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Modulation of Pro-Inflammatory Cytokines by Ethanol Leaf Extract and Aqueous Fraction of Rothmannia longiflora Salisb

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ABSTRACT

Background: *Rothmannia longiflora* leaf is is a plant found in Nigeria and in most African countries which is used in the management of pain and inflammation in herbal medicine. Previous studies had demonstrated its analgesic and anti-inflammatory properties but there is no further study on pro-inflammatory cytokine, hence the need for this study.
Objectives: To investigate the anti-inflammatory role of ethanol leaf extract and the residual aqueous fraction of *Rothmannia longiflora* Salisb on pro-inflammatory cytokines.
Methods: The qualitative phytochemical evaluation, acute toxicity (LD_{50}) and anti-inflammatory potentials of ethanol leaf extract of *R. longiflora* and its residual aqueous fraction on pro-inflammatory cytokines were investigated by Trease and Evans guidelines, OECD 423 and ELISA kits methods respectively. The animals received graded doses of 250, 500 and 1000 mg/kg for groups 1, 2 and 3 respectively of ethanol extract of *R. longiflora* (EERL) and its residual aqueous fraction (RAQF) while group 4 received distilled water 1 mL/kg as a negative control and group 5 received Aspirin 300 mg/kg as a positive control.
Results: The results of qualitative evaluation revealed the presence of flavonoids, tannins, saponins, steroids glycosides, anthraquinones and phenols. The acute oral toxicity (LD_{50}) of the EERL and RAQFRL in rats was found to be greater than 5000 mg/kg body weight. The EERL and RAQFRL demonstrated anti-inflammatory activity significantly ($p<0.01$ and $p<0.001$) and dose-dependently decreased paw oedema of the animals compared to the negative control; The anti-inflammatory activity of the EERL significantly ($p<0.001$) inhibited pro-inflammatory cytokines; interleukins-1β and tumor necrosis factor-α (IL-1β and TNF-α) and significantly ($p<0.05$) increased the concentrations of interleukin-6 (IL-6) compared to the negative control. Thus, confirming its anti-inflammatory properties.
Conclusion: The EERL and RAQFRL were found to decrease the concentrations of pro-inflammatory cytokines (IL-1β and TNF-α) and decrease the paw oedema of the animals thus validating its anti-inflammatory properties.

INTRODUCTION

Inflammation is a natural biological response produced by the tissues within the body as a reaction to harmful stimuli in order to eradicate the necrotic cells and initiate the tissue repairing process. The cardinal signs of inflammation include the hot inflamed sites due to increase in blood flow towards the region, redness and swelling due to vascular permeability pain caused by the activation and sensitization of primary afferent neurons and lasting

loss of function. The localized inflammatory response then induces the release of free arachidonic acid (AA) from the phospholipids which are converted into prostaglandins (PG) via the cyclooxygenase (COX) pathways[1]. Pain from the inflammation can be classified into two; chronic and acute pain. The main anti-inflammatory drugs (betamethasone, prednisolone, dexamethasone, aspirin, diclofenac, ibuprofen, indomethacin, naproxen, and celecoxib)[2] used in the management of inflammatory diseases such as osteoarthritis and rheumatoid arthritis are either steroids or non-steroidal anti-inflammatory drugs (NSAIDs)[3]. The prolong use of these drugs is associated with various side effects; for example, steroidal drugs cause adrenal atrophy, osteoporosis, suppression of response to infection and injury, euphoria, cataract and glaucoma[4] while the non-steroidal drugs peptic ulcers and bronchospasm due to blockade of both the physiological inflammatory prostaglandins and concurrent production of leucotrienes[5]. Due to these adverse effects[6] and their high cost, the search for new anti-inflammatory agent from the wealth of herbal sources becomes necessary, with the aim of obtaining better efficacy, greater safety and less expensive drugs for effective management of inflammatory diseases. Phytomedicine has gain recognition as an epitome of alternative medicine and have been utilized for the search of many bioactive substances used as medicine[8]. *R. longiflora* has been scientifically proven to possess significant anti-inflammatory and analgesic properties[7], however, further study of the role of anti-inflammatory property of ethanol leaf extract and the residual aqueous fraction of *R. longiflora* Salisb on pro-inflammatory cytokines have not been investigated. Many medicinal plants have demonstrated anti-inflammatory activities but the mechanism of action of most of them have not been investigated. Therefore, the aim of this study is to investigate the mechanism of anti-inflammatory role of ethanol leaf extract and the residual aqueous fraction of R. longiflora Salisb on pro-inflammatory cytokines.

