In vitro anti−inflammatory, anti−oxidant and *in vivo* anti−arthritic properties of stem bark extracts from *Nauclea pobeguinii* (Rubiaceae) in rats

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ABSTRACT

Objective: To explore the immunomodulatory, anti-inflammatory, anti-oxidant and anti-arthritic activity of aqueous and methanolic extracts of *Nauclea pobeguinii* stem bark.

Methods: For *in vitro* assays, the production of reactive oxygen species (chemiluminescence technique), the proliferation of T cells (liquid scintillation counter method), as well as the inhibition of cyclooxygenase, lipoxygenase, protein denaturation, and free radicals [DPPH, ABTS and nitric oxide (NO) inhibition methods] were evaluated. For *in vivo* assays, a polyarthritis model was induced by complete Freund's adjuvant in rats. The aqueous and methanolic extracts of *Nauclea pobeguinii* stem bark were administered orally at 150 and 300 mg/kg. After 28 days of treatment, the total blood was taken to quantify the hematological parameters and the serum was used to evaluate the biochemical parameters (alanine aminotransferase, aspartate transaminase, phenylalnine ammonialyase, and proteins) and oxidative stress parameters (malondialdehyde, catalase, superoxide dismutase, glutathione and NO), and then the knee joint was removed for histological analysis.

Results: The extracts of *Nauclea pobeguinii* significantly reduced the production of intra- and extracellular reactive oxygen species and decreased T cell proliferation. They had an inhibitory effect on cyclooxygenase, lipoxygenase, and protein denaturation, and both extracts had antioxidant capacity on DPPH, ABTS and NO. Both extracts alleviated joint inflammation and pain sensitivity after complete Freund's adjuvant injection, reduced alanine aminotransferase, aspartate transaminase, alkaline phosphatase, NO and malondialdehyde levels, increased protein concentration, superoxide dismutase, catalase and glutathione activity, and restored the cytoarchitecture of the joint after complete Freund's adjuvant injection.

Conclusions: The aqueous and methanolic extracts of *Nauclea pobeguinii* have immunomodulatory, anti-inflammatory, anti-oxidant and anti-arthritic properties.

KEYWORDS: *Nauclea pobeguinii*; Immunomodulation; Antioxidant; Anti-inflammatory; Anti-arthritic

1. Introduction

As a physiological process to protect the body against external or internal aggression, inflammation could limit and repair damage, reduce the size of the lesions, and restore the homeostasis of damaged tissues. Nevertheless, when the inflammatory process is prolonged, it is very harmful to the body and can lead to the development of many inflammatory diseases including rheumatoid arthritis[1]. Inflammatory, systemic, autoimmune and multifactorial rheumatism involves endocrine, psychological, genetic and environmental factors that are particularly responsible for joint destruction, pain, destruction of bone and cartilage with consequent functional disability[2]. In the rheumatoid synovium, the activation of polymorphonuclear cells, macrophages, and synoviocytes leads to the excessive production of T cells and then triggers the inflammatory process, during which leukocytes and many pro-inflammatory cytokines (IL-1 beta and/or TNF alpha) are produced. These could lead to the production of proteolytic enzymes that are responsible for the damage to the joint such as destruction of cartilage tissue, chronic cell proliferation and bone resorption[2].

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During the development of rheumatoid arthritis, the proteins in the articular cartilage can lose their structures (secondary and/or tertiary), thus cause the denaturation and the aggravation of the pathology[3]. Similarly, the phospholipids in the cartilage can be denatured by phospholipase A2 to produce arachidonic acid. This process takes place in a double way: the cyclooxygenase pathway with a production of thromboxane A2, prostacyclin, and prostaglandins E_2 (PGE_2) and F_2, as well as the lipooxygenase pathway with leukotriene production[4].

In the physiopathology mechanism of rheumatoid arthritis, reactive oxygen species (ROS) play a key role in chronic inflammation. Indeed, the onset of the phagocytosis process by polymorphonuclear neutrophils, which represent more than 90% of the cells in the synovial fluid during rheumatoid arthritis, requires an increase in oxygen consumption with the direct consequence of the production of several potentially toxic free radicals (oxygenated water, superoxide, radical hydroxyl), which are capable of destabilizing cell membranes promoting cytolysis[5]. This process can either lead to a significant decrease in anti-oxidant defense systems including the reduced glutathione (GSH) (non-enzymatic antioxidant), catalase (CAT) and superoxide dismutase (SOD) (enzymatic antioxidants) or a significant increase in malondialdehyde (MDA) (for lipid peroxidation rate)[6]. In addition, many liver disorders may occur during the development of rheumatoid arthritis, such as changes in some liver enzymes (transaminases, alkaline phosphatase, and gamma transferases)[7].

The debilitating and uncomfortable pathological status results in the cessation of work in more than 50% of affected people and this disease affects nearly 1% of the world's population[8]. The knowledge of rheumatoid arthritis, its mediators as well as the molecular and cellular mechanisms involved in its pathophysiology has made it possible to develop multitude of therapeutic targets for its control and relief. Corticoids and nonsteroidal anti-inflammatory drugs are used for symptomatic treatments, while biotherapies and immunosuppressants are used in combination with disease-modifying anti-rheumatic drugs. But these drugs have numerous undesirable effects such as heartburn, digestive bleeding, renal insufficiency, hypertension and even increased risk of microbial infection[9]. Besides, the costs of these treatments are high, so many scientists are turning to alternative treatments that are accessible and have no side effects.

For both developing countries and low-income populations in developed countries, plants are important sources of herbal treatment for many diseases. Traditional medicine remains the main recourse for approximately 85% of the African population[10]. The efficacy and safety of these plant species have become the subject of many pharmacological and clinical investigations. Belonging to the family Rubiaceae, *Nauclea pobeguinii* (*N. pobeguinii*) is a widespread plant in Cameroon, where it is widely used in traditional medicine to relieve stomach pains, fevers, joint pain, and even inflammation. Several studies have already been carried out on this plant and have shown that this plant has anti-diabetic[11], antibacterial[12], analgesics and anti-inflammatories activities[13]. In addition, Xu *et al*[14] showed the presence of flavonoids, tannins, terpenoids, alkaloids, and steroids in this plant in a phytochemical study. The effects of *N. pobeguinii* on polyarthritis, immunological changes

and oxidative stress parameters related to polyarthritis have not yet been demonstrated. Thus, this study aims to evaluate anti-arthritic properties *in vivo* in polyarthritis rats induced by complete Freund's adjuvant (CFA); and to evaluate *in vitro* immunomodulatory, anti-inflammatory and antioxidant potential.

2. Materials and methods

2.1. Plant material and extraction

N. pobeguinii (fresh barks, leaves and flowers) was harvested in August 2016 in Mbalmayo, Department of Nyon and So'o, Central Cameroon region, and was then identified at the National Herbarium of Cameroon (Youndé) by simple comparison with an already existing sample [R. Letouzey sample No. 11,367 (28,335/SRFcam)]. After identification, the barks were dried in the shade (shelter from the sun) and ground (roping) to give a fine powder. Part of this powder (500 g) was soaked in distilled water (5 L), and the mixture was boiled for 30 min. The another part (500 g) was quenched in methanol (2.5 L), then the mixture was macerated for 72 h. At the end of each preparation, the two mixtures were filtered *via* filter paper. The aqueous filtration was evaporated in an oven at 40 ℃ while the methanol filtration was concentrated using a rotary evaporator at 65 ℃ (reduced pressure). This operation yielded 22 g of aqueous extract (4.4% yield) and 42 g of methanolic extract (8.4% yield).

2.2. Reagents, chemicals, and equipments

1,1-Diphenyl-2-picrylhydrazyl (DPPH) was procured from Sigma Aldrich (St. Louis, MO, USA). Sodium nitroprusside was purchased from Merck Ltd. India, Mumbai. Phosphoric acid, sodium hydroxide, sodium linoleate, sulfanilamide, sulfuric acid, linoleic acid, ascorbic acid, and arachidonic acid were purchased from S D Fine Chem. Ltd, Mumbai. Thiobarbituric acid, lipopolysaccharide, hemoglobin, ethylenediaminetetraacetic acid and bovine albumin were procured from Central Drug House Pvt. Ltd, New Delhi. Casein and trypsin were procured from Hi-Media Lab. Ltd, Mumbai. *N*-(1-Naphthyl) ethylenediamine dihydrochloride, potassium persulfate, potassium phosphate buffer, glutathione, and benzene were obtained from LOBA CHEME Pvt. Ltd. Mumbai. Phytohemagglutinin and RPMI 1640 medium were from HIMEDIA. CFA was purchased from Sigma Chemical Co. (St Louis, MO, USA), while diclofenac (Olfen-100 SR) was bought in a locally certified pharmacy. All the other chemicals and reagents were of pure analytical grade and obtained from the local suppliers.

2.3. Phytochemical assay of N. pobeguinii extracts

To determine the chemical compounds in the aqueous and methanolic extracts of *N. pobeguinii*, several tests were used based on visual observation of a formed precipitate and/or changes in the coloration of the solution after addition of one or more characteristic reagents. The presence of chemical compounds such as flavonoids, saponins, tannins, anthraquinones, terpenoids, steroids, and alkaloids

was verified using the method described by Matos[15].

2.3.1. Tannins
A solution of FeCl₃ (a few drops) was mixed with 2 mL of each extract dissolved in 2 mL of distilled water, and the appearance of a green precipitate indicated the presence of tannins.

2.3.2. Saponins
The presence of saponins is generally demonstrated by the formation of a stable foam after stirring a solution. A solution of 5 mL of distilled water and 5 mL of each extract was vigorously shaken to check the presence of saponins.

2.3.3. Flavonoids
By mixing 1 mL of lead acetate solution (10%) with 1 mL of each extract, the appearance of a yellow precipitate indicated the presence of flavonoids.

