Justicia secunda leaf aqueous fraction suppressed NF-κB, TNF-α, IL-6 and COX-2 in arthritic rat

ABSTRACT

Aims: This study evaluated the effect of aqueous fraction of *Justicia secunda* Vahl leaves on selected inflammatory markers (Tumor necrosis factor- alpha (TNF-α), nuclear factor–kappa B (NF-κB), interleukin-6 (IL-6) and cyclooxygenase-2 (COX-2) and its possible mechanism of action.

Study design*: J. secunda* aqueous leaf fraction (JSALF) was tested on carrageenan, arachidonic and Complete Freund's Adjuvant (CFA) induced rat paw inflammation models, respectively.

Methodology: Ninety male albino rats (Wistar strain) were randomly distributed into 6 groups of 5 rats each. Group I: normal, administered with 1 mL NaCl, Group II: untreated control, no treatment, Group III: administered with 10 mg/kg body weight (*b.wt.*) diclofenac sodium (reference drug), Group IV, V and VI: administered with 200, 400 and 600 mg/kg *b.wt. J. secunda* leaf aqueous fraction (JSALF), respectively. TNF-α, NF-κB, IL-6 and COX-2 levels were analysed using enzyme-linked immunosorbent assays (ELISA). Gas Chromatography-Mass Spectrometry (GC-MS) analysis was carried out.

Results: JSALF treated animals had significantly ($P < .05$) suppressed carrageenan, arachidonic and CFA-induced paw edema when compared with untreated control animals. Serum levels of TNF-α, NF-κB, IL-6 and COX-2 were significantly reduced ($P < .05$) in JSALF-treated animals. GC-MS detected 10 suspected anti-inflammatory compounds.

Conclusion: Findings from this study indicate that *J. secunda* aqueous leaf fraction substantially suppressed pro-inflammatory mediators and COX-2 pathways in arthritic rats which justifies its folkloric use in the management of inflammatory diseases.

Keywords: Anti-inflammation, complete freund's adjuvant, cyclooxygenase-2, interleukin-6 and *Justicia secunda*

1. INTRODUCTION

Inflammation participates essentially in the initiation and progression of arthritis. Chronic inflammation gives rise to inflammatory diseases which is typified in the generation of nitrogen species, reactive oxygen species and pro-inflammatory mediators which include lipid mediators, particularly leukotrienes, prostaglandins and cytokines including tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1) and interleukin-6. Provocation of these mediators proceeds to cause extreme damage to the host tissue [1].

When activated by inflammation, nuclear factor-κappa B which is a transcription factor commonly found in the cell cytoplasm moves to the nucleus in order to regulate the expression of various genes such as COX-2, inducible nitric oxide synthase and cytokines (IL-6, TNF-α, IL-1β, IL-8)

[2,3,4]. NF-κB plays varied roles in the development and continuation of rheumatoid arthritis. Stimulated NF-κB remains the usual feature found in the synovial membrane of arthritic individuals. Patients with rheumatoid arthritis present high levels of serum pro-inflammatory cytokines, such as TNFα, IL-1 and IL-6 recognized to be the targeted genes for NF-κB, indicating the stimulation of the signalling pathway of disease [4, 5].

The usage of non-steroidal anti-inflammatory drugs (NSAIDs) including aspirin, diclofenac and ibuprofen for pain relief has been the conventional treatment for rheumatoid arthritis. More so, various attempts have been made through the usage of corticosteroid or disease-modifying anti-rheumatic drugs (DMARDs) such as methotrexate (immune system suppressant). Despite their potency, there are limitations on their clinical long-term usage due to the adverse aftermath of these drugs on the digestive system, kidney and liver [3, 6]. Hence, medicinal plants are being relied on as an alternative source of therapy against inflammatory disorders as a result of its purported limited side effects.

J. secunda is a colourful plant with arboraceous stems, green leaves and pink flowers. It has a length of 90 – 200 cm. It is a long-lasting plant that survives in damp areas. It is commonly found in uncultivated bushes, jungles and alongside streams and gathered for native use as a treatment for disease [7]. The leaves of this plant are brewed like tea and are used traditionally to cleanse the womb after miscarriage and treat afterbirth related diseases in women. In addition, the tea is used by natives to relieve menstrual pain, treatment of fever, measles, anaemia, cold, cough, whooping cough, wound healing, abdominal pain and diabetes [8]. It is commonly referred to as "Blood root" and "Sanguinaria" in Barbados and Venezuela respectively [9]. Nigeria locally calls it "blood leaf" or "blood tonic". The South-Eastern region in Nigeria refers to it as either "Ulogwu Di Anya" (meaning: hospital too far) or "Obara bu ndu" (meaning: blood is life). Ogbia clan in South-South region of Nigeria refer to it as "Asindiri or Ohowaazara" (meaning: medicine that gives blood). Conventionally, hypertensive and anaemic conditions are ameliorated through the use of the red aqueous leaf extract of *J. secunda* by some communities in the Niger Delta region of Nigeria and other African countries [10]. Some religious organizations in South-Eastern Nigeria, Congo and South Co^te-d'Ivoire administer the leaf decoction to manage anaemic conditions [11].

Phytochemical assessment of *J. secunda* extract by researchers reveals the presence of the bioactive compounds such as anthocyanin, flavonoids, tannins, saponins, steroids and alkaloids [10, 11]. In addition, compounds including luteolin, auranamide, quindoline aurantamide acetate and pyrrolidone derivatives such as secundarellone A, B and C have been isolated from *J. secunda* [10, 12]. The plant's essential oil contains an abundant of polyenoic fatty acids and fatty acid methyl esters [13]. As a result, it is being used in the treatment of hyperlipoidemia and atherosclerosis [14]. The major compound 9,12,15-Octadecatrienoic acid methyl ester identified in *J. secunda* essential oil is reported to possess, cancer preventive, hypocholesterolemic, nematicide, anti-inflammatory, insectifuge, hepatoprotective, anti-histaminic, anti-eczemic, anti-acne, anti-arthritic, anti-androgenic and anti-coronary properties[13]. *J. secunda* possesses anti-sickling, haematinic and anti-hypertensive activities [11, 15, 16] The anti-nociceptive, antioxidant and anti-inflammatory effects of methanol leaf extracts of *J. secunda* has also been reported [16, 17].

