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Minoue Kuum Marc Germain
1. Department of Psychology, Faculty of Letters and Human Social Sciences, P.O. Box, University of Douala, Cameroon
2. Department of Animal Biology and Physiology, Faculty of Sciences, P.O Box, University of Yaounde I, Cameroon

Tchadji Jules Colince
1. Department of Animal Biology and Physiology, Faculty of Sciences, P.O Box, University of Yaounde I, Cameroon
2. Laboratory of Vaccinology / Biobanking of The Chantal Biya International Reference Center for research on the prevention and management of HIV/AIDS (CIRCB), BP, Messa Yaounde, Cameroon

Mezui Christophe
Department of Biological Sciences, Higher Teachers' Training College, University of Yaounde I, P.O. Box 047, Cameroon

Dimo Théophile
Department of Animal Biology and Physiology, Faculty of Sciences, P.O Box, University of Yaounde I, Cameroon

Corresponding Author:
Minoue Kuum Marc Germain
1. Department of Psychology, Faculty of Letters and Human Social Sciences, P.O. Box, University of Douala, Cameroon
2. Department of Animal Biology and Physiology, Faculty of Sciences, P.O Box, University of Yaounde I, Cameroon

The properties of *Albizia ferruginea* (Mimosaceae) stem bark aqueous extract on pro-inflammatory cytokines and hematological parameters among sub-chronic inflammation-induced rats

Minoue Kuum Marc Germain, Tchadji Jules Colince, Mezui Christophe and Dimo Théophile

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Abstract
The present research was carried out to evaluate the properties of *Albizia ferruginea* aqueous stem bark extract (AFASBE) on the variation of some pro-inflammatory cytokines and haematological parameters during sub-chronic inflammation. Sub-chronic inflammation was induced in rats by the insertion of cotton pellets and then treated with AFASBE. Determination of the concentrations of TNF-α, IL-1β, IL-6 and INF-γ was carried out by the ELISA technique "Enzyme Linked Immuno Sorbent Assay" using the Quantikines kits (France). Haematological parameters were quantified using the Coulter S-Plus IV/D. The levels of TNF-α, IL-1β, IL-6 and INF-γ significantly (p<0.001) increased in the negative control serum compared to normal rats. AFASBE (100 and 200 mg/kg) and Diclofenac (4 mg/kg) induced significant (p<0.001) decrease of TNF-α, IL-1β, IL-6 and INF-γ levels compared to the negative control. Haematological analysis revealed that AFASBE (100 mg/kg and 200 mg/kg) and diclofenac (4 mg/kg) significantly (p<0.05) reduced the number of white blood cells. The number of platelets decreased significantly (p<0.05), in all groups treated with AFASBE and diclofenac, compared to control. *Albizia ferruginea* aqueous stem bark extract is believed to have immunomodulatory potential on the inhibition of the expression of inflammatory mediators in serum during sub-chronic inflammation.

Keywords: Cytokines, sub-chronic inflammation, *Albizia ferruginea*, immuno-modulator, mimosaceae

1. Introduction
Granulomatosis is a subchronic inflammatory reaction secondary to the persistence of the etiological agent. This agent, which is poorly eliminated or degraded, maintains a persistent inflammatory reaction, which is a source of lesion. Tissue lesions cause a cascade of cell response in the injured zone, with the liberation of inflammatory cytokines such as IL-6, IL-1β, TNF-α and INF-γ and other substances [1]. Chronic or cellular inflammation can take different forms: diffuse or granulomatous inflammation. Blood and blood components are essential to maintaining homeostasis. However, low impairment of standard levels of blood components can lead to serious illnesses or disorders [2]. The haematological system allows the detection of numerous damage and toxic effects when xenobiotics are administered and inflammatory responses. The inflammatory reaction, a physiological response to an aggression, involves a complex network of mediators and cells involved in a variable way depending on the causal agent, the site and the individual himself, taking into account possible variations in the genetic terrain [3]. By going beyond its homeostatic goal, the inflammatory reaction becomes chronic and can cause cellular and tissue damage. Knowledge of the intimate mechanisms involved is increasing thanks to technological progress, while at the same time making it possible to envisage a better targeted therapeutic approach [4]. The effectiveness of anti-inflammatory therapy is all the better as pharmacological intervention takes place at an early stage, touching the elements responsible for the various potential lesions as early as possible [5]. Cytokines implicated in natural immunity: IL-6, IL-1β, TNF-α and INF-γ. In response to inductor factors, there is a cascade of inflammation activation involving different cells.