2.3.4. Anthraquinone
The presence of free anthraquinones and/or anthraquinone derivatives in a mixture is indicated by a pink, violet or red coloration in the lower phase (ammoniacal phase) of the mixture after stirring. Thus, two methods were used:
(i) stirring a solution prepared in the following manner: extract (3 mL) and benzene (3 mL), then filtrating and adding 5 mL ammonia (10%) in the filtrate.
(ii) Bring a boiling mixture of extract (3 mL) and sulfuric acid (3 mL), filtering while hot and adding benzene (3 mL) to the filtrate followed by stirring. After separating the benzene layer, adding ammonia prepared at 10% (3 mL).

2.3.5. Terpenoids
A solution of chloroform (2 mL) and each extract (2 mL) was prepared and evaporated to dryness, then the concentrated sulfuric acid (2 mL) was added to the solution and the mixture was heated for 2 min. The appearance of a greyish color was characteristic of the presence of terpenoid.

2.3.6. Steroids
The presence of steroids was characteristic of a greenish color. A total of 2 mL of each extract was dissolved in 2 mL of chloroform, then acetic and sulfuric acids were added.

2.3.7. Alkaloids
A solution of 3 mL of each extract, 3 mL of HCl with the addition of Wagner's reagents and Layers was stirred and the appearance of turbidity of the precipitate indicated the presence of alkaloids.

2.4. Anti–inflammatory effects of aqueous and methanolic extracts of *N. pobeguinii*

2.4.1. Inhibition of protein denaturation
To evaluate the anti-inflammatory properties of *N. pobeguinii* extract, the method previously described by Padmanabhan and Jangle[16] with small modifications was used. Briefly, 1 mL of different extracts or diclofenac sodium of different concentrations

(100, 200, 500 and 1 000 μg/mL) was mixed with 5% bovine serum albumin solution (1 mL), then the mixture was incubated 27 ℃ for 1 h. The control tube consisted of a mixture of distilled water and bull serum albumin. Denaturation of the proteins was initiated by placing the mixture in water for 10 min at 70 ℃. After cooling the solution to room temperature, the activity of each mixture was measured at 660 nm. Each test was performed in three repetitions. The formula below was used to calculate the percent inhibition.

$$\% \text{ Inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

2.4.2. Assay of cyclooxygenase and 5–lipoxygenase inhibition

To grow human peripheral lymphocytes, RPMI 1640 medium (HIMEDIA) supplemented with heat-inactivated fetal calf serum (20%) and antibiotics (penicillin and streptomycin) were used. Cell proliferation was stimulated by phytohemagglutinin (HIMEDIA). Under aseptic conditions, after filtration of the culture (cellulose acetate filter, 0.2 μm of the pore), it was incubated for 72 h and activated after 24 h by addition of lipopolysaccharide (1 μL), then incubated again for 24 h. Then, aqueous and methanolic extracts, as well as ibuprofen at 100, 200, 500 and 1 000 μg/mL, were added and incubated for 24 h. After centrifugation at 6 000 rpm for 10 min, the supernatant was isolated, then 50 μL of cell lysis buffer was added followed by further centrifugation at 6 000 rpm for 10 min. The supernatant was isolated and the inflammation test was performed as a pellet suspended in a small amount of supernatant[17].

Tris-HCl, glutathione, hemoglobin and an enzyme were used to prepare the mixing solution. After the addition of the arachidonic acid in this mixture followed by incubation at 37 ℃ for 20 min, 0.2 mL of 10% trichloroacetic acid in 1 N HCl and 0.2 mL of thiobarbituric acid were added to the solution, and heated in a boiling water bath for 20 min, and then centrifuged at 1 000 rpm for 3 min. The supernatant was used to evaluate cyclooxygenase activity at 632 nm[17].

Linoleic acid (70 mg) and an equal weight of interpolation were dissolved in 4 mL of oxygen-free water and pipetted. Sodium hydroxide (0.5 N) and water without oxygen (25 mL) were added, then the solution was divided into 0.5 mL portions, rinsed with nitrogen and stored in ice until further use. A quartz cuvette at 25 ℃ was used for the reaction. Tris buffer (2.75 mL, pH 7.4), sodium linoleate (0.2 mL) and enzyme (50 mL) constituted the assay mixture. Optical density was measured at 234 nm[17]. To calculate the percent inhibition, the following formula was used:

$\% \text{ inhibition} = [(\text{Abs}_{control} - \text{Abs}_{sample})/\text{Abs}_{control}] \times 100$

2.5. Antioxidant activity

2.5.1. DPPH radical scavenging activity
To determine the DPPH radical scavenging activity of aqueous and methanolic extracts of *N. pobeguinii*, the method described by Brand-Williams *et al.*[18] was used with some modifications. DPPH (24 mg) dissolved in 100 mL of methanol was used as the stock solution. The working solution was obtained by diluting the stock solution

with methanol until an absorbance of (0.98 ± 0.02) at 517 nm was obtained. The working solution of DPPH (3 mL) was mixed with 100 μL of each extract (1 mg/mL) or standard in a test tube, while the control contained only 100 μL of methanol. The absorbance was measured for 30 min at 517 nm. The following formula was used to calculate the antioxidant activity:

% Antioxidant activity = $[(A_c-A_s)/A_c] \times 100$

Where, A_s and A_c are the absorbance of the sample and the control, respectively.

2.5.2. ABTS$^{\bullet+}$ discoloration test

The ABTS$^{\bullet+}$ bleaching test was performed using the procedure described by Re et al[19] with some modifications. For this, 9.5 mL of ABTS reagent (7 mmol/L) was prepared with 245 μL of potassium persulfate (100 mmol/L), then the volume was supplemented to 10 mL with distilled water. The solution was diluted with potassium phosphate buffer (0.1 mol/L, pH 7.4) to an absorbance of (0.70 ± 0.02) at 734 nm after being kept in the dark at room temperature for 18 h, then samples of each extract were prepared in methanol with dilutions at 0.1, 0.25, 0.5 and 1 mg/mL. A sample (10 μL) was placed in a test tube and thoroughly mixed with 2.99 mL of ABTS radical working solution. The absorbance of the resulting clear mixture was recorded at 734 nm. The percentage of antioxidant activity was determined using the following formula:

% Antioxidant activity = $[(A_c-A_s)/A_c] \times 100$

Where A_s and A_c are the absorbance of the sample and the control, respectively.

2.5.3. Nitric oxide (NO) trapping test

Sodium nitroprusside (0.5 mL, 5 mmol/L, pH 7.4) was combined with the test compounds (0.1, 0.25, 0.5 and 1 mg/mL), ascorbic acid or an equivalent amount of methanol and incubated for 180 min at 25 ℃. The Griess reagent (1.5 mL) consisting of sulfanilamide (1%), phosphoric acid (2%) and N-(1-naphthyl)ethylenediamine dihydrochloride (0.1%) was added and the mixture was again incubated for 30 min. The absorbance was measured at 546 nm and the percentage of activity was determined relative to the standard[20]. The activity was expressed as percent inhibition using the following equation:

$$\% \text{ NO scavenging} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

2.6. Immunomodulatory effects of aqueous and methanolic extracts of N. pobeguinii

2.6.1. Isolation of polymorphonuclear neutrophil

To isolate the polymorphonuclear neutrophil, 10 mL of venous blood was aseptically removed from an adult person (voluntary and healthy), then introduced into a tube with heparin as an anticoagulant. The lymphocytes and Hanks Balanced Saline (HBSS) were added to the tube and the tube was stored at room temperature for 30 min. Then the supernatant was removed using the pipette, slowly introduced into centrifuge tubes containing lymphocytes (5 mL) and centrifuged at 400 g for 20 min at room temperature. After

centrifugation, the supernatant of each tube was poured and 1 mL of distilled water was added. One minute precisely after the addition of the distilled water, HBSS⁻ (2×) (1 mL) was added; 5 mL of HBSS was then added to the tubes and these tubes were centrifuged at 300 g for 10 min at 4 ℃. The supernatant of each tube was decanted, 1 mL of HBSS was reintroduced and the tube was kept in ice. The trypan blue exclusion method was used to evaluate the viability of the cells and the cells were counted using a hemocytometer. For the different tests, a cell concentration of 1×10^6 cells/mL was used[21].

2.6.2. Isolation of macrophages on mice

To isolate macrophages in Naval Medical Research Institute mice, the technique previously described by Mbiantcha et al[21] was used. Three adult mice (20-25 g, 4 months) were immunized by intraperitoneal injection of fetal calf serum (1 mL). These animals were observed for 72 h and killed by cervical dislocation. The body of each mouse was soaked in ethanol (70%) for decontamination, then RPMI (Roswell Park Memorial Institute Institute) (10 mL, 10%) was injected into the peritoneal cavity, followed by a massage of the peritoneal cavity for 2 min. The abdominal skin was cut to expose the peritoneal cavity. A sterile syringe was introduced into the peritoneum of each mouse and RPMI-containing macrophages were collected and introduced into a dry sterile tube and then centrifuged at 400 g for 20 min at 4 ℃. Then, the supernatant was removed, 5 mL of RPMI was reintroduced into the tube and the tube was centrifuged at 300 g for 10 min at 4 ℃. The supernatant was again decanted, 1 mL of RPMI/HBSS (1 mL) was added to the tube and then the tube was kept in ice. The viability of the cells was verified using the trypan blue exclusion method and the cells were counted using a hemocytometer. For the different tests, a cell concentration of 1×10^6 cells/mL was used.