METHODS

Materials

Leaves of *R. longiflora*, animal cages, mortar and pestle, digital weighing balance (Mettler-Toledo GmbH, Switzerland), digital vernier caliper (Shinwa Rules Co., Ltd, Japan), syringes, needles, oral cannula, hand gloves, and animal cages.

Laboratory animals

Wistar rats (120–180g) were obtained from the Animal House Facility of the Department of Pharmacology and Therapeutics, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria, Nigeria. The animals were maintained at room temperature and were fed with rodent Chikun feed, Kaduna and water *ad libitum.* The experiments were carried out after securing approval from the Ahmadu Bello University Committee on Animal Use and Care (ABUCAUC) with approval number: ABUCAUC/2021/067.

Chemicals and reagents

Carrageenan, Ethanol, Ethyl acetate, n-Butanol, Hexane (Sigma Aldrich, Saint Louis, Missouri, USA), Sodium hydroxide, Magnesium chips, Hydrochloric acid, Drangedorff's reagent, Wagner's reagent, Mayer's reagent, Glacial acetic acid, Ferric chloride, Sulphoric acid, Acetic anhydride, Chloroform, Ammonia, Molish reagent (Loba Chemie, Mumbai, India).

Collection and identification of plants

The plant sample comprising of the stem, fruits and the leaves were collected from Bagauda forest along Kaduna-Kano Road in October, 2019 and were identified by a Taxonomist, Mal. Namadi Sanusi in the Department of Botany, Faculty of Life Sciences, Ahmadu Bello University, Zaria, Nigeria by comparing with the existing specimen (voucher number: ABU037195) previously deposited in the herbarium.

Extraction and fractionation of the plant material

The extraction and fractionation of the plant material were carried out according to the method described by Abubakar and Haque[10]. The leaves of *R. longiflora* were air-dried at room temperature for several days until constant weight was obtained. The dried material was size reduced into powder with the aid of an electrical blender. The powdered material (2 kg) was extracted with 70% (2.8 L) ethanol and 30% water (1.2 L) by cold maceration for 48 hours with occasional shaking. The ethanol solvent was recovered through rotary evaporator. The macerated plant filtrate was evaporated to dryness in the oven at 40 ^{o}C to obtain solid ethanol extract. The ethanol extract was then stored in a bottle in a refrigerator prior to use, solution of the extract was freshly prepared with distilled water for each study. The percentage yield of the extract was calculated using the formula below:

$$\text{Percentage yield} = \frac{\text{Weight of the extract}}{\text{Weight of powdered material}} \text{ X } 100$$

One hundred grams (100 g) of the ethanol extract was weighed and fractionated by suspending in water (500 mL / 5 times) and partitioned with Hexane (500 mL /5 times) to obtain the Hexane fraction. The aqueous layer was further partitioned with Dichloromethane (500 mL /5 times) to obtain the Dichloromethane fraction (DF), and Ethyl acetate (500 mL /5 times) to obtain the Ethyl acetate fraction (EAF). The aqueous layer was further partitioned with n-Butanol (500 mL /5 times) to obtain the n-Butanol fraction (n-BUTF) and the aqueous residue was concentrated to obtain residual aqueous fraction (RAQF). The n-Butanol and residual aqueous fractions were concentrated on water bath set at 40°C while the Hexane, Dichloromethane and Ethyl-acetate fraction were air dried. The concentrated and dried fractions were stored in separate desiccators in the refrigerator ready for use. Solutions of the fractions were prepared freshly with distilled water for each study.

