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ORIGINAL ARTICLE

# Anti-inflammatory activities of crude ethanol extract of *Combretum zenkeri* Engl. & Diels leaves

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## Abstract

**Background** To augment orthodox anti-inflammatory drugs, which are scarce in some areas, local dwellers in some parts of Nigeria and Asia rely on herbal products such as *Combretum zenkeri* Engl. & Diels leaves for the treatment of inflammatory diseases.

**Aim** This study investigated the anti-inflammatory activities of crude ethanol extract of *C. zenkeri* leaves (EECZ).

**Materials and methods** EECZ was prepared and subjected to anti-inflammatory activity tests. The ability of EECZ to inhibit the release of inflammatory mediators in rats was used as a measure of its anti-inflammatory property. The inhibitory effects of EECZ on phospholipase A2 (PLA2) activity, platelet aggregation and haemolysis of human red blood cells (HRBCs) were determined using standard methods. The phytochemical constituents and acute toxicity profile were also evaluated.

**Results** EECZ at oral doses of 50 and 100 mg/kg b.w. and at 6 h inhibited egg albumin-induced rat paw oedema by 67.36% and 86.19% respectively. The extract similarly inhibited PLA2 activity causing 58.23% and 60.24% inhibition at concentrations of 400 and 800 μg/ml, respectively. At 100–800 μg/ml, EECZ also significantly inhibited haemolysis of HRBCs. In a concentration-dependent manner, EECZ significantly inhibited $CaCl_2$-induced platelet aggregation. The presence of flavonoids, terpenoids, alkaloids, phenol, saponins, steroids, tannins and glycosides were detected in EECZ. The acute toxicity test showed that EECZ is safe at doses less than 5,000 mg/kg b.w.

**Conclusion** This study shows that EECZ possess anti-inflammatory activities that could be through the inhibition of PLA2 activity and platelet aggregation, and membrane stabilization.

**Keywords** Medicinal plants · *Combretum zenkeri* Engl. & Diels · Acute toxicity · phytochemicals · Phospholipase A2 · Anti-platelet aggregation · Membrane stabilization

**Abbreviations**

| | |
|---|---|
| EECZ | Ethanol extract of *Combretum zenkeri* Engl. & Diels |
| PLA2 | Phospholipase A2 |
| COX | Cyclooxygenase |
| LTs | Leukotrienes |
| LOX | Lipoxygenase |
| HRBC | Human red blood cell |
| EDTA | Ethylenediamine tetraacetic acid |
| PRP | Platelet-rich plasma |
| TXA2 | Thromboxane A2 |

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## Introduction

Inflammation refers to the body's protective response to harmful stimuli. Interactions of cells of the immune system regulate certain aspects of acute and chronic inflammation that are associated with diseases of many organs including cardiovascular disease, cancer, periodontal diseases and arthritis (Xiao 2017; Wongrakpanich et al. 2018). These non-communicable chronic diseases contribute immensely to the growing global death rate. The management of these diseases using anti-inflammatory drugs has been hampered by worrisome side effects associated with these agents (Modi et al. 2012;

Waljee et al. 2017). This necessitates the continuous search for new and safer drugs especially from natural products.

Some plants have been strongly linked with reduced risk to inflammatory conditions like rheumatoid arthritis (Ogbole et al. 2010; Ogbonna et al. 2016). An example of these plants is *Combretum zenkeri* Engl. & Diels of Combretaceae family (Ujowundu et al. 2010), a medicinal plant that is widely distributed throughout the plant world especially in tropical West African countries from southern Nigeria to Guinea and Cameroon (Ekeke et al. 2014). It is a climbing shrub or scandent forest liane of 27 m high with white flowers occurring over the tip during the raining season. The stem is cylindrical, woody and covered with short, soft hair while the leaves are opposite, short petiole, oblong-elliptic, acute to obtuse at apex, 8–15 cm long and 4–7 cm wide and prominently nerved. Also, the upper surface is covered with dense short brown hairs, especially along the nerves. Pollination of *C. zenkeri* is usually by mammals, insects and birds (Christenhusz and Byng 2016). *C. zenkeri* is known as bushwillows, other common names include *Ubi* (Mbaise) or *Ubegwu* (Owerri) both in Eastern Nigeria, *Ọ̀gàn* (Yoruba, Nigeria), *Anyi foro* (Ivory Coast) and *Adangme* (Ghana). The fresh leaves serve as vegetable for habitats of Umuahia, South-Eastern Nigeria while Mbaise natives (South East, Nigeria) use them as spice to prepare delicacies for nursing mothers (Ujowundu et al. 2015). Previous studies have shown that extract of *C. zenkeri* is rich in phytochemicals, minerals and vitamins (Ujowundu et al. 2010) and has antioxidant activity (Ujowundu et al. 2015). The ethno-botanical observations revealed that the leaves of *C. zenkeri* are used as anti-inflammatory agents (Ogbole et al. 2010; Ajibesin 2012; Wande and Babatunde 2017). Other Combretaceae species are widely reported in literature for the treatment of inflammation, cancer, urinary infections, bacterial infections, heart diseases, skin diseases and malaria among several others (Tan et al. 2002; Wande and Babatunde 2017). It is therefore necessary to establish the scientific basis of the folkloric application of the leaves of *C. zenkeri* in the management of inflammation.

One of the established models of assessing the anti-inflammatory activity of a drug against acute inflammation is by (measuring) its ability to decrease local oedema induced by egg albumin (an irritant/phlogistic agent) (Anosike et al. 2009). Subcutaneous injection of egg albumin into the rat paw produces oedema as a result of leukocyte migration, exudation and adherence (Enechi and Nwodo 2015). Egg albumin-induced oedema formation is a triphasic event simultaneous to release of various inflammatory mediators such as vasoactive amine/peptide and eicosanoids. Histamine and serotonin mediate the first phase of acute inflammation which ranges from 0 to 2 h after the administration of irritant; the second phase (2–4 h) is characterized by the release of kinnin while the later phase occurring between 4 and 6 h after the administration of the irritant is due to release of prostaglandins, cyclooxygenases (COX), protease, bradykinin and lysosome (Fullerton and Gilroy 2016). This method was adopted in this study because the mechanism of inducing inflammation is known, and the model is widely accepted.

## Materials and methods

### Study animals

The animals used for this study were thirty healthy adult male albino mice (7-week-old mice and body weight 26–30 g) for acute toxicity study and twenty healthy adult male albino rats (8-week-old rats and body weight 160–180 g) for *in vivo* anti-inflammatory test. They were obtained from the Animal Breeding Unit, Department of Zoology and Environmental Biology, University of Nigeria, Nsukka, and were acclimatized to laboratory conditions at the Animal House of Department of Biochemistry, University of Nigeria, Nsukka for 1 week prior to the commencement of the study. They were maintained on portable water and commercial rodent diet (Growers mash from Vital® feeds Nig. Ltd.). All the institutional, national and international guidelines for care and use of laboratory animals were adhered to throughout the course of this study. The study was approved by the Faculty of Biological Sciences Research Ethical Committee, University of Nigeria, Nsukka.