Therefore, this study investigated the effect of aqueous fraction of *J. secunda* leaf on COX-2, TNF-α, IL-6 and NF-κB in arthritic rat and possibly proffer some mechanistic insight into the anti-inflammatory action.

2. MATERIALS AND METHODS

2.1 Plant Material

Fresh leaves of *J. secunda* were collected from an uncultivated farmland in Isiala-Ngwa North Local Government Area of Abia State, Nigeria. A specimen of the leaves was deposited, identified and certified at the Forestry Herbarium Ibadan, Oyo state, Nigeria with voucher number: FHI.112568.

2.1.1 Preparation of J. secunda extract and fractions

J. secunda leaves were removed from the plant, washed and dried in an oven at 40^{o}C for 3 days**.** The dried leaves were then pulverized using an electric grinder (Panasonic MX-AC 300, India) to a fine powdery form. The powdered leaf samples were extracted using 70% methanol in the ratio 1:8 w/v. For each 80 g of pulverised plant sample, 640 mL of methanol was used for the extraction which lasted for 3 days with random vibration with the use of mechanical shaker (Water Bath Thermostat Vibrator Model DKZ-2, China) at 40^{o}C. The methanol extract then was filtered with Whatman No. 1 filter papers and subsequently concentrated with rotary evaporator at 40^{o}C. The concentrated extract was reconstituted with distilled water in the ratio 1:2 and further partitioned using Kupchan [18] modified method of successive solvent partitioning in the following order of fraction: hexane (JSHLF), ethyl acetate (JSELF), n-butanol (JSBLF) and aqueous (JSALF) fractions. The various fractions were concentrated with rotary evaporator at 40^{o}C and were placed in the refrigerator at 4^{o}C for further use.

2.2 *In vitro* anti-inflammatory activity

2.2.1 Anti-protein denaturation assay

The effect of different fractions of *J. secunda* leaves on heat-induced protein denaturation was studied following the method described by Mizushima et al.[19] and Leelaprakash et al.[20] with minor modifications.

The mixture consisted of 50 µL of different concentrations of standard drug (diclofenac sodium), test fractions and methanol serving as control was contained in the separate test tubes. Bovine serum albumin (BSA) (450 µL) was added into each test tube and incubated for 20 minutes at 37^{o}C. Heat was applied by the use of water bath at 57^{o}C for 3 minutes. Phosphate buffer saline (2.5 mL, pH 6.3) was added into each tube after it was cooled. Measurement of the absorbance was done at 660 nm. The concentration sufficient to obtain 50% inhibition of BSA denaturation (IC_{50}) was estimated. The procedure was performed in triplicate. The percentage inhibition of protein denaturation was calculated as follows:

$$\mathrm{I}(\%) = \frac{(Ac - As)100}{Ac}$$

Where
I = % Inhibition
Ac = Abs control
As = Abs sample

2.3 Membrane stabilization assay

2.3.1 Preparation of red blood cell suspension

Ten millilitre of human whole blood was drawn and transferred to a vacuum tube containing anticoagulant (heparin) and centrifuged at 3000 revolution per minutes (rpm) for 10 minutes and were rinse with 10 mL of 0.9% NaCl up to three times. The blood was reconstituted as 10% v/v suspension with 0.9% NaCl to yield human red blood cell (HRBC) suspension [21].

2.4 Hypotonicity- induced haemolysis

The effect of different fractions of *J. secunda* leaves on hypotonicity- induced haemolysis were studied following the method as described by Chippada et al.[22].
One millilitre of phosphate (0.015 M, pH 7.4), 2 mL of hyposaline (0.36%), 0.5 mL HRBC suspension (10%), 0.5 mL of *J. secunda* extracts or reference drug (Diclofenac sodium) of varied concentrations of 50, 100, 250, 500 and 1000 µg/mL were mixed in test tubes. Distilled water was used as control to obtain 100% haemolysis. The mixture was incubated at 37^{o}C for 30 minutes and centrifuged at 3000 rpm. The haemoglobin content in the suspension was measured with UV-Visible spectrophotometer (Schimadzu Double Beam UV-2600, Japan) at 560 nm. The experiment was done in triplicate. Percentage inhibition of haemolysis was calculated using the formula:

$$\mathrm{I}(\%) = \frac{(Ac - As)100}{Ac}$$

Where
I = % Inhibition
Ac = Abs control
As = Abs sample

2.5 Experimental animals

Ninety-six male albino rats (Wistar strain) weighing 150 - 250 g were procured from Babcock University Animal Facility. Ninety animals were used for the 3 adopted models of inflammation while 6 rats were used for the acute toxicity. Acclimatization for the animals after procurement of animals was done in the animal facility for 14 days, housed in aerated plastic cages with natural lighting condition at room temperature. They were fed with pelleted rat chow and water *ad libitum*. All the experiments with the animals were conducted in conformity with the National Institute of Health (NIH) Guideline for Care and Use of Laboratory animals. In addition, Institutional ethical clearance was obtained from Babcock University Health Research Ethics Committee with certificate number: BUHREC 603/19.

2.6 Acute toxicity test

The up and down method of acute toxicity was used to determine the toxic level *of J. secunda* aqueous leaf fraction (JSALF) according to the Organisation for Economic Co-operation and Development guidelines for the testing of chemicals [23]. The animals were fasted overnight before the experiment. Random selection and weighing of six rats was done and placed in a cage. Two rats were orally administered with 2 g/kg of JSALF. They were observed for any signs of increased rate of breathing, fur removal or change in colour and mortality for 30 minutes, 240 minutes, 1 day and

2 days. Subsequently, the dosage was increased to 3.5 g/kg and 5 g/kg, respectively for the four remaining rats and subjected to the same scrutiny for 48 hours.

