Ziziphus abyssinica hydro-ethanolic root bark extract attenuates acute inflammation possibly through membrane stabilization and inhibition of protein denaturation and neutrophil degranulation

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ABSTRACT

Background: Despite the widespread use of *Ziziphus abyssinica* (ZAE) roots and claims of its efficacy against diverse inflammatory conditions in traditional medicine, there is paucity of information on the scientific basis for its folkloric use.

Objectives: The current study evaluated the anti-inflammatory property of the root bark extract of ZAE and its possible mechanism(s) of action in *in vivo* and *in vitro* experimental models.

Methods: Anti-inflammatory activity of ZAE was assessed *in vitro* using heat and hypotonic solution – induced haemolysis as well as egg albumin (EA) and bovine serum albumin (BSA) denaturation assays. Carrageenan and formalin-induced paw oedema and carrageenan-induced peritonitis in rats were used to evaluate the anti-inflammatory property of the extract *in vivo*.

Results: ZAE (100, 300 and 1000 µg/mL) significantly inhibited heat and hypotonic solution-induced haemolysis as well as EA and BSA-induced denaturation. ZAE (300 mg/kg, *p.o.*) similar to diclofenac (10 mg/kg, *p.o.*), significantly ($P<0.05$) reduced paw oedema by 40.77±6.82 and 54.81 ± 3.74% respectively in carrageenan-induced paw oedema test. The percentage inhibitions produced by ZAE (30, 100 and 300 mg/kg, *p.o.*) were 3.31±22.12, 49.89±2.98 and 76.98±0.50 % respectively compared to 80.51±0.53 % produced by diclofenac (10 mg/kg, *p.o.*) in the formalin-induced paw oedema test. Massive recruitment of leukocytes (mainly neutrophils) into the peritoneal cavity of the rats by carrageenan was significantly ($P<0.01$) reduced by ZAE (30, 100 and 300 mg/kg *p.o.*) and dexamethasone (5 mg/kg, *p.o.*).

Conclusions: The inhibitory effect of ZAE against acute inflammation in this study provides scientific basis for its use in folk medicine and reveals its potential as a source of novel anti-inflammatory agent.

Keywords: *Ziziphus abyssinica*, carrageenan-induced peritonitis, formalin-induced paw oedema, acute inflammation, in vitro, in vivo, anti-inflammatory property

L'extrait d'écorce de racine hydro-éthanolique de *Ziziphus abyssinica* atténue l'inflammation aiguë possiblement par la stabilisation de la membrane et l'inhibition de la dénaturation des protéines et de la dégranulation des neutrophiles

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RESUME

Contexte : Malgré l'utilisation répandue des racines de *Ziziphus abyssinica* (ZAE) et les affirmations de son efficacité contre diverses conditions inflammatoires en médecine traditionnelle, il y a peu d'informations sur la base scientifique de son utilisation folklorique.

Objectifs : La présente étude a évalué la propriété anti-inflammatoire de l'extrait d'écorce de racine de ZAE et son (ses) mécanisme(s) d'action *in vivo* et *in vitro* modèles expérimentaux.

Méthodes : L'activité anti-inflammatoire de ZAE a été évaluée *in vitro* en utilisant la chaleur et l'hémolyse induite par une solution hypotonique ainsi que des tests de dénaturation de l'albumine d'œuf (EA) et de la sérumalbumine bovine (BSA). L'œdème de la patte induit par le carragheen et le formol et la péritonite induite par la carragheen chez le rat ont été utilisés pour évaluer la propriété anti-inflammatoire de l'extrait *in vivo*.

Résultats : ZAE (100, 300 et 1000 µg/mL) inhibe significativement la chaleur et l'hémolyse induite par la solution hypotonique ainsi que la dénaturation induite par EA et BSA. ZAE (300 mg/kg, *p.o.*) similaire au diclofénac (10 mg/kg, *p.o.*), significativement ($P<0,05$) réduisent l'œdème de la patte de 40,77±6,82 et 54,81±3,74% respectivement le test de l'œdème des pattes induit par la carragheen. Les inhibitions en pourcentage produites par ZAE (30, 100 et 300 mg/kg, *p.o.*) étaient respectivement de 3,31±22,12, 49,89±2,98 et 76,98±0,50% comparées à 80,51±0,53% produites par le diclofénac (10 mg/kg, *p.o.*) dans le test de l'œdème des pattes induit par le formol. Le recrutement massif de leucocytes (principalement neutrophiles) dans la cavité péritonéale des rats par la carragheen était significativement ($P<0,01$) réduit par ZAE (30, 100 et 300 mg/kg *p.o.*) et dexaméthasone (5 mg/kg, *p.o.*).

Conclusion : L'effet inhibiteur de ZAE contre l'inflammation aiguë dans cette étude fournit une base scientifique pour son utilisation dans la médecine populaire et révèle son potentiel en tant que source de nouvel agent anti-inflammatoire.

Mots-clés : *Ziziphus abyssinica*, péritonite induite par la carragheen, œdème de la patte induit par la formaline, inflammation aiguë, *in vitro, in vivo*, propriété anti-inflammatoire

INTRODUCTION

Inflammatory responses are the body's defense mechanism to many harmful stimuli. However, its dysregulation especially in devastating chronic conditions is known to trigger various disease complications such as neurodegenerative diseases, asthma, arthritis, sepsis, autoimmune disorders, infectious disease, trauma, transplant rejection, obesity, allergy, cancer and atherosclerosis.[1, 2] Arthritis alone is a primary source of debility of the working force around the world and has been recognised as the 'king of human miseries'.[3]

Steroidal and non-steroidal anti-inflammatory drugs which constitute the mainstay of treatment for several inflammatory conditions only ameliorate the symptoms but do not completely treat the underlying disease. Despite rendering only temporary relief, they also cause severe adverse effects such as gastrointestinal bleeding, renal impairment, immune-suppression among others.[4, 5] Because of these reasons, patients with acute or chronic inflammatory disorders are prone to seek alternative methods for relief and are among the highest users of complementary and alternative medicines particularly herbal medicines.[6, 7] The factors responsible for the continual and widespread usage of these herbal remedies are their stress-free availability, effectiveness, inexpensiveness, comparatively less toxic effects and insufficiency of practitioners of modern medicine in rural areas.[8]