2.6.3. Determination of production of ROS

The production of ROS was evaluated according to the method previously described by Mbiantcha et al[21] with some adjustments. Plates (96 wells) were used for this experiment. A total of 25 μL of each extract (3.1 to 100 μg/mL) or ibuprofen were mixed (same volume) with whole blood, polymorphonuclear neutrophils (1×10^6 cells/mL) or macrophages (2×10^6 cells/mL) diluted in HBSS^{++} (1:50), while control wells with only HBSS^{++}, blood or polymorphonuclear neutrophils or macrophages. The plate was incubated at 37 ℃ for 30 min in the thermostatic chamber of the luminometer. Then, 25 μL of zymosan/phorbol myristate acetate and/or 25 μL of luminol/lucigenin (7×10^5 mol/L) were added. In this test, the results were obtained in relative light units (RLU). The following formula was used to calculate the percent inhibition (%) of each extract[21]:

$$\text{Percentage inhibition (\%)} = \frac{\text{RLU}_{\text{control}} - \text{RLU}_{\text{test}}}{\text{RLU}_{\text{control}}} \times 100$$

2.6.4. T cell proliferation assay

A volume of 50 μL of each extract (2, 10 and 50 μg/mL) or prednisolone diluted in RPMI (5%) were introduced into plates (96 wells), then the isolated T cells (50 μL, 2×10^6 cells/mL) and phytohemagglutin (PHA)-L (PHAL) (50 μL, 7.5 μg/mL) were

added. Negative control wells received only certain cells (50 μL) and RPMI (5%, 150 μL), while positive control wells received cells (50 μL), PHA (50 μL) and RPMI (5%, 100 μL). After incubating for 72 h at 37 ℃ in a CO_2 incubator (5%), the culture was stimulated with 25 μL of 0.5 μCi/well (methyl 7 3H) thymidine solution. Then the plates were incubated for 18 h and the cells were harvested using a filter (fiberglass). The level of thymidine integrated into the cells was determined using an LS65000 liquid counter. Inhibition percentage was determined using the number per minute (CPM) of each well[21]:

$$\text{Percentage inhibition (\%)} = \frac{CPM_{control} - CPM_{test}}{CPM_{control}} \times 100$$

2.6.5. Cytotoxicity assay

The cytotoxicity of the aqueous and methanolic extracts was evaluated using the *in vitro* assay method described by Mbiantcha *et al*[21]. Briefly, 100 μL of cell (Hep G2 cells) suspension (6×10^4 cells/mL) were incubated for 24 h at 37 ℃, 5% CO_2 in 96-well plates. The supernatant was removed in each well, then each extract (3.1 to 100 μg/mL) and complete DMEM were added to each well to give a final volume of 200 μL. The standard well contained cells (100 μL) and complete DMEM, while 2 μL of triton×100 (0.5%) was added to the negative control well. The supernatant was transferred after incubation for 48 h at 37 ℃ in a CO_2 incubator and 50 μL of MTT (0.5 mg/mL) diluted in PBS (5 mg/mL) were completed in each well. Then the plates were incubated for 4 h, the MTT was carefully inhaled, 100 μL of DMSO was added as stirring for 10 to 15 min in an orbital shaker. The spectrophotometer was used and the absorbance was increased to 540 nm. The percentage inhibition of cell viability was obtained using the following formula[21]:

$$\text{Percentage inhibition (\%)} = 100 - \frac{OD_{test} - OD_{blank}}{OD_{control} - OD_{blank}} \times 100$$

2.7. In vivo assay

2.7.1. Animal

Wistar rats that weighed between 160 and 200 g (3-4 months) and were raised under normal conditions (temperature 19-23 ℃, brightness 12 h) at the Animal Physiology and Animal Physiology Research Unit Phytopharmacology of the Department of Biology of the University of Dschang (Cameroon) were used. These animals were housed in cages with six animals per cage.

2.7.2. Induction of rheumatoid arthritis

The animals were selected and grouped into 7 groups with 6 rats in each group according to their weight. The doses were based on our previous work[13]. After acclimation for 3 days, the animals were anesthetized with ether vapor, and the polyarthritis was induced by a double injection of CFA (10 mg/mL, Sigma Chemical Co. St Louis, MO, USA) (100 μL on day 1 and 50 μL on day 2) in the caudal vein except group 1 (normal control). The rats were distributed as follows: group 2: negative control (CFA + NaCl), group 3: positive control (CFA + Diclofenac 5 mg/kg), group 4: treated (CFA + aqueous extract at 150 mg/kg), group 5: treated (CFA + aqueous extract at 300 mg/kg), group 6: treated (CFA + methanol extract at 150 mg/kg), group 7: treated (CFA + methanol extract at 300 mg/kg). The different treatments were administered orally from the 14th day after the first CFA injection, then the treatment continued until the 28th day[22].

2.7.3. Measurement of joint diameter, mechanical hyperalgesia, body weight, and arthritic score

The diameter of the joints was measured with Digital Vernier caliper (Mitutoyo, Japan) on day 0 before the injection of CFA which served as the base value, days 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25 and 27 after the first injection of CFA[22].

Hyperalgesia was measured with an algesimeter (UGO Basil, Italy) on day 0 before CFA injection and on days 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26 and 28 after the first CFA injection[22].

The arthritic score was assessed by the number of infected legs, based on leg inflammation, leg distortion and the animal's inability to use the legs on days 0, 7, 14, 21 and 28, while body weight was measured daily using a scale[21].

2.7.4. Weight of the organs, hematological parameters, biochemical parameters, and oxidative stress

At the end of treatment, each rat was anesthetized by intraperitoneal injection of thiopental (50 mg/kg) and euthanized. The blood was removed by catheterization of the abdominal artery and introduced into two tubes. Blood in tube contained ethylenediaminetetraacetic acid as an anticoagulant was used to evaluate the hematological parameters (red blood cells, globules white, hemoglobin, hematocrit, platelets) by the usual standardized laboratory method[21]. Then blood in a second tube without anticoagulant was centrifuged at 2 500 rpm for 15 min, the serum was taken for the evaluation of biochemical parameters such as transaminases [alanine aminotransferase (ALT), aspartate transaminase (AST)], total proteins, creatinine, alkaline phosphatase[21] and oxidative stress parameters (NO, MDA, CAT, SOD and GSH)[23]. The organs (liver, kidneys, spleen, lungs, and thymus) were removed, cleaned with 0.9% NaCl, dehydrated and weighed with a sensitive balance.

2.7.5. Histological analysis

The joints were gently removed, stripped of all fat, and fixed with PBS-buffered formalin (10%) for histological analysis. This analysis was performed according to the standard histology method using color staining (hematoxylin and eosin). The section was observed using a microscope (Scientico STM-50) equipped with digital camera (Celestron 44421) connected to a computer (Toshiba Tecra A9). The digital microscope software (Suit 2.0) was used for taking microphographs with a magnification (×100).

2.8. Statistical analyses

All data of *in vitro* tests were expressed as mean ± standard deviation in triplicate, whereas for the *in vivo* test, the data were

presented as mean ± SEM. The differences between the groups were evaluated by ANOVA (unidirectional and bidirectional) followed by the Bonferroni test, the significant differences were considered at *P* <0.05.

2.9. Ethical statement

Experimental procedures were in accordance with NIH publication Nos.85–23 on "Laboratory Animal Care Principles" and approved by the Laboratory Committee (Laboratory of Animal Physiology and Phytopharmacology, Department of Animal Biology, University of Dschang, Cameroon) (Protocol No. 1209004). The experiments also conformed to the guidelines for the study of pain in awake animals established by the International Association for the Study of Pain, Ministry of Scientific Research and Technology, which has adopted the European Union on Animal Care and Experimentation (CEE Council 86/609) guidelines.

For the donation of human blood samples, all processes were accepted by an independent ethics committee, ICCBS, University of Karachi (N°:ICCBS/IEC-008-BC-2015/Protocol/1.0). The blood donors provided informed approval for the use in this study.

3. Results

3.1. Phytochemical screening

Phytochemical test indicated that both extracts of *N. pobeguinii* were very rich in secondary metabolites, including flavonoids, tannins, saponins, steroids, and alkaloids, while terpenoids were present only in the methanolic extract.

3.2. Immunomodulatory effects of aqueous and methanolic extracts of N. pobeguinii

For intracellular ROS, the extracts of *N. pobeguinii* stem bark inhibited the production of intracellular ROS in whole blood (IC_{50} = 6.57 μg/mL for aqueous extract and IC_{50} = 3.73 μg/mL for methanolic extract), polymorphonuclear neutrophils (IC_{50} = 4.27 μg/mL for aqueous extract and IC_{50} = 3.27 μg/mL for methanolic extract) and macrophages (IC_{50} = 6.10 μg/mL for aqueous extract and IC_{50} = 4.61 μg/mL for methanolic extract). Concerning the extracellular ROS, the aqueous extract inhibited their production with IC_{50} of 6.42 μg/mL (whole blood), 5.51 μg/mL (polymorphonuclear neutrophils) and 5.11 μg/mL (mouse peritoneal macrophages), while methanol extract with IC_{50} of 3.86 μg/mL (whole blood), 3.15 μg/mL (polymorphonuclear neutrophils) and 3.74 μg/mL (mouse peritoneal macrophages).

Concerning the proliferation of T cells, the methanolic extract, the aqueous extract and prednisolone (reference substance) showed an antiproliferative activity with IC_{50} of 1.15, 4.10 and <3.10 μg/mL, respectively.

The aqueous and methanolic extracts of *N. pobeguinii* did not show any toxic effect on the cells as compared to the cyclohexamide (0.1 μg/mL). Aqueous extract had IC_{50} greater than 100 μg/mL, while the methanolic extract had IC_{50} of 80.54 μg/mL.

3.3. Antioxidant effects of aqueous and methanolic extracts of N. pobeguinii

Table 1 shows that these extracts had a remarkable antioxidant effect. DPPH assay result revealed that aqueous and methanolic extracts at 1 000 μg/mL had maximum inhibition percentages of 91.01% and 78.80%, respectively. With regard to the ABTS discoloration test, the aqueous extract at 1 000 μg/mL had a maximum percentage inhibition of 82.39%, while the methanolic extract exhibited a maximum inhibition percentage of 79.08%. For the NO inhibition test, the maximum inhibition percentages of 94.66% (aqueous extract) and 88.31% (methanolic extract) were obtained at a concentration of 1 000 μg/mL.