2.7 Animal experimental designs

Each animal model consisted of thirty male rats were randomly separated into six groups of 5 animals per group. Group I (normal) rats were administered with 1mL NaCl. Group II (negative control) were induced with edematogen and administered with 1mL of NaCl with no treatment. Group III (positive control) rats were induced with edematogen and treated with 10 mg/kg body weight (*b.w*.) diclofenac sodium (reference drug). The remaining three groups were induced with edematogen and treated with varied doses of JSALF at 200, 400 and 600 mg/kg *b.w.* respectively. The drug and JSALF were dissolved in normal saline and oral administration was carried out using an oral cannula half an hour before inducing inflammation by the injection of the edematogen into the subplantar region of the rat left hind paw.

2.8 Induction of inflammation protocol

2.8.1 Model I: Carrageenan-induced rat paw edema

The effect of JSALF on carrageenan-induced paw edema in rats was evaluated in accordance with the method described by Winter et al.[24] with minor modification.
Edema was induced by injecting carrageenan (1% w/v carrageenan in normal saline, 0.1 mL) into the sub-plantar surface of the left hind paw of each rat. The hind paw thickness was measured before injection at 0, 30, 60, 120, 180 and 240 minutes after carrageenan injection with a micrometer screw gauge. The percentage inhibition of swelling was calculated using the following formula by Olukunle [25].

$$\text{Ip}(\%) = \frac{(\Delta vc - \Delta v)}{\Delta vc} \times 100$$

Where
Ip = % Inhibition of paw edema
Δvc = (Vt-Vo) control
Δv = (Vt-Vo)
Vt = the right hind paw thickness in mm at time t
Vo = the right hind paw thickness in mm before carrageenan injection

2.8.2 Model II: Arachidonic-induced paw edema

The effect of JSALF on arachidonic acid-induced paw edema in rats was evaluated using the method described by Bulani et al.[26]. Rats paw edema was produced by injecting 0.1 mL of 0.5% arachidonic acid prepared in 0.2 M carbonate buffer (pH 8.4) into the sub plantar region of the right hind paw of rats. The paw thickness was measured using a micrometer screw gauge prior to and immediately after arachidonic acid injection at 30, 60, 90 and 120 minutes.

$$\text{Ip}(\%) = \frac{(\Delta vc - \Delta v)}{\Delta vc} \times 100$$

Where
Ip = % Inhibition of paw edema
Δvc = (Vt-Vo) control

Δv = (Vt-Vo)
Vt = the right hind paw thickness in mm at time t
Vo = the right hind paw thickness in mm before carrageenan injection

2.8.3 Model III: Complete Freund's adjuvant-induced paw arthritis

The effect of JSALF on Complete Freund's Adjuvant (CFA)-induced arthritis in rats was evaluated using the method described by Miller & Tainter [27]. Arthritis was produced in rats by injecting CFA (0.1 mL of 10 mg suspension of heat killed *Mycobacterium tuberculosis* cells in mineral oil) into the right hind paw. Treatment lasted for 21 consecutive days and the hind paw of each rat was measured using micro meter screw gauge

2.9 Animal sacrifice and collection of blood samples

Animals were sacrificed on the 22nd day of Complete Freund's adjuvant-induced inflammation model study and blood samples were collected via cardiac and ocular punctures using capillary tubes into ethylenediamine tetraacetic acid (EDTA) and plain bottles. Blood samples in EDTA bottles were used for haematological analysis. Serum was prepared by centrifugation for 10 minutes at 3,000 rpm to obtain the clear supernatant for Enzyme-Linked Immunosorbent assay (ELISA) analysis.

2.10 *In vivo* anti-inflammatory assays

Measurement of cyclooxygenase (COX)-2, interleukin (IL)-6, tumor necrosis factor (TNF)-alpha, nuclear factor kappa (NFκ)- B cells were done using a quantitative sandwich enzyme-linked immunosorbent assay (ELISA) (Biotuva, USA) following the manufacturer's protocol.

2.11 Haematological analysis

The blood samples collected into EDTA bottles were used for the haematological analysis to determine white blood cell (WBC), hematocrit, haemoglobin, neutrophils, eosinophil, basophil and monocytes counts using an autoanalyzer (Swelab Alfa 3-Part Hematology Analyzer, Boule Medicals, Spanga, Sweden) at Babcock University Teaching Hospital Medical Laboratory .

2.12 Gas chromatography-Mass Spectrometry (GC-MS) analysis

The aqueous leaf fraction of *J. secunda* (JSALF) which exhibited the most active anti-inflammatory property was subjected to GC-MS analytical method. This was carried out at Shimadzu Training Centre for Analytical Instruments (STC) Lagos Nigeria. The GC-MS Specification was: GCMS-QP2010SE Shimadzu, Japan, initial temperature = 60 °C held for 2 min, final temperature = 300 °C at the rate of 13 °C/min, 1 µL of 0.2 g/mL fraction was injected. Temperature of heater was 250 °C, pressure was: 144.4 kPa, mode type splitless and carrier gas (flow rate = 3.0 mL/min; linear velocity = 46.3 cm/sec). The constituent compounds were determined by comparing the retention times and mass spectrum of the authentic samples obtained by GC with the mass spectra from the National Institute of Standards and Technology (NIST) Version 2.0 MS database library.

2.13 Statistical analysis

GraphPad Prism® version 8 Software was used to determine the difference between means using one way analysis of variance followed by Tukey Kramer post hoc test. Fifty percent inhibitory concentration (IC_{50}) values were measured using nonlinear regression. Data were reported as mean ± standard deviation and the significance level was set at $P < .05$.