Ziziphus abyssinica (Hochst Ex A. Rich) is a member of the rhamnaceae family together with over 900 other species majority of which are known medicinal plants and widely distributed in many parts of the world especially the tropics and warm temperate regions.[9] It is commonly called larukluror' (Sisaala, Ghana,) 'magariya' (Hausa), catch thorn (English) 'Jujubier sauvage' (French), among others.[10, 11] Qualitative phytochemical investigations have revealed that the aqueous and methanol fruit extracts of Z. abyssinica contain saponins, tannins, sterols and steroids, alkaloids, flavonoids and reducing sugars.[12] Also, the presence of saponins, carbohydrates, glycosides, alkaloids, tannins, steroids and anthraquinones were detected in aqueous root extract of the plant.[13] We have recently reported that the hydroethanlolic leaf extract of the plant contains tannins, phenols, alkaloids, triterpenes, flavonoids, phytosterols as well as reducing sugars.[14] Extracts from various parts of the plant have exhibited antioxidant, antibacterial and antifungal activities.[12, 15, 16] The plant has been reported to have antiplasmodial activity.[17] Additionally, extracts from the roots have been reported to possess anti-ulcerogenic [13] and anti-diarrhoeal [18] and analgesic [14, 19] properties.

Despite the fact that several ethnobotanical surveys conducted on the roots of Ziziphus abyssinica reveal its widespread use in managing inflammatory conditions, there are no scientific data or reports that authenticate this usage. The present study, therefore, is not only important in authenticating or providing scientific basis for the folkloric use of Ziziphus abyssinica against inflammatory conditions but also investigating its anti-inflammatory property with a view to exploring its potential as a source of novel anti-inflammatory drug. It would also be interesting to evaluate anti-inflammatory properties of an anti-ulcerogenic medicinal plant like Ziziphus abyssinica considering the fact that most anti-inflammatory agents cause gastric ulcers.

METHODS

Plant collection

Fresh root barks of Ziziphus abyssinica were collected from Ejura (7°23'00.16"N, 1°22'00.00"W) in the Ashanti Region of Ghana in the month of November, 2016. It was authenticated at the Herbarium Unit of the Faculty of Pharmacy of the Kwame Nkrumah University of Science and Technology (KNUST). A voucher specimen (KNUST/HM/2016/R003) was subsequently deposited at the herbarium.

Plant extraction

Fresh root barks of Ziziphus abyssinica were air dried at room temperature for three weeks and pulverized into fine powder with the aid of a hammer mill. A portion (800 g) of the powdered roots was extracted with 5 L of 70 % $^{v}/_{v}$ ethanol for a 48 h period using Soxhlet Extraction Apparatus (Aldrich®, St. Louis, MO, USA). The extract obtained was labelled as ZAE and subsequently concentrated using a rotary evaporator (Rotavapor R-215 model, BÜCHI Labortechnik AG, Flawil, Switzerland) under reduced pressure and temperature (70°C). This was further dried on a water bath and then preserved in a desiccator containing activated silica until it was ready for use. The yield obtained was 8.7 % $^{w}/_{w}$.

Phytochemical Screening of the plant parts

Qualitative phytochemical analysis was conducted on Ziziphus abyssinica root extract to determine the presence of the various phytoconstituents. For qualitative investigation, 500 mg of the extracts was

dissolved in hydro-alcoholic solvent. Phytochemical analysis was carried out by a procedure based on previous reports by Tiwari et al. [20]

Drugs and chemicals

Diclofenac sodium, aspirin, dexamethasone, carrageenan, bovine serum albumin were of analytical grade and purchased from Sigma-Aldrich Inc, St. Louis, MO, USA.

Animals

Male Sprague - Dawley rats (170 - 250 g) were purchased from Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, Ghana. They were kept in stainless cages (34 × 47 × 18) cm^3 in groups of six rats at the animal house facility of the Department of Biomedical Sciences, University of Cape Coast, Ghana. They were fed with normal commercial diet bought from Floor Mills of Ghana Limited, Tema, Ghana and water was given ad libitum. All procedures and techniques employed for the study were in accordance with established public health guidelines in 'Guide for Care and Use of Laboratory Animals'. [21]

Hypotonic solution-induced haemolysis

Whole blood was collected into heparinized vacutainer from a healthy human volunteer who had not taken any non-steroidal anti-inflammatory drugs (NSAIDs) for 2 weeks prior to the experiment. The blood was washed three times with 0.9 % saline and centrifuged intermittently for 10 min at 3000 rpm. The packed cells were washed with 0.9 % saline and 10 % $^{v}/_{v}$ human red blood cells (HRBC) suspension was prepared using 0.9 % saline. [22] Hypotonic solution-induced haemolysis test was then performed as previously described by Laboni et al. [23] Test samples consisted of 0.5 mL of stock HRBC mixed with 4.5 mL of hypotonic solution (0.45 % NaCl) containing varying concentrations of ZAE (100, 300 and 1000 µg/mL). The negative control sample contained 0.5 mL HRBC suspension mixed with 0.45 mL of hypotonic solution alone. The positive control sample was prepared using 0.5 mL of the HRBC suspension and 4.5 mL of the hypotonic solution containing varying concentrations of diclofenac sodium (100, 300 and 1000 µg/mL). The experiment was carried out in triplicates. Mixtures were incubated for 10 min at room temperature, centrifuged for 10 min at 3000 rpm and haemoglobin content of the supernatant was measured spectrophotometrically (Jenway model 6715 UV/Visible Spectrophotometer) at 560 nm. The percentage inhibition of haemolysis was calculated

$$\%\text{inhibition of haemolysis} = \left(\frac{\text{Optical density of control - Optical density of test}}{\text{Optical density of control}}\right) \times 100$$

Heat-induced haemolysis

The test was carried out as has been previously described. [24] The reaction mixture (2 mL) consisted of 1.0 mL of 10 % HRBC (described above) and 1.0 mL of various concentrations of ZAE (100, 300 and 1000 µg/mL). The negative control sample consisted of 1.0 mL of 10 % HRBC and 1.0 mL of normal saline. Positive control samples comprised of 1.0 mL of 10 % HRBC and 1.0 mL of different concentrations of diclofenac sodium (100, 300 and 1000 µg/mL). The experiment was carried out in triplicates. The samples were heated at 56°C for 30 min then cooled to room temperature followed by centrifugation at 3000 rpm for 10 min. The supernatants were collected, and their absorbance measured at 560 nm using Jenway model 6715 UV/Visible Spectrophotometer. Percentage inhibition of haemolysis was calculated as:

$$\%\text{ inhibition} = \left(\frac{\text{Absorbance of control - Absorbance of test}}{\text{Absorbance of control}}\right) \times 100$$