It begins with the synthesis of the first cytokines of the IL-1β and TNF-α cascade, produced mainly by monocytes [6]. Both cytokines then incite the production of IL-6 by the stromal cells that form the supporting structures of the tissues. Interleukin 6 (IL-6) also contributes to systemic effects by stimulating the liver and the synthesis of acute-phase proteins like CRP. The pathophysiological mechanisms of inflammatory diseases involve a variable contribution of B and T cells and inflammatory cytokines such as IL-6 and TNF [7]. They stimulate B lymphocyte production and differentiation. IL-6 is also anti-inflammatory as it inhibits TNF-α production. Interferon α (IFNα), also produced by fibroblasts, shows antiretroviral properties. Traditional doctors use many natural plant and unnatural as treatment alternatives for a large variety, including subchronic and chronic inflammation [8]. Natural products are believed to be an important source of new chemical substance with future therapeutic applicability [9]. Cameroon is a rich root of medicinal plants, many of which are used against diseases in traditional medicine. Many plant species are traditionally used as analgesics, anti-inflammatories and antipyretics. *A. ferruginea* is an extensive tree in Cameroon and other African countries. Traditionally, the stem barks of *Albizia ferruginea* are used to treat inflammation, pain, abdominal and tooth pain, abdominal and tooth pain, headache, rheumatism, diarrhoea and reduced fever [10]. Phytochemical work shows in the plant the presence of carbohydrates, triterpenes, heart glycosides, saponins, tannins, anthraquinones and flavonoids, and these molecules have many effects, mainly known as anti-inflammatories. Flavonoids and flavonols present inhibition of arachidonic acid peroxidation, which are chemical mediators involved in chronic inflammation [11]. Likewise, some studies have shown anti-inflammatory effects of this plant on cotton pellet, formalin and complete Freund's Adjuvant (CFA)-induced sub-chronic and chronic inflammation in rats [10, 12]. But no systematic research has been done on the research of the effects of this plant on pro-inflammatory cytokines and hematological parameters during inflammation therefore, the present study was designed to evaluate the anti-inflammatory properties of aqueous extract of the stem bark of *A. ferruginea* on pro-inflammatory cytokines and haematological parameters in rats induced by subchronic inflammation.

2. Materials and Methods

2.1 Plant material

The stem bark of *A. ferruginea* was collected in the village of Angallé, in the southern region of Cameroon. The plant matter was identified by Dr Barthélémy TCHIENGUE of the National Herbarium of Cameroon, where a reference specimen of the plant was deposited under number 49871.

2.2 Preparation of plant extract

The peel on fresh stems has been air-dried and reduced to a fine powder. The powder (500 g) was macerated with 2.5 L of distilled water over a period of 24 hours. The blend was filtered with Whatman N°3 filter paper, concentrated under reduced pressure and freeze-dried at 50 °C for 48 hours. A dark brown solid (84 g) representing the stem barks aqueous extract of *A. ferruginea* was obtained (yield of 16.8%).

2.3 Qualitative and quantitative phytochemical analysis

Qualitative phytochemicals studies of the aqueous bark of the stem of *A. ferruginea* have been performed for alkaloids, flavonoids, saponins, phenols, steroids, glycosides and tannins, by our research team [10, 12] using standard methods previously described [13, 14].

2.4 Animals

Male and female Wistar albino rats (200-250 g) and Swiss albino mice (25-30 g) obtained from the Animal Unit of the Faculty of Science at the University of Yaoundé I, Cameroon. They were maintained under standard environmental conditions with a dark and bright 12/12 o'clock circle. They were fed commercial quality feed and water was supplied ad libitum. The innovative protocol complied with the guidelines of the Cameroon National Ethics Committee on the use of laboratory animals in scientific research (CEEC Council 86/609).