### Plant material

Fresh leaves of *C. zenkeri* were collected from a habitat in Obinze, Owerri-West Local Government Area of Imo State, Nigeria. The plant materials were authenticated by Mr. Alfred Ozioko, a taxonomist with the Bioresources Development and Conservation Programme (BDCP) Research Center, Nsukka, Enugu State, Nigeria. Voucher specimen with reference number Intercedd/16022 was deposited at the herbarium of the Center for future references. They were cleaned and shade-dried with regular turning to avoid decaying, until crispy. The dried leaves were pulverised into coarse form using a mechanical grinder, and 250 g of the pulverised leaves was macerated in 1000 ml of ethanol. The suspension was left for 48 h with occasional stirring, after which it was filtered using a Whatman No. 4 filter paper. This procedure was repeated twice. The filtrates collected were mixed and concentrated using a rotary evaporator under reduced pressure at 45°C to obtain crude ethanol extract of *C. zenkeri* leaves (EECZ**)** with percentage yield of 16.42%. EECZ was stored in a reagent bottle at 4 °C until used.

## Reference drugs and standards

Indomethacin used as reference drug for paw oedema, membrane stabilization and anti-platelet aggregation assays was a product of Yangzhou Pharmaceutical Co. Ltd., while prednisolone used as the reference drug for the PLA2 activity assay was a product of Kunimed Pharmaceutical Ltd. They were purchased from reputable drug stores in Nsukka, Enugu State, Nigeria. Standard phytochemicals used for this study (digitoxin, gallic acid, tannic acid, cucurbitacin, berberine, aescin, linalool and quercetin) were products of Merck, Germany.

## Microorganism

The fungal organism used as source of enzymes, strains of *Aspergillus niger*, was obtained from the Department of Microbiology, University of Nigeria, Nsukka.

## Blood samples

The human blood samples used for the membrane stabilization assay, platelet aggregation tests and phospholipase A2 activity were collected from three apparently-healthy voluntary donors. The blood donors were free from drug treatments for 1 month prior to sample collection.

## Ethical statement

The experimental procedures were carried out in accordance with the institutional, national and international ethical recommendations for the use of human subjects for research (Helsinki declaration), as well as for the care and use of laboratory animals (NIH publication no. 85-23, revised in 1985; NAS 2011) in experiments, clinical studies and biodiversity rights.

## Phytochemical analysis of the extract

The phytochemical constituents of the extract were qualitatively and quantitatively determined using previously reported methods (Evans 2009; Aiyegroro and Okoh 2010; Yadav and Agarwala 2011).

## Detection and estimation of the concentration of flavonoids

To detect the presence of flavonoids in the extract, 0.5 g of EECZ was dissolved in distilled water and filtered. Using sodium hydroxide test, 2 ml of 10% aqueous sodium hydroxide was added to 4 ml of the filtrate to produce a yellow colouration. A change in colour from yellow to colourless on addition of dilute hydrochloric acid was indicative of the presence of flavonoids. Also, in the aluminium chloride test, 4 ml of the filtrate was added 1 ml of 1% aluminium chloride; the observation of a yellow colouration indicated the presence of flavonoids. To estimate the quantity of flavonoid in the extract, 1 g of EECZ was macerated in 20 ml of ethyl acetate and filtered. To 5 ml of the filtrate was added 5 ml of 28% ammonia. The mixture was shaken and the upper layer was collected and its absorbance measured at 490 nm. The concentration of the flavonoid in the extract was determined from standard curve using quercetin as a standard.

## Detection and estimation of terpenoid concentration of the extract

EECZ (0.5 g) was dissolved in ethanol, followed by the addition of 1 ml acetic anhydride and concentrated sulphuric acid. A change in colour from pink to violet was indicative of the presence of terpenoids. Thereafter, 1 g of the extract was macerated in 20 ml of ethanol and then centrifuged for 5 min. The supernatant was transferred into a tube and 1 ml of 5 % phosphomolybdic acid solution added. Concentrated sulphuric acid (1 ml) was gradually added and mixed. The mixture was allowed to stand for 30 min followed by the addition of 2 ml of ethanol. The absorbance was read at 700 nm against blank. The concentration of the terpenoid in the extract was determined from standard curve using linalool as standard.

## Detection and estimation of the concentration of saponins

EECZ (1 g) was boiled with 5 ml of distilled water and filtered while still hot. For frothing test, 2 ml of the filtrate was added 4 ml of distilled water and the mixture shaken vigorously for 5 min. A persistent froth was indicative of the presence of saponins. For emulsion test, two drops of olive oil were added to 1 ml of the filtrate with concomitant shaking. The formation of emulsion indicated the presence of saponins. To quantify the saponin content, 1 g of the extract was macerated in 20 ml of methanol and centrifuged for 5 min. A known volume, 2 ml, of the supernatant was then transferred into test-tubes. It was evaporated to dryness over a water bath. A volume (2 ml) of ethyl acetate was added and allowed to dissolve. Then, 1 ml of 50 % sulphuric acid in ethyl acetate and 1 ml of 0.5 formaldehyde were added. It was allowed to stand for 30 min and absorbance taken at 430 nm. The concentration of the saponin in the extract was extrapolated from standard curve using aescin as standard.

## Detection and estimation of alkaloids concentration in the extract

EECZ (0.5 g) was stirred with 5 ml of 1% aqueous hydrochloric acid on a water bath and filtered. The appearance of orange-red precipitate when two drops of Dragendorff's reagent, creamy-white coloured precipitate when two drops of Mayer's reagent and reddish-brown precipitate when two drops of Wagner's reagent, respectively were separately added to 1 ml of the filtrate in different test tubes were indicative of the presence of alkaloids. Thereafter, EECZ (1 g) was macerated with 10 ml of ethanol and 10 ml of 20 % sulphuric acid and allowed to stand for 1 h. The suspension was centrifuged at 3000 rpm for 5 min and filtered. To 1 ml of the filtrate were added 5 ml of 60% sulphuric acid and 5 ml of 0.5 % formaldehyde. This was allowed to stand for 3 h after which the absorbance was measured at 565 nm. The concentration of the alkaloid in the extract was determined from standard curve using berberine as standard.

## Detection and estimation of the concentration of steroids

The detection of the presence of steroids in the extract was done using Liebermann-Burchard test. Extract (0.2 g) was added to 2 ml of acetic anhydride; the solution was cooled in ice, followed by the addition of 1 ml of concentrated sulphuric acid. Colour development from violet to blue or bluish-green was indicative of the presence of a steroid. To estimate the quantity of steroid in the extract, 1 g of extract was macerated in 20 ml of ethanol and filtered. To 2 ml of the filtrate, 2 ml of colour reagent was added and the solution left to stand for 30 min. The absorbance was read at 550 nm. The concentration of the steroid in the extract was determined from standard curve using cucurbitacin as standard.

## Detection and estimation of the concentration of tannins

EECZ (0.5 g) was macerated with 10 ml of distilled water and filtered. The filtrate was divided into two portions. For ferric chloride test, on addition of a few drops of $FeCl_3$, a greenish-black colour was observed, indicating the presence of tannins while for lead acetate test, a reddish colour was observed after a few drops of lead acetate was added, indicating the presence of tannin. To quantify the tannin content of the extract, 1 g of the extract was macerated in 20 ml of distilled water and filtered. To 5 ml of the filtrate, 0.3 ml of 0.1 N ferric chloride in 0.1 N hydrochloric acid and 0.3 ml of 0.0008 M potassium ferricyanide was added. The absorbance was read at 720 nm. The concentration of the tannin in the extract was extrapolated from standard curve using tannic acid as standard.