Egg albumin denaturation assay

The test was carried out as was previously described by Test samples (5 mL) consisted of 0.2 mL of egg albumin (from fresh egg), 2.8 mL of phosphate-buffered saline (PBS, pH 6.4) and 2 mL of varying concentrations (100, 300 and 1000 µg/mL) of the extract. Negative and positive control samples contained the same volume of egg albumin and PBS but the extract was replaced with either 2 mL of distilled water or diclofenac (100, 300 and 1000 µg/mL) respectively. The mixtures were incubated at 37±2°C for 15 min and then heated at 70°C for five min. After cooling, absorbance of reaction mixture was measured using a spectrophotometer (Jenway model 6715 UV/Visible Spectrophotometer) at 660 nm. The experiment was performed in triplicates and the percentage inhibition of protein denaturation was calculated using the formula below

$$\%\text{ inhibition} = \left(\frac{\text{Absorbance of control - Absorbance of test}}{\text{Absorbance of control}}\right) \times 100$$

Bovine serum albumin (BSA) denaturation model

A method previously described by [22] was used. The reaction mixtures consisted of 0.5 mL of 1 % BSA fraction and 0.5 mL of either normal saline (negative control), diclofenac (100, 300 and 1000 µg/mL) or ZAE (100, 300 and 1000 µg/mL). Samples were incubated at 37°C for 20 min and then heated at 51°C for 20 min. After cooling the samples, the turbidity was measured

Spectrophotometer, Jenway Gransmore Green Felsted, Dunmow Essex CM6 3LB ENGLAND) at 660 nm. The experiment was performed in triplicate. Percentage inhibition of protein denaturation was calculated as follows

$$\% \text{ inhibition} = \left(\frac{\text{Absorbance of control - Absorbance of test}}{\text{Absorbance of control}} \right) \times 100$$

Carrageenan – induced paw oedema in rats

Acute anti-inflammatory activity of the extract was evaluated in rats using a method described previously.[25] Five groups of male Sprague Dawley rats (n = 5) were used for the study. Paw oedema was induced by the administration of 0.1 mL of 1 % suspension of carrageenan in 0.9 % sterile saline solution into the planter region of the rats' right hind paw. This was preceded by pre-treatment of different groups with ZAE (30, 100, 300 mg/kg, *p.o*.), diclofenac (10 mg/kg, *p.o*.) or normal saline (10 mL/kg, *p.o*.) 1 h before paw oedema was induced. Rat's paw thickness was measured using Starrett 798A – 6 / 150 Electronic Digital Callipers before intraplantar injection of carrageenan and at hourly intervals for 5 h post oedematous injury. Raw scores of foot oedema were individually normalized as percentage of change in their paw diameter at time 0 and then averaged for each treatment group. This was used to plot a time course curve for the 5 h period. Total oedema response for each treatment was then calculated as area under the time course curves (AUC). The effect of the drugs was evaluated using percentage inhibition of oedema calculated as:

$$\% \text{ inhibition} = \left(\frac{AUC_{control} - AUC_{treatment}}{AUC_{control}} \right) \times 100$$

Formalin-induced paw oedema in rats

The test was performed as described by Choudhary et al. [26] with some modifications. Five groups of male Sprague Dawley rats (n=5) were used for the study. Inflammation of the right hind paws was induced by intraplantar injection 0.1 mL of formaldehyde (2 % $^{v}/_{v}$) in normal saline. The point of injection was marked in order to maintain consistency in measurement of the paw circumference. All animals received treatment via oral gavage. One hour post formalin injection, rats in groups I, II and III received ZAE at doses of 30, 100 and 300 mg/kg *p.o.* respectively. Group IV rats received diclofenac (10 mg/kg, *p.o.*) whereas group V rats received distilled water (10 mL/kg *p.o.*). Paw oedema was measured with Electronic Digital Callipers (Starrett 798A – 6 / 150) once every day for ten days, starting from day one, after induction of inflammation. The treatment also continued once daily for the entire duration of the experiment. Paw oedema response was calculated from the difference between final and basal average paw diameters at different time intervals. Percent inhibition of oedema was calculated using the formula:

$$\% \text{ inhibition} = \left(\frac{\text{Paw diameter at time } t}{\text{Paw diameter at time } 0} \right) \times 100$$

Carrageenan-induced peritonitis

Method previously described by [27] was used to assess the effect of ZAE against carrageenan-induced peritonitis in rats. Twenty-four (24) Sprague-Dawley rats were used for this study. The rats were assigned to 6 groups of four rats per group. Three groups out of the 5 served as the experimental groups with the remaining three representing the positive, negative and normal control groups respectively. Animals in the three experimental groups were pre-treated with ZAE (30, 100 and 300 mg/kg *p.o.*) 1 h before intraperitoneal injection of 1 mL of 1% $^{w}/_{v}$ carrageenan. Positive control rats were pre-treated with dexamethasone (5 mg/kg, *p.o*.) while those in the negative and normal control groups were given normal saline (*p.o.*) before i.p. injection of 1% carrageenan (500 µg/mL). All drugs were administered at a reference volume dose of 10 mL/kg.

Five hours after induction of inflammation, the rats were euthanized under chloroform anaesthesia and peritoneal fluids were collected by abdominal laparoscopy. Five (5) mL of phosphate-buffered saline (PBS, pH 7.4) was injected into the peritoneal cavity of the rats. The abdomen was carefully massaged for approximately 10 - 15 s. A total of 3 mL fluid was withdrawn from the peritoneal cavity of each animal and centrifuged at 1000 rpm for 5 min. The resulting cell pallet was gently suspended in 1.0 mL of phosphate-buffered saline (PBS, pH 7.4). Total leukocytes count was assayed using the 1.0 mL cell suspension and determined using Neubauer's chamber. Cells in each square corner of the chamber were counted and their average calculated. For differential cell counts, Hema[3] stain was used to stain the cytospin preparations. Differential cell counts were then performed by counting the cells and they were classified as either mononuclear or polymorphonuclear cells, based on conventional morphological criteria.

