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ORIGINAL RESEARCH

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Anti-inflammatory properties and gas chromatography-mass spectrometry analysis of ethyl acetate fraction of *Crateva adansonii* DC leaves

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ABSTRACT

Aim: This study was designed to determine the most active anti-inflammatory fraction of *Crateva adansonii* leaves and characterize the bioactive compounds.

Methods: Dried and pulverized *C. adansonii* leaves (CAL) were extracted with 70% methanol, followed by successive solvent partitioning into hexane, ethyl acetate (CALE), butanol, and aqueous leaf fractions. *In vitro* antioxidant assays carried out on the extract/fractions were 1,1-diphenyl 2-picryl hydrazyl (DPPH), hydrogen peroxide, ferric reducing antioxidant power (FRAP), nitric oxide scavenging, total antioxidant capacity (TAC), total phenol, and flavonoid (TF) assays. *In vitro* anti-inflammatory assays investigated were heat-induced bovine serum albumin (BSA) denaturation and human red blood cell (HRBC) membrane stabilization against hypotonicity-induced hemolysis. Formaldehyde-induced inflammation model in rats was carried out, followed by paw edema measurement, as well as serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities assays. The CALE fraction was subjected to gas chromatography-mass spectrophotometry (GC-MS) analytical method.

Results: CAL extract and fractions substantially inhibited BSA denaturation and stabilized HRBC membrane against hypotonicity-induced hemolysis; however, CALE exhibited the highest activity. CALE also suppressed formaldehyde-induced rat paw edema and significantly ($p < 0.001$) reduced serum ALT and AST activities. In addition, CALE demonstrated significantly high ($p < 0.05$) antioxidant activities in DPPH and nitric oxide scavenging activities when compared with the other test samples. CALE also exhibited an appreciable amount in FRAP, TAC, and TF. GC-MS analysis of CALE indicated the presence of 19 tentatively identified compounds with anti-inflammatory and/or antioxidant properties.

Conclusion: Ethyl acetate fraction of *C. adansonii* leaves exhibited anti-inflammatory activities which could be through antioxidant effect and perhaps through suppression of pro-inflammatory mediators. Hence, ethyl acetate fraction of *C. adansonii* leaf could serve as an important source of a lead drug candidate for pharmaceutical drug development in the management of inflammation.

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Introduction

Inflammation is the immune system protective response triggered by body tissues against infection, autoimmune injury, trauma, and harmful agents. However, chronic inflammation could result in pathological immune cell infiltration to the localized site of tissue damage mainly through the excessive production of free radicals and pro-inflammatory mediators [1]. Several chronic disorders are associated with inflammation, marked by excessive reactive oxygen species signals [2]. Inflammatory disorders such as rheumatoid

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arthritis is an autoimmune disorder that mainly affects skeletal joints and characteristically results in heat, redness, edema, and pains [3]. Pathogenesis of rheumatoid arthritis is thought to encompass genetic and environmental factors [4,5]. The major goals for the treatment of inflammatory disorders include pain suppression, decreasing inflammation, and improving the functioning of body systems using steroids, non-steroidal anti-inflammatory drugs, and disease-modifying anti-rheumatic drugs to retard the progression of disease [5,6].

However, the search for anti-inflammatory agents of plant origin used for the treatment and prevention of numerous diseases, particularly those induced by reactive oxygen species (ROS), is on the increase. ROS play an important role that is related to the degenerative processes of various diseases, including aging, cancer, coronary heart disease, neurodegenerative disorders, and inflammation [7]. In many inflammatory disorders, there is elevated activation of phagocytes with increased generation of superoxide anion, hydroxyl radicals, and hydrogen peroxide leading to oxidative damage to surrounding tissues [7].

Herbal remedies used in the management of several inflammatory disorders are being evaluated by several researchers as potential sources of valuable drugs through isolation and characterization of active components of these plants. This could be due to the perception that herbal medications are effective with minimal side effects [8]. Several medicinal plants have been used in ethnomedical practice as a therapeutic agent to suppress and prevent inflammation with very little scientific understanding [8,9].