## Detection and estimation of the concentration of glycosides

An aliquot of the extract (0.5 g) was dissolved in 30 ml of distilled water and heated on a water bath for 5 min after which it was filtered. To 5 ml of the filtrate was added 0.2 ml of Fehling's solutions A and B until it turned alkaline. This was heated on a water bath for 2 min. A brick red precipitate was indicative of the presence of glycosides. Thereafter, a known quantity of the extract (1 g) was macerated in 20 ml of distilled water followed by the addition of 2.5 ml of 15 % lead acetate and filtered. To the filtrate was added 2.5 ml of ethanol and the mixture shaken vigorously. The lower layer was collected and evaporated to dryness. The residue was dissolved with 3 ml of glacial acetic acid. To this was added 0.1 ml of 5% ferric chloride and 0.25 ml of concentrated sulphuric acid. This mixture was kept in the dark for 2 h and the absorbance measured at 530 nm. The concentration of the glycoside in the extract was extrapolated from standard curve using digitoxin as standard.

## Detection and estimation of phenols in the extract

The presence of phenols in the extract was detected using the sodium hydroxide test. Briefly, extract (0.5 g) was dissolved in 5 ml of water and filtered. To 1 ml of the filtrate was added 2 ml of 10 % aqueous sodium hydroxide to produce a yellow colouration. A change in colour from yellow to colourless on addition of dilute hydrochloric acid was indicative of the presence of phenols. The amount of phenol in the aqueous extract was determined by Folin-Ciocalteu reagent method with some modifications. A mixture of 2.5 ml of 10% Folin-Ciocalteu reagent, 2 ml of 2% solution of $Na_2CO_3$ and 1 ml of EECZ was prepared and incubated for 15 min at room temperature. The absorbance of the mixture was read at 765 nm. The concentration of phenol in the extract was extrapolated from a standard curve using gallic acid as standard.

## Acute toxicity study on the extract

The acute toxicity profile (median lethal dose ($LD_{50}$) and safe dose) of EECZ was defined using modification of a previously reported method (Lorke 1983). This study was carried out in two phases and involved a total of 30 albino mice. In the first phase, 15 mice were grouped into three groups of five mice each and were orally administered graded doses of EECZ (10, 100 and 1000 mg/kg b.w. respectively). The mice were observed for 24 h for sign of toxicity such as nervousness, dullness, in-coordination and/or death. In the second phase, the remaining 15 mice

were divided into three groups of five mice each received 1600, 2900 and 5000 mg/kg b.w. respectively. The mice were observed for 24 h as above. The $LD_{50}$ of the plant was calculated using the formula below:

$$LD50 = \sqrt{\text{highest dose that produced no mortality} \times \text{lowest dose that produced mortality}}$$

## Anti-inflammatory effect of the extract using egg albumin-induced rat paw oedema

The method of Winter et al. (1962) was adopted. The increase in the right hind paw size of the rats injected with freshly collected egg albumin into the sub-plantar was used as a measure of acute inflammation. Egg-albumin induces the release of mediators of acute inflammation which cause oedema. The ability of the ethanol extract to inhibit the release of these mediators of inflammation is a measure of its anti-inflammatory property. Briefly, a total of 20 adult male Wistar albino rats (average body weight of 175 g and 8 weeks old) were used for the study. They were divided into four groups of five rats each and treated as follows: group 1 received 1 ml/kg b.w. of normal saline. Group 2 received 50 mg/kg b.w. of standard drug (indomethacin). Groups 3 and 4 received 50 and 100 mg/kg b.w. of EECZ, respectively. The experimental rats were fasted for 18 h before the experiment to ensure uniform hydration and minimize variability in oedematous response, after which the right hind paw size of the rats at time 0 (before oedema induction) was measured using a Vernier caliper. This was followed by oral administration of the extract as outlined above. One hour after the administration, acute inflammation was induced by injecting 0.1 ml of freshly collected egg albumin into the sub-plantar surface of the right hind paw of rats. The size of right hind paw size of rats was subsequently measured at 0.5, 1, 2, 3, 4, 5 and 6 h after albumin injection. The difference between the paw size of the injected paws at time 0 and at different times after albumin injection was used to assess the formation of oedema. These values were used in the calculation of the percentage inhibition of oedema for each dose of the extract and for indomethacin at the different time intervals using the relation below:

$$\text{Paw oedema} = (V_t - V_o)$$

where $V_o$ = Paw oedema at time zero; $V_t$ = Paw oedema at time t (0.5, 1, 2, 3, 4, 5 and 6 h)

$$\text{Percentage inhibition of oedema} = \left(\frac{(\text{Vt}-\text{Vo})\ \text{control} - (\text{Vt}-\text{Vo})\ \text{treated groups}}{(\text{Vt}-\text{Vo})\ \text{control}}\right) \times 100$$

## Anti-inflammatory effect of EECZ using inhibition of phospholipase A2 activity model

The effect of the extract on phospholipase A2 (PLA2) activity was determined using methods of Vane (1971) and Morimoto et al. (1979). PLA2 activity was assayed using its action on erythrocyte membrane. The enzyme releases free fatty acids from the membrane phospholipids thereby causing leakage, allowing haemoglobin to flow into the medium in the process. PLA2 activity is thus directly related to the amount of haemoglobin in the medium; this was measured at 418 nm since haemoglobin absorbs maximally at this wavelength.

**Enzyme preparation** Fungal enzyme preparation was obtained from *Aspergillus niger* strain culture. The organism was cultured in Sabouraud dextrose broth (SDA) for 3 days with intermittent shaking. The culture was transferred into a test-tube containing 3 ml phosphate-buffered saline (PBS) and centrifuged at 3000 rpm for 10 min. The fungal cells settled at the bottom of the test tube while the supernatant contained the crude enzyme preparation. The supernatant was then decanted and used for enzyme assay.

**Substrate preparation** Fresh human whole blood samples were collected from the first healthy volunteer who was free from drug treatment for at least 1 month into anticoagulant-containing blood sample bottle and centrifuged at 3000 rpm for 10 min and the supernatant (plasma) discarded. The red cells (pellets) were re-suspended in normal saline of equal volume to that of the plasma, centrifuged at 3000 rpm for 10 min and the supernatant discarded. The red blood cells were reconstituted as 40% (v/v) suspension with normal saline and this served as the substrate for PLA2. Aliquots (0.2 ml) of red blood cell suspension (substrate), normal saline (1.2 ml), calcium chloride solution (0.2 ml), 1 ml varying concentrations of the extract (100, 200, 400, 600 and 800 μg/ml) dissolved in normal saline and 0.2 ml of the enzyme preparation were incubated at 37 °C for 1 h. The control tube contained 0.1 ml of the erythrocytes, 1.2 ml normal saline, 0.2 ml calcium chloride and 0.2 ml of the enzyme preparation. The blanks contained everything in the test except the enzyme. The incubated reaction of various mixtures was centrifuged at 3000 rpm for 10 min. The absorbances of the supernatants

were read against the blank at 418 nm. Prednisolone (200 and 400 μg/ml), a known inhibitor of the enzyme, was used as the standard control. The % inhibition of PLA2 activity was calculated thus:

$$\text{The\%inhibition of enzyme activity} = \frac{\text{AC}-\text{AT}}{\text{AC}} \times 100$$

where AC = absorbance of the control; AT = absorbance of the test drug/extract

## Anti-inflammatory effect of EECZ using inhibition membrane stabilization models

The effect of EECZ on membrane stabilization was determined using both heat and hypotonicity-induced haemolysis of human red blood cells (HRBCs).

## Inhibition of heat-induced haemolysis of human red blood cells

This was evaluated using the method of Shinde et al. (1989) with slight modification. The principle of this test is based on the susceptibility of RBC's membrane to oxidative damage, due to high content of polyunsaturated fatty acids in the membranes leading to haemolysis. Haemoglobin and other internal cell components are thus released into the surrounding fluids. Absorbance readings at 418 and 540 nm are thus reflection of the amount of haemoglobin in the medium. High absorbance values thus imply high haemolysis and less stability of the RBC's membrane.