Statistical analysis

Time-course curves were subjected to two-way (*treatment x time*) analysis of variance (ANOVA) with Dunnet's *post hoc*. One-way ANOVA followed by Bonferroni's *post hoc* test was used to compare differences between treatment groups (AUCs). GraphPad® Prism for Windows Version 7.0 (Graphpad Software, San Diego, CA, USA, 2016) was used for all statistical analysis. $P < 0.05$ was considered statistically significant for all tests.

RESULTS

Phytochemical analysis

Phytochemical screening conducted on the plant revealed the presence of phenols, triterpenes, alkaloids, phytosterols, reducing sugars, tannins, flavonoids, proteins and amino acids.

Hypotonic solution-induced haemolysis of human red blood cells

From the results presented in Figure 1a, ZAE significantly ($P<0.010$) and concentration-dependently inhibited HRBC haemolysis in hypotonic solution. The percentage inhibitions increased from 42.98, 81.58 to 93.86% at a concentration of 100, 300 and 1000 µg/mL respectively. The percentage inhibition was comparable with diclofenac which were 50.95, 68.89 and 86.73% at concentrations of 100, 300 and 1000 µg/mL respectively.

Heat-induced haemolysis

ZAE also inhibited heat-induced haemolysis by 61.8%, 65.3% and 85.2% at 100, 300 and 1000 µg/mL respectively. The effect was similar to diclofenac which gave percentage inhibitions of 66.03, 68.33 and 86.49% at concentrations of 100, 300 and 1000 µg/mL respectively as shown in Figure 1b.

Egg albumin denaturation

Data presented in Figure 1c shows a concentration-dependent inhibition of protein (albumin) denaturation by ZAE by 43.5, 52.7 and 65.9% at concentration 100, 300 and 1000 µg/mL respectively. This effect was comparable to the which produced 50.08, 54.48 and 72.23 percentage inhibitions at concentrations of 100, 300 and 1000 µg/mL respectively.

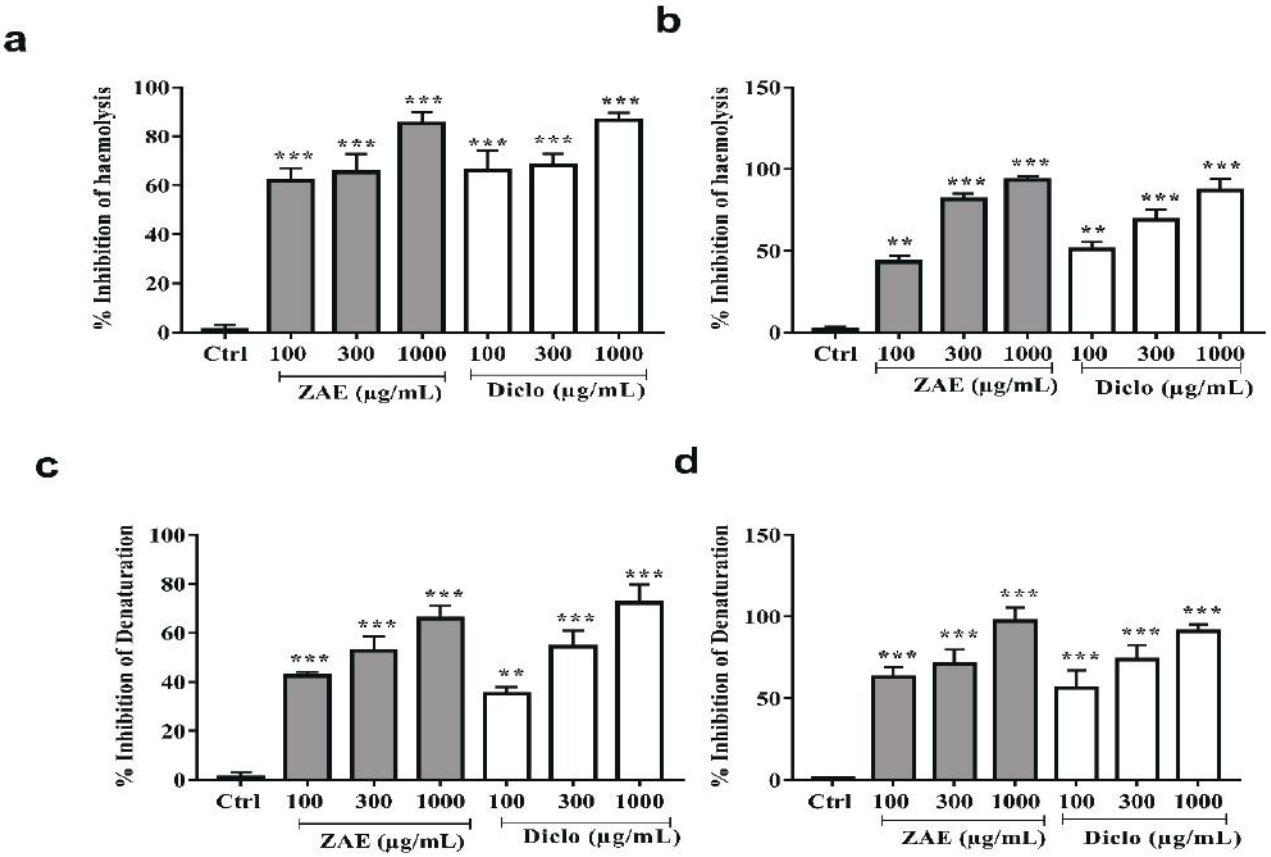

Figure 1: Effect of *Ziziphus abyssinica* root bark extract (ZAE) on (a) hypotonic solution-induced hemolysis (b) heat-induced hemolysis (c) egg albumin and (d) bovine serum albumin denaturation assay. Data is presented as mean ± standard error of mean (n = 3). $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to control group (one-way ANOVA followed by Bonferronis's *post hoc*). ZAE: *Ziziphus abyssinica* extract, Diclo: diclofenac.