Qualitative phytochemical screening

The Ethanol leaf extract of *R. longiflora* (EERL) and RAQFR, were subjected to phytochemical screening according to the methods described by Trease and Evans[11], Sofowora[12]. They were screened for the presence of flavonoids, alkaloids, saponins, glycosides, tannins, steroids, carbohydrates, anthraquinones and triterpenes.

Acute toxicity study

Median lethal dose (LD_{50}) determination was conducted using Organization for Economic Co-operation and Development (OECD 423) guidelines in rats. In this method, two groups of three animals each were fasted before dosing (food but not water was withheld for 3 hours). The fasted body weight was determined for each animal and the dose was then calculated according to the body weight. In the first phase of LD_{50} determination, 2000 mg/kg of EERL and RAQFRL were administered to each rat using oral cannula and the rats were observed for 48 hours for clinical sign and symptoms of toxicity including death. The second phase was conducted similarly to the first but with dose of 5000 mg/kg. At the end of this test, rats that survived were euthanized with diethyl ether and subsequently incinerated.

Acute anti-inflammatory study in rats (Carrageenan-induced oedema)

Anti-inflammatory activity was determined using the method of Winter[13]. Rats were divided randomly into five groups each consisting of 5 rats. Group 1 received distilled water 1 mL/kg (negative control), Group 2 received 250 mg/kg, Group 3 received 500 mg/kg, Group 4 received 1000 mg/kg of EERL and Group 5 received Acetylsalicylic acid (ASA) 300 mg/kg (positive control). The test drugs were administered orally sixty minutes before carrageenan injection. Rats were injected with 0.1 ml 1% carrageenan on the right hind paw. The hind paw thickness was measured and recorded at 0, 1, 2, 3, 4, and 5 hours using vernier caliper. The percent inhibition of edema was calculated for each group with respect to its vehicle-treated control group. The same procedure was repeated for the residual aqueous fractions with the same doses as in the ethanol leaf extract above.

% inhibition =

$$\frac{\text{Mean increase in paw volume of control} - \text{Mean increase in paw volume of treated}}{\text{Mean increase in paw volume of control}} \times 100$$

Investigating involvement of inflammatory cytokines in the anti-inflammatory activities of *R. longiflora*

The method described by Santos[14] was used to investigate the effects of EERL and its RAQF on inflammatory cytokines. Twenty-five rats were divided into 5 groups containing 5 rats in each group and were orally administered distilled water 1 mL/kg, Group 1, ASA 300 mg/kg, Group 2, Minocycline 30 mg/kg, Group 3, RAQF 1000 mg/kg and EERL 1000 mg/kg Group 5. One hour after the treatments, each rat was injected with 0.1 ml of 1% carrageenan into the planter surface of the rat's right hind paw[13]. Six hours after carrageenan injection rats were euthanized using diethyl ether and the hind paws were removed at the level of the calcaneus bone. The paws were homogenized using phosphate buffered saline and centrifuged at 10,000 RCF for 10 minutes and oedema fluid were collected for quantification of the concentrations of the cytokines (IL 1β, IL 6 and TNF-α) by ELISA method.

Quantify of interleukins (IL)-6, IL-1β, and Tumour Necrosis Factor-alpha (TNF-α) using the ELISA method, a suitable biological sample must be collected, such as serum or tissue homogenate, store it appropriately, then perform a quantitative sandwich ELISA following the manufacturer's protocol. This involves immobilizing antibodies on a plate, adding the sample, followed by a detection antibody, and finally a substrate that produces a colour. The colour intensity, measured by a microplate reader, is then compared to a standard curve to determine the concentration of each cytokine.