3.4. Anti−inflammatory effects of aqueous and methanolic extracts of N. pobeguinii

Table 2 shows the effect of aqueous and methanolic extracts of *N. pobeguinii* on protein denaturation, inhibition of cyclooxygenase and inhibition of 5-lipoxygenase. The extracts of *N. pobeguinii*, as well as diclofenac, inhibited the denaturation of proteins in a concentration-dependent manner. Diclofenac, aqueous and methanolic extracts at 1 000 μg/mL had maximum inhibition of 89.19%, 80.13%, and 60.39%, respectively. For cyclooxygenase, the aqueous and methanolic extracts as well as ibuprofen at 1 000 μg/mL exhibited a maximum percentage inhibition of 91.14%, 70.23%, and 97.88%, respectively. With respect to 5-lipoxygenase, the aqueous and methanolic extracts of *N. pobeguinii* as well as ibuprofen showed a maximum inhibition of 94.56%, 78.17% and 95.31%, respectively.

Table 1. *In vitro* antioxidant property of aqueous and methanolic extracts of *Nauclea pobeguinii*.

Compounds	Inhibition (%)		
	DPPH	ABTS	NO
Vitamin C (μg/mL)			
100	85.38 ± 0.71	54.30 ± 0.09	52.86 ± 1.90
250	88.28 ± 0.45	62.51 ± 0.25	76.27 ± 2.21
500	96.02 ± 0.41	68.08 ± 0.39	88.56 ± 0.47
1 000	98.31 ± 0.04	75.64 ± 0.28	94.08 ±0.80
Aqueous extract (μg/mL)			
100	66.34 ± 0.88	58.45 ± 0.26	61.11 ± 0.78
250	73.06 ± 1.01	69.55 ± 0.43	72.77 ± 0.98
500	88.12 ± 0.72	75.35 ± 0.25	84.67 ± 3.99
1 000	91.01 ± 1.11	82.39 ± 0.21	94.66 ± 0.46
Methanolic extract (μg/mL)			
100	56.28 ± 1.09	55.38 ± 0.51	61.02 ± 1.99
250	67.13 ± 0.78	69.03 ± 0.29	71.34 ± 1.22
500	77.47 ± 0.69	76.82 ± 0.12	80.61 ± 1.93
1 000	78.80 ± 0.62	79.08 ± 0.19	88.31 ± 0.85

The percentage values were obtained using various concentrations of test compounds and readings are presented as mean ± SD of triplicates.

Table 2. *In vitro* antiinflammatory property of aqueous and methanolic extracts of *Nauclea pobeguinii*.

Compounds	Inhibition (%)		
	Protein denaturation	Cyclooxygenase	5-lipoxygenase
Diclofenac (µg/mL)			
100	74.41 ± 1.56	-	-
200	76.72 ± 2.01	-	-
500	81.48 ± 2.12	-	-
1 000	89.19 ± 2.20	-	-
Ibuprofen (µg/mL)			
100	-	83.46 ± 1.90	82.03 ± 1.32
200	-	89.90 ± 1.97	88.63 ± 1.23
500	-	93.39 ± 2.12	92.96 ± 1.54
1 000	-	97.88 ± 2.22	95.31 ± 1.87
Aqueous extract (µg/mL)			
100	60.64 ± 1.11	64.19 ± 1.45	67.25 ± 1.23
200	66.43 ± 1.23	71.03 ± 1.89	70.17 ±1.34
500	71.13 ± 1.34	78.78 ± 1.49	81.97 ± 1.39
1 000	80.13 ± 1.54	91.14 ± 1.25	94.56 ± 1.24
Methanolic extract (µg/mL)			
100	42.64 ± 1.87	44.50 ± 1.32	47.51 ± 1.59
200	44.57 ± 1.27	51.82 ± 1.89	57.25 ± 0.98
500	49.07 ± 1.31	61.30 ± 1.11	67.57 ± 0.93
1 000	60.39 ± 1.45	70.23 ± 1.29	78.17 ± 1.27

The percentage values were obtained using various concentrations of test compounds and readings are presented as mean ± SD of triplicates.

3.5. In vivo test results

3.5.1. Effect of aqueous and methanolic extracts of N. pobeguinii on the joint diameter

Figure 1A shows the effect of stem bark extracts of *N. pobeguinii* on the change in joint diameter. The injection of CFA significantly increased ($P<0.01$) the diameter of the joint from the 9th day after the administration compared to the normal control. Oral administration of the aqueous and methanolic extracts of *N. pobeguinii* as well as diclofenac decreased significantly the diameter of the joint from the first administration until the 28th day. Aqueous extract at 300 mg/kg on the 27th day showed the maximum inhibition as 68.39%, while the methanolic extract at 300 mg/kg on the 19th day showed 48.92%. Moreover, the maximum effect of diclofenac (40.20%) was observed on the 17th day.

3.5.2. Effect of aqueous and methanolic extracts of N. pobeguinii on mechanical hyperalgesia

The CFA injection significantly reduced ($P<0.001$) the pain threshold assessed with the analgesimeter in all groups from the 6th day after CFA injection. Treatment with *N. pobeguinii* extracts at different doses significantly increased ($P<0.05$) the pain threshold (Figure 1B). This activity reached a maximal level on the 28th day by aqueous extract at 300 mg/kg (75.00%) and the 24th day by the
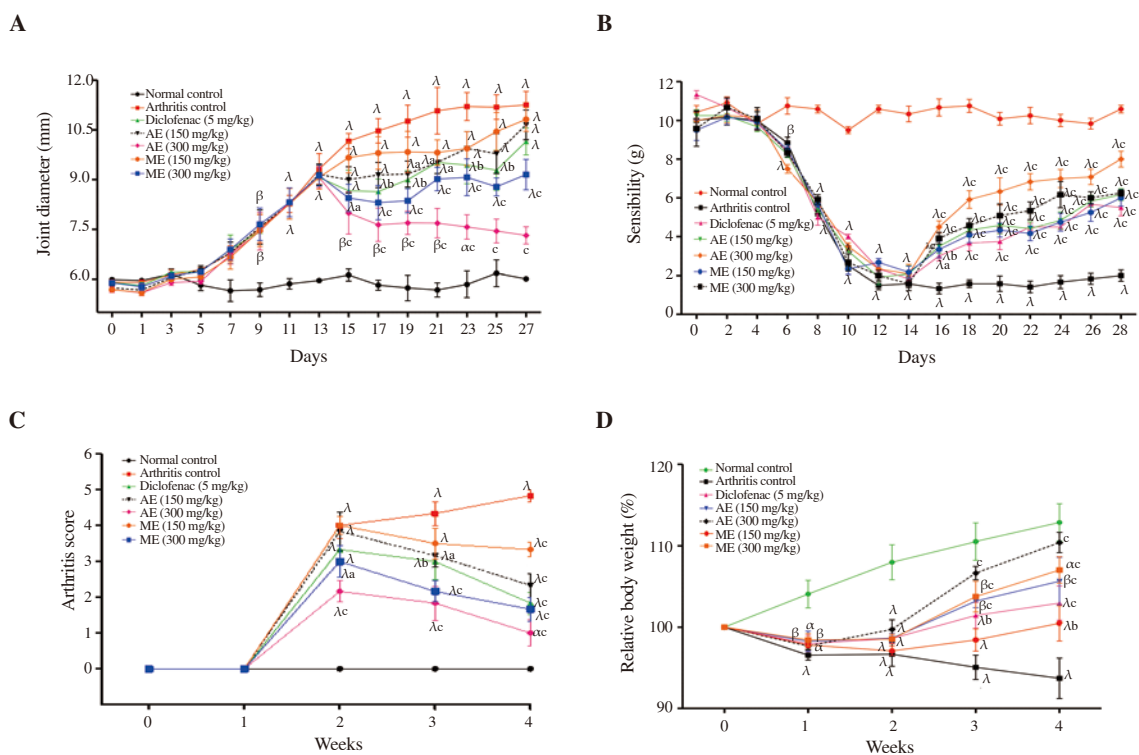
Figure 1. Effect of aqueous (AE) and methanolic (ME) extracts of *Nauclea pobeguinii* on joint diameter (A), mechanical hyperalgesia (B), arthritis score (C) and body weight (D) in complete Freund's adjuvant-induced arthritis. Values are expressed as mean± SEM for six animals and analyses by two-way ANOVA (One-way analysis of variance) followed by Bonferroni *post–hoc* test, [a]$P<0.05$; [β]$P<0.01$, [λ]$P<0.001$ when compared to the normal control, [a]$P<0.05$, [b]$P<0.01$, [c]$P<0.001$ when compared to the arthritis control group.

Table 3. Effect of aqueous and methanolic extracts of *Nauclea pobeguinii* on organ weight.

Groups	Dose (mg/kg)	Liver (g)	Kidney (g)	Spleen (g)	Thymus (g)	Lung (g)
Normal control	-	6.86 ± 0.34	0.61 ± 0.04	0.85 ± 0.05	0.38 ± 0.04	1.53 ± 0.04
Arthritis control	-	9.34 ± 0.38[β]	0.96 ± 0.03[Λ]	1.25 ± 0.03[Λ]	0.22 ± 0.03[β]	1.10 ± 0.03[β]
Diclofenac	5	7.33 ± 0.41[a]	0.70 ± 0.02[c]	0.97 ± 0.07[a]	0.32 ± 0.02	1.46 ± 0.05[a]
Aqueous extract	150	8.10 ± 0.52	0.79 ± 0.04[βb]	1.06 ± 0.10	0.34 ± 0.03	1.46 ± 0.03
	300	6.57 ± 0.26[c]	0.60 ± 0.01[c]	0.86 ± 0.06[b]	0.42 ± 0.02[c]	1.70 ± 0.07[c]
Methanolic extract	150	8.66 ± 0.42	0.80 ± 0.03[Λb]	1.08 ± 0.04	0.33 ± 0.02	1.52 ± 0.03[b]
	300	6.97 ± 0.34[b]	0.68 ± 0.02[c]	0.88 ± 0.06[b]	0.41 ± 0.04[c]	1.78 ± 0.20[c]

Values are expressed as mean ± SEM for six animals and analyses by one-way ANOVA followed by Bonferroni *post–hoc* test, [β]$P<0.01$, [Λ]$P<0.001$ when compared to the normal control, [a]$P<0.05$, [b]$P<0.01$, [c]$P<0.001$ when compared to the arthritis control.