Bovine serum albumin denaturation

Results presented on Figure 1d shows the inhibitory effect of ZAE and diclofenac on heat-induced bovine serum albumin denaturation. At concentrations of 100, 300 and 1000 µg/mL of ZAE, the mean percentage inhibitions were 63, 70 and 97.15% respectively. Diclofenac similarly, showed concentration-dependent inhibition of protein denaturation of 56.2, 73.77 and 99.02% at 100, 300 and 1000 µg/mL.

Carrageenan-induced paw oedema

Time course curves [two-way ANOVA (*treatment* x *time*)] as shown in Figure 2 revealed a significant effect of drug treatments on the percentage change in paw oedema ($F_{4, 90}$ =30.25, P < 0.001). ZAE (300 mg/kg, *p.o.*) significantly (P<0.05) reduced paw oedema with a maximum percentage inhibition of 40.8 ± 6.8%. Diclofenac (10 mg/kg, *p.o.*) also produced significant (P < 0.01) decrease in paw oedema (54.81 ± 3.74).

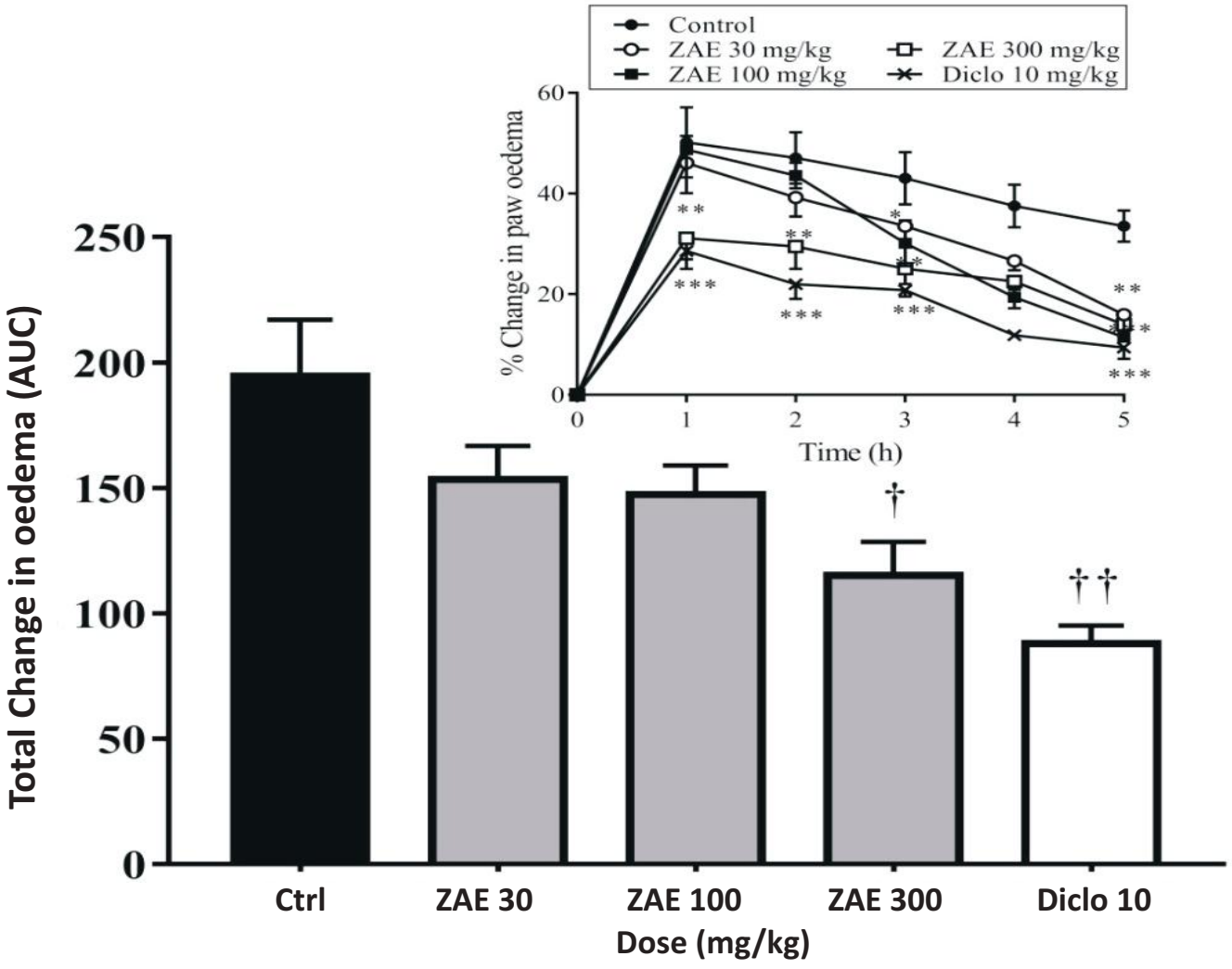

Figure 2: The effects of *Ziziphus abyssinica* root bark extract (ZAE, 30 - 300 mg/kg, *p.o.*) and diclofenac (Diclo 10 mg/kg, i.p) on total change in paw oedema (calculated as AUCs) in carrageenan-induced paw oedema test in rats. † P < 0.05, †† P < 0.01 and ††† P < 0.001 compared to control (ctrl) group (one-way ANOVA followed by Bonferronis's *post hoc*). Insert: Percentage change in paw oedema and over 5 h peroid. Each data represents mean ± standard error of mean, n = 5: *P < 0.05, **P < 0.01 and ***P < 0.001 compared to control group (two – way ANOVA followed by Dunnet's *post hoc*).

Formalin induced inflammation

Intraplantar injection of formaldehyde into the right hind paws of the rats produced prominent increase in paw oedema beginning in the first hour of injection (Figure 3). This effect was sustained throughout the entire duration of the experiment in the vehicle treated group. The mean total anti-oedematous effect (calculated as areas under the curve in Figure 3 insert) obtained for ZAE (30, 100 and 300 mg/kg *p.o*.) were 151.8±35.72, 250±15.35 and 539±12.15 respectively. Diclofenac (10 mg/kg *p.o*.) produced a mean anti-oedematous effect of 637.2±16.4 whereas the negative control group had 123.9±11.2 The percentage inhibitions calculated from the total anti-oedematous effect of ZAE (30, 100, 300 mg/kg) and diclofenac were 3.31±22.12, 49.89±2.98 and 76.98±0.50 and 80.51±0.53% respectively.