3. RESULTS

3.1 Acute toxicity study

There was no observable sign of toxicity such as increased rate of breathing, lethargy, fur removal or change in colour and mortality up to the dosage level of 5000 mg/kg *b.w*.

3.2 In vitro anti-inflammatory assays

Data in Table 1 shows that JSALF (IC_{50} = 74.828 µg/mL) exhibited high stabilization of HRBC membrane against hypotonicity-induced haemolysis followed by JSHLF (IC_{50} =79.029 µg/mL), JSELF (82.936 µg/mL) and JSBLF (IC_{50} = 119.143 µg/mL). In addition, JSALF (IC_{50} = 34.443 µg/mL) had the highest anti-protein denaturation effect while JSBLF (IC_{50} = 40.398 µg/mL) had the lowest activity.

Table 1: Fifty percent inhibitory concentration (IC_{50}) of *J. secunda* leaf fractions on hypotonicity-induced hemolysis and protein denaturation

Assay	Fractions of *J. secunda*	IC_{50} (µg/mL)	Concentration (µg/mL)
Hypotonicity-Induced Hemolysis	Hexane	79.029	**50- 1000**
	Butanol	119.143	
	Ethyl Acetate	82.936	
	Aqueous	74.828*	
	Diclofenac	34.994	
5% BSA	Hexane	35.802	**50- 500**
	Butanol	40.398	
	Ethyl Acetate	38.242	
	Aqueous	34.443*	
	Diclofenac	34.198	

BSA = bovine serum albumin; * indicates fraction with highest IC_{50} value

3.3 Gas chromatography- mass spectrometry of *J. secunda* leaf aqueous fraction

The GC – MS analysis of JSALF detected 10 compounds which have been reported to possess anti-inflammatory activity. The compounds detected were: hexadecanoic acid, methyl ester, n-hexadecanoic acid, phytol, 1,2-15,16-diepoxyhexadecane, benzofuran, 2,3-dihydro- (Coumaran), 9,12-octadecadienoic acid, methyl ester, hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester, 9,12-octadecadienoic acid (Z,Z)-)-, 2-hydroxy-1-(hydroxymethyl)ethyl (Linoleic acid ester), 2-methyl-9-.beta.-d-ribofuranosylhypoxanthine, 9,12-octadecadienoic acid (Z,Z)-(Linolenic acid)

3.4 Acute anti-inflammatory study

3.4.1 Carrageenan-induced paw edema

Data in Figure 1 indicated that the animals induced with inflammation using carrageenan and treated with 10 mg/kg *b.w*. diclofenac sodium and JSALF at doses of 200, 400 and 600 mg/kg *b.w*. at the 6th hour significantly (P < .05) inhibited paw edema by 70.13%, 71.57% and 80%, respectively compared with the untreated control group (0%).

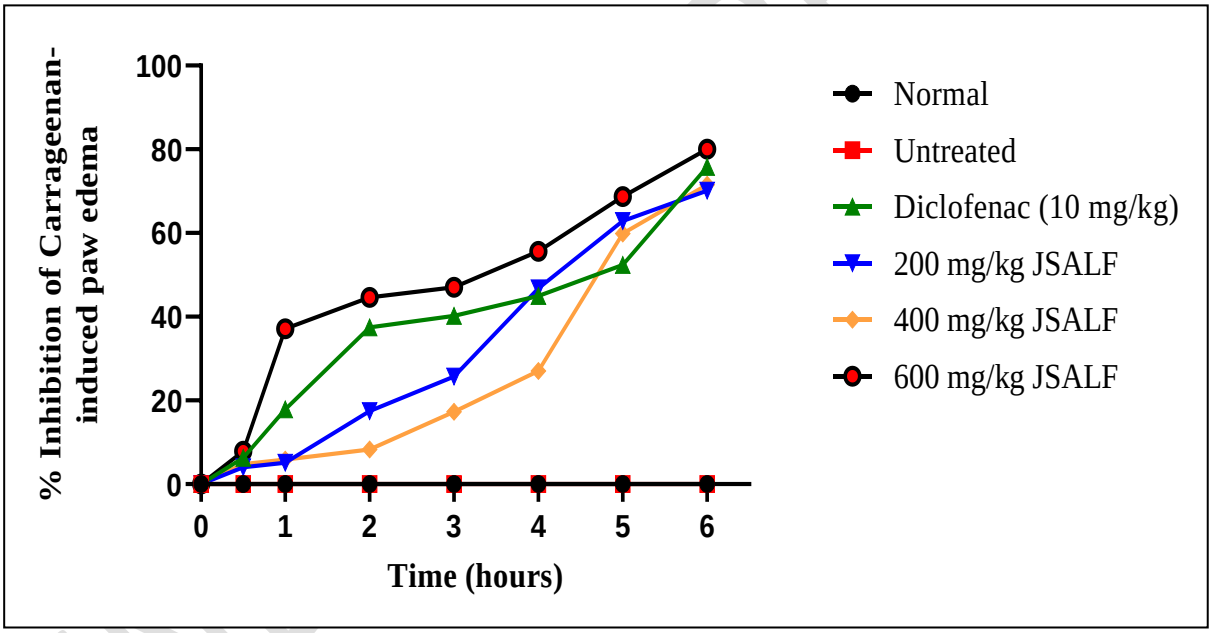

Figure 1: Effect of different doses of *J. secunda* aqueous leaf fraction on carrageenan-induced paw edema in rats. **Normal** – animals without induction, **Untreated** – animals induced with edema, **JSALF** - *J. secunda* aqueous leaf fraction.