Table 4. Effect of aqueous and methanolic extracts of *Nauclea pobeguinii* on hematological parameters.

Groups	Dose (mg/kg)	WBC (10^9/L)	RBC (million/μL)	Hemoglobin (g/dL)	Platelet (%)	Hematocrit (%)
Normal control	-	6.20 ± 0.26	8.11 ± 0.53	17.78 ± 0.46	607.50 ± 35.28	40.85 ± 1.05
Arthritis control	-	11.83 ± 0.40[Λ]	5.54 ± 0.42[α]	15.03 ± 0.65	969.00 ± 41.19[Λ]	27.25 ± 1.55[Λ]
Diclofenac	5	8.15 ± 0.32[αc]	6.41 ± 0.27	15.38 ± 0.33	709.75 ± 24.82	39.90 ± 1.39[b]
Aqueous extract	150	8.88 ± 0.27[Λc]	6.21 ± 0.52	15.68 ± 0.73	822.25 ± 37.67[β]	30.38 ± 3.37[β]
	300	6.53 ± 0.26[c]	8.11 ± 0.52[a]	19.85 ± 0.52[c]	614.25 ± 31.10[c]	41.88 ± 1.15[c]
Methanolic extract	150	6.80 ± 0.41[c]	5.87 ± 0.49[α]	15.43 ± 0.47	856.75 ± 33.81[β]	34.18 ± 1.54
	300	6.83 ± 0.56[c]	7.42 ± 0.39	17.70 ± 0.75	701.75 ± 39.39[c]	40.75 ± 1.31[c]

Values are expressed as mean ± SEM for six animals and analyses by one-way ANOVA followed by Bonferroni *post–hoc* test, [a]$P<0.05$, [β]$P<0.01$, [Λ]$P<0.001$ when compared to the normal control, [a]$P<0.05$, [b]$P<0.01$, [c]$P<0.001$ when compared to the arthritis control. WBC: White blood cell; RBC: Red blood cell.
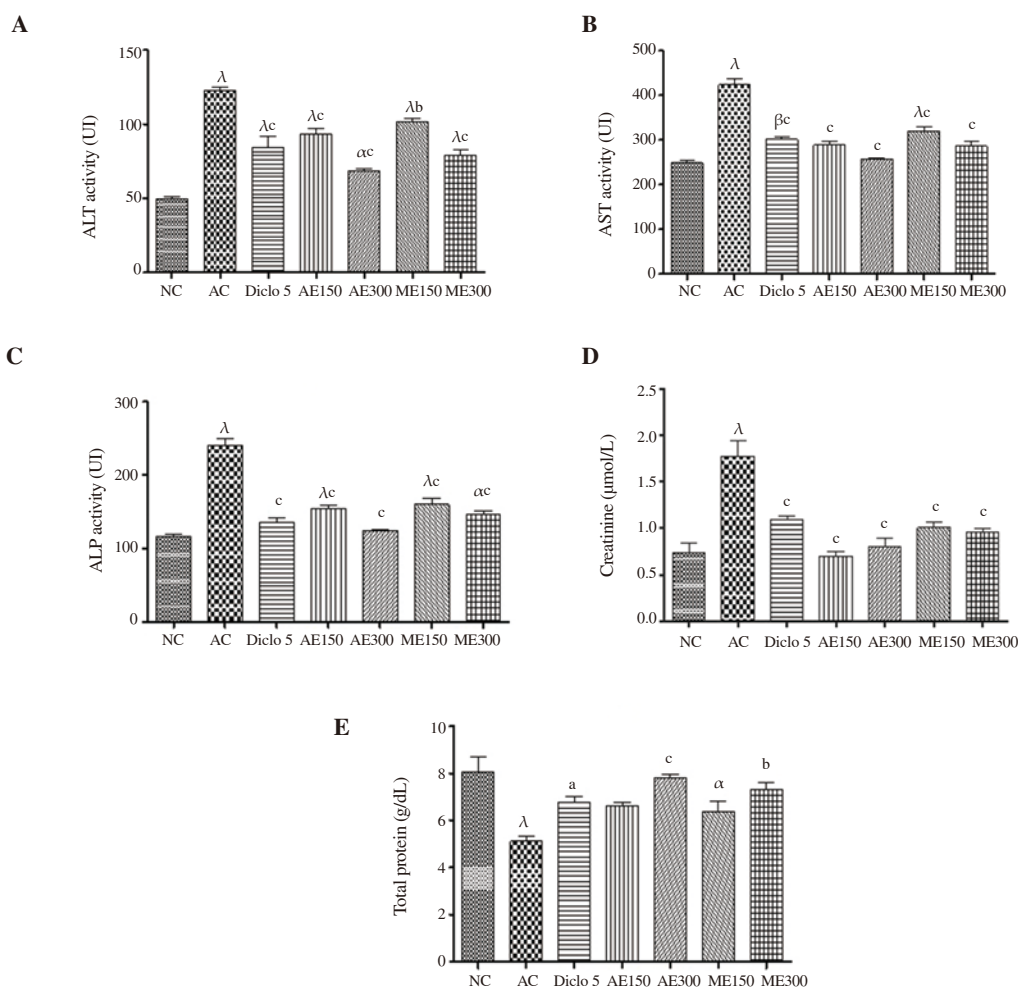
Figure 2. Effect of aqueous and methanolic extracts of *Nauclea pobeguinii* on serum parameters. Values are expressed as mean ± SEM for six animals and analyses by one-way ANOVA followed by Bonferroni *post–hoc* test, [a]$P<0.05$, [β]$P<0.01$, [Λ]$P<0.001$ when compared to the normal control, [a]$P<0.05$, [b]$P<0.01$, [c]$P<0.001$ when compared to the arthritis control. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; NC: Normal control; AC: Arthritis control; Diclo: Diclofenac; AE 150: Aqueous extract (150 mg/kg); AE 300: Aqueous extract (300 mg/kg); ME 150: Methanolic extract (150 mg/kg); ME 300: Methanolic extract (300 mg/kg).

methanolic extract at 300 mg/kg (58.91%). Diclofenac used at a dose of 5 mg/kg induced a maximum inhibition on day 26 (38.80%).

3.5.3. Effect of aqueous and methanolic extracts of N. pobeguinii on arthritis score

The arthritic score represented by the number of affected limbs increased significantly ($P<0.001$) in all groups compared to the normal group two weeks after CFA injection. Both extracts of *N. pobeguinii* significantly decreased ($P<0.05$) the arthritic score in treated animals one week after treatment. Both extracts at 300 mg/kg showed greater effects than that of diclofenac (5 mg/kg) (Figure 1C).

3.5.4. Effect of aqueous and methanolic extracts of N. pobeguinii on the body weight and organ weight

Figure 1D shows the effect of different treatments on the body weight 4 weeks after CFA injection. In arthritis animals, body weight decreased gradually and the decrease became significant ($P<0.01$) from the 1st week compared to the normal control. Aqueous and

methanolic extracts (300 and 150 mg/kg) and diclofenac (5 mg/kg) had an increase in body weight from week 2, and this increase was significant the in the 4th week.

Table 3 shows the effect of the extracts on the weight of the organs. CFA administration significantly increased the weights of liver ($P<0.01$), kidney ($P<0.001$), spleen ($P<0.001$) and significantly reduced the weights of thymus ($P<0.01$) and lungs ($P<0.01$). Oral administration of the aqueous and methanolic extracts of *N. pobeguinii* significantly improved the weights of these organs.