General Steps for ELISA Quantification: 1. Sample Collection and Preparation Collection: Collect blood or other relevant biological samples (e.g., urine, tissue). Processing: Centrifuge blood samples to separate serum or plasma, or homogenize tissue to

extract proteins. Storage: Store samples at very low temperatures (-70°C or -80°C) to prevent degradation and interference. 2. ELISA Procedure (following manufacturer's instructions) Antibody Immobilization: Antibodies specific to the target cytokine (IL-6, IL-1β, or TNF- α) are coated onto the wells of a microplate. Sample Addition: Add the prepared samples and a standard curve of known cytokine concentrations to separate wells. Incubation: Incubate to allow the target cytokines in the samples and standards to bind to the immobilized antibodies. Washing: Wash the wells to remove unbound materials. Detection Antibody: Add a biotinylated secondary antibody that binds to the captured cytokine. Washing: Wash again to remove unbound detection antibodies. Enzyme Conjugation: Add streptavidin conjugated to an enzyme (like horseradish peroxidase, HRP). Washing: Wash to remove unbound enzyme conjugate. Substrate Addition: Add a substrate solution that reacts with the enzyme to produce a coloured product. 3. Measurement and Calculation: Reading the Plate: Read the optical density (colour intensity) of each well using a microplate reader. Standard Curve: Create a standard curve by plotting the absorbance values of the known standards against their respective concentrations. Concentration Determination: Determine the cytokine concentrations in the samples by comparing their absorbance values to the standard curve.

Statistical Analysis

Data were expressed as Mean ±Standard Error of Mean and were presented as Figures and Tables. Data were analyzed using One way or Repeated Measure Analysis of Variance (ANOVA) using SPSS version 20 followed by Bonferroni's post-hoc test where appropriate. Values of $p<0.05$ were considered significant.

RESULTS

The weight and percentage yield of the extract and the fractions were determined and were expressed (Table 1).

Table 1. Weight and percentage yield of the fractions of ethanol leaf extract of *Rothmannia longiflora*

Extract/Fractions	Weight (g)	Percentage yield (%)
Ethanol Leaf Extract (EERL)	500.0	25.0
Dichloromethane	120.0	6.0
Ethyl acetate	72.0	3.6
n-Butanol	100.0	5.0
Residual aqueous	108.0	5.4

Qualitative phytochemical evaluation

The result of the preliminary phytochemical screening of EERL and its fractions indicated the presence of various phytochemicals such as alkaloids, saponins, flavonoids, tannins, anthraquinones, carbohydrate, glycosides, triterpenes and steroids (Table 2)

Table 2. Phytochemical constituents of the ethanol leaf extract of *R. longiflora and its fractions*

Chemical constituents	Ethanol Extract	Dichloromethane Fraction	Ethyl acetate Fraction	n-Butanol Fraction	Residual Aqueous Fraction
Alkaloids	+	+	+	+	+
Saponins	+	+	+	+	+
Flavonoids	+	+	+	+	+
Tannins	+	+	+	+	+
Carbohydrate	+	+	+	+	+
Glycosides	+	-	-	-	+
Anthraquinones	+	+	+	+	+
Steroids	+	+	+	+	+
Triterpenes	+	+	+	+	+

+ = Present - = Absent

Acute oral toxicity test

In the acute toxicity test, the oral median lethal doses (LD_{50}) of EERL and all its RAQF) were estimated to be greater than 5,000 mg/kg in rats according to OECD 423 guidelines.

Effect of Ethanol Leaf Extract of *R. longiflora* and its Fraction in the Carrageenan-induced Paw Oedema Test in Rats

EERL and RAQF significantly ($p<0.05$, $p<0.01$, $p<0.001$) and dose-dependently (250, 500 and 1000 mg/kg) decreased paw oedema of the animals with maximum inhibition at the 3rd hour compared to the control (Table 3 and 4).