**Preparation of HRBC suspension** Fresh blood sample (6 ml) was collected from a second healthy adult volunteer into an ethylene diamine tetraacetic acid (EDTA) bottle and was divided into two. The first 3 ml which was used for the inhibition of heat-induced haemolysis was centrifuged at 3000 rpm for 10 min and the supernatant discarded. A volume of normal saline equivalent to that of the supernatant was used to re-suspend the RBCs. The volume of the re-suspended RBCs was measured and reconstituted as a 40% (v/v) suspension with normal saline. The reconstituted RBCs were used for the test.

Samples of the extract and indomethacin used were dissolved in isotonic phosphate buffer solution. A set of five test tubes containing respectively 5 ml graded concentration of the extracts (100, 200, 400, 600 and 800 μg/ml) were arranged in triplicate (three sets per concentration). For the control, a set of two test tubes containing 5 ml of the vehicle (phosphate buffer) and 5 ml graded doses of indomethacin (200 and 400 μg/ml) was also arranged in triplicates. HRBC suspension (0.1 ml) was added to each of the tubes and mixed gently. A pair of the tubes was incubated in a regulated water bath at 54 °C for 20 min while the other pair was maintained at 10 °C in the freezer for 20 min. Afterwards, the tubes were centrifuged at 1300 rpm for 3 min and the absorbance read at a wavelength of 540 nm. The blanks contained varying concentration of the extract, indomethacin and vehicle (for control) without HRBC suspension. The percentage (%) inhibition of haemolysis by the ethanol extract and indomethacin was calculated thus

$$\text{\%Inhibition of Haemolysis by extract} = 1-\left(\frac{\text{OD2}-\text{OD1}}{\text{OD3}-\text{OD1}}\right) \times 100$$

where OD1 = absorbance of test sample (extract, indomethacin) unheated, OD2 = absorbance of test sample heated and OD3 = absorbance of control sample heated

## Inhibition of hypotonicity-induced haemolysis of HRBCs

This was determined using the method of Oyedepo et al. (1997) as modified by Ezekwesili and Nwodo (2000). Hypotonicity-induced haemolysis of HRBCs occurs due to osmotically coupled water uptake by the cells, leading to the swelling and subsequently lysis of RBCs. This results in the extracellular release of haemoglobin. The stability of absorbance at 418 nm reflects the stability of RBC's membrane. Hence, comparing the absorbance at 418 nm, a measure of erythrocytic haemolysis in the presence and absence of the extract indicates erythrocytic membrane stabilization potential of the extract.

**Preparation of human red blood cells suspension** A portion of the fresh whole blood sample (3 ml) collected from the second healthy volunteer was centrifuged at 3000 rpm for 10 min and supernatant (plasma) discarded. The pellet was washed twice by re-suspending it in normal saline equal to the volume of the supernatant (plasma). The blood volume was measured and reconstituted as a 40% (v/v) suspension with normal saline.

Samples of the extract and indomethacin used were dissolved in distilled water (hypotonic solution) and normal saline (isotonic solution). The hypotonic solution (5 ml) contained aliquot (1 ml) of varying concentrations of the extract (100, 200, 400, 600 and 800 μg/ml) and indomethacin (200 and 400 μg/ml) and 0.1 ml erythrocyte suspension. The isotonic solution (5 ml) also contained aliquot (1 ml) of varying concentrations of the extract (100, 200, 400, 600, 800 μg/ml) and indomethacin (200 and 400 μg/ml) and 0.1 ml erythrocyte suspension. Two control tests were used for this test. A control tube (5 ml) contained distilled water and 1 ml of erythrocyte suspension while the other contained normal saline and 1 ml erythrocyte suspension. The various reaction contents were gently mixed and incubated for 1 h at room temperature (37 °C). After incubation, the reaction mixture for each tube was centrifuged at 3000 g for 10 min. The

absorbance (OD) of the supernatant was determined at 418 nm using Jenway spectrophotometer. The tests were carried out in triplicates with blank tubes containing 1 ml varying concentrations of extract or indomethacin without HRBCs suspension in hypotonic or isotonic medium. The percentage inhibition was calculated assuming haemolysis produced in the presence of distilled water was 100%. The percentage inhibition of haemolysis was calculated using the relation below:

$$\%\text{Inhibition of Haemolysis} = 1-\left(\frac{\text{OD2}-\text{OD1}}{\text{OD3}-\text{OD1}}\right)\times 100$$

where OD1 = absorbance of test sample (extract, indomethacin) in isotonic solution, OD2 = absorbance of test sample (extract, indomethacin) in hypotonic solution and OD3 = absorbance of control sample in hypotonic solution.

### Anti-inflammatory effect of EECZ using inhibition of platelet aggregation model

This was achieved following a modification of the method of Rein et al. (2000). The aggregation of platelets leads to increased transmittance and therefore less absorbance of light. $CaCl_2$-induced platelet aggregation is thus shown by reduced absorbance at 520 nm. Any substance that has anti-aggregatory effect would thus lead to increased absorption by the medium.

**Preparation of PRP** Fresh blood sample (3 ml) was drawn from the third healthy volunteer into EDTA bottles and centrifuged at 3000 rpm for 10 min. The supernatant which is rich in platelets was collected and diluted twice its volume with normal saline, then transferred to a clean plastic tube and thereafter used as the platelet-rich plasma.

An aliquot of platelet-rich plasma (PRP) (0.2 ml) was put into each of a set of four plastic test tubes containing 1 ml each of varying concentrations of extract (100, 200, 400, 600 and 800 μg/ml). Also, another test tube contained an aliquot (0.2 ml) of PRP and 1 ml of 200 and 400 μg/ml indomethacin. The contents of the respective tubes were made up to 2.2 ml with the vehicle (normal saline). A control tube contained 2 ml of the vehicle and 0.2 ml of PRP. The tubes were allowed to incubate after which platelet aggregation was induced by the addition of 0.4 ml of 1.37 % calcium chloride ($CaCl_2$) solution. This test was performed in triplicates. Changes in the absorbance of the solutions were taken at intervals of 30 s for 2 min at 520 nm. The blanks contained the extract or indomethacin without PRP.

### Statistical analysis

The data obtained were analysed using Statistical Product and Service Solutions (SPSS), version 18, and were expressed as mean ± standard deviation (SD). Tests of statistical significance were carried out using analysis of variance (ANOVA). Means with $p$ values < 0.05 were considered statistically significant.

## Results

### Phytochemical constituents of EECZ

The qualitative phytochemical analyses of the EECZ revealed the presence of some phytochemicals in varied proportion as shown in Table 1. The bioactive compounds such as terpenoids (17.67 ± 0.26 mg/g), saponins (10.24 ± 0.21 mg/g) and flavonoids (18.24 ± 0.33 mg/g) were found in relatively high concentrations; alkaloids (13.94 ± 0.29 mg/g), phenols (10.57 ± 0.06 mg/g) and steroids (8.27 ± 0.51 mg/g) were present in moderate concentrations, while tannins (1.03 ± 0.12 mg/g) and glycosides (0.41 ± 0.20 mg/g) were slightly present.

### Acute toxicity profile of the EECZ

The acute toxicity test of EECZ showed that the extract did not cause death with doses up to 2900 mg/kg per body weight (b.w.). However, the administration of 5000 mg/kg b.w. of the extract provoked sedation, weakness and dullness, resulting to one death within 24 h of administration. Table 2 shows the observations in phases I and II of the acute toxicity test of the extract. These observations reveal that only a single mortality was recorded in both phases of the experiment and the $LD_{50}$ was calculated as 3807.89 mg/kg b.w.