3.4.2 Arachidonic acid-induced paw edema

Data in Figure 2 showed that animals induced with inflammation using arachidonic acid and treated with 10 mg/kg *b.w*. diclofenac and JSALF at 200, 400 and 600 mg/kg *b.w*. had significant (P < .05) decrease in paw edema by 60.87%, 60.93%, 67.08% and 65.72%, respectively when compared with untreated control animals (0%). JSALF treated group at 400 mg/kg *b.w*. had the highest paw edema decrease (1.07 ± 0.46 mm) while 200 mg/kg JSALF had the least percentage decrease (1.27 ± 0.40 mm) when compared with untreated control group (3.26 ± 0.71 mm).

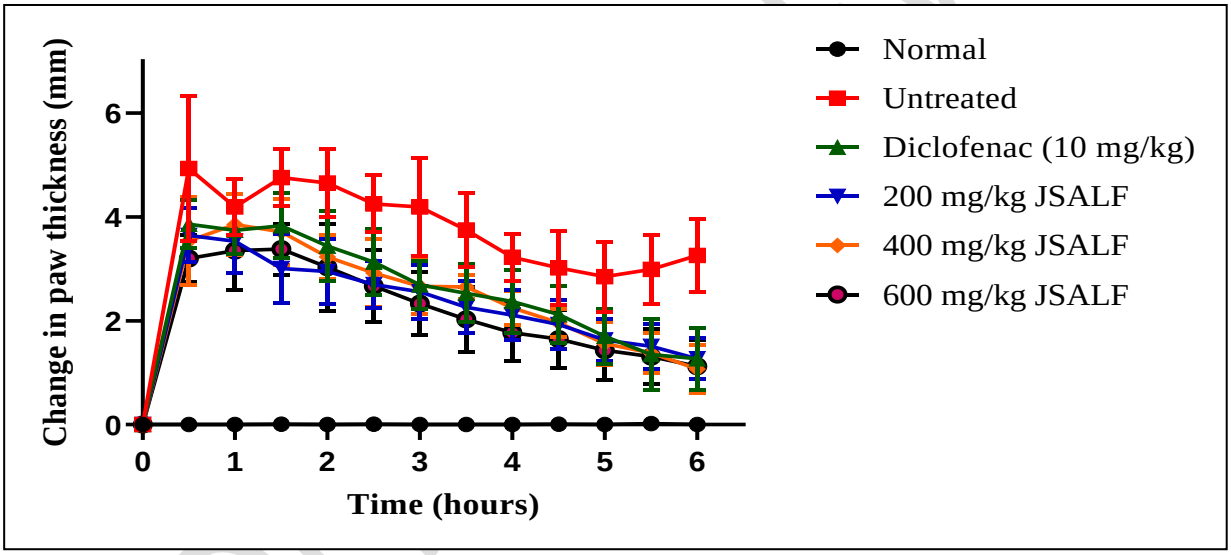

Figure 2: Change in rat paw thickness in arachidonic acid-induced paw edema by various doses of JSALF. **Normal** – animals without induction, **Untreated** – animals induced with edema but not treated, **JSALF** - *J. secunda* aqueous leaf fraction.
Data were expressed as Mean ± SEM.

3.4.3 Complete Freund's Adjuvant (CFA) - induced paw edema

Data in Figure 3 showed that animals induced with arthritis using CFA and treated with 100 mg/kg *b.w.* aspirin, 200, 400 and 600 mg/kg *b.w.* JSALF had significant ($P < .05$) decrease in paw edema by 48.28%, 32.58%, 34.92% and 62.58%, respectively when compared with untreated control animals (0%) at the 21st day. JSALF treated group at 600 mg/kg had the highest decrease (0.53 ± 0.21 mm) while 200 mg/kg JSALF had the least decrease (0.92 ± 0.56 mm) when compared with untreated control group (2.00 ± 0.10 mm).

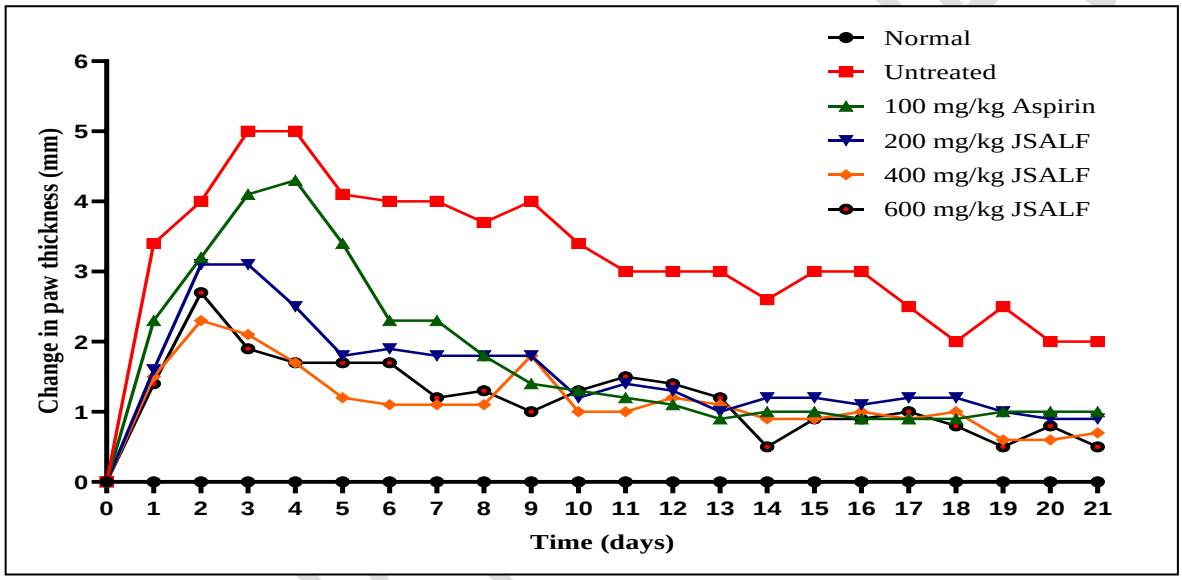

Figure 3: Change in rat paw thickness in Complete Freund's Adjuvant-induced paw edema by various doses of JSALF. **Normal** – animals without induction, **Untreated** – animals induced with edema but not treated, **JSALF** - *J. secunda* aqueous leaf fraction.
Data were expressed as Mean ± SEM.

