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Formulated hexane fraction of *Costus afer* leaves balm suppressed xylene induced topical inflammation in rat model

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ABSTRACT

Objective: This study was designed to evaluate the effects of topically applied hexane fraction and aqueous extract of *Costus afer* leaves balm and cream formula on xylene-induced ear edema.
Methods: *In vitro* anti-inflammatory study was carried out using thermally induced protein denaturation and human erythrocyte membrane stabilization assays while *in vivo* anti-inflammatory study was performed using xylene-induced ear edema in rats.
Results: Hexane fraction of *C. afer* leaves significantly inhibited thermally induced protein denaturation and stabilized human erythrocyte membrane against hypotonicity-induced hemolysis when compared with aqueous extract of *C. afer* leaves. Furthermore, *in vivo* study showed that 10% and 20% w/w hexane fraction of *C. afer* leaves balm formula at 360 minutes significantly suppressed xylene-induced ear edema by 82.02% (0.16 ± 0.01 mm) and 96.62% (0.03 ± 0.01 mm), respectively, when compared with 10% and 20% w/w aqueous extract of *C. afer* leaves cream formula with 55.06% (0.40 ± 0.01 mm) and 67.42% (0.29 ± 0.02 mm) inhibitions, respectively.
Conclusion: The findings from this study revealed that hexane fraction of *C. afer* leaves balm formula exhibited higher anti-inflammatory activity against xylene-induced topical inflammation.

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Anti-inflammation; balm; cream; *C. afer*; topical inflammation

Introduction

Inflammation is a multifaceted biological response of body tissues to harmful stimuli including injuries, irritants, damaged cells, and pathogens [1]. The process of inflammation is associated with increased blood flow to the localized site of tissue damage, enhanced permeability of capillaries, migration of macrophages, and neutrophils from capillaries to the interstitial spaces [2]. As more fluids continue to build up in the interstitial spaces, the damaged tissue site begins to swell [3]. This swelling mark is one of the cardinal signs of inflammation, and other signs include redness, joint pain, heat, and loss of tissue function [4].

The conventional drug treatment strategy used in the management of inflammation involves the use of nonsteroidal anti-inflammatory drugs (NSAIDs) and steroidal anti-inflammatory drugs. However, the use of these drugs has been associated with severe side effects including ulceration, hemorrhage, gastric tissue damage, delay in muscle regeneration, and death [5,6]. Furthermore, data from the National Kidney Foundation estimated that 10% of the kidney failures are positively correlated with the abuse of NSAIDs per year [7]. These findings have led to the quest for an alternative source of anti-inflammatory agent from medicinal plants.

Costus afer Ker Gawl. (family of Costaceae) is a plant that is located at river banks and shady or moist forests of tropical areas of West Africa. It is commonly known as monkey sugar cane, gingerlily, or bush cane [8]. *C. afer* is a moderately tall herbaceous monocot, which is unbranched with creeping rhizome [9]. In tropical Africa, *C. afer* is used as an herbal remedy, particularly for the treatment of rheumatoid arthritis, cough, and hepatic disorders

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[10]. Phytochemical analysis of *C. afer* plant showed that it contains diosgenin, dioscin, flavonoid glycoside kaempferol, saponin aferosides, paryphyllin C, and 3-O-α-L-rhamnopyranoside [11]. Data from gas chromatography-mass spectrometry analysis had shown that hexane fraction of *C. afer* leaves contains naphthalene-2,3-dimethyl, naphthalene-1,6-dimethyl, phenol-2,4-bis(1,1-dimethylethyl)-, phytol, 2(4H)-benzo-furanone,5,6,7,7a-tetrahydro-4,4,7a-trimethyl, pentadecanoic acid, hexadecanoic acid methyl ester, n-hexadecanoic acid, linoleic acid, α-linolenic acid, and cis-vaccenic acid [12]. The previous study from our laboratory had shown that fraction of *C. afer* leaves exhibited higher anti-inflammatory activity than the stem fraction [13]. Therefore, this study was designed to evaluate the effects of topically applied *C. afer* leaves balm and cream formula on xylene-induced ear edema in a rat model. This study was carried out with the rationale to identify the most active *C. afer* leaves formula for the treatment of topical inflammation.