### Effect of the EECZ on egg albumin-induced rat paw oedema

The result of anti-inflammatory effect of EECZ evaluated by inhibition of egg albumin-induced rat paw oedema method is presented in Table 3. Experimental rats in various groups

**Table 1** Phytochemical constituents of ethanol extract of *Combretum zenkeri* leaves

| Phytochemicals | Relative presence | Composition (mg/g) |
|---|---|---|
| Tannins | + | 1.03 ± 0.12 |
| Flavonoids | +++ | 18.24 ± 0.33 |
| Saponins | ++ | 10.24 ± 0.21 |
| Alkaloids | +++ | 13.94 ± 0.29 |
| Terpenoids | +++ | 17.67 ± 0.26 |
| Steroids | ++ | 8.27 ± 0.51 |
| Glycosides | + | 0.41 ± 0.20 |
| Phenols | ++ | 10.57 ± 0.06 |

Values indicate mean ± SD ($n$ = 3)

+ minimally present, ++ Moderately present, +++ Highly present

**Table 2** The acute toxicity profile of ethanol extract of *Combretum zenkeri* leaves

| Group | Dosage (in mg/kg b.w) | Mortality |
|---|---|---|
| Phase I | | |
| Group 1 | 10 | 0/5 |
| Group 2 | 100 | 0/5 |
| Group 3 | 1000 | 0/5 |
| Phase II | | |
| Group 4 | 1600 | 0/5 |
| Group 5 | 2900 | 0/5 |
| Group 6 | 5000 | 1/5 |
| Median lethal dose ($LD_{50}$) = 3807.89 mg/kg b.w | | |

Group 1–3 = mice treated with 10, 100 and 1000 mg/kg b.w. of extract respectively for phase I while groups 4–6 = mice treated with 1600, 2900 and 5000 mg/kg b.w. of extract respectively

treated with the extract and standard drug had significant reduction in paw oedema, in a dose and time-dependent manner when compared with the (normal) control. At time 2 h, the groups treated with 50 and 100 mg/kg b.w of extract inhibited rat paw oedema by 51.17% and 64.02% respectively as compared to 50 mg/kg b.w. of indomethacin (49.53%). At 6 h, the inhibition of rat paw oedema for the groups treated with 50 and 100 mg/kg b.w of extract increased to 67.36% and 86.19% respectively. Similarly, there were significant reductions in the mean paw oedema volume of rats in all experimental groups at 0, 1, 2, 3, 5 and 6 h as shown by the progressive increase in the percentage inhibition of rat paw oedema. Groups 2 and 3 showed non-significant reduction in the mean paw volumes only at the time range of 0.5–5 h.

### Effect of the EECZ on phospholipase A2 activity

As shown in Table 4, the ethanol extract significantly inhibited PLA2 activity in a concentration-related manner, provoking inhibition comparable to that of indomethacin. This was shown by the reduced absorbances of the supernatant solution. High percentage inhibition of activity (55.67% and 58.23%) was obtained for concentrations of 200 and 400 μg/ml of the extract as compared to 39.36% and 49.43% inhibition of activity for that of prednisolone at the same concentrations. At higher concentrations of 600 and 800 μg/ml of extract, the percentage of inhibition obtained was 59.77% and 60.24% respectively.

### Effect of the EECZ on membrane stabilization using hypotonicity-induced haemolysis of human red blood cells

From the data presented in Table 5, EECZ protected the human erythrocyte membrane against lyses induced by hypotonic solution compared to the control by significantly inhibiting the liberation of haemoglobin. When erythrocytes were suspended in water and later centrifuged, the supernatant was found to have a mean absorbance of 0.459 ± 0.002 at 540 nm. On the other hand, suspension of the erythrocytes in normal saline, given the same treatment as in the case of water, gave an absorbance of 0.257 ± 0.002. The result show that in the hypotonic (water) environment, there was liberation of haemoglobin and hence the high absorbance reading. On the other hand, when the ethanol extract was introduced, there was a concentration-dependent decrease in absorbance readings. High percentages inhibition of haemolysis (57.9% and 59%) were obtained at 600 and 800 μg/ml concentrations of the extract respectively comparable to those of the indomethacin (51.1% and 81.5%) at the same concentrations.

### Effect of the EECZ on heat-induced haemolysis of human red blood cells

The ethanol extract, like indomethacin, significantly reduced the lysis of RBCs induced by heat when compared to the control (Table 6). When erythrocytes were heated and later centrifuged, the supernatant was found to have a mean absorbance of 0.812 ± 0.002 at 418 nm. On the other hand, when the suspension of the erythrocytes was cooled, given the same treatment as in the case of heat, it gave an absorbance of 0.410 ± 0.002. However, when the extract was introduced, there was decrease in absorbance readings; all groups treated with the extract had significantly higher inhibitions of heat-induced haemolysis in concentration-independent conditions compared to indomethacin. High percentage inhibition of haemolysis (90.4% and 90.6%) was obtained at 200 μg/ml and 800 μg/ml doses of the plant extract respectively comparable to that of the standard drug, indomethacin (76.8% and 77%) at 200 μg/ml and 400 μg/ml.

### Effect of the EECZ on platelet aggregation

The mean anti-platelet aggregation of HRBC for various groups treated with indomethacin and EECZ at different time intervals are shown in Table 7. The platelet aggregation of all the groups treated with EECZ and indomethacin were significantly higher from 0 to 120 s when compared to control. The rate of inhibition of $CaCl_2$-induced platelet aggregation by the extract-treated groups (groups 2–6) increased in a concentration-dependent fashion when comparable with the indomethacin-treated group (positive control).

## Discussion

In this study, the anti-inflammatory activity and the possible mechanisms of action of EECZ were reported using *in vivo*

**Table 3** Effect of ethanol extract of *Combretum zenkeri* leaves on egg albumin-induced rat paw oedema

| Treatment/time (h) | Δ Paw volume (oedema) mm, % inhibition of oedema and time (h) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0.5 | 1 | 2 | 3 | 4 | 5 | 6 |
| Group 1 (1 ml/kg b.w. of normal saline) | 3.31 ± 0.19bC | 4.19 ± 0.10cDE | 4.28 ± 0.07cE | 3.99 ± 0.24cD | 3.32 ± 0.07cC | 2.62 ± 0.18cB | 2.39 ± 0.12cA |
| Group 2 (50 mg/kg b.w. of indomethacin) | 3.20 ± 0.51bF (3.32) | 2.63 ± 0.36bE (37.23) | 2.16 ± 0.37bD (49.53) | 1.85 ± 0.23bCD (53.64) | 1.57 ± 0.09bBC (53.71) | 1.27 ± 0.24bB (54.52) | 0.56 ± 0.16abA (76.51) |
| Group 3 (50 mg/kg b.w. of EECZ) | 3.09 ± 0.19bF (6.65) | 2.37 ± 0.03bE (43.44) | 2.09 ± 0.65bD (51.17) | 1.81 ± 0.08bC (54.64) | 1.50 ± 0.05bB (54.82) | 1.33 ± 0.04bB (59.23) | 0.78 ± 0.24bA (67.36) |
| Group 4 (100 mg/kg b.w. of EECZ) | 2.56 ± 0.04aF (22.66) | 1.65 ± 0.05aE (60.62) | 1.54 ± 0.03aDE (64.02) | 1.44 ± 0.01aD (63.91) | 1.00 ± 0.23aC (69.88) | 0.60 ± 0.09aB (77.01) | 0.33 ± 0.10aA (86.19) |