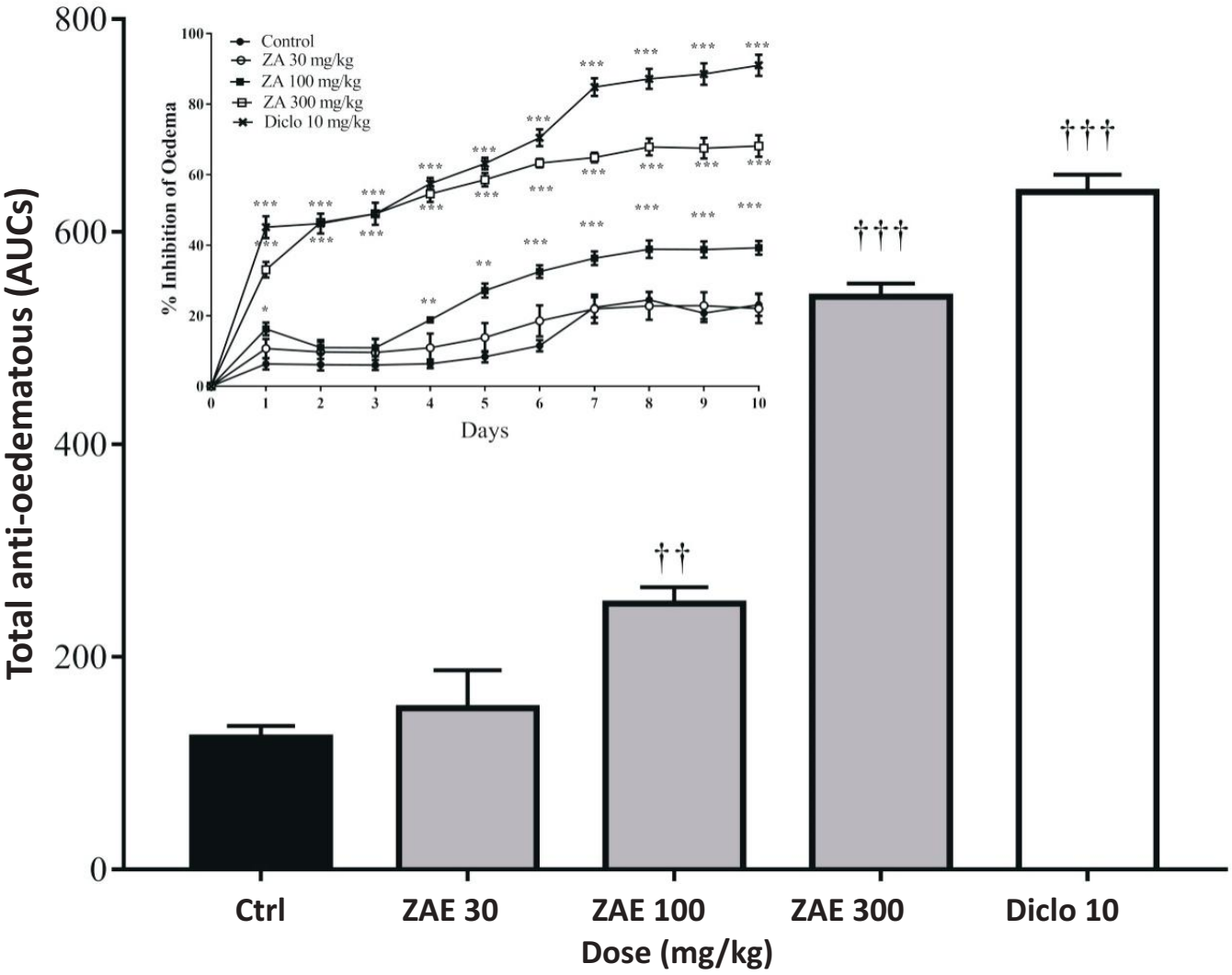

Figure 3: The effects of *Ziziphus abyssinica* root bark extract (ZAE, 30 - 300 mg/kg, *p.o*.) and diclofenac (Diclo 10 mg/kg, i.p) on (A) percentage inhibition of paw oedema and (B) total anti-oedematous effect (calculated as AUCs) in formalin-induced paw oedema test in rats. Each data represents mean ± standard error of mean, n = 5: † P < 0.05, †† P < 0.01 and ††† P < 0.001 compared to control group (two – way ANOVA followed by Dunnet's *post hoc*). P < 0.05 compared to control (ctrl) group (one-way ANOVA followed by Bonferronis's *post hoc*).

Carrageenan-induced peritonitis

This study evaluates the inhibitory effect of the plant extract on leukocyte recruitment to inflammatory sites. Intense inflammation was provoked in the rats by the injection of 1% carrageenan characterized by massive recruitment of leukocytes (mainly neutrophils) into the peritoneal cavity of the rats. ZAE (30, 100 and 300 mg/kg, *p.o.*) and dexamethasone (5 mg/kg, *p.o.*) showed a significant ($P < 0.01$) reduction in the total number of cells compared to the saline treated group as shown on Figure 4. The differential count was performed using basic cell morphology to differentiate between mast cells, neutrophils, macrophages, lymphocytes and basophils. Pre-treatment of the rats with ZAE (30, 100 and 300 mg/kg) also caused marked ($P < 0.001$) reduction in the number of neutrophils recruited to the peritoneal cavity compared to saline-treated rats (Figure 5). There was also a significant ($P<0.05$) decrease in the number of mononuclear cells in the peritoneal cavity of the animals that received ZAE (300 mg/kg) and dexamethasone (5 mg/kg).