2.5 Pharmacological tests

2.5.1 Subchronic inflammation caused by the insertion of cotton granules and treatment with aqueous extract of the bark of *Albizia ferruginea*

The antiproliferative effects of aqueous extract of *A. ferruginea* were evaluated inflammation due to cotton pellet implantation as described in our previous article [10, 12] according to the method described by Sapna and Manish [15]. Briefly, animals were separated into four groups of five animals each. Animals were anaesthetised with subcutaneous valium (10 mg/kg) and ketamine (50 mg/kg). Two cotton pellets weighing 10±1mg each were sterilized and implanted under the skin on each side of the animal's abdomen through a small ventral incision. The animals in the different groups were treated as in the formalin test. The different substances were administered in a single dose daily by mouth for 7 days following the protocol described in the previous article on anti-inflammatory effects of the stem barks from *A. ferruginea* on chronic inflammation induced in rats, published by our research group [10, 12]. At the end of the experimental period (on the 8th day), the animals were sacrificed under anaesthesia (valium and ketamine), by decapitation, and arterovenous blood was collected. The arteriovenous blood was collected in two types of tubes: the EDTA tube for the complete blood count (CBC) and the dry tube without anticoagulant for the preparation of the serum which was stored at -4°C for the ELISA test (measurement of cytokines). In addition, the cotton pellets and fibrovascular tissue constituting the granuloma were collected and weighed. The wet and dry weights of the granuloma were determined. The exudates were carefully removed using a 10 mL syringe and the volumes noted. The percentage inhibition was determined according to the following formula just to ensure the inflammatory effect (Results not presented in this paper):

$$\%Pi = (1-\overline{mt}/\overline{me}) \times 100$$

Pi = Percentage of inhibition
$\overline{mt}$ = mean mass of cotton pellets in control animals
$\overline{me}$ = mean mass of cotton pellets in test animals

2.5.2 Quantification of haematological parameters

The haematological parameters (haemoglobin level, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin content, mean corpuscular haemoglobin concentration and white blood cell count, lymphocytes, monocytes, granulocytes, red blood cells and blood platelets) were measured from blood samples collected in EDTA tubes, using the Coulter S-Plus IV/D which is a multi-parameter automated hematological analyser.

2.5.3 Cytokine assessment

The determination of the concentration of IL-6, IL-1β, TNF-α and INF-γ was carried out in the Serum by the "Enzyme Linked Immuno Sorbent Assay" (ELISA) technique using the quantikines kits (France) following the manufacturer's instructions for sample collection, storage and assay procedure.

2.5.3.1 Principle

The ELISA technique allowed specific investigation of proteins by "sandwiching" the antigen between two special antibodies, the primary antibody at the bottom of the wells, and the secondary antibody added to the reaction medium after the samples had been deposited. A biotin was conjugated to the second antibody, allowing revelation by streptadivine-conjugated peroxidase.

2.5.3.2 Operating procedure

Fifty microliters of the RD1-42 dilution solutions for TNF-α, RD1-21 for IL-1β, RD1-54 for IL-6 and RD1-21 for INF-γ were introduced respectively into each well of a 70-well plate. Next, 50 µL of normal, control, or sample were added respectively to the wells of the upper column of the chips. The mixture was homogenized by gently tapping the frame plate for 1 minute. The microplates were incubated for 2 hours at 37 °C. After incubation, each well was washed 5 times with Wash Buffer (400µL). Capture antibody, specific for each biotin-conjugated protein was added to each well at 100µL for IL-6, IL-1β, TNF-α and INF-γ, and the whole was incubated for 2 hours at 37 °C and washed 5 times with Wash Buffer (100 µL). 100 µL of peroxidase-coupled streptadivine substrate was added to each well and the mixture incubated for 30 minutes at room temperature and protected from light. The enzymatic reaction was stopped by adding 100 µL of stop solution (HCl). Absorbance was measured at 450 nm with a microplate reader. The concentrations of each cytokine (TNF-α, IL-1β, IL-6 and INF-γ) were expressed in pg/mL and determined from their respective calibration curves shown in Figures 1.