Results are expressed in means ± SD ($n = 5$). Values in parenthesis are percentage inhibition of inflammation calculated relative to control. Mean values having different lower cases letters from top to bottom of the column (groups) and those having different upper case letters across the row (time) are considered significant < 0.05

inhibition of egg-albumin-induced rat-paw oedema and *in vitro* inhibition of the activity of phospholipase $A_2$, membrane stabilization and platelet aggregation approaches. The presence of bioactive compounds such as presence of phenols, alkaloids, flavonoids, tannins, steroids, glycosides, terpenoids and saponins in this extract suggests that it might have anti-inflammatory activities. Studies have shown that these phytoconstituents are present in many known plants with anti-inflammatory properties such as *Curcumma longa* (turmeric), *Zingiber officinale* (ginger) and *Rosmarinus offininalis* (rosemary) (Anosike et al. 2009; Mahluji et al. 2013). Furthermore, flavonoids have been shown to have anti-inflammatory activity via inhibition of PLA2 (Nair et al. 2006; Rosillo 2012). Steroids-containing plants are associated with several biological activities such as anti-inflammatory (Ahmadiania et al. 2001) and anti-tumour (Dushimemaria et al. 2017). In general, therapeutic actions of bioactive compounds are linked to their antioxidant effects (Laveti et al. 2013). Bioactive compounds act as reducing agents, hydrogen donors and singlet oxygen scavengers, and hence, reduce inflammatory signals and key mediators of inflammation which activate signal transduction cascades. Activation of signal transduction cascades leads to changes in transcription factors that regulate immediate cellular stress responses and inflammation (Laveti et al. 2013). Previously, *C. zenkeri* was found to exert free radical scavenging activities against radicals of nitric oxide ($NO^{\cdot}$) and hydroxyl ($OH^{\cdot}$), and ferric-reducing antioxidant activities (Ujowundu et al. 2015), suggesting a possible anti-inflammatory effect.

In other to determine the least concentration of extract that elicits harmful effects, acute toxicity tests, which are performed in short durations under monitored conditions and are very crucial in assessing the safety of drugs in biological systems, were carried out. Results from the acute toxicity test suggest that EECZ at the dose of 5000 mg/kg b.w was harmful and caused a single mortality. However, there was no mortality recorded at 2900 mg/kg b.w and below and the $LD_{50}$ for EECZ was calculated to be 3807.89 mg/kg b.w. Substances with $LD_{50}$ values above 1000 mg/kg b.w. have been reported to be relatively safe (Clarke and Clarke 1977). Okwu et al. (2014) previously reported no mortality upon oral administration of ethanol extract of *C. zenkeri* up to 5000 mg/kg b.w. dose. The difference in the results could be as a result of variations in the extraction procedure, and type, age and body weight of experimental animals used. Mice with body weight of 26 to 30 g were used in this study as against rats of weighing 80–100 g employed by Okwu et al. (2014). The $LD_{50}$ of *C. micranthum*, a plant from the same genus as *C. zenkeri,* was reported to be 3807.9 mg/kg b.w in mice and 5000 mg/kg in rats (Abdullahi et al. 2014). Generally, the Combretaceae family is considered to be relatively safe. Based on the result of acute toxicity test, the doses administered in the anti-inflammatory study were therefore selected.

**Table 4** Effect of ethanol extract of *Combretum zenkeri* leaves on phospholipase $A_2$ activity

| Treatment | Concentration (μg/ml) | Mean absorbance | % Inhibition of enzyme activity |
|---|---|---|---|
| Control | – | 0.496 ± 0.001h | – |
| Extract | 100 | 0.224 ± 0.002a | 54.94 |
| | 200 | 0.220 ± 0.002b | 55.67 |
| | 400 | 0.207 ± 0.002c | 58.23 |
| | 600 | 0.200 ± 0.001d | 59.77 |
| | 800 | 0.197 ± 0.002e | 60.24 |
| Indomethacin | 200 | 0.301 ± 0.002f | 39.36 |
| | 400 | 0.251 ± 0.002g | 49.43 |

Results are expressed in means ± SD ($n$ = 3); Mean values with different letters in a column are considered significant at $p < 0.05$

EECZ at 50 and 100 mg/kg b.w. showed good anti-inflammatory activity by significantly inhibiting egg albumin-induced rat paw oedema at all phases (0 to 6 h) of acute inflammation; the highest percentage inhibition was observed during the last phase (4 to 6 h). This was evident due to the progressive reduction in the rat paw volume of groups administered with EECZ with time. The inhibition of all three phases of oedema formation accords with the behaviour of another species from the same genus, *C. fragrans* reported by Mbiantcha et al. (2018) and *C. micranthum* (Abdullahi et al. 2014). The reduction of oedemogenesis in all the three phases of oedema formation suggest that the anti-inflammatory activity of the plant extract may be linked to the inhibition of inflammatory mediators such as histamine, serotonin and kinnin release and suppression of COX activity which are responsible for the emancipation of metabolites of arachidonic acid. Alternatively, it may be due to increase in cell membrane stability thereby suppressing the release of blood cellular components (Bartnik and Facey 2017).

EECZ at concentrations of 100 to 800 μg/ml was found to significantly exhibit high membrane stabilization effect against both hypotonicity and heat-induced haemolysis of HRBCs when compared to indomethacin. Although this is the first study that reported the membrane stabilization effect on *C. zenkeri* leaves (an indicator of the possible mechanism of anti-inflammatory effect), Ujowundu et al. (2015) showed that the ethanol extract of *C. zenkeri* exhibited free radical scavenging activities against nitric oxide ($NO^{\cdot}$) and hydroxyl ($OH^{\cdot}$) radicals and ferric-reducing antioxidant power, supporting the opinion that the anti-inflammatory effect of EECZ correlates with its redox properties. When the production of free radicals outweighs their elimination, oxidative stress ensues. The mast cells, macrophages and leukocytes are summoned to the site of injury during inflammation. This causes a respiratory burst resulting from an increased uptake of oxygen, and hence, an elevation in elevated production and accumulation of free radicals at the injury site (Reuter et al. 2010). In addition, some standard anti-inflammatory drugs function via membrane stabilization by suppressing iNOS and inhibition of the production of free radicals. These cause the suppression of cellular signalling pathways such as tumour necrotic factor alpha (TNF-α) and nuclear factor

**Table 5** Inhibition of hypotonicity-induced haemolysis by ethanol extract of *Combretum zenkeri* leaves

| Treatment | Concentration (μg/ml) | Absorbance (nm) | | |
|---|---|---|---|---|
| | | Hypotonic solution | Isotonic solution | % Inhibition of haemolysis |
| Control | – | 0.459 ± 0.002d | 0.257 ± 0.002h | – |
| Extract | 100 | 0.326 ± 0.002c | 0.014 ± 0.002a | 29.9 |
| | 200 | 0.295 ± 0.002bc | 0.017 ± 0.003b | 37.1 |
| | 400 | 0.249 ± 0.003abc | 0.020 ± 0.002c | 47.9 |
| | 600 | 0.206 ± 0.003a | 0.022 ± 0.002d | 57.9 |
| | 800 | 0.203 ± 0.002a | 0.025 ± 0.001e | 59 |
| Indomethacin | 200 | 0.298 ± 0.002ab | 0.146 ± 0.004g | 51.4 |
| | 400 | 0.197 ± 0.003a | 0.137 ± 0.002f | 81.5 |

Results are expressed in means ± SD ($n$ = 3). Mean values with different letters in a column are considered significant at $p < 0.05$