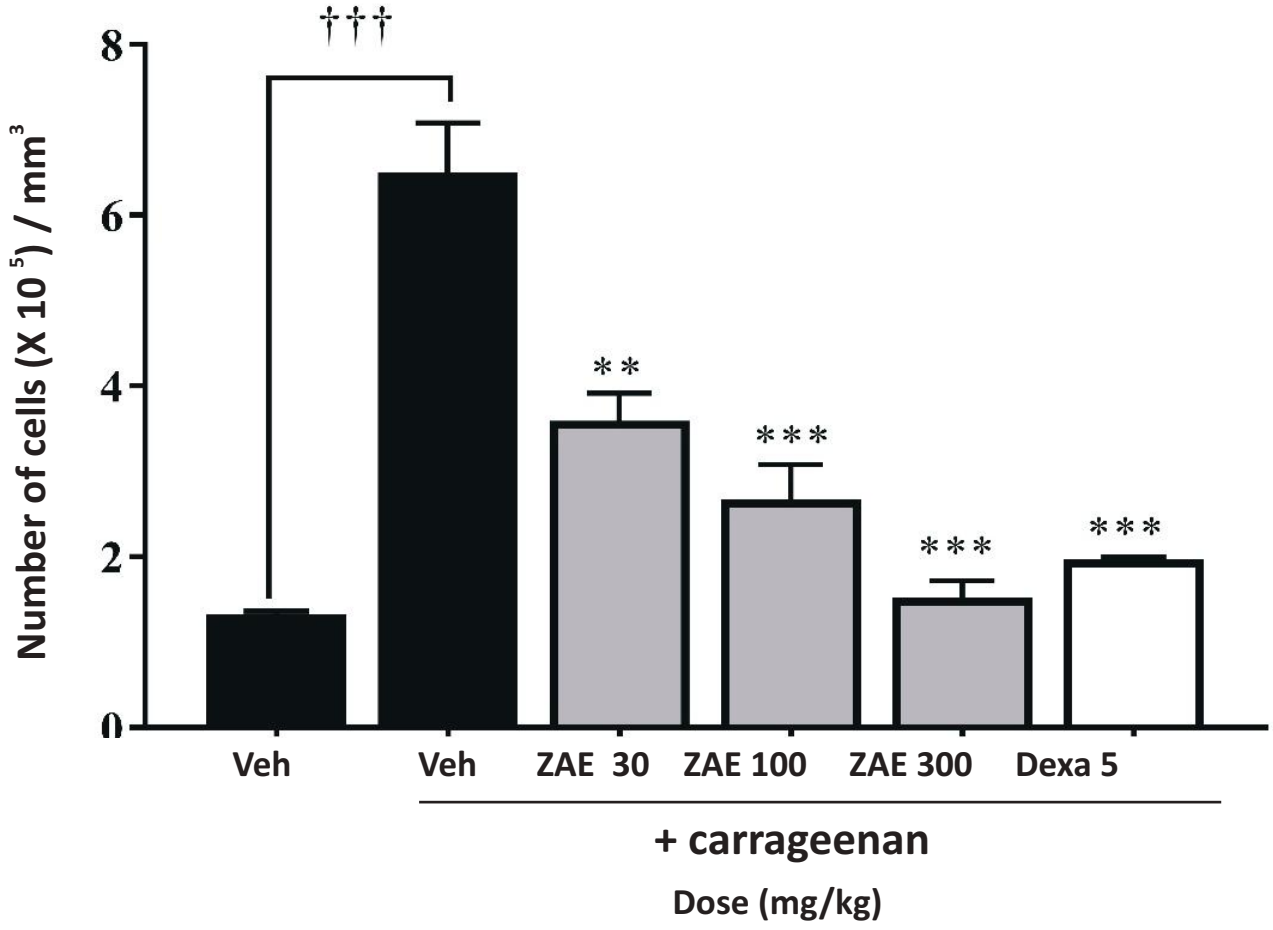

Figure 4: Effects of the administration of *Ziziphus abyssinica* root bark extract (ZAE, 30 - 300 mg/kg), Dexamethasone (Dexa, 5 mg/kg) or vehicle (Negative control) on acute carrageenan-induced peritonitis, measured by the number of cells in the peritoneal exudate. Results are presented as mean ± S.E.M. of cells/peritoneal cavity for n=4 rats. ***$p < 0.001$ and **$p < 0.01$ when compared with negative control (NC) group respectively (one-way ANOVA followed by Bonferronis's *post hoc*).

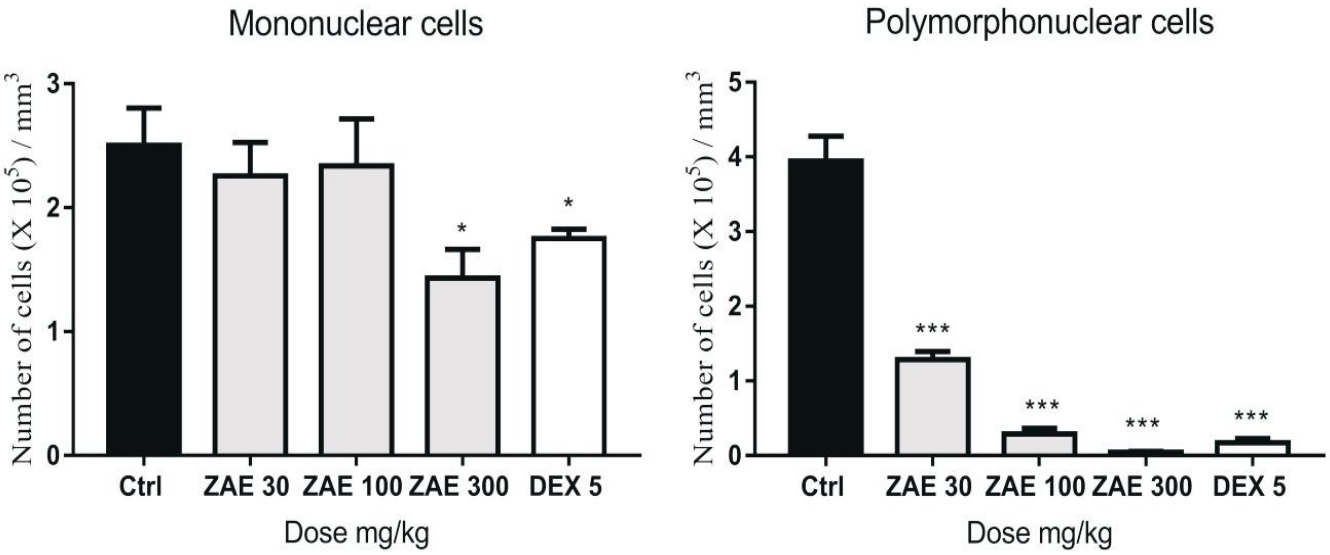

Figure 5: Differential leukocyte counts in the peritoneal cavity of rats pretreated with vehicle, *Ziziphus abyssinica* root bark extract (ZAE) or dexamethasone (DEX) in the peritonitis model induced by carrageenan. Results are presented as mean ± S.E.M. of cells/peritoneal cavity for n=4 rats. Where *** represents $p < 0.001$ compared to negative control (ctrl) group (one-way ANOVA followed by Bonferronis's *post hoc*).