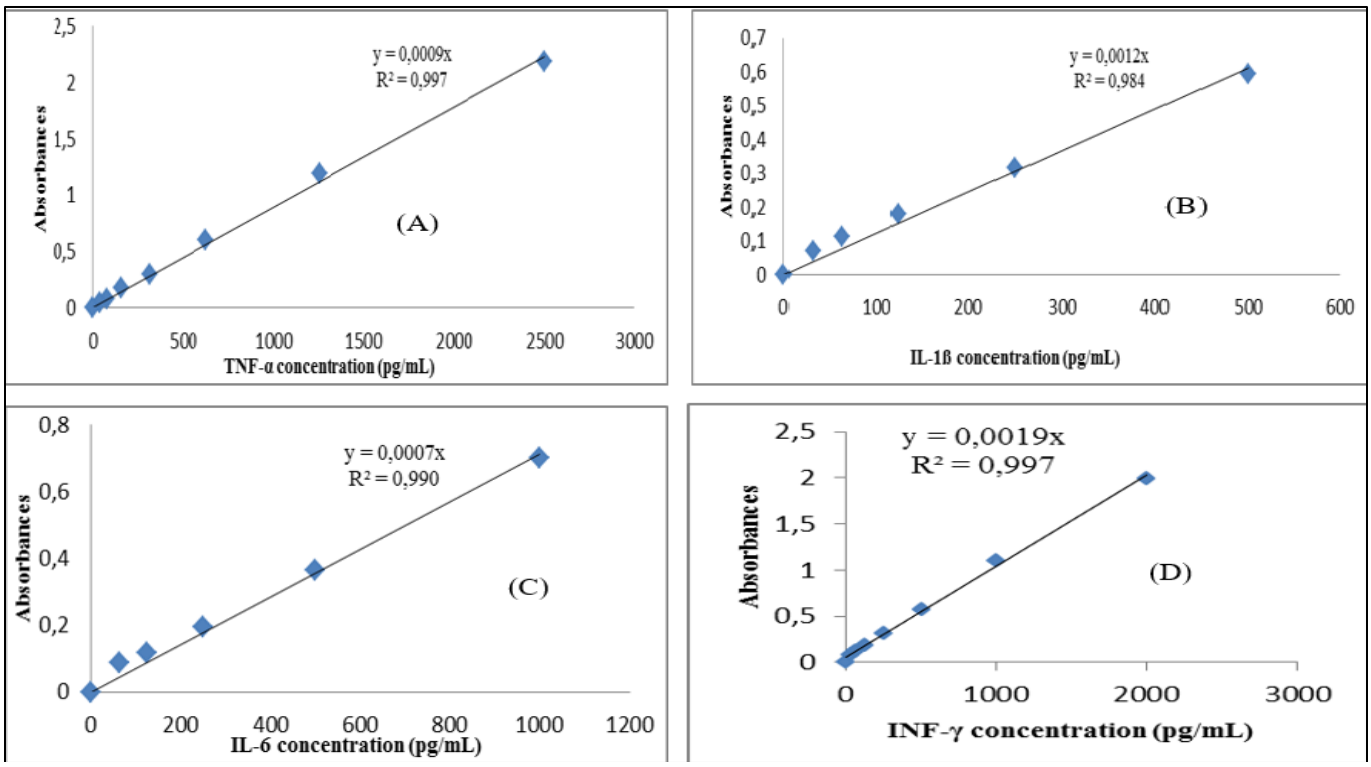


Fig 1: Calibration curve for. A: TNF-α dosage; B: IL-1β dosage; C: IL-6 dosage and D: IFN-γ dosage

2.6 Ethics

The experimental protocol was consistent with the guidelines of the National Ethics Committee of Cameroon on the use of laboratory animals for scientific research (CEEC Council 86/609).

2.7 Statistical Analysis

All the results were expressed as Mean ± SEM. The data were analyzed statistically by one way ANOVA by Dunnett's test using Graph pad prism (5.03) software. P values below 0.05 were regarded as statistically significant.