Materials and Methods

Collection of plant material

C. afer plant was obtained from a farmland at Irolu, Ikenne Local Government Area, Ogun State, Nigeria. *C. afer* plant was identified and authenticated by Professor O.A. Denton, a crop scientist in the Department of Agronomy and Landscape Design, Babcock University, Ilishan-Remo, Ogun State. A voucher sample of *C. afer* leaves with the number FHI-108001 was deposited at Forestry Herbarium Ibadan, Oyo State, Nigeria.

Plant processing, extraction, and solvent partitioning

C. afer leaves were plucked out of the stem, washed with distilled water, and subsequently oven-dried at 40°C. Dried leaves were pulverized using a mechanical grinder. Fifty grams of ground sample was macerated in 400 ml distilled water and another 50 g ground leaf sample was also macerated in 400 ml 70% methanol in the ratio of 1:8, with intermittent shaking for 48 hours. The aqueous and methanol suspensions were filtered separately using Whatman No.1 filter papers. Filtrates obtained were subsequently concentrated using a rotary evaporator at 30°C (Buchi Rotavapor RE; Switzerland). Aqueous concentrate was completely dried in an oven for 24 hours at 40°C. After drying, aqueous extract from *C. afer* leaves was weighed, stored in McCartney bottle and preserved in a refrigerator until further use. More so, 70% methanol extract was reconstituted in distilled water in the ratio of 1:2, and subsequently, partitioned using n-hexane with the aid of separating funnel and concentrated in a rotary evaporator at 30°C. Hexane concentrate obtained was collected in McCartney bottle and stored at 4°C until further use.

Drugs

Diclofenac sodium and diclomol gel tube were purchased from a pharmacy store in Ilishan-Remo, Ogun State. The diclomol gel composed of diclofenac sodium (1% w/w), linseed oil (3% w/w), methyl salicylate (10% w/w), and menthol (5% w/w).

In vitro anti-inflammatory assays

Anti-denaturation of protein assay

Effects of hexane fraction and aqueous extract of *C. afer* leaves on thermally induced denaturation of protein were studied using a modified method described by Sakat et al. [14]. The reaction mixtures contained 50 µl of varying concentrations of diclofenac sodium (standard) and test samples dissolved in 10% dimethyl sulfoxide (DMSO) while DMSO alone served as a control in test tubes. Bovine serum albumin (450 µl, 5% w/v) was added into the test tubes and subsequently incubated at 37°C for 20 minutes, then heated at 57°C for 3 minutes, and allowed to cool. After cooling the test tubes, 2.5 ml phosphate buffered saline at pH 6.3 was added to each tube and absorbance of these solutions were measured using a double beam UV-Visible spectrophotometer (T80 model PG Instrument, UK) at a wavelength of 660 nm.

Human erythrocyte membrane stabilization assay

Effects of hexane fraction and aqueous extract of *C. afer* leaves against hypotonicity-induced hemolysis of erythrocyte membrane were performed in accordance with the method described by Oyedapo [15]. Human blood (10 ml) was obtained from a subject by venipuncture of the arm vein using 5 ml hypodermal syringe and the blood was immediately transferred into a bottle containing ethylenediaminetetraacetic acid as anti-coagulant. Whole blood was centrifuged for 10 minutes at 3,000 revolutions per minute and supernatant was carefully removed while the packed red blood cells were

washed in freshly prepared 0.9% w/v NaCl. The process of washing and centrifugation was repeated until a clear supernatant was attained. Human red blood cell (10% v/v) was prepared in 0.9% w/v NaCl. The assay mixture was prepared to contain 1 ml sodium phosphate buffer (pH 7.4, 0.15 mol/L), 2 ml 0.36 % w/v NaCl, 0.5 ml stock human erythrocyte suspension (10% v/v) with 0.5 ml diclofenac sodium or test fractions of varying concentrations in test tubes dissolved in 10% DMSO. For the control, distilled water was used in place of NaCl (0.36%, w/v) in order to achieve 100% hemolysis induction. The different test tubes were incubated at 56°C in a water bath (Uniscope, SM801A England) for 30 minutes and then centrifuged at 5,000 rpm. The hemoglobin content in each tube was then estimated using a double beam UV-Visible spectrophotometer (T80 model PG Instrument, UK) at 560 nm.