DISCUSSION

Ziziphus abyssinica is undoubtedly one of the least researched species of over 100 species within the *Ziziphus* genus for their chemical and therapeutic properties. Our recent studies appear to suggest that anti-inflammatory mechanisms may underpin its analgesic activity, thus necessitating the need for further studies.[18] We, therefore, report on the anti-inflammatory effect of the root extract of *Ziziphus abyssinica* and the possible involvement of membrane stabilization, inhibition of protein denaturation and neutrophil degranulation in its mode of action.

Considering the fact that researches involving animal studies are fraught with ethical challenges particularly when there are available and appropriate *in vitro* models,[28] it was expedient for the anti-inflammatory activity of ZAE to be first assessed using *in vitro* models. As such, the human red blood cell membrane stabilization models were employed due to the fact that the erythrocyte membrane is analogous to the lysosomal membrane hence the stability of the erythrocyte membrane could be extrapolated to the stabilization of lysosomal membrane.[29] The haemolytic effect of hypotonic solution is related to excessive accumulation of fluid within the cell resulting in the rupturing of its membrane. This form of injury causes secondary damage through free radical induced lipid peroxidation.[30, 31] NSAIDs are known to exert their anti-inflammatory activities partly by stabilizing lysosomal membrane to prevent the release of enzymes into the extracellular matrix.[32] In the hypotonic solution and heat - induced haemolysis test, ZAE and the standard drug showed dose-dependent stabilization of red blood cells. Although the precise mechanism of this membrane stabilization is yet to be established, it is possible that the extract exerted its effect on the surface area/volume ratio of the cells, probably through expansion of the membranes or shrinkage of the cells and a subsequent interaction with membrane proteins.[33]

One of the well-recognised causes of arthritics and inflammatory diseases is denaturation of tissue proteins. Production of auto-antigens in certain arthritic diseases may be due to denaturation of proteins.[28, 34] The antigenic property of the denatured proteins leads to diseases such as rheumatoid arthritis, glomerulonephritis, serum sickness and systemic lupus erythematosus.[35] NSAIDs in addition to their inhibitory effect on prostaglandin synthesis by blocking the cyclooxygenase pathway, they also have the ability to prevent protein denaturation which contributes to their anti-inflammatory effects.[36] This gives an indication that plant extracts which have inhibitory effect on protein denaturation may possess anti-inflammatory effects. In

this present study, ZAE exhibited anti-denaturation property in both egg albumin and BSA-induced denaturation assays and this is an indication of anti-inflammatory and anti-rheumatoid properties.

In order to be sure that the observed *in vitro* anti-inflammatory activity of ZAE is applicable in *in vivo* studies, the root extract of the plant was further assessed using the carrageenan-induced rat paw oedema test. It is a suitable and most widely used model for evaluating anti-inflammatory effects of plant extracts and their possible underpinning mechanisms.[37] Intraplantar injection of carrageenan induces a biphasic inflammation in which the first phase occurs mostly one hour post carrageenan injection and it is characterized by symptoms such as oedema, erythema and pain. The induction results in a subsequent release of pro-inflammatory mediators including histamine, serotonin, tachykinins, bradykinin, reactive oxygen species (ROS) and complement proteins.[38] Prostaglandins are known to mediate the late phase of the oedema via the action of cyclo-oxygenase-2 (COX-2) together with inducible nitric oxide synthase (iNOS).[39] During the late phase also, IL-6, IL-1β, TNF-α, and MCP-1 levels are also enhanced.[40, 41] Oral administration of the *Ziziphus abyssinica* extract suppressed the oedematous response one hour after carrageenan injection and this effect was sustained throughout the entire 5 h duration of the experiment. This suggests a possible activity of the extract on both phase-1 and phase-2 inflammatory mediators.

The anti-inflammatory potential of ZAE was also investigated using formaldehyde - induced paw oedema model, which is one of the most common methods for screening of agents for anti-arthritic properties.[26, 42] In the present study, ZAE at 100 and 300 mg/kg markedly decreased the paw oedema similar to diclofenac 10 mg/kg. The observed activity of ZAE may be due to certain alterations in the in?ammatory response comparable with the mechanism of the standard drug diclofenac which has anti-arthritic potential through the inhibition of inducible COX-2.

In the carrageenan-induced peritonitis study, we observed that the anti-inflammatory property of the ZAE was mediated through the inhibition of neutrophils recruitment to the site of inflammation. Carrageenan is known to induce neutrophil migration into peritoneal cavity through an indirect mechanism that involves the activation of macrophages and the release of cytokines into the peritoneal cavity.[43] The plant extract may have inhibited carrageenan activation of macrophages leading to down regulation of IL-1β and a subsequent inhibition of neutrophil recruitment. However, the exact mechanism needs to be explored in further studies.

Phytochemical screening conducted on the plant revealed the presence of phenols, triterpenes, alkaloids, phytosterols, reducing sugars, tannins, flavonoids, proteins and amino acids. The results obtained were similar to those obtained in earlier studies either on the roots or on other parts of the plant.[12, 13, 14] The presence of these phytochemicals is known to be responsible for the pharmacological effects of medicinal plants.[20, 44, 45, 46, 47, 48, 49]

The present study is not without some limitations and we believe that these challenges will create perspectives for future direction. First, due to ethical consideration, we were constrained to using five animals per group for the *in vivo* aspects of our study. The strength of data interpretation and statistical significance may be influenced by this sample size, although reliance on statistical significance in biological experiments remains controversial. Second, resource constraints at the moment also limited the *in vivo* aspect of the study to a single mammalian species (Sprague Dawley rats) and did not permit characterization of the plant material investigated. With improved funding, we hope to investigate the influence of species variation on the anti-inflammatory property of *Ziziphus abyssinica* root bark extract reported in this study and determine the specific compound(s) responsible for this action in future studies.

CONCLUSION

The inhibitory effect of ZAE against acute inflammation in this study provides scientific basis for its use in folk medicine and reveals its potential as a source of novel anti-inflammatory agents.

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