3. Results

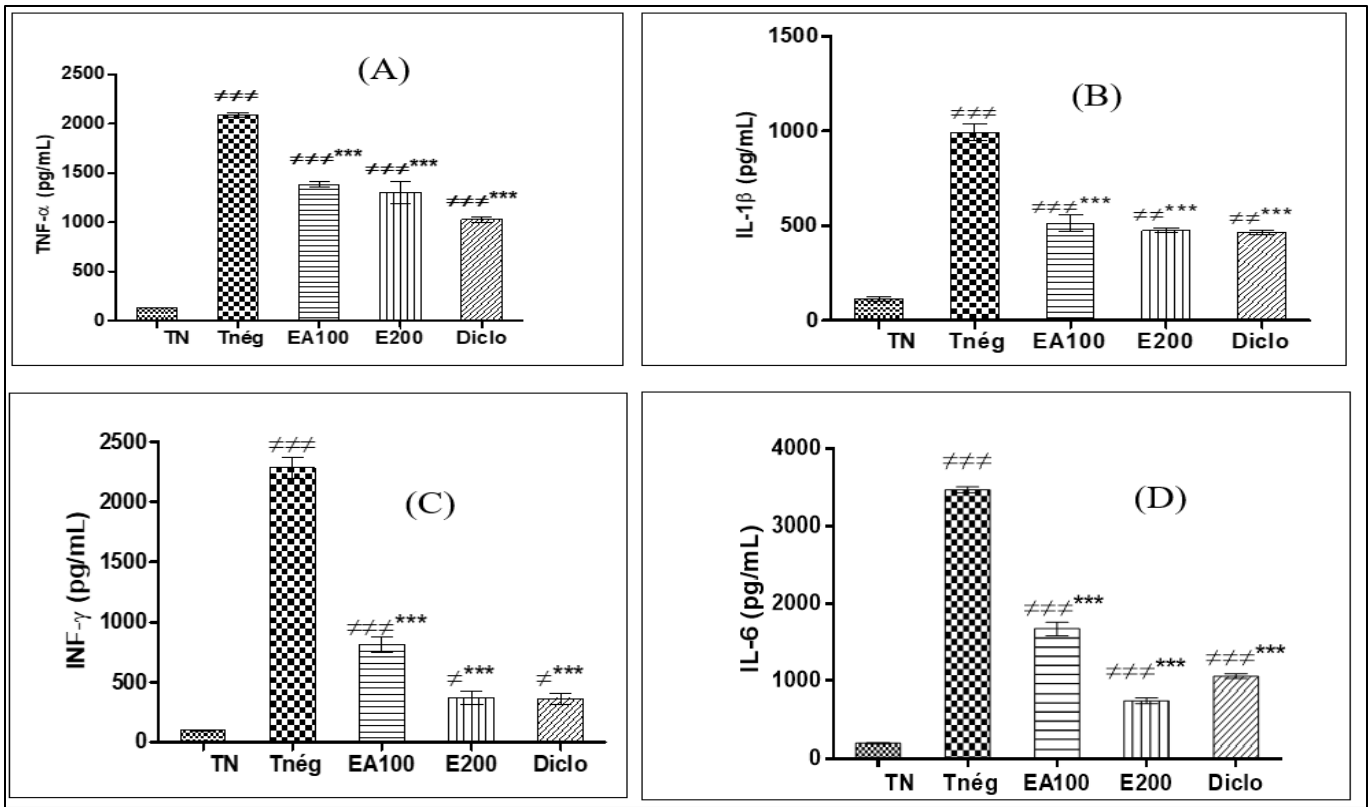
3.1 Effects of the aqueous extract of *Albizia ferruginea* bark on some cytokines markers (IL-6, IL1-β, TNF-α and INF-γ) in inflammation induced by cotton pellets