Table 3. Effect of ethanol leaf extract of *R. longiflora* in carrageenan-induced hind paw oedema in rats

Treatment mg/kg	Mean paw oedema index (mm)					
	T_0	T_1	T_2	T_3	T_4	T_5
D/W 1 mL/kg	2.40±0.09	2.93±0.08	3.52±0.07	3.97±0.05	3.67±0.08	3.47±0.08
EERL 250	2.35±0.10	2.78±0.07 (5.12)	3.22±0.10 (8.52)	3.82±0.10 (3.78)	3.50±0.07 (4.63)	3.38±0.08 (2.60)
EERL 500	2.42±0.05	2.78±0.05 (5.12)	3.00±0.06** (14.77)	3.23±0.08*** (18.64)	3.12±0.10*** (15.00)	3.00±0.09*** (13.55)
EERL 1000	2.42±0.08	2.50±0.09** (14.68)	2.83±0.08*** (19.60)	2.98±0.07***# (24.94)	2.87±0.08***# (21.80)	2.72±0.07*** (21.61)
ASA 300	2.43±0.05	2.47±0.07** (15.70)	2.98±0.05** (15.34)	3.37±0.05*** (15.11)	3.20±0.05*** (12.81)	2.98±0.05*** (14.12)

Values presented as Mean± S.E.M., ** = $p<0.01$, *** = $p<0.001$ compared to D/W, # = $p<0.05$ compared to T1. Oedema index was analysed using Repeated Measure ANOVA followed by Bonferroni's post hoc test, n = 6, D/W = Distilled water, EERL = Ethanol Extract of *R. longiflora* Leaf ASA = Acetylsalicylic acid. Figures in parenthesis represent the percentage inhibition of oedema paw.

Table 4. Effect of residual aqueous fraction of *R. longiflora* on carrageenan-induced inflammation in rats

Treatment mg/kg	Mean Increase in Rats Paw Diameter (mm)					
	T0	T1	T2	T3	T4	T5
D/W 1 (mL/kg)	2.53±0.10	2.93±0.09	3.52±0.10	4.35±0.11	3.87±0.10	3.61±0.14
RAQFRL 250	2.28±0.10	2.92±0.10 (0.34)	3.10±0.12** (11.93)	3.35±0.08*** (22.99)	3.18±0.11*** (17.83)	3.08±0.12*** (14.68)
RAQFRL 500	2.37±0.10	2.90±0.10 (1.02)	3.02±0.12** (14.21)	3.28±0.08*** (24.60)	3.15±0.11*** (18.61)	3.05±0.12*** (15.51)
RAQFRL 1000	2.28±0.10	2.33±0.10*** (20.48)	2.52±0.12*** (28.41)	2.78±0.08***# (36.09)	2.63±0.11***# (32.04)	2.52±0.12*** (30.19)
ASA 300	2.42±0.10	2.62±0.10* (10.58)	2.98±0.12*** (15.34)	3.37±0.08*** (22.53)	3.20±0.11*** (17.31)	2.98±0.12*** (17.45)

Values presented as Mean± S.E.M, Data were analysed using Repeated Measure ANOVA followed by Bonferroni's post hoc test, * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$ compared to D/W, # = $p<0.05$ compared to T1, n = 6, Figures in parentheses represent percentage of oedema inhibition, D/W = Distilled water, RAQF =Residual aqueous fraction of *R. longiflora*, ASA = Acetylsalicylic acid.

Effect of Ethanol Leaf Extract of *R. longiflora* and its Aqueous Fraction in Carrageenan-induced Alteration on Cytokines in Rats

EERL and RAQF (1,000 mg/kg) significantly ($p<0.05$ decreased the concentrations of TNF-α and IL-1β and significantly ($p<0.05$) increased the concentration of IL-6. The standard drug, ASA (300 mg/kg), significantly ($p<0.05$ reduced the concentration of TNF-α and IL-1β respectively compared with the control (Figure 1).