Herbal balm and cream formula preparation

Balm and cream formula was prepared according to the following procedures:

Herbal balm formula

Hexane fraction was measured at 0.125, 0.250, and 0 mg into separate beakers. This was followed by addition of 50 ml castor oil and 14.17 g dissolved beeswax and subsequently heated at 35°C with constant stirring for 1 minute. Menthol (2.5 ml 10% w/v) was added to mixtures and thoroughly mixed. The resultant mixtures were poured into containers and allowed to cool and solidify to form herbal balms. The herbal balms were covered and preserved in a refrigerator until further use.

Herbal cream formula

Aqueous extract of *C. afer* leaves at 0.125, 0.250, and 0 mg was added into separate beakers. Distilled water (15 ml) was added and stirred to dissolve the extracts. Subsequently, 50 ml castor oil and 14.17 g dissolved beeswax were added to the extracts and heated with constant stirring until uniform mixtures were formed and allowed to cool. On cooling, 2.5 ml 10% w/v menthol was added and thoroughly stirred until the creams were formed. The formulated herbal creams were covered and preserved in a refrigerator until further use.

Animals

Forty-five male albino rats (Wistar strain) weighing between 95 and 180 g were purchased from an inbred colony at the Animal Facility, Babcock University. Animals were allowed to acclimatize for 2 weeks prior to the study, maintained, and cared for following the National Research Council/ National Institute of Health Guide for the Care and use of Laboratory Animals [16]. Institutional ethical approval was obtained with certificate reference as NHREC/17/2013/BUHREC 383/17.

In vivo anti-inflammatory study

Xylene-induced edema on right ears of albino rats

Effects of hexane fraction of *C. afer* leaves balm and aqueous extract of *C. afer* cream on xylene-induced edema on the right ears of albino rats were investigated following the procedure described by Hosseinzadeh et al. [17]. Animals were randomly distributed into nine groups of five rats per group. Right ears of rats in each group were induced with edema using 0.02 ml xylene and topically treated with hexane fraction of *C. afer* leaves balm, aqueous extract of *C. afer* leaves cream, balm base alone, cream base alone, and standard diclomol gel, respectively. Change in the thickness of rat ear was measured for 6 hours after xylene induction of edema using micrometer screw gauge.

Experimental protocol was as follows. Group I: normal rats; Group II: untreated control rats induced with edema using xylene; Group III: rats induced with edema using xylene and treated with 10% w/w balm base only; Group IV: rats induced with edema using xylene and treated with 10% w/w cream base only; Group V: standard rats induced with edema using xylene and treated with 10% w/w diclomol gel; Group VI: rats induced with edema using xylene and treated with 10% w/w hexane fraction of *C. afer* leaves balm; Group VII: rats induced with edema using xylene and treated with 20% w/w hexane fraction of *C. afer* leaves balm; Group VIII: rats induced with edema using xylene and treated with 10% w/w aqueous extract of *C. afer* leaves cream; and Group IX: rats induced with edema using xylene and treated with 20% w/w aqueous extract of *C. afer* leaves cream. Percentage inhibition of ear thickness was calculated using the following formula:

$$\text{Percentage inhibition of ear thickness} = (1 - \frac{v_t}{v_c} \times 100)$$

where v_t is the average ear thickness in treated groups and v_c is the average ear thickness of the control group.