The levels of the cytokine markers (TNF-α, IL-1β, IL-6 and INF-γ) are shown in Figure 5. The level of TNF-α in the serum of negative control rats increased significantly (p<0.001) compared to normal rats. The level of TNF-α of 132.85±1.86 pg/mL in the serum of the normal control increased to 2090.17±8.71pg/mL in the negative control (Figure 1.A). The aqueous extract at 100 and 200 mg/kg resulted in a significant (p<0.001) decrease in TNF-α levels of 33.76% and 37.81% respectively compared to the negative control. Diclofenac at 4 mg/kg resulted in a significant (p<0.001) decrease in TNF-α levels of 50.80%. Negative control rats showed a significant (p<0.001) increase in the amount of IL-1β in serum compared to normal rats; The IL-1β level of 114.08±1.17pg/mL in the serum of the normal control increased to 992.66±4.78 pg/mL in the negative control (Figure 1.B). The extract at 100 and 200 mg/kg caused a significant (p<0.001) decrease

in IL-1β levels of 48.40% and 52.15%, respectively, compared to the negative control. Diclofenac at 4 mg/kg caused a significant (p<0.001) decrease in serum IL-1 levels of 52.98%. Implantation of cotton pellets under the rat skin induced a significant (p<0.001) increase in serum IL-6 levels compared to the normal control. The IL-6 level of 190.60±1.01pg/mL in the normal control increased to 3471.99±4.53 pg/mL in the negative control (Figure 1.C). The extract at 100 and 200 mg/kg induced a significant (p<0.001) decrease in serum IL-6 levels of 51.82% and 78.43% respectively compared to the negative control. Diclofenac caused a significant (p<0.001) decrease in serum IL-6 levels of 69.48%. Negative control rats showed a significant (p<0.001) increase in the level of INF-γ in the serum compared to the normal control. The INF-γ level of 94.45±0.84pg/mL in the normal control increased to 2286.84±9.78pg/mL in the negative control (Figure 1.D). The extract at 100 and 200 mg/kg induced a significant decrease (p<0.001) in serum INF-γ levels of 64.57% and 84.02%, respectively, compared to the negative control. Diclofenac resulted in a significant (p<0.001) decrease in serum INF-γ levels of 84.21%.

3.2 Effects of the aqueous extract of *Albizia ferruginea* on some haematological Parameters

The effects of the plant extract of the bark of *A. ferruginea* on some haematological parameters of the animals are shown in Table 1. Haematological analysis revealed in the animals that received the aqueous extract at the dose of 100 and 200 mg/kg, a significant reduction in total white blood cell counts of 28.58% and 29.61% respectively (p<0.01), lymphocytes by 45.68% and 46.14% (p<0.05), monocytes by 47.89% (p<0.05) and 53.15% (p<0.01) and granulocytes by 42.85% and 43.27% (p<0.05) compared to the control group, respectively. A significant increase in red blood cell count was observed in animals given the aqueous extract at 100 mg/kg and 200 mg/kg, by 40.23% (p<0.01) and 48.42% (p<0.001) respectively. Haemoglobin levels increased by 10.38% and 12.88% (p<0.05) and haematocrit by 26.92% and 43.60 (p<0.05)% at these two respective doses of the extract compared to the control group. The blood platelet level decreased by 20.69% and 21.18 (p<0.01) compared to the control lot. Group having received diclofenac, at a dose of 4 mg/kg, resulted in a significant reduction in total white blood cell count of 56.02% (p<0.01), lymphocyte count of 25.64% (p>0.05), monocyte count of 53.68% (p<0.01) and granulocyte count of 42.01% (p<0.05) compared to the control group. An increase in red blood cell count of 15.12% and haemoglobin of 15.38% was observed in animals treated with diclofenac. The haematocrit level increased by 19.21% compared to the control group while the blood platelet level decreased by 19.22% (p<0.05) compared to the control group.



Each bar represents the mean of each ESM group, n = 5, ###p<0.001, ##p<0.01, ##p<0.05 significant difference from normal control and ***p<0.001 significant difference from negative control. TN = Normal control; Tneg = Negative control; EA100, EA200 = Aqueous extract of the bark of *Albizia ferruginea* at 100 mg/kg and 200 mg/kg respectively. Diclo = Diclofenac (4 mg/kg). TNF-α = Tumor Necrosing Factor-alpha; IL-1β = Interleukin-1 beta; IL-6 = Interleukin-6; and INF-γ = Interferon-gamma.