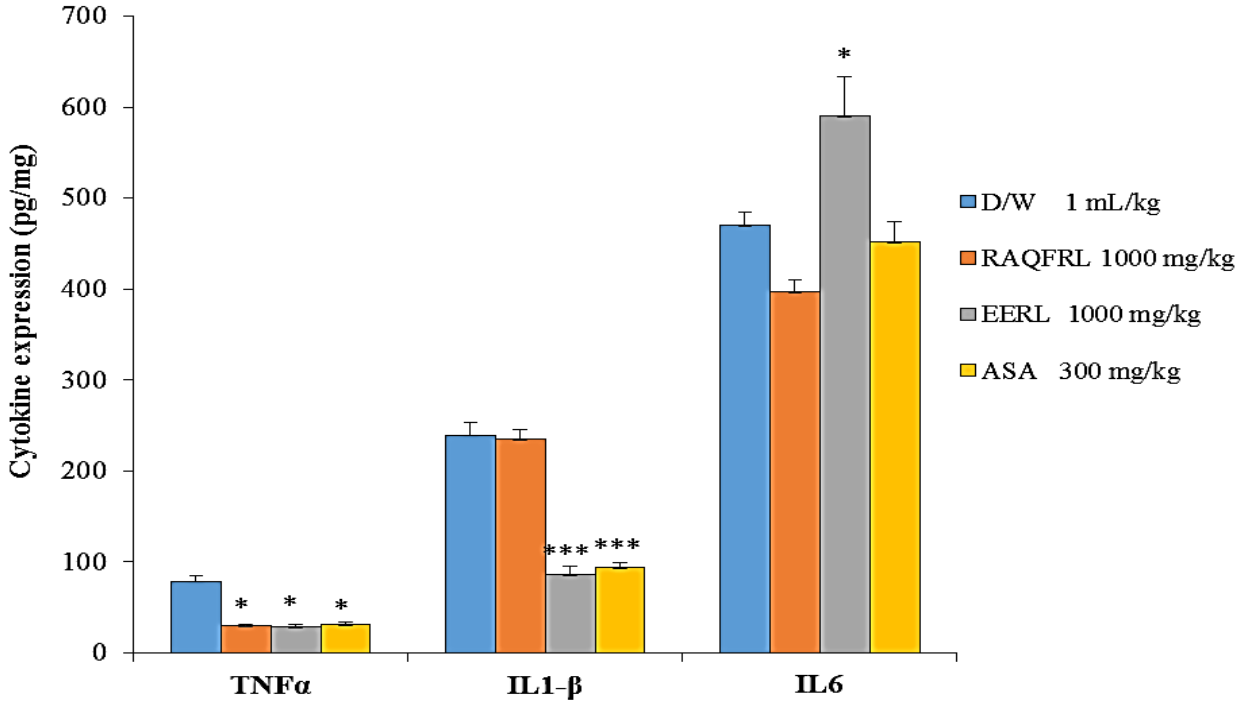

Figure 1: Effect of *R. longiflora* leaf extracts and its aqueous fraction on pro-inflammatory cytokines. Data were analyzed using One-way Analysis of Variance (ANOVA) followed by Bonferroni post-hoc test, n = 6, * = $p<0.05$, *** = $p<0.001$ compared D/water, IL = Interleukin, TNF-α =Tumor necrosis factor alpha.