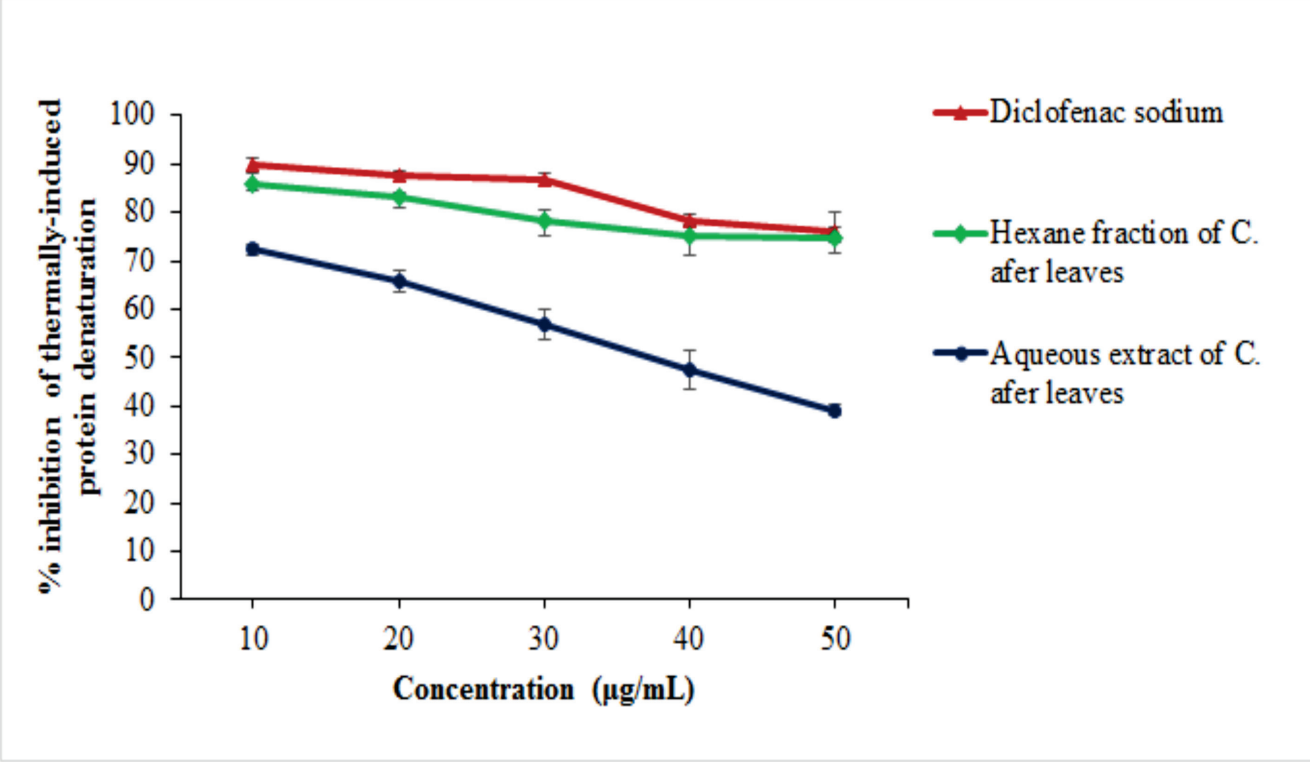

Figure 1. Effects of hexane fraction and aqueous extract of *C. afer* leaves against thermally induced protein denaturation.

Statistical analysis

Statistical analysis was carried out using SPSS for Windows; SPSS Inc., Chicago, standard version 17.0 to determine the difference between mean using one-way analysis of variance. This was followed by *post hoc* analysis using least significance difference analytical test. Non-linear regression was carried out to determine 50% inhibitory concentration (IC_{50}) for hexane fraction and aqueous extract of *C. afer* leaves using GraphPad Prism® 7.00. Graphical presentations were plotted using Microsoft Excel 2013 version and Microsoft Office Suite 2013. $P < 0.05$ and 0.01 were considered significant as applicable. All values were expressed as mean ± SEM of triplicate readings.

