects such as vasodilatation, vascular permeability, chemotaxis and pain, and thus preventing inflammation. This inhibition also implies that the FRFPML has potentials in curing cancer and atherosclerosis as PLA_2 has been implicated in their etiology [38]. The mechanism of inhibition of PLA_2 by the fraction could be similar to that of steroidal anti-inflammatory drugs (corticosteroids) which induce lipocortin [46]. This inhibitory effect of the plant fraction could be attributed to the presence in the fraction of phytochemical constituents such as flavonoids [47].

Injury to tissues during inflammation produces free radicals such as ROS and reactive nitrogen species (RNS) which impair cellular functions [48]. These radicals are highly reactive and cause lipid peroxidation as well as oxidative damage to nucleic acids and proteins in cellular membranes. Overproduction of free radicals without increased radical scavenging ability result in oxidative stress [49]. Flavonoids posses' anti-inflammatory activity due to its inhibitory activity on free radical production and capacity to scavenge RNS, ROS and other reactive species [50, 51]. The FRFPML significantly inhibited ($p<0.05$) DPPH and H_2O_2 radicals as shown in tables 8 and 9 respectively.

5. Conclusion

Findings from the study reveal that the FRFPML possesses remarkable anti-inflammatory and anti-oxidant activities. This study therefore shows that FRFPML has modulatory effect on the vascular changes that occur during inflammation. Many anti-inflammatory plants and agents modify inflammatory responses by accelerating the destruction of or antagozing the action of the mediators of inflammatory reaction. The anti-inflammatory and anti-oxidant activities of the plant fraction may also be due to the synergistic activity of various photochemical compounds present. The results indicate that the plant can be a potential source of anti-inflammatory agents if utilized.

6. Suggestions for Further Study

1. Further toxicity studies; sub-acute and chronic toxicity tests using different animal should be carried out in order to determine the long-term effects of the plant extract.
2. Further studies to elucidate the exact mechanism of action of the plant extract need to be carried out.
3. Further studies ought to be carried out to purify, isolate and characterize the bio-active compounds present in the plant extract.

Credit Authorship Contribution Statement

OCE: Conceptualization, Supervision and Editing of the manuscript.

CCA: Methodology, Supervision, Writing of the original draft of the manuscript, Editing of the manuscript, Analyzed and Interpreted the Data.

JIO: Editing of the manuscript.

UCO: Editing of the manuscript.

ECO: Funding acquisition, Methodology, Investigation, Writing of the original draft of the manuscript.

ENO: Funding acquisition, Methodology, Investigation.

CFE: Funding acquisition, Methodology, Investigation.

CFO: Funding acquisition, Methodology, Investigation.

SOM: Funding acquisition, Methodology, Investigation.

CEF: Funding acquisition, Methodology, Investigation.

NHO: Funding acquisition, Methodology, Investigation.

Data Availability

The numerical data used to support the findings of this study are available from the corresponding author upon request.

Declaration of Competing Interest

The authors declare that they have no known competing interest that could have appeared to influence the work reported in this paper.

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