Crateva adansonii DC of the family of Capparidaceae is commonly called sacred garlic pea or temple plant. *Crateva adansonii* is widespread in tropical regions of the world, including Japan, Australia, and the South Pacific Islands. *Crateva adansonii* tree has three foliated leaves and grows up to 3–10 m in height. The flowers are white, while fruits are spherical in shape and edible with a size of about 3.5–5 mm [10].

The plant extract has been ethnomedically claimed to cure fever, headache, swellings, gastrointestinal complaints, rheumatoid arthritis, sterility, jaundice, and yellow fever among others [11]. The plant has also been shown to inhibit the growths of pathogenic fungi and bacterial species. A similar plant *C. religiosa* has been shown to express antimutagenic activity and immune suppressing activity [12,13].

Despite the pharmacological reports and ethnomedical uses of the *C. adansonii* plant, scientific information with regards to its anti-inflammatory activity appears scanty in the scientific literature. Therefore, this study was designed to evaluate the anti-inflammatory property, as well as bioactive compound characterization of active *C. adansonii* leaves with the aim of proffering scientific validation for its ethnomedical use in the management of inflammatory diseases.

Materials and Methods

Collection of plant material

Crateva adansonii plant was obtained from a garden in Ilishan-Remo, Ogun State, Nigeria. It was authenticated and assigned a voucher number LUH; 6988 at the Herbarium of Botany Department, University of Lagos.

Animals

Thirty-six male albino rats (Wistar strain) weighing 180–200 g were purchased from the Animal Facility, Babcock University. Acclimatization of the animals was allowed for 14 days before the commencement of study within the Animal Facility. All animals used in this study were maintained and well cared in accordance with the National Institute of Health Animal Care Guidelines (NRC 2011). Ethical consent was obtained from the Babcock University Health Research and Ethics Committee with the certificate number NHREC/17/12/2013 BUHREC436/17.

Plant processing and extraction

Crateva adansonii leaves (CAL) were separated from the whole plant and air-dried under shade. Dried leaf samples were ground into a powder using an electric blender and stored in well-stoppered bottles. Pulverized leaf sample (100 g) was soaked in 70% methanol with discontinuous shaking over 8 hours at room temperature. Subsequently, it was filtered using Whatman No. 1 filter paper. This procedure was done twice on the residue and the filtrates obtained were pulled together and concentrated under reduced pressure at 45°C using a rotary evaporator (Buchi Rotavapor RE, Switzerland). The concentrate was dried completely by gentle aeration from a pump and the residue was kept in a freezer until further use. Part of the CAL residue was reconstituted in

distilled water and subjected to successive solvent partitioning to obtain various fractions of increasing polarity in the following order: n-hexane (CALH), ethyl acetate (CALE), butanol (CALB) while the leftover suspension was considered as the aqueous fraction (CALA). The crude methanol extract (CALM) and partitioned fractions were subjected to anti-inflammatory and antioxidant assays.

In vitro anti-inflammatory studies

Protein denaturation assay

The effects of CAL extract and fractions on heat-induced bovine serum albumin (BSA) denaturation were investigated using a modified method of Williams et al. [14]. The reaction mixtures containing 0.5 ml of 1.5 mg/ml BSA and varying concentrations of extract, fractions, or diclofenac standard were incubated at 37°C for 20 minutes. The incubated reaction mixtures were then heated at 57°C for 3 minutes. This was immediately followed by the addition of 2.5 ml of 0.5 M phosphate buffer, pH 6.3. Subsequently, 1 ml of the reaction mixture was transferred into test tubes with the addition of 1 ml copper-alkaline reagent and 1 ml of 1% Folin–Ciocalteu's reagent was added and incubated at 55°C for 10 minutes. After incubation, the tubes were allowed to cool and absorbance was read at 650 nm against the reagent blank. Assays were carried out in triplicate. The quantity of protein left (QP) and the percentage inhibition of denaturation were calculated as follows:

$$(\mathrm{QP}) = \frac{\text{Absorbance of sample} - \text{Absorbance of blank}}{\text{Absorbance of standard} - \text{Absorbance of blank}}$$

$$\text{Percentage inhibition of denaturation} = \frac{\mathrm{QP}}{\text{Total protein}} \times 100$$

$$\text{Total protein}\,(\mathrm{TP}) = \frac{\text{Absorbance of sample} \times \text{dilution factor} \times \text{BSA concentration}}{\text{BSA concentration}}$$