Results

Data in Figure 1 show that diclofenac sodium, hexane fraction, and aqueous extract of *C. afer* leaves inhibited thermally induced protein denaturation in a reverse concentration-dependent manner. Hexane fraction of *C. afer* leaves (IC_{50} = 48.77 ± 2.15 µg/ml) exhibited a significantly ($p < 0.01$) high inhibition of protein denaturation when compared with aqueous extract of *C. afer* leaves (IC_{50} = 85.01 ± 3.35 µg/ml) (Table 1). Similarly, Figure 2 shows that diclofenac sodium, hexane fraction, and aqueous extract of *C. afer* leaves stabilized erythrocyte membrane against hypotonicity-induced hemolysis in a concentration-dependent manner. In addition, hexane fraction of *C. afer* leaves (IC_{50} = 1.08 ± 0.02 µg/ml) exhibited a significantly ($p < 0.05$) high stabilization of erythrocyte membrane against hypotonicity-induced hemolysis when compared with aqueous extract of *C. afer* leaves (IC_{50} = 2.80 ± 0.01 µg/ml) (Table 1).

Table 1. Fifty percent inhibitory concentrations of hexane fraction and aqueous extract of *C. afer* leaves against thermally induced protein denaturation and stabilization of erythrocyte membrane against hypotonicity-induced hemolysis.

Test samples	IC_{50} (µg/ml)	
	Anti-protein denaturation	Stabilization of erythrocyte membrane
Diclofenac sodium	37.00 ± 1.45*	0.34 ± 0.02**
Hexane fraction of *C. afer* leaves	48.77 ± 2.15*	1.08 ± 0.02**
Aqueous extract of *C. afer* leaves	85.01 ± 3.35	2.80 ± 0.01

* indicates significantly different at $p < 0.01$; ** indicates significantly different at $p < 0.05$

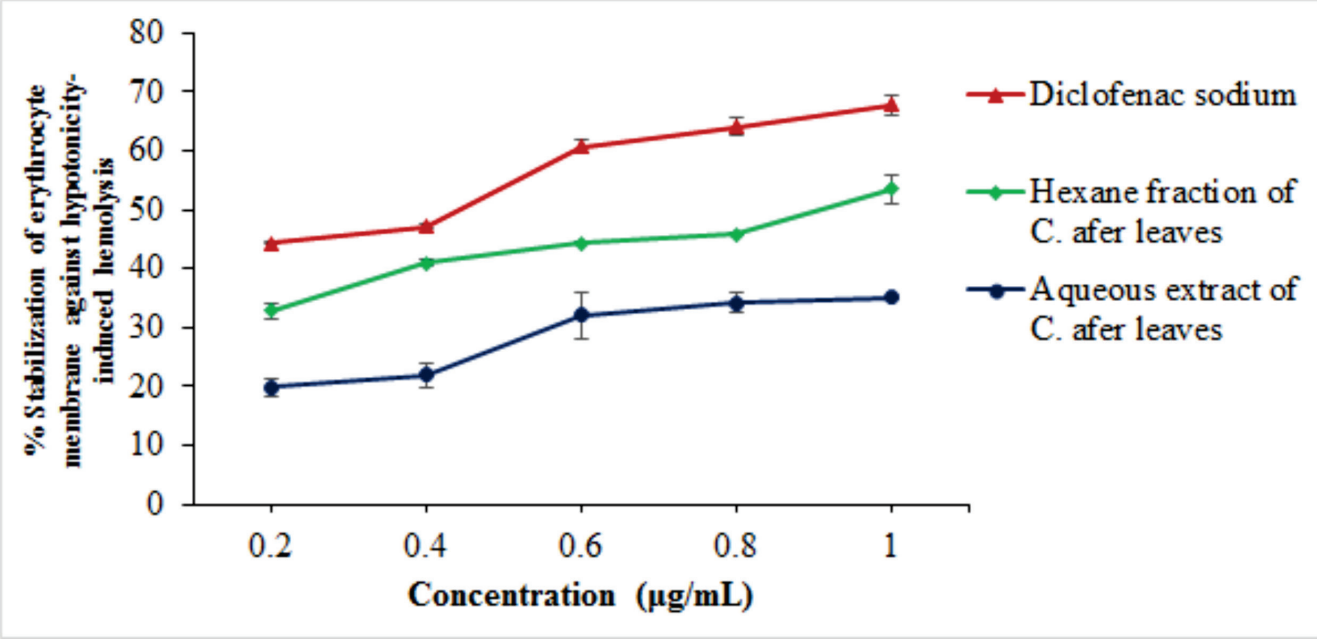

Figure 2. Effects of hexane fraction and aqueous extract of *C. afer* leaves on erythrocyte membrane against hypotonicity-induced hemolysis.