**Table 6** Inhibition of heat-induced haemolysis by ethanol extract of *Combretum zenkeri* leaves

| Treatment | Concentration (μg/ml) | Absorbance (nm) | | % Inhibition of haemolysis |
|---|---|---|---|---|
| | | Unheated solution | Heated solution | |
| Control | – | 0.410 ± 0.002 | 0.812 ± 0.002h | – |
| Extract | 100 | 0.077 ± 0.002 | 0.155 ± 0.001a | 89.4 |
| | 200 | 0.122 ± 0.002 | 0.188 ± 0.002b | 90.4 |
| | 400 | 0.130 ± 0.001 | 0.198 ± 0.002c | 90.2 |
| | 600 | 0.134 ± 0.002 | 0.206 ± 0.001d | 89.5 |
| | 800 | 0.149 ± 0.002 | 0.211 ± 0.001e | 90.6 |
| Indomethacin | 200 | 0.080 ± 0.001 | 0.247 ± 0.002g | 76.8 |
| | 400 | 0.066 ± 0.002 | 0.238 ± 0.002f | 77 |

Results are expressed in means ± SD ($n$ = 3); Mean values with different letters are considered significant at $p < 0.05$

kappa-B (NF-κB) which are transcription factors for pro-inflammatory proteins (Yahfoufi et al. 2018). The presence of phytochemicals with known antioxidant activities in *C. zenkeri* may account for its membrane stabilization effects (Suvanto et al. 2017).

The cleavage of membrane phospholipids by PLA2 releases free fatty acids such as arachidonic acid which are acted upon by the COX, lipoxygenases (LOX) and other enzymes to produce mediators of inflammation (Burke and Dennis 2009). The observed concentration-dependent inhibition of PLA2 activity by EECZ implies that the extract inhibited the release of free fatty acids from membrane phospholipids, synthesis of inflammatory mediators and inflammation. This mechanism of action is also unique to steroidal anti-inflammatory drugs (Coutinho and Chapman 2011). The anti-inflammatory activities of many steroids and their application in treating inflammatory diseases have been linked with their inhibitory effects on phospholipase A2 (Patel and Savjani 2015), an enzyme involved in production of inflammatory mediators. This suggests that EECZ which is rich in steroids has the potentials for treating inflammatory diseases.

This study also showed that EECZ, in a concentration-dependent manner, significantly inhibited $CaCl_2$-induced platelet aggregation compared to control. To the best of the knowledge of the researchers, this is the first study that reported anti-platelet aggregatory effect of *C. zenkeri*. However, Suleiman et al. (2010) reported a significant inhibition of platelet aggregation by methanol extract of *C. vendae*—a plant from the same genus as *C. zenkeri*. Platelets are a component of blood whose function along with the coagulation factors is to stop bleeding by initiating blood clot (Thomas and Storey 2015).

## Conclusion

Results from this study shows that the ethanol extract of *C. zenkeri* leaves (EECZ) possesses anti-inflammatory

**Table 7:** Inhibition of platelet aggregation by ethanol extract of *Combretum zenkeri* leaves

| Group | Antiplatelet aggregation ($A_{520nm}$) (s) | | | | |
|---|---|---|---|---|---|
| | 0 | 30 | 60 | 90 | 120 |
| Group 1 | 0.144 ± 0.002aE | 0.139 ± 0.002aD | 0.135 ± 0.002aC | 0.129 ± 0.001aB | 0.122 ± 0.001aA |
| Group 2 | 0.443 ± 0.002gD | 0.403 ± 0.002hC | 0.390 ± 0.003hB | 0.384 ± 0.002hA | 0.384 ± 0.001hA |
| Group 3 | 0.393 ± 0.002fE | 0.376 ± 0.003gD | 0.372 ± 0.002gC | 0.366 ± 0.002gB | 0.360 ± 0.001gA |
| Group 4 | 0.341 ± 0.002eC | 0.337 ± 0.002fBC | 0.334 ± 0.002fB | 0.333 ± 0.007fB | 0.320 ± 0.001fA |
| Group 5 | 0.273 ± 0.056cB | 0.241 ± 0.002cAB | 0.232 ± 0.001cA | 0.232 ± 0.001cA | 0.232 ± 0.001cA |
| Group 6 | 0.276 ± 0.002cdC | 0.265 ± 0.001dB | 0.266 ± 0.002eB | 0.262 ± 0.002eA | 0.260 ± 0.001eA |
| Group 7 | 0.217 ± 0.003bC | 0.202 ± 0.003bB | 0.192 ± 0.002bA | 0.194 ± 0.002bA | 0.193 ± 0.001bA |
| Group 8 | 0.310 ± 0.002deE | 0.272 ± 0.003eD | 0.261 ± 0.002dC | 0.252 ± 0.003dB | 0.248 ± 0.002dA |

Results are expressed in means ± SD ($n$ = 3). Mean values having different lowercase letters from top to bottom of the and those having different uppercase letters from left to right (across the row) are considered significant ($p < 0.05$). Group 1 = normal control; groups 2–8 = 100, 200, 400, 600 and 800 μg/ml of ethanol extract of *C. zenkeri* leaves respectively; groups 7 and 8 = 200 and 400 μg/ml of indomethacin (standard drug) respectively

activity and its mechanisms of action could be via the inhibition of the synthesis of inflammatory mediators, achieved by inhibiting the activities of phospholipase A2 and aggregation of platelets, and stabilization of erythrocyte membrane. The diverse mechanisms of action of *C. zenkeri* leaves could be due to the synergistic actions of some of the identified phytoconstituents in the leaf extract. This research provides scientific evidence to support the use of this plant in the management of inflammatory conditions. The isolation and further characterization of the anti-inflammatory agents in the extract is warranted.

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## Compliance with ethical standards

**Conflict of interest** The authors declare no conflict of interest.

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## References

Abdullahi MH, Anuka JA, Yaro AH, Musa A (2014) Analgesic and anti-inflammatory effects of aqueous leaf extract of *Combretum micranthum*. g Don (Combretaceae). Bayero J Pure Appl Sci 7: 78–85

Ahmadiania A, Javana M, Semnanianb S, Barata E, Kamalinejada M (2001) Anti-inflammatory and antipyretic effects of *Trigonellafoenum graecum* leaves extract in the rat. J Ethnopharmacol 75:283–286

Aiyegroro OA, Okoh AI (2010) Preliminary phytochemical screening and *in vitro* antioxidant activities of aqueous extract of *Helichrysum longifolium* DC. BMC Complement Altern Med 10:21

Ajibesin KK (2012) Ethnobotanical survey of plants used for skin diseases and related ailments in Akwa Ibom State, Nigeria. Ethnobot Res Appl 10:463–522

Anosike CA, Obidoa O, Ezeanyika LUS, Nwuba MM (2009) Anti-inflammatory and anti-ulcerogenic activity of ethanol extract of ginger (*Zingiber officinale*). Afr J Biochem 3:379–384

Bartnik M, Facey PC (2017) Glycosides. In: Badal S, Delgoda R (eds) Pharmacognosy- fundamentals, application and strategies. Academic Press, USA, pp 101–161

Burke JE, Dennis EA (2009) Phospholipase A2 structure/function, mechanism, and signaling. J Lipid Res 50: S237–S242

Christenhusz MJM, Byng JW (2016) The number of known plants species in the world and its annual increase. Phytotaxa Magnolia Press 261:201–217

Clarke EGC, Clarke ML (1977) Veterinary toxicology, 1st edn. Bailliere Tindal, New York, p 10

Coutinho AE, Chapman KE (2011) The anti-inflammatory and immunosuppressive effects of glucocorticoids, recent developments and mechanistic insights. Mol Cell Endocrinol 335:2–13