Fig 2: Effects of the aqueous extract of *Albizia ferruginea* bark on some cytokines Markers (TNF-α (A), IL-1β (B); IL-6 (C) and INF-γ (D)) in inflammation induced by cotton pellets.

Table 1: Effects of the aqueous extract of *Albizia ferruginea* on some haematological Parameters

	Animal treatment			
Haematological parameters	**Control**	**AF 100 mg/kg**	**AF 200 mg/kg**	**Diclofenac**
White blood cells (10^3/µL)	8.78 ± 0.58	6.27 ± 0.35**	6.18 ± 1.64**	6.32 ± 0.06**
Lymphocytes (10^3/µL)	1.56 ± 0.08	1.27 ± 0.06*	1.20 ± 0.07*	1.36 ± 0.07*
Monocytes (10^3/µL)	1.90 ± 0.09	0.99 ± 0.06*	0.89 ± 0.08*	0.88 ± 0.06**
Granulocytes (10^3/µL)	4.76 ± 0.16	2.72 ± 0.28*	2.70 ± 0.24*	2.76 ± 0.08*
Red blood cells (10^6/µL)	6.96 ± 0.07	9.76 ± 0.43**	10.33 ± 0.31***	7.53 ± 0.29
Haemoglobin (g/dL)	10.40 ± 0.69	11.48 ± 0.48*	11.74 ± 0.47*	11.12 ± 0.39
Haematocrit (%)	39.38 ± 0.64	44.68 ± 0.91	47.88 ± 1.63	39.68 ± 1.02
Blood platelets (10^3/µL)	4.06 ± 0.39	3.22 ± 0.38*	3.20 ± 0.20*	3.28 ± 0.69*

Each value represents the average ± ESM, n=5, *p<0.05, **p<0.01 and *** p<0.001 significant difference from negative control.
AF=Albizia ferruginea.

4. Discussion

The cotton pellet-induced inflammatory granuloma model is widely used to evaluate the transudative and proliferative elements of sub-chronic inflammation. It is an indication of the proliferative phase of inflammation which involves the multiplication of macrophages, neutrophils and fibroblasts that are the basis of granuloma formation [15]. The inflammatory response to cotton granuloma is established in 3 phases [16]. The initial phase occurs within 3 hours after the cotton is implanted and is characterized by increased vascular permeability leading to the leakage of fluid from the blood vessels. The exudative phase continues from 3 hours to 72 hours and results in a leakage of proteins around the granuloma due to the repair mechanisms of the altered vascular permeability. The proliferative phase lasts from 3 to 6 days and is characterized by the development of the granuloma as a consequence of the release of pro-inflammatory mediators. The increase in the water weight of the cotton pellet represents the transudative phase while the proliferative phase is represented by an increase in the dry weight of the granuloma. During this inflammation, cell proliferation occurs. These cells can be buried in the formed granuloma. This test highlights the ability of a substance to prevent the infiltration of leukocytes into the inflammatory site and the formation of exudate during sub chronic inflammation [17]. In the present study, oral administration of the extract at 100 mg/kg and 200 mg/kg resulted in a significant decrease in exudate and granuloma mass. These results are similar to those obtained by Parmar and associates when evaluating the anti-inflammatory potential of *Kigelia pinnata* extract in rats [18]. Diclofenac also significantly reduced the mass of the granuloma and the volume of exudate. NSAIDs decrease the size of the granuloma as a result of the cellular response by inhibiting leukocyte infiltration, preventing collagen fiber generation and suppressing mucopolysaccharides [15]. The plant extract of the trunk bark of *A. ferruginea* is thought to have a potential inhibitory effect on cell infiltration and proliferation during sub chronic inflammation. The results showed that the aqueous extract of *A. ferruginea* as well as diclofenac is capable of significantly reducing the level of leukocytes in the exudate. These results indicate that the aqueous extract would possess substances capable of preventing not only the infiltration of leukocytes into the inflammatory site, but also the proliferation of inflammatory cells and the synthesis of the constituents of the extracellular matrix. Granulomatous inflammation is a subacute inflammatory response secondary to the persistence of the etiological agent. The latter is badly eliminated or badly degraded; maintaining a persistent inflammatory reaction, source of lesion. Tissue damage induces a cascade of cellular response in the injured area, accompanied by the release of inflammatory cytokines such as TNF-α, IL-1, IL-6 and IL-8 and other substances [1]. In this model, a significant increase in serum levels of cytokines was observed compared to the normal control. It is known that NSAID-activated receptor complexes migrate into the nucleus and bind to regulatory sequences that regulate gene transcription, either to inhibit or activate them. NSAID-receptor complexes can also bind to certain transcription factors, causing their inhibition. The consequences will be a decrease in the synthesis of cytokines (IL-1, IL-6 and TNF-α) and lipid mediators (prostaglandins, leukotrienes) [19, 20]. Alkaloids are known to enhance the immune response through their immunostimulant properties. Alkaloids also increase immune function by 50% [21]. Terpenoids have a dual effect on the immune system; improving antibody production and inhibiting the T cell response [22]. The results obtained are similar to those obtained by Wagner and collaborators when evaluating the immunostimulant potential of bark extract and *Uncaria tomentosa* roots in rats [21]. The aqueous extract from the bark of the trunk of *A. ferruginea* is thought to have an immunomodulatory potential on the inhibition of the expression of inflammatory mediators in serum during sub-chronic inflammation. The results showed that the aqueous extract of *A. ferruginea* as well as diclofenac are capable of significantly reducing the levels of cytokines (TNF-α, IL-1, IL-6 and IFN-γ) in the serum. These results suggest that the plant extract possesses substances competent of not only preventing the immunoproliferation of leukocytes in the inflammatory site, but also has immunostimulatory effects on the inflammatory and regulatory cells of the antigen-presenting cells of the receptors involved in lymphocyte activation.