Data in Figure 3 show that the right ears of rats induced with edema using xylene and treated with hexane fraction of *C. afer* balm formula, aqueous extract of *C. afer* cream formula, and diclomol gel had significantly ($p < 0.05$) suppressed ear edema from 10 to 360 minutes in a concentration-dependent manner when compared with untreated control, balm base, and cream base alone treated

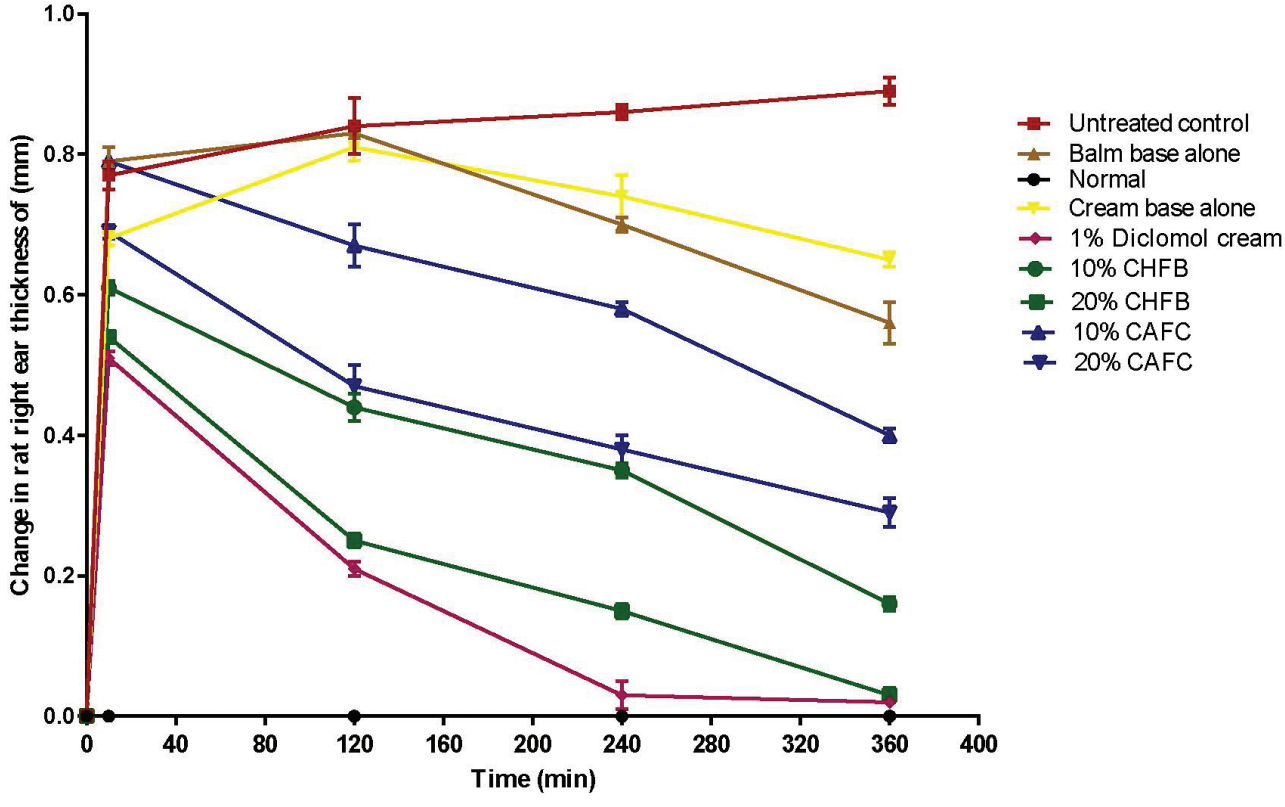

Figure 3. Change in ear edema thickness by hexane fraction of *C. afer* leaves balm and aqueous extract of *C. afer* leaves cream formula on xylene-induced inflammation on the right ear of rats.