DISCUSSION

The anti-inflammatory activity observed with the Ethanol extract of *R. longiflora* leaf (EERL) and its residual aqueous (RAQF) fraction suggest that they might be producing their anti-inflammatory effect through the inhibition of inflammatory mediators such as prostaglandins, serotonin and histamine, which is in agreement with the study carried out by Owolabi and colleagues[15], which demonstrated that the ethanol root bark of *Feretia apondanthera* (Rubiaceae) possess anti-inflammatory activity against carrageenan-induced pain[15]. Another study by Ekpo and colleagues also demonstrated that aerial parts of *Diodia samentosa* possesses anti-inflammatory activity. This further confirmed that medicinal plants that are from the same species, genera or family may have similar medicinal activity. Carrageenan is the phlogistic agent of choice for testing anti-inflammatory drugs as it is none antigenic and devoid of systemic effect. Carrageenan-induced inflammation is commonly used as an experimental animal model for screening the anti-inflammatory potency of compounds and natural products. It is a distinctive model of acute inflammation with greater reproducibility[16]. After Carrageenan injection, oedema develops mainly in two phases: the first 30 minutes after the injection, and the second begins at the end of the first hour and lasts until the third hour. The first phase has been attributed to the release of histamine, serotonin and bradykinin on vascular permeability and the later phase has been due to over production of prostaglandins in tissues and it is linked with the activation of the cyclooxygenase pathway[17],[18]. The model determines the oedema-inhibitory effects of pharmacological agents that inhibit inflammatory and pro-inflammatory chemical substances such as prostaglandin, histamine, leukotrienes, bradykinin and TNF-α[19]. The EERL and its aqueous fraction reduced the concentrations of TNF-α and IL-1β. These reductions in concentrations may be due to direct inhibition by EERL and its aqueous fraction or by IL-6 anti-inflammatory cytokine. Cytokines are modulators of inflammation; they participate in acute and chronic inflammation in a complex network of interactions. The functional definition of a pro-inflammatory cytokine is the ability of the cytokine to induce inflammation and that of anti-inflammatory cytokine is the ability to inhibit the synthesis of major pro-inflammatory cytokines such as TNF-α and IL-1β[20]. Histamine will in turn trigger early vasodilation and increase in vascular permeability. IL-6 has both pro and anti-inflammatory properties but possesses more of anti-inflammatory properties[17]. It inhibits the production of TNF-α and IL-1β and other pro-inflammatory cytokines[20]. The oral administration of EERL and its fraction at doses up to 5,000 mg/kg did not show any sign of toxicity or death in both rats. This suggests that the EERL and its fraction is relatively safe when administered orally. Acute toxicity study is carried out to determine the range of doses that could be toxic to the animal and also used

to estimate the therapeutic index of the drugs[21]. All the doses selected and subsequently used in this experiment were below 30% of the estimated LD50 which have been previously reported to be safe in ethnopharmacological study[21]. EERL and its residual fraction revealed the presence of phytochemicals such as alkaloids, saponins, flavonoids, tannins, glycosides, steroids, triterpenes and carbohydrates. These could be the reason for the anti-inflammatory activities of EERL and its fraction. Preliminary phytochemical screening gives an idea about the type of phytochemical constituents present in plant extracts or fractions[22]. These phytochemical constituents are known to be responsible for several pharmacological activities. Flavonoids, tannins, alkaloids, saponins, glycosides, steroids and triterpenes have been found to possess analgesic and anti-inflammatory activities[23]. Flavonoids including quercetin, bioflavonoids, luteolin and triterpenoids inhibit cyclooxygenase (COX), lipoxygenase, phospholipase A2 and C enzymes production[22].

CONCLUSION

The Ethanol leaf extract of *R. longiflora* and its residual aqueous fraction and demonstrated anti-inflammatory activities associated with inhibition of pro-inflammatory cytokines (IL-1β and TNF-α).

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AUTHORS' CONTRIBUTION

Research conceptualization: Danjuma Mallam, Nuhu Mohammed Danjuma, Mohammed Garba Magaji and Ben Ahmed Chindo; Experimentation: Danjuma Mallam. Data collection: Danjuma Mallam. Data Analysis: Abdullahi Balarabe Nazifi. Manuscript writing: Danjuma Mallam. Proofreading: Abdulfatai Adetunji Jimoh and Amina Busola Olorukooba. Supervision: Nuhu Mohammed Danjuma, Mohammed Garba Magaji and Ben Ahmed Chindo. Graphics' editing: Micah Timothy.

CONFLICT OF INTEREST

All authors declare no conflict of interest

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