Sub-chronic inflammation is a complex process that is mediated by activated inflammatory or immune cells. Non-steroidal anti-inflammatory drugs inhibit the migration of polynuclear cells and macrophages during the cell phase. The cotton pellets model is widely used to evaluate the cellular and immune constituents of sub chronic inflammation [28]. These results indicate that the aqueous extract of *A. ferruginea* is capable of improving the sub chronic inflammatory response induced by cotton pellets. Similar results were obtained by Kim and collaborator on the study of the effects of *Aureobasidum pullulans* on formalin-induced sub-chronic inflammation [24]. The blood count (CBC) showed a significant increase in white blood cells, lymphocytes, red blood cells and granulocytes in animals given the extract at 100 and 200 mg/kg. These results suggest that the extract would act on haematopoietic differentiation cells, for the production of leukocytes and/or stimulate defence cells. Indeed, according to Adeneye and

collaborators, certain essences (*Spondias pinnata* and *Musanga cecropioides*) could have an action on haematopoietic differentiation of cells [25] by stimulating B lymphocytes [26] and their transformation into B lymphocytes to become T lymphocytes by proliferation. The granulocyte population was high in the groups that received the plant extract. These results could be explained by the fact that the granulocytes, which are defence cells, were activated by the extract. Equivalent results were obtained by Ukpabi and associates on the effects of *A. ferruginea* on the correction of anaemia [27].

5. Conclusion

The extract significantly decreased the level of cytokines (TNF-α, IL1-β, IL-6 and IFN-γ). These results showed that both the aqueous extract of *A. ferruginea* and diclofenac were able to significantly reduce the levels of cytokines (TNF-α, IL-1β, IL-6 and IFN-γ) in the serum. These results suggest that the plant extract may possesses substances efficient of not only preventing the immunoproliferation of leukocytes in the inflammatory site, but could also has immunostimulatory effects on the inflammatory and regulatory cells of the antigen-presenting cells of the receptors involved in lymphocyte activation. The aqueous extract of the trunk bark of *A. ferruginea* is believed to have immunomodulatory potential on the inhibition of the expression of inflammatory mediators in serum during sub-chronic inflammation. Still in sub-chronic treatment, the results indicated that this extract could cause an inhibition of cyclooxygenase and lipooxygenase in the model of sub-chronic inflammation caused by formalin.

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