3.5.5. Effect of aqueous and methanolic extracts of N. pobeguinii on hematological parameters

Table 4 shows the effect of different treatments on hematological parameters 28 days after the CFA administration. CFA significantly elevated levels of white blood cells ($P<0.001$), platelets ($P<0.001$), and significantly reduced levels of red blood cells ($P<0.05$) and hematocrit ($P<0.001$) compared with animals of the normal control group. Oral administration of the extracts significantly reduced the
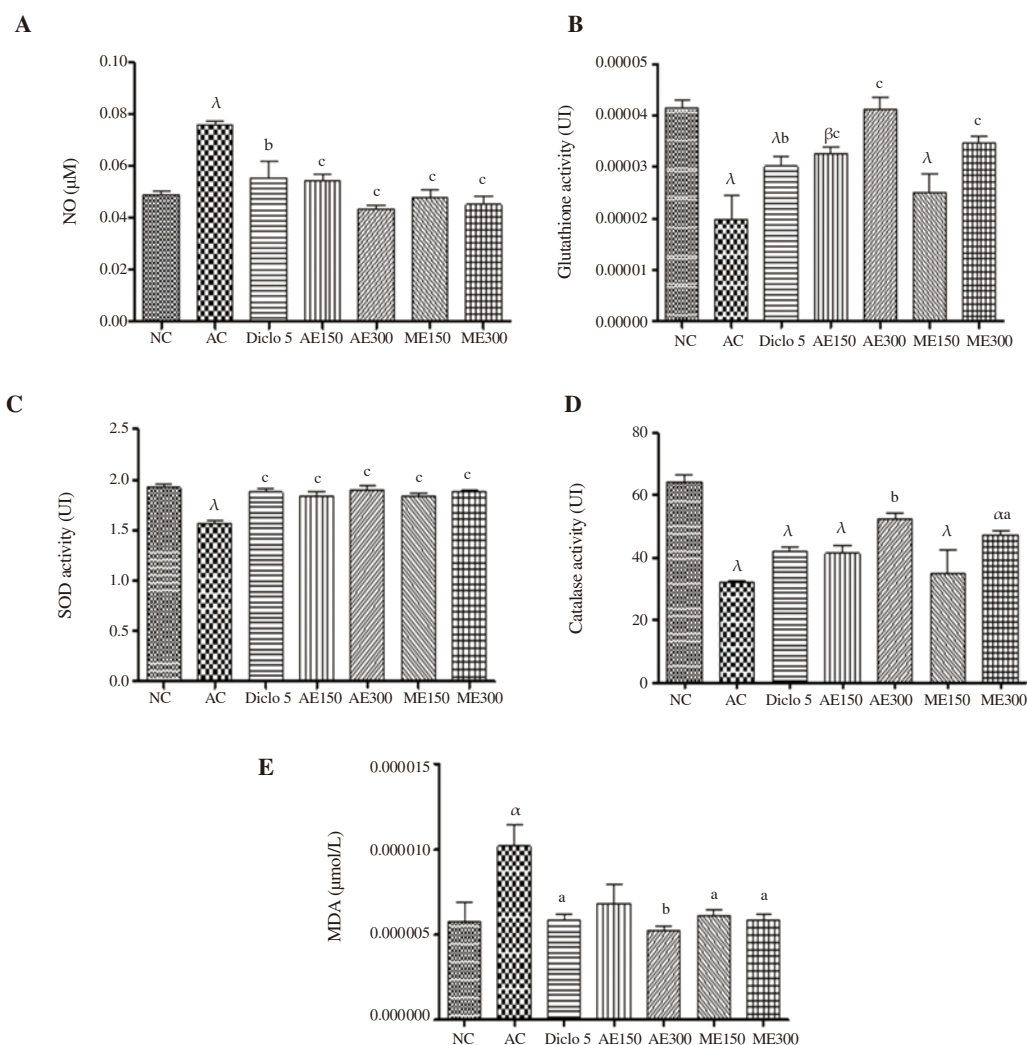
Figure 3. Effect of aqueous and methanolic extracts of *Nauclea pobeguinii* on some parameters of oxidative stress. Values are expressed as mean ± SEM for six animals and analyses by one-way ANOVA followed by Bonferroni *post–hoc* test, [α]$P<0.05$, [β]$P<0.01$, [λ]$P<0.001$ when compared to normal control, [a]$P<0.05$, [b]$P<0.01$, [c]$P<0.001$ when compared to arthritis control. NO: nitric oxide; SOD: superoxide dismutase; MDA: malondialdehyde; GSH: glutathione; NC: Normal control; AC: Arthritis control; Diclo: Diclofenac; AE 150: Aqueous extract (150 mg/kg); AE 300: Aqueous extract (300 mg/kg); ME 150: Methanolic extract (150 mg/kg); ME 300: Methanolic extract (300 mg/kg).

number of leukocytes and platelets and significantly increased red blood cell and hematocrit levels. The effect of the extracts at 300 mg/kg was more significant than that of diclofenac (5 mg/kg).

3.5.6. Effect of aqueous and methanolic extracts of N. pobeguinii on biochemical parameters

Arthritic group significantly elevated serum ALT ($P<0.001$) (Figure 2A), AST ($P<0.001$) (Figure 2B), ALP ($P<0.001$) (Figure 2C) and creatinine ($P<0.001$) (Figure 2D), and significantly decreased the level of proteins ($P<0.001$) (Figure 2E). Both extracts of *N. pobeguinii* significantly reduced ALT, AST, ALP, and creatinine levels and significantly increased total protein level. Extracts at 300 mg/kg showed greater effects than diclofenac on all evaluated parameters.

3.5.7. Effect of aqueous and methanolic extracts of N. pobeguinii on oxidative stress parameters

The effect of aqueous and methanolic extracts of *N. pobeguinii* on the oxidative stress is illustrated by Figure 3. Th extracts and diclofenac markedly reduced ($P<0.05$) NO and MDA activities, and significantly increased ($P<0.05$) CAT, SOD and GSH activities compared to arthritic control group.

### 3.5.8. Effect of aqueous and methanolic extracts on histopathology

The histopathology of the joints in the normal control (Figure 4A) showed cartilage and intact bones without infiltration of leucocytes; while arthritic control (Figure 4B) showed infiltration of leucocytes, bone destruction, and cartilage. Animals treated with diclofenac (5 mg/kg) (Figure 4C), aqueous extract (150 and 300 mg/kg) (Figures 4D, 4E) and methanolic extract (150 and 300 mg/kg) (Figures 4F, 4G) showed a significant reduction in bone erosion, leukocyte infiltration and destruction of articular cartilage.

4. Discussion

This study aims to evaluate the effects of aqueous and methanolic extracts of *N. pobeguinii* via *in vitro* and *in vivo* studies. The *in vitro* results show that the extracts of *N. pobeguinii* possess anti-inflammatory (inhibition of the denaturation of proteins, the activity of cyclooxygenase, the activity of 5-lipoxygenase, the proliferation of T lymphocytes, production of intracellular and extracellular ROS) and antioxidants properties (DPPH, ABTS and NO), while *in vivo* results show that the extracts have anti-arthritic activity using arthritis models induced by CFA.

Rheumatoid arthritis is systemic inflammatory rheumatism characterized by joint pain, inflammation of the synovial membrane, bone erosion and destruction of articular cartilage. Articular cartilage is made up of proteins, whose denaturation can cause rheumatoid arthritis and can cause the aggravation of the pathology[24]. These proteins can lose their secondary and tertiary structures, which can cause a constraint generated in the cell and cause the modification of the hydrogen bonds[6,24]. The aqueous and methanolic extracts of *N. pobeguinii* inhibited protein denaturation in this study. These effects would be related to the ability of these extracts to
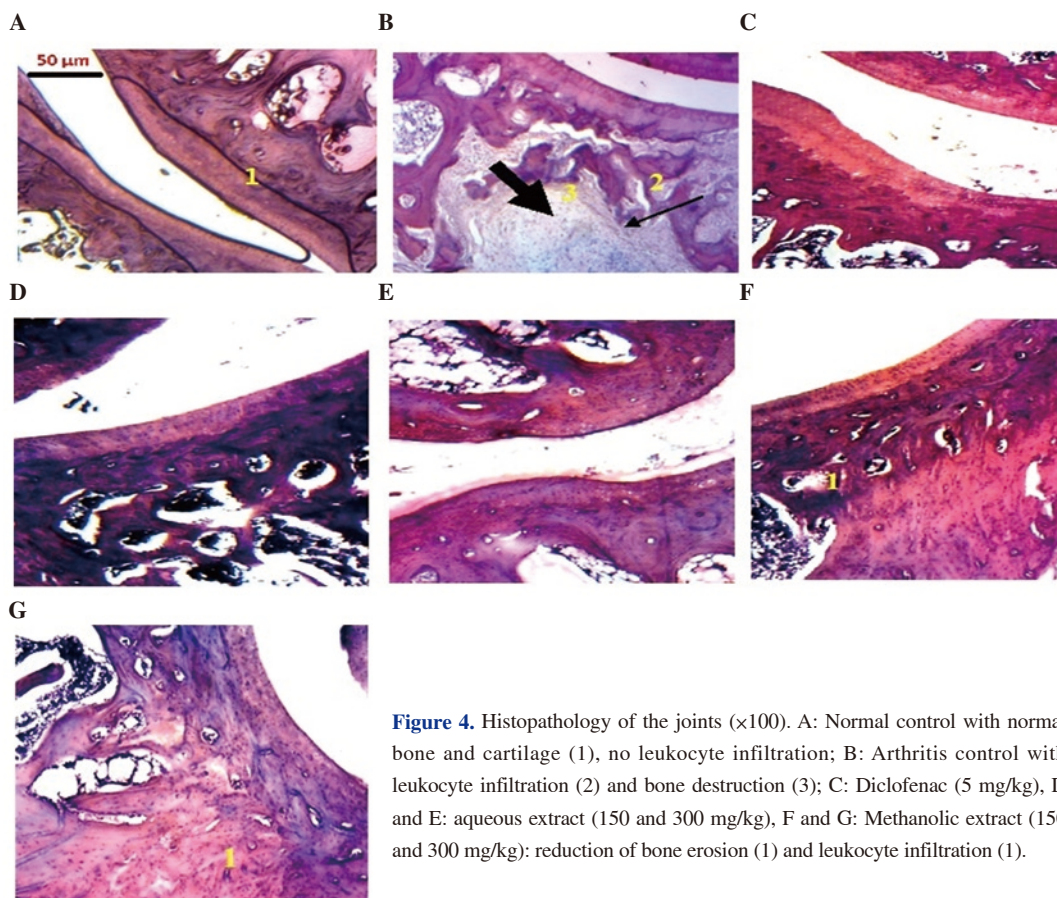
Figure 4. Histopathology of the joints (×100). A: Normal control with normal bone and cartilage (1), no leukocyte infiltration; B: Arthritis control with leukocyte infiltration (2) and bone destruction (3); C: Diclofenac (5 mg/kg), D and E: aqueous extract (150 and 300 mg/kg), F and G: Methanolic extract (150 and 300 mg/kg): reduction of bone erosion (1) and leukocyte infiltration (1).