3.5 Nuclear factor, tumor necrosis factor, interleukin-6 and cyclooxygenase assays

Data in Table 2 showed that animals administered with 100 mg/kg *b.w.* aspirin, 200, 400 and 600 mg/kg *b.w.* JSALF had significantly ($P < .05$) reduced serum concentrations of NF-κB, TNF-α, IL-6 and COX-2 when compared with the untreated control animals.

Table 2: Effects of aspirin and different doses of *J. secunda* aqueous fraction on nuclear factor, tumor necrosis factor, interleukin-6 and cyclooxygenase activities.

Parameters	Normal	Untreated	100mg/kg Aspirin Treated	200mg/kg JSALF Treated	400mg/kg JSALF Treated	600mg/kg JSALF Treated
NF-κB (pmol/L)	4.1 ± 0.04	30.7 ± 0.13$^{\alpha}$	4.4 ± 0.04$^{\beta}$	5.5 ± 0.03$^{\gamma}$	3.7 ± 0.07$^{\omega}$	4.0 ± 0.02$^{\epsilon}$

TNF-α (ng/mol)	16.9 ± 0.01	55.5± 0.06$^{\alpha}$	22.4 ± 0.03$^{\beta}$	27.4 ± 0.06 $^{\gamma}$	15.1 ± 0.02 $^{\omega}$	25.4 ± 0.05$^{\epsilon}$
IL-6 (pg/ml)	57.1 ± 0.09	78.2 ± 0.08$^{\alpha}$	45.1 ± 0.05$^{\beta}$	66.7 ± 0.07 $^{\gamma}$	58.5 ± 0.04 $^{\omega}$	35.0 ± 0.03$^{\epsilon}$
COX-2 (U/ml)	48.6 ± 0.11	117.4 ± 0.14$^{\alpha}$	34.7 ± 0.18$^{\beta}$	9.6 ± 0.00 $^{\gamma}$	10.1 ± 0.00 $^{\omega}$	11.1 ± 0.06$^{\epsilon}$

NF-κb-nuclear factor,**TNF-α**-tumor necrosis factor, **IL-6** - interleukin-6, **COX-2-** cyclooxygenase, **NORMAL** – animals without induction, **UNTREATED** – animals induced with edema but not treated, **JSALF** - *J. secunda* aqueous leaf fraction. Different symbols along rows indicate significant difference ($P < .05$).

3.6 Haematological Analysis

Table 3 showed that animals administered with 100 mg/kg aspirin and 600 mg/kg *b.w.* JSALF had a significantly ($P < .05$) reduced haematocrit and haemoglobin counts when compared with the untreated. White blood cell count analysis showed that animals treated with 100 mg/kg aspirin was significantly ($P < .05$) elevated when compared with the control and the different test groups. On the other hand, JSALF significantly ($P < .05$) reduced the white blood cell count of animals treated with 600 mg/kg dose when compared with the untreated control animals. Neutrophil count was significantly ($P < .05$) reduced in animals treated with 100 mg/kg aspirin, 200 and 400 mg/kg JSALF compared with the untreated control animals. Animals treated with specific JSALF and aspirin showed no significant difference in lymphocyte count when compared with the untreated control animals. JSALF at 200, 400 and 600 mg/kg *b.w.* treated animals exhibited a significant ($P < .05$) reduction in monocytes, basophil and eosinophil counts when compared with the untreated control animals.

Table 3: Effect of aspirin and different concentration of *Justicia. secunda* aqueous fraction on hematocrit, hemoglobin, WBC, leucocytes, monocytes, neutrophils, basophils and eosinophils

Parameters	Normal	Untreated	100mg/kg Aspirin Treated	200mg/kg JSALF Treated	400mg/kg JSALF Treated	600mg/kg JSALF Treated
HCT (%)	43.0 ± 3.74	46.0±5.39^{a}	38.4±4.1^{b}	43.4±1.67^{a}	41.0±1.63^{a}	39.5±5.8^{c}
HB (gm/dl)	14.28 ±1.23	15.26±1.7^{d}	12.76±1.36^{e}	14.44±0.56^{d}	13.6±0.49^{d}	13.13 ± 1.93^{f}
WBC (mm^3)	5.2±2.64	5.44±1.42^{g}	9.32±2.95^{h}	5.6 ± 1.07^{g}	5.18 ±0.64^{g}	4.62 ± 2.39^{i}
NEU (%)	27.6 ± 5.59	35.6 ± 4.34^{j}	29.2 ± 5.40^{k}	31.0 ± 5.66^{k}	28.0 ± 10.13^{k}	34.0 ± 2.52^{j}
LYMPH (%)	62.8 ± 5.93	64.4 ± 4.56^{l}	67.2 ± 3.63^{l}	65.6 ± 5.18^{l}	65.00 ± 8.72^{l}	64.00 ± 1.63^{l}
BEM	1.4 ± 1.67	2 .0± 1.41^{m}	2.0 ± 2.19 ^{m}	0.4 ± 0.89^{n}	2.0 ± 1.63^{m}	1.0 ± 2.0^{o}

(%)

HCT – haematocrit, **HB –** haemoglobin, **WBC –** blood cells, **NEU –** neutrophils, **LYMPH –** lymphocytes, **BEM –** basophils, eosinophils and monocytes. **JSALF –**aqueous fraction of *Justicia secunda* leaves. **NORMAL** – animals without induction, **UNTREATED** – animals induced with edema but not treated, **JSALF** - *J. secunda* aqueous leaf fraction.
Different letters along rows indicate significant difference ($P < .05$).