groups. At 360 minutes, 10% and 20% w/w hexane fraction of *C. afer* leaves balm formula significantly ($p < 0.05$) suppressed ear edema induced with xylene by 82.02 ± 0.01% and 96.62 ± 0.01% when compared with 55.06 ± 0.01% and 67.42 ± 0.02% of 10% and 20% aqueous extract of *C. afer* leaves cream formula, respectively. Ten percent balm base and cream base suppressed ear edema by 37.08 ± 0.03% and 26.97 ± 0.01%, respectively. Furthermore, at 360 minutes, there was no significant difference ($p > 0.05$) between 20% hexane fraction of *C afer* balm formula and 10% diclomol gel (97.75 ± 0.00%).

Discussion

Drug discovery and development studies have used different medicinal plant products as potential sources of therapy against inflammation *in vitro* and *in vivo*. In this study, hexane fraction of *C. afer* leaves significantly inhibited thermally induced protein denaturation when compared with aqueous extract of *C. afer* leaves. This suggests that hexane fraction of *C. afer* leaves could have anti-inflammatory activity. Protein denaturation has been well associated with inflammatory response event in different types of arthritis [18]. It involves an alteration in the electrostatic, disulfide, hydrophobic, and hydrogen bonds of protein tertiary structures [19]. Hence, compounds capable of preventing alterations in protein tertiary structure during denaturation could serve as an anti-inflammatory agent. More so, the previous study had shown that conventional anti-inflammatory agents possess the capacity to inhibit thermally induced protein denaturation [19]. In this study, the inhibition of thermally induced protein denaturation by the hexane fraction of *C. afer* leaves was in a reverse concentration-dependent manner. This trend is in agreement with the report of Williams et al. that plant extracts with anti-inflammatory activity tend to inhibit thermally induced protein denaturation at lower concentrations [20].

Furthermore, hexane fraction of *C. afer* leaves exhibited a higher membrane stabilization effect against hypotonicity-induced hemolysis of erythrocyte membrane when compared with aqueous extract of *C. afer* leaves. This observation strengthens the previous notion in this study that hexane fraction of *C. afer* leaves could possess anti-inflammatory activity. The previous study had shown that the composition of erythrocyte membrane is similar to lysosomal membrane, hence the adoption of erythrocyte membrane in this study [15]. In the body system, the stabilization of lysosomal membrane by anti-inflammatory agents plays an important role as a limiting step in the cascade of pro-inflammatory mediators.Further study on the effects of hexane fraction of *C. afer* leaves balm formula and aqueous extract cream formula on xylene-induced ear edema in rats for 6 hours showed that 10% and 20% w/w hexane fraction of *C. afer* leaves balm formula significantly suppressed ear edema when compared with aqueous extract of *C. afer* leaves cream formula. This observation was found to be comparable to 10% diclomol gel which indicated that the hexane fraction of *C. afer* balm formula substantially exhibited anti-inflammatory activity topically. Similar topical anti-inflammatory effect has been reported for a herbal gel containing *Trigonella foenum* seed extract [21].

Previous study had shown that damage to tissues triggers an early inflammatory phase mediated by substance P and kinin-like substances and tissue mediated responses including histamine, five hydroxytryptamine, and increased prostaglandin synthesis at the localized site of tissue injury within 2 hours. The later inflammatory phase involving bradykinins, leukotrienes, polymorphonuclear cells, and prostaglandins occurs within 3 hours [22].

Most conventional anti-inflammatory drugs including diclomol gel suppress inflammatory responses at the later phase of inflammation [23]. In this study, hexane fraction of *C. afer* balm formula suppressed xylene-induced ear thickness from 10 to 360 minutes which suggested that the anti-inflammatory activity of hexane fraction of *C. afer* balm formula could be through modulation of arachidonic acid metabolism. Thus, data from this study demonstrated that topically applied hexane fraction of *C. afer* leaves balm formula on rat ear induced with edema using xylene exhibited higher anti-inflammatory activity than the other test formula. In addition, these findings provided an insight into the possible mechanism of action of *C. afer* leaves used as therapy against topical inflammation by traditional medicine practitioners.

Conflict of interest

The authors declare no known conflict of interest associated with this work.

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