protect the structure of the protein and prevent the change of the aforementioned bonds. Actually, flavonoids, tannins, and alkaloids are able to interact with the aliphatic region around the lysine residue on albumin protein and prevent protein denaturation[25]. During the inflammatory process, activation of protein kinase C promotes the activity of phospholipase A2 which causes the denaturation of membrane phospholipids to form arachidonic acid[25]. This arachidonic acid is responsible for the formation of eicosanoids which will stimulate enzymes (cyclooxygenase and 5-lipoxygenase) causing the release of inflammatory mediators (PGE$_2$, thromboxanes, and leukotrienes). Our results show that these extracts have an inhibitory effect on the activities of cyclooxygenase and 5-lipoxygenase, and these properties could be attributed to the presence of secondary metabolites belonging to the flavonoids, terpenoids and/or saponins classes. Some of these compounds are able to affect the metabolism of arachidonic acid in several ways, then to specifically inhibit the activities of cyclooxygenase and/or lipoxygenase[26]. Polymorphonuclear neutrophils, macrophages and T cells induce activation of the immune response in patients with rheumatoid arthritis. These agents provoke an immune response by activation of the toll-like receptor, and triggering an inflammatory reaction with cascade release of many proinflammatory cytokines, particularly TNF-α and interleukin 1β. These mediators released by the immune system play an important role in the initiation and progression of rheumatoid arthritis. They are likely to cause phosphorylation and the deterioration of proteins following activation of energy nuclear factor kappa B which is likely to influence the activity of immune cells[27]. Thus, to evaluate the effects of aqueous and methanolic extracts of *N. pobeguinii* on the modulation of the immune system, the T cell proliferation assay was used. The results show that these extracts significantly inhibit the proliferation of T lymphocytes. It is known that some flavonoids and/or saponins are able to inhibit or block PI3k/Akt/mTOR and NF-κB pathways, thus significantly reduce cell proliferation[28]. Moreover, during the progression of polyarthritis, proinflammatory cytokines (particularly TNF-α and IL-1β) are capable of activating the nicotinamide adenine dinucleotide phosphate-oxidase which through the respiratory chain could cause the release of the superoxide and then induce oxidative stress. Increased production of free radicals and proinflammatory cytokines is responsible for accelerated joint cartilage degradation, bone destruction and osteoclast stimulation[29]. Thus, the antioxidant potential of the extracts was tested *in vitro* by DPPH, ABTS and NO assays. It shows that both extracts significantly reduce DPPH, ABTS discoloration and NO production. These results suggest that *N. pobeguinii* has antioxidant properties *in vitro*. Compounds such as flavonoids and saponins in the two extracts have already shown *in vitro* antioxidant activities by reducing DPPH, ABTS discoloration and NO production[30].

ROS are involved in the pathophysiology of inflammatory diseases such as rheumatoid arthritis. They lead to the release of cytokines and the activation of proinflammatory enzymes such as cyclooxygenase, lipoxygenase and inducible nitric oxide[7]. Lack of control of these ROS is responsible for cartilage destruction, bone erosion, and nuclear factor expression. The results of our study show a significant inhibitory effect of the extracts on the production of intracellular and extracellular ROS. On the one hand, these extracts had the inhibitory effect on NF-κB phosphorylation, preventing it from releasing proinflammatory cytokines. This effect could be attributed to some terpenoids, flavonoids and/saponins, which are able to inhibit NF-κB/AP1 axes and reduce TNF-α, IL-1β, NO and PGE$_2$ production[31,32]. On the other hand, these extracts could inhibit the denaturation of proteins. The activities of cyclooxygenase and 5-lipoxygenase are potent mediators of inflammation, since these enzymes are capable of forming the hydroperoxides precursors of leukotrienes.

Cyclooxygenase is an enzyme that catalyzes the biosynthesis of prostaglandins, an anti-pain, and anti-inflammatory agent, while free radicals oxidize the lipids of the cell membrane, resulting in the formation of lipid peroxidation[31]. The injection of CFA into the rat causes the activation of these enzymes and the activation of lipid peroxidation. The injection in the paw is associated with the development of a monoarthritis whereas the injection in the tail is associated with the development of a polyarthritis in Wistar rats and Swiss albino mice[32]. CFA-induced arthritis is widely used to test anti-arthritic potential of many substances[23]. This systemic injection is characterized by the migration of leukocytes and lymphocytes into the joint, resulting in the development of joint inflammation on the ninth day after injection[23]. In this study, *N. pobeguinii* extract significantly reduced joint diameter and arthritis score, which could be associated with the ability of these extracts to inhibit lipoxygenase and ROS mediators[33], and with the antiproliferative power of these extracts[18]. Some flavonoids and/or terpenoids are able to act as a nonsteroidal anti-inflammatory drug (diclofenac) by inhibiting the thromboxane-prostanoid receptor, affecting the arachidonic acid release and uptake, inhibiting lipoxygenase enzymes and inducing antiproliferative effect[28]. Inflammatory stimuli such as CFA result in a cascade of proinflammatory mediators such as cytokines and PGE$_2$. These mediators are responsible for the hypersensitization of nociceptors with subsequent hyperalgesia[33]. The analgesic effect of the extract of *N. pobeguinii* was evaluated by measuring the pain with an analgesimeter and these extracts showed an antihyperalgesic effect. This activity of *N. pobeguinii* is attributed to the inhibition of the production and/or activity of proinflammatory mediators released during the development of hyperalgesia, or to the ability to inhibit cyclooxygenase, which is responsible for the production of PGE$_2$. In fact, the nonsteroidal anti-inflammatory drug (diclofenac) and some compounds (flavonoids, saponins and/or triterpenoids) could diminish prostaglandin synthesis by inhibiting cyclooxygenase, which catalyzes the formation of prostaglandin precursors from arachidonic acid[34].

Variation in body weight is an important indicator of health conditions. Rheumatoid arthritis is associated with reduced muscle mass, loss of appetite, or metabolic disorders caused by the inflammatory response[35]. The weight loss observed in untreated animals after CFA injection was alleviated after the administration of the aqueous and methanolic extract of *N. pobeguinii* as well as diclofenac. All treated animals showed an increase in body weight after treatment. People with rheumatoid arthritis typically develop anemia characterized by elevated white blood cell counts and reduced hemoglobin levels, decreased erythropoietin levels, or bone

marrow activity[36]. In addition, an increase in platelet levels, a reduction in red blood cells and hematocrit levels are also observed in these patients. Patients with rheumatoid arthritis also have significant variation in liver enzyme activity such as transaminases (ALT and AST), ALP[37], creatinine and total proteins, with hepatic and renal damage. These enzymes (transaminases and ALP) are usually released into the circulation during bone resorption. They are involved in localized bone loss such as bone erosion and periarticular osteopenia and are also considered as precursors of some proinflammatory mediators such as serotonin, bradykinin, and histamine, which are responsible for the development and maintenance of inflammation[38]. In this study, the results show that extracts of *N. pobeguinii* reduced the number of white blood cells and increased hemoglobin levels. They also improved the variations in serum parameters. It is known that compounds as alkaloid, flavonoids, tannins, terpenes, and steroids have the ability to protect the erythrocytes from oxidative damage, and possess erythropoietin stimulatory, immune-stimulatory and thrombopoietin stimulatory activities[39].

Free radicals are known to destroy membrane lipids, proteins, DNA and articular cartilage. In patients with rheumatoid arthritis, polymorphonuclear neutrophils and macrophages produce ROS that lead to cartilage damage, lipid peroxidation and increase NO-catalyzed reaction or antioxidant defense[40]. In this study, extracts of *N. pobeguinii* significantly decreased MDA and NO levels, and increased activity of SOD, GSH, and CAT. The presence of compounds such as flavonoids and saponins could justify the antioxidant activity of the plant because it is known that these compounds are able to significantly increase the activities of SOD, CAT, and glutathione-*S*-transferase as well as levels of ascorbic acid and GSH, while significantly decrease MDA level[41]. In the present study, histopathology of the joints showed that animals treated with aqueous and methanolic extracts of *N. pobeguinii* have joints with little cellular infiltration, bones and cartilage are less damaged than the untreated animal, suggesting that these extracts would have restored the normal architecture of the joint. Some flavonoids could inhibit the release of NO, TNF-α, IL-1, IL-6 as well as T-cell proliferation[42], significantly suppress secondary inflammatory paw swelling, pain response, and polyarthritis index in rats by inhibiting production of IL-1, TNF-α, and PGE$_2$ in synoviocytes and significantly reduce the joint damage[43]. These effects would be related to the inhibitory activities of these extracts on the proliferation of T cells and the production of ROS, which are considered as the main factors for deterioration and destruction of bones and cartilage[9]. Besides, these effects may also be related to the inhibition of proinflammatory cytokines, which have been shown to be responsible for inflammation.

The *in vitro* study shows that the aqueous and methanolic extracts of *N. pobeguinii* have important anti-arthritic properties by inhibition of protein denaturation, cyclooxygenase, 5-lipoxygenase, T cell proliferation and production of free radicals. Moreover, *in vivo* study also shows that they also could reduce inflammation of the joint, hyperalgesia, improve hematological parameters, reduce liver enzymes, regulate oxidative stress parameters, and restore inflammatory joint. These results confirm the analgesic, anti-

inflammatory and antiarthritic activities of this plant as a traditional medicine.

Conflict of interest statement

The authors declare that they have no competing interests.

Authors' contributions

TEG, MM and AG designed the work. DNSF, MM, TEG, AAD and MMMV conducted the work, collected and analysed the data. MM, NYW, AG and TTH drafted the manuscript and revised it critically. All authors agree to be accountable for all aspects of the work.

References

[1] Straub RH, Schradin C. Chronic inflammatory systemic diseases: An evolutionary trade-off between acutely beneficial but chronically harmful programs. *Evol Med Public Health* 2016; **1**: 37–51.

[2] Ghozlani I, Achemlal L, Rezqi A, Mounach A, Bezza A, El Maghraoui A. Physiopathologie de la polyarthrite rhumatoïde. *Revue Marocaine de Rhumatol* 2012; **19**: 6-9.

[3] Volluri SS, Bammidi SR, Chippada SC, Vangalapati. *In vitro* anti-arthritic activity of methanolic extract of *Bacopa monniera. Int J Chem Environ Pharm Res* 2011; **2**(2-3): 156-159.

[4] Martel-pelletier J, Lajeunesse D, Reboul P, Pelletier JP. Therapeutic role of dual inhibitors of 5-LOX and COX, selective and non-selective non-steroidal anti-inflammatory drug. *Ann Rheum Sci* 2003; **62**: 501-509.