4. DISCUSSION

Rheumatoid arthritis, one of the inflammatory disorders affects about 1% of the global population [28]. The traditional use of *J. secunda* plant to treat different diseases including rheumatoid arthritis has fuelled the interest to isolate and evaluate the fraction of *J. secunda* leaf with the most active anti-inflammatory activity and perhaps gain some mechanistic insight. In this study, successive solvent partitioning of 70% methanolic extract of *J. secunda* leaf indicated that JSALF had the highest yield compared to the other fractions. This could be due to the extractive ability of water to isolate aqueous phytoconstituents. Studies have shown that aqueous extract of most plants contain high quantity of polar phytoconstituents such as glycosides, tannins, flavonoids and polyphenols [29, 30].

In vitro anti-inflammatory assays showed that JSALF had high HRBC stabilization when compared with other test fractions. Stabilization of HRBC membrane assay was modelled after lysosomal membrane. Lysis of lysosomes occurs during inflammation resulting to the release of its contents causing several debilitating disorders [31]. Its stabilization is paramount in supressing inflammation by preventing the lysosomal components of triggered neutrophil from escaping which include bacterial enzymes and proteases thereby resulting to additional inflammation and tissue damage [6, 31]. Hence, the stabilization of membrane from lysis by anti-inflammatory agents could suffice as a mechanism to safeguard cells from inflammatory agents. Several synthetic anti-inflammatory drugs have the benefit of either preventing or stabilizing the lysosomal content release [6, 31]. Subjection of erythrocyte to hazardous medium such as phenylhydrazine, hypotonic solution, heat and methyl salicylate leads to the lysis of the membrane along with oxidization and destruction of haemoglobin haemoglobin [6, 32]. It could then be deduced that JSALF possess anti-inflammatory activity.

A well-documented result of inflammation is denaturation of proteins whereby protein loses its secondary and tertiary structure by an external stressor [21, 33]. Majority of the proteins' biological activities are lost when they are denatured [34]. Inhibition of protein denaturation remains the generally accepted *in vitro* tests to assess anti-inflammatory drugs [21, 33]. JSALF also had the highest anti-denaturation activity when compared with the other test fractions in a reverse concentration dependent manner. Previous reports have demonstrated that plant extracts usually show their ability to inhibit the denaturation of proteins at lower concentrations [35].

Subjection of JSALF to GC-MS analysis revealed presence of compounds suspected to exhibit anti-inflammatory activities. These suspected compounds are: hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester,[36] 9,12-octadecadienoic acid (Z,Z)-(Linolenic acid), 9,12-octadecadienoic acid, methyl ester (Linoleic methyl ester), [37] 9-octadecenoic acid (Z)-, methyl ester,[38] phytol (diterpenes),[39] 1,2-15,16-diepoxyhexadecane,[40] hexadecanoic acid, methyl ester (palmitic acid),[41] 2-methyl-9-.beta.-d-ribofuranosylhypoxanthine,[42] benzofuran, 2,3-dihydro- (coumaran)[43]. In the animal study, carrageenan induced paw edema model serves as a general and appropriate *in vivo* model utilized in establishing the anti-inflammatory capability of drugs or natural products and to ascertain the possible mechanisms necessary in inflammation due

to its association with variety of mediators [44]. Oral administration of 200, 400 and 600 mg/kg b.w JSALF and 10 mg/kg b.w diclofenac sodium inhibited paw edema from 1 hour to 6 hours post carrageenan injection. Due to this suppression of paw edema during the early phase (1 hour), there is the possibility that the fraction could either block the pro-inflammatory mediators including prostaglandin, histamine, bradykinin, serotonin and leukotrienes or prevent the synthesis or release of these mediators. This finding also corroborate the results obtained from the *in vitro* anti-inflammatory study that JSALF exhibits anti-inflammatory activity and serve as scientific evidence for its traditional use as a treatment for arthritis. Perhaps phytol, 1, 2-15, 16-diepoxyhexadecane, 9, 12-octadecadienoic acid, methyl ester and 2-methyl-9-.beta.-d-ribofuranosylhypoxanthine obtained from GC-MS may account for this phenomenon.

Arachidonic acid-induced paw edema in rats is employed as an *in vivo* procedure to differentiate between cyclooxygenase (COX) and lipoxygenase (LOX) inhibitors since it detects LOX inhibitor much more than COX inhibitors [45, 46]. The arachidonic-induced swelling is both insensitive to selective cyclooxygenase inhibitors and noticeably decreased by arachidonic acid metabolism inhibitors and inhibitors of corticosteroids [47, 48]. Furthermore, prostaglandins and leukotrienes can be formed through cyclooxygenase and lipoxygenase pathways. In this study, the injection of arachidonic acid into the subplantar region of rat paw produced swelling as early as 30 minutes after injection reaching its peak at 1 hour 30 minutes. The results revealed that JSALF significantly ($P < .05$) reduced paw edema at doses of 200, 400 and 600 mg/kg in a dose dependent manner when compared with the diclofenac group. This indicates that the mechanistic action of JSALF could be connected to the prevention of leukotriene and prostaglandins production through lipoxygenase and cyclooxygenase pathways since the sensitivity of arachidonic acid to LOX is more than that of COX enzyme [45, 46]. Two-fold inhibitors of both LOX and COX enzymes such as diclofenac and 2,3-diarylxanthones have the capability to prevent the production of leukotriene B_4 (LTB_4) as well as Prostaglandin E_2 (PGE_2) [49, 50]. LTB_4 mediates leukocytes activation in inflammation whereas PGE_2 produces the five manifestations of inflammation which includes fever, swelling, redness, immobility and pain.