Dushimemaria F, Du Preez CI, Mumbengegwi DR (2017) Randomized anticancer and cytotoxicity activities of *Guibourtia coleosperma* and *Diospyros chamaethamnus*. Afr J Tradit Complement Altern Med 14(4):1–7

Ekeke C, Agbagwa IO, Okoli BE (2014) Numerical taxonomy of *Combretum Loefl* from Southeastern Nigeria. J Plant Sci 9:25–31

Enechi OC, Nwodo OFC (2015) The mechanisms of anti-inflammatory action of *Buchholzia coriacea* seed extract. Jokull J 65:10–13

Evans WC (2009) Trease and Evans' pharmacognosy, 16th edn. Saunders Elsevier, London, p 385

Ezekwesili CN, Nwodo OFC (2000) Anti-inflammatory properties of *Vigna unguiculata* seed extract. J Med Lab Sci 9:141–145

Fullerton JN, Gilroy DW (2016) Resolution of inflammation: a new therapeutic frontier. Nat Rev Drug Discov 15:551–567

Laveti D, Kumar M, Hemalatha R, Sistla R, Naidu VG, Talla V, Verma V, Kaur N, Nagpal R (2013) Anti-inflammatory treatments for chronic diseases: a review. Inflamm Allergy Drug Targets 12:349–361

Lorke D (1983) A new approach to practical acute toxicity testing. Arch Toxicol 55:275–287

Mahluji S, Ostadrahimi A, Mobasseri M, Attari VE, Payahoo L (2013) Anti-inflammatory effects of *Zingiber officinale* in type 2 diabetic patients. Adv Pharm Bull 3:273–276

Mbiantcha M, Almas J, Dawe A, Faheem A, Sidra Z (2018) Analgesic, anti-inflammatory and anticancer activities of combretin A and combretin B isolated from *Combretum fragrans* F. HOFFM (Combretaceae) leaves. Inflammopharmacology 26:1429–1440

Modi CM, Mody SK, Patel HB, Dudhatra GB, Kumar A, Avale M (2012) Toxicopathological overview of analgesic and anti-inflammatory drugs in animals. J Appl Pharm Sci 2:149–157

Morimoto S, Kazuna S, Kawai K (1979) Effects of some anti-rheumatic agents on copper-catalysed thermal aggregation of gamma albulin. Agents Actions 9:375–380

Nair MP, Mahajan S, Reynolds JL, Aalinkeel R, Nair H, Schwartz SA, Kandaswami C (2006) The flavonoid quercetin inhibits proinflammatory cytokine (tumor necrosis factor alpha) gene expression in normal peripheral blood mononuclear cells via modulation of the NF-KB system. Clin Vaccine Immunol 13:319–328

National Academy of Sciences [NAS] (2011) Guide for the care and use of laboratory animals, 8th edn. Institute for Laboratory Animal Research, Division on Earth and Life Studies, National Academy of Sciences, Washington DC

Ogbole OO, Gbolade AA, Ajaiyeoba EO (2010) Ethnobotanical survey of plants used in treatment of inflammatory diseases in Ogun State of Nigeria. Eur J Sci Res 43:183–191

Ogbonna CU, Ujowundu CO, Okwu GN, Emejulu AA, Alisi CS, Igwe KO (2016) Biochemical and histological evaluation of benzo[a]pyrene induced nephrotoxicity and therapeutic potentials of *Combretum zenkeri* leaf extract. Afr J Pharm Pharmacol 10: 873–882

Okwu GN, Ogbonna CU, Ujowundu CO, Igwe KO, Igwe CU, Emejulu AA (2014) Protective effect of ethanol leaf extract of *Combretum zenkeri* on liver functions of albino rats following benzo(A)pyrene exposure. Biol Chem Res 2014:16–25

Oyedepo OO, Akindele VR, Okunfolami OK (1997) Effect of extracts of *Olax subsorpoides* and *Aspila Africana* on bovine red blood cells. Phytother Res 11:305–306

Patel SS, Savjani JK (2015) Systematic review of plant steroids as potential antiinflammatory agents: Current status and future perspectives. J Phytopharmacol 4:121–125

Rein D, Paglieroni TG, Wun T (2000) Cocoa inhibits platelet activation and function. Am J Clin Nutr 72:30–35

Reuter S, Gupta SC, Chaturvedi MM, Aggarwal BB (2010) Oxidative stress, inflammation, and cancer: How are they linked? J Free Radic Biol Med 49:23–28

Rosillo MA, Alcaraz MJ, Sánchez-Hidalgo M, Fernández-Bolaños JG, Alarcón-de-la-Lastra C, Ferrándiz ML (2014) Anti-inflammatory and joint protective effects of extra-virgin olive-oil polyphenol extract in experimental arthritis. J Nutr Biochem 25: 1275–1281

Shinde UA, Phadke AS, Nari AM, Mungantiwar AA, Dikshit VJ, Saraf MN (1989) Membrane stabilization activity - a possible mechanism of action for the anti-inflammatory activity of *Cedrus deodora* wood oil. Fitoterapia 70:251–257

Suleiman MM, McGaw LJ, Naidoo V, Eloff JN (2010) Detection of antimicrobial compounds by bioautography of different extracts of leaves of selected South African tree species. Afr J Trad CAM 7:64–78

Suvanto J, Nohynek L, Seppänen-Laakso T, Rischer H, Salminen JP, Puupponen-Pimiä R (2017) Variability in the production of tannins and other polyphenols in cell cultures of 12 Nordic plant species. Planta 246:227–241

Tan FX, Shi SH, Zhong Y, Gong X, Wang YG (2002) Phylogenetic relationships of Combretoideae (Combretaceae) inferred from plastid, nuclear gene and spacer sequences. J Plant Res 115:475–481

Thomas MR, Storey RF (2015) The role of platelets in inflammation. Thromb Haemost 114:449–458

Ujowundu CO, Okafor OE, Agha NC, Nwaogu LA, Igwe KO, Igwe CU (2010) Phytochemical and chemical composition of *Combretum zenkeri* leaves. J Med Plant Res 4:965–968

Ujowundu CO, Ogbonna CU, Okwu GN, AIisi CS (2015) Free radicals scavenging and neuroprotective effects of ethanolic leaf extract of *Combretum zenkeri* leaf. Annu Res Rev Biol 6:133–141

Vane JR (1971) Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. Nat New Biol 231:232–235

Waljee AK, Rogers MAM, Lin P, SingalAmit G, Stein JD, Marks RM (2017) Short term use of oral corticosteroids and related harms among adults in the United States: population based cohort study. Br Med J 357:j1415

Wande OM, Babatunde SB (2017) *In vitro* screening of ten Combretaceae plants for antimalarial activities applying the inhibition of beta-hematin formation. Int J Biol Chem Sci 11:2971–2981

Winter CA, Risley EA, Nuss GV (1962) Carrageenin-induced oedema in hind paw of the rat as an assay for anti-inflammatory drugs. Proc Soc Exp Biol Med 111:544–547

Wongrakpanich S, Wongrakpanich A, Melhado K, Rangaswami J (2018) A comprehensive review of non-steroidal anti-inflammatory drug use in the elderly. Aging Dis 9(1): 143–150

Xiao TS (2017) Innate immunity and inflammation. Cell Mol Immunol 14:1–3

Yadav RNS, Agarwala M (2011) Phytochemical analysis of some medicinal plants. J Phytol 3(12):10–14

Yahfoufi N, Alsadi N, Jambi M, Matar C (2018) The immunomodulatory and anti- inflammatory role of polyphenols. Nutrients 10:1618–1632