[5] Dewar CL, Harth M. Superoxide production from cytokine-treated adherent rheumatoid neutrophils. *Clin Invest Med* 1994; **17**: 52-60.

[6] Bonnefont-Rousselot D, Beaudeux J, Thérond P, Peynet J, Legrand A, Delattre J. Diabète sucré, stress oxydant et produits de glycation avancée. *Ann Pharm Fr* 2004; **62**: 147-157.

[7] Falgaron G, Nahon P, Boissier MC. *Atteintes hepatiques de la polyarthrite rhumatoide*. Elsevier Mason; 2013; p. 11-13.

[8] Majjad A, Toufik H, Djossou JH, El Khattary M, El Maghraoui A. Les traitements de la polyarthrite rhumatoïde et la pneumopathie interstitielle diffuse. *Rev Mar Rhum* 2018; **43**: 5-10.

[9] Cheen K, Nolan J, Shariq S, Cho L, Pal A, Arnold J. Fatal case of disseminated BCG infection in an infant born to a mother taking infliximab for Crohn's disease. *J Crohns Colitis* 2010; **4**: 603-605.

[10] CSTR/OUA. *Pharmacopée Africaine*. 1ère édition. Nigeria, Lagos: CSTR/OUA; 1985, p. 4-8.

[11] Agnaniet H, Mbot EJ, Keita O, Fehrentz JA, Ankli A, Gallud A, et al. Antidiabetic potential of two medicinal plants used in Gabonese folkmedicine. *BMC Complement Altern Med* 2016; **16**: 71.

[12] Seukep JA, Sandjo LP, Ngadjui BT, Kuate V. Antibacterial activity of the extract and compounds from *Nauclea pobeguinii* against Gram negative multi drug. *BMC Complement Altern Med* 2016; **16**: 193.

[13] Mbiantcha M, Tsafack EG, Ateufack G, Nana YW, Bomba TFD, Djuichou NSF, et al. Analgesic, anti-inflammatory and anti-arthritic properties of aqueous and methanolic stem bark extracts from *Nauclea*

pobeguinii (Rubiacee) in rats. *J Complement Integr Med* 2018; **15**(4): 1-13.

[14]Xu YJ, Foubert K, Dhooghe L, Lemiere F, Cimanga K, Mesia K. Chromatographic profiling and identification of two new iridoid-indole alkaloids by UPLC-MS and HPLC-SPE-NMR analysis of an antimalarial extract from *Nauclea pobeguinii. Planta Med* 2012; **78**(11): 316.

[15]Matos FJA. *Introducao à Fitoqu´ımica experimental*. 2nd ed. Fortaleza, Brazil; 1997, p. 1-150.

[16]Padmanabhan P, Jangle SN. Evaluation of *in–vitro* anti-inflammatory activity of herbal preparation, a combination of four medicinal plants. *Int J Basic Appl Med Sci* 2012; **2**(1):109-116.

[17]Viji V, Helen A. Inhibition of lipoxygenases and cyclooxygenase-2 enzymes by extracts isolated from *Bacopa monniera* (L.) Wettst. *J Ethnopharmacol* 2008; **118**: 305–311.

[18]Brand-Williams W, Cuvelier ME, Berset C. Use of a free radical method to evaluate antioxidant activity. *LWT–Food Sci Technol* 1995; **28**: 25–30.

[19]Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Rad Biol Med* 1999; **26**: 1231–1237.

[20]Benzie IFF, Strain JJ. Ferric reducing/antioxidant power assay: Direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. *Methods Enzymol* 1999; **299**: 15–27.

[21]Mbiantcha M, Almas J, Shabana US, Nida D, Aisha F. Anti-arthritic property of crude extracts of *Piptadeniastrum africanum* (Mimosaceae) in complete Freund's adjuvant-induced arthritis in rats. *BMC Complement Altern Med* 2017; **17**:111.

[22]Mbiantcha M, Almas J, Atsamo AD, Ateufack G, Shabana US, Bomba Tatsinkou DF, et al. Antiinflammatory and antiarthritic effects of methanol extract of the stem bark of *Boswellia dalzielii* Hutch (Burseraceae) in rats. *Infammopharmacology* 2018; **26**: 1383-1398.

[23]Geetha T, Varalakshmi P. Anticomplement activity of triterpenes from *Crataeva nurvala* stem bark in adjuvant arthritis in rats. *Gen Pharmacol* 1999; **32**: 495–497.

[24]Williams LAD, O'Connar A, Latore L, Dennis O, Ringer S, Whittaker JA, et al. The *in vitro* anti-denaturation effects induced by natural products and non-steroidal compounds in heat treated (immunogenic) bovine serum albumin is proposed as a screening assay for the detection of anti-inflammatory compounds, without the use of animals. *West Indian Med J* 2008; **57**(4): 327-351.

[25]Falcão S, Baptista P, Freire C, Vilas-Boas M, Ferreira I. Antioxidant activity of *Agaricus* sp. mushrooms by chemical, biochemical and electrochemical assays. *Food Chem* 2016; **111**: 61-66.

[26]Guan F, Wang H, Shan Y, Chen Y, Wang M, Wang Q, et al. Inhibition of COX-2 and PGE_2 in LPS-stimulated RAW264.7 cells by lonimacranthoide VI, a chlorogenic acid ester saponin. *Biomed Rep* 2014; **2**(5): 760–764.

[27]Castrillo A, De las Heras B, Hortelano S, Rodriguez B, Villar A, Boscar L. Inhibition of nuclear factor kappa B (NF-kappa B) pathway by tetracyclic kaurene diterpenes in macrophages: Specific effects on NF-kappa B inducing kinase activity and on the coordinate activation of ERK and p38MA PK. *J Biol Chem* 2001; **276**: 15854-15860.

[28]Joby J, Sudheesh S, Sumesh KTM, Sony J, Jayadevi VE. Study of *in vitro* immunomodulatory effect of flavonoid isolated from *Phyllanthus niruri*

on human blood lymphocytes and evaluation of its antioxidant potential. *Inter J Pharmacogn Phytochem Res* 2014; **6**(2): 284-289.

[29]Ilhami G, Vakhtang M, Akçahan G, Rim E. Antioxidant activity of saponins isolated from ivy: Alpha-hederin, hederasaponin-C, hederacolchiside-E and hederacolchiside-F. *Planta Med* 2004; **70**(6): 561-563.

[30]Teixeira TS, Vale RC, Almeida RR, Ferreira TPS, Guimarães LGL. Antioxidant potential and its correlation with the contents of phenolic compounds and flavonoids of methanolic extracts from different medicinal plants. *Rev Virtual Quim* 2017; **9**(4): 1546-1559.

[31]Tanas S, Odabasoglu F, Halici Z, Cakir A, Aygum H, Aslan A, et al. Evaluation of anti-inflammatory and antioxidant activities of *Peltigera rufuscens* lichen species in acute and chronic inflammation models. *J Nat Med* 2010; **64**: 42-49.

[32]Mythilypriya R, Shanthi P, Sachdanandam P. Salubrious effect of Kalpaamruthaa, a modified indigenous preparation in adjuvant-induced arthritis in rats – A biochemical approach. *Chem Biol Interact* 2008; **173**: 148–158.

[33]Lee JH, David SA, Mary K, Janine E, Klaus B, Paul HG, et al. Antiproliferative effect of nonsteroidal antiinflammatory drugs against human colon cancer cells. *Cancer Epidemiol Biomarkers Prev* 1994; **3**: 433-438.

[34]Gan TJ. Diclofenac: An update on its mechanism of action and safety profile. *Curr Med Res Opin* 2010; **26**(7): 1715-1731.

[35]Paval J, Kaitheri SK, Potu BK, Govindan S, Kumar RS, Narayanan SN, et al. Anti-arthritic potential of the plant *Justicia gendarussa. Burm F Clinics* 2009; **64**(4): 357-362.

[36]Jalacpure S, Mandavkar Y, Khalure P, Shinde G, Shelar P, Shah A. Antiarthritic activity of various extracts of *Mesua ferrea* linn. seed. *J Ethnopharmacol* 2011; **132**: 700-704.

[37]Bihani GV, Rojatkar SR, Bodhankar SL. Anti-arthritic activity of methanol extract of *Cyathocline purpurea* (whole plant) in Freund's complete adjuvant-induced arthritis in rats. *Biomed Aging Pathol* 2014; **4**: 197–206.

[38]Jorum OH, Piero NM, Machocho AK. Haematological effects of dichloromethane-methanolic leaf extracts of *Carissa edulis* (Forssk.) vahl in normal rat models. *J Hematol Thrombo Dis* 2016; **4**: 232.

[39]Moris G, Maes M. Mitochondrial dysfunction in myalgic encephalomyelitis: Chronic fatigue syndrome explained by activated immuno-inflammatory oxidative and nitrosative stress pathway. *Metab Brain Dis* 2014; **29**(1): 19-36.

[40]Ebenezer TO, Ayokanmi O, Oluwatobi AA, Olaniyi SO, Olaoluwa OO, Roseline CE. Quercetin, a flavonoid antioxidant, ameliorated procarbazine-induced oxidative damage to murine tissues. *Antioxidants* 2015; **4**: 304-321.

[41]Han Y. Rutin has therapeutic effect on septic arthritis caused by *Candida albicans. Int Immunopharmacol* 2009; **9**: 207–211.

[42]Tang LQ, Wei W, Wang XY. Effects and mechanisms of catechin for adjuvant arthritis in rats. *Adv Ther* 2007; **24**: 679–690.

[43]Jun Z, Tao L, Fang X, Shuping Y, Fang X, Chenyang L, et al. Anti-arthritic effects of total flavonoids from *Juniperus sabina* on complete freund's adjuvant induced arthritis in rats. *Pharmacogn Mag* 2016; **12**(47): 178–183.