CFA-induced polyarthritic model of chronic inflammation was utilized to evaluate the anti-inflammatory potentials of JSALF. Injection of CFA in rats causes gradual swelling to develop with the influence of inflammatory cells which degenerate the tissues and results in bone destruction. This model can be regarded as having close resemblance to human rheumatoid disease. Results in this study showed that oral administration of JSALF at doses 200, 400, 600 mg/kg and 100 mg/kg aspirin to CFA induced groups reduced paw thickness significantly ($P < .05$) when compared with the control group. This indicates that JSALF exhibited anti-arthritic activities. This effect could be attributed to the anti-inflammatory and antioxidant properties of JSALF. Activated neutrophils, macrophages and dendritic cells have been demonstrated to generate reactive oxygen species (ROS) in large amounts in response to the presence of CFA induced in rats [51]. These ROS generated by lymphocytes, endothelial cells and macrophages are responsible for the destruction of cartilage, hence the JSALF anti-inflammatory compounds could suppress the pro-inflammatory mediators that would eventually activate the inflammatory cells and possibly counteract the ROS generated with its antioxidant compounds in a multi-targeted approach.

Determination of the serum pro-inflammatory cytokines in JSALF-treated arthritic animals showed that the level of nuclear factor kappa B (NF-κB) decreased significantly ($P < .05$) in rats administered with 100 mg/kg aspirin, 200, 400 and 600 mg/kg b.w JSALF when compared with the untreated control group. This suggests that JSALF has the capacity to reduce the level of serum NF-κB. When activated, NF-κB which is a transcription factor commonly found in the cell cytoplasm moves to the nucleus in order to regulates the expression of various genes such as COX-2, inducible nitric oxide synthase and cytokines (IL-6, IL-1β, IL-8, TNF-α) [2, 3]. NF-κB plays

varied roles in the development and continuation of rheumatoid arthritis. Stimulated NF-κB remains the usual feature found the synovium of arthritic individuals. Patients with rheumatoid arthritis present high levels of serum pro-inflammatory cytokines, such as TNFα, IL-1 and IL-6 recognized to be the targeted genes for NF-κB, indicating the stimulation of the signalling pathway of disease [5].

Further analysis revealed that there was a significant ($P < .05$) decrease in serum levels of TNF-α, IL-6 and COX-2 in JSALF-treated rats when compared with untreated control animals. Several studies had shown that key factors involved in stages of inflammatory process remains the cytokines which are remarkably elevated during the first phase of inflammation [2]. TNF-α is extremely activated in inflammatory cells in which it deploys and activate leukocytes, enhances the natural killer cell cytotoxicity and the multiplication of B lymphocytes, Thymus cells (T-cells) and playing a vital role in the physiological reaction to endotoxins [52, 53]. IL-6 alongside TNF-α stimulate the acute phase response, with IL-6 being the major initiator of the generation of majority of acute proteins in response to diverse stimuli, in which interleukins stimulate the synthesis of acute-phase proteins in the liver such as coagulation factors, protease antagonist, complement constituents and complex proteins [52]. Researchers have reported that IL-6 plays a major part in the pathogenesis of rheumatoid arthritis and other related changes [53, 54, 55]. In this study, JSALF significantly reduced IL-6 and TNF-α and this further strengthened literature positions which reported that *J. secunda* leaf extract has the capacity to reduce inflammation probably by suppression of TNF-α and ROS levels as well as by immunomodulatory activity [16, 17].

COX-2 enzyme is a major player in the production of pro-inflammatory prostaglandins and arachidonic acid metabolism in which it converts arachidonic acid to a hydroperoxyendoperoxide (PGG2), then to prostaglandin H2 from which prostacyclins, prostaglandins and thromboxanes are synthesized [46]. Elevated levels of COX-2 are observed when the body is under the influence of stimuli such as infection and injury and this enzyme is regarded as the target for the non-steroidal anti-inflammatory drugs such as diclofenac and aspirin [46]. COX-2 is induced by pro-inflammatory cytokines including tumor necrosis factor to catalyse the transformation of arachidonic acid to prostaglandins which then results in pains. Studies have shown that high Cox-2 expression and prostaglandin production have been seen in rheumatoid arthritis synovial fibroblast and is considered to be the major cellular source of COX-2 in arthritic patients [56, 57]. Critical suppression of COX-2 and the counterbalance of prostaglandin E2 can alleviate rheumatoid arthritis inflammation [57, 58]. JSALF reduced the expression of COX-2 in animals induced with inflammation. This suggests that the anti-inflammatory effect of JSALF could be via the prevention of TNF release and the down regulation of COX-2 expression.

Furthermore, haematological analysis showed that the animals administered with 100 mg/kg aspirin, 200, 400 and 600 mg/kg body weight JSALF for 21 days had a significant ($P<.05$) reduction in haematocrit, haemoglobin, white blood cells, neutrophils, monocytes, basophil and eosinophil with the exception of lymphocytes when compared with the untreated control animals. There was no significant difference in lymphocytes count in JSALF-treated animals and aspirin treated animals when compared with the untreated control animals. This proposes that JSALF has the capability to block the release of inflammatory cells since that is also the functions of many non-steroidal anti-inflammatory drugs including diclofenac and aspirin. Inflammation causes the leukocytes including eosinophils, monocytes and neutrophils to be activated and then move directly from the vein to location of damage with neutrophils being the foremost inflammatory cells usually engaged at the acute inflammation sites, [51, 59] however, after 24 to 48 hours, monocytic cells which differentiate into macrophages in tissues becomes the most prominent directed by chemotactic factors [60, 61].

5. CONCLUSION

Findings from this study indicated that JSALF exhibited a substantial anti-inflammatory activity, which mechanistically could have been through stabilization of membranes and suppression of protein denaturation, pro-inflammatory mediators, COX-2 pathways as well as immunomodulatory activity. JSALF should be further studied to isolate the GC-MS determined anti-inflammatory compounds and channel into pharmaceutical drug discovery.

ETHICAL APPROVAL

Institutional ethical clearance was obtained from Babcock University Health Research Ethics Committee with certificate number: BUHREC 603/19.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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