Human red blood cell membrane stabilization assay

The stabilization effects of CAL extract/fractions on hypotonicity-induced hemolysis of human red blood cells (HRBC) were investigated, following the procedure described by Oyedapo et al. [15]. Erythrocyte suspension was prepared by collecting blood samples from the arm vein with a 5 ml hypodermal syringe and transferred into an ethylenediamine tetra-acetate bottle to avoid clotting. The blood was immediately centrifuged at 3,000 g for 10 minutes and the plasma layer removed. Subsequently, the erythrocytes were washed in fresh 0.85% w/v NaCl and centrifuged successively until the supernatant layer became clear. The washed HRBC was suspended in 0.85% NaCl to yield 10% suspension. The reaction mixture containing 1 ml phosphate buffer (pH 7.4, 0.15 M), 2 ml 0.36% NaCl, 0.5 ml HRBC suspension (10% v/v), and 0.5 ml of CAL extract/fractions or diclofenac sodium of varying concentrations was incubated at 37°C for 30 minutes and then centrifuged. The hemoglobin content in the supernatant was determined at 560 nm. The percentage hemolysis in the presence of distilled water was assigned 100%. This study was carried out in triplicates. Percentage stabilization of HRBC membrane was calculated using the formula stated below.

$$\text{Percentage stabilization} = 100 - \left[\frac{\text{Optical density of test solution}}{\text{Optical density of control}}\right] \times 100$$

Animal study

Formaldehyde-induced arthritis assay

Effect of ethyl acetate fraction of *C. adansonii* leaf extract on formaldehyde-induced arthritic rats was investigated using a modified method described by Choi and Hwang [16]. The fraction (100–300 mg/kg) and diclofenac sodium (10 mg/kg) were administered orally to different groups of rats 30 minutes before induction of arthritis using formaldehyde. Formaldehyde (0.2 ml of 2% v/v) was injected on the days 1 and 3 into the left hind paw of rats beneath the *plantar aponeurosis*. The experimental design involved six groups of six rats each as presented below:

- Normal group: rats were administered with 1 ml carboxymethyl cellulose (CMC)
- Control group: rats were administered with 1 ml CMC + induction with arthritis
- Standard group: rats were administered with 10 mg/kg diclofenac sodium using CMC as a vehicle + induction with arthritis
- Test group 1: rats were administered with 100 mg/kg b.w. CALE using CMC as vehicle + induction with arthritis.
- Test group 2: rats were administered with 200 mg/kg b.w. CALE using

CMC as vehicle + induction with arthritis.

Test group 3: rats were administered with 300 mg/kg b.w. CALE using CMC as vehicle + induction with arthritis.

The different doses of CALE and diclofenac sodium were orally administered to rats daily for 7 days. Rat paw thickness was measured using a micrometer screw gauge, before induction of rats with arthritis and at 30 minutes, 1, 2, 3, 4, and 6 hours after the induction. The percentage inhibition of the increase in paw thickness was estimated as follows.

$$\text{Percentage inhibition} = \left(1 - \frac{P_t}{P_c}\right) \times 100$$

P_t is the mean paw thickness of rats in treated groups

P_c is the mean paw thickness of rats in the control group.

Determination of aspartate aminotransferase and alanine aminotransferase activities

The effects of CALE on serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were investigated with the aid of Randox kits. Twenty-four hours after the end of the 7 days administration of the leaf fraction and standard diclofenac, the rats were anesthetized with chloroform and blood samples were obtained through cardiac puncture and the blood transferred into plain bottles. The obtained blood samples were immediately centrifuged at 3,000 rpm for 10 minutes to obtain serum for the analysis of AST and ALT activities.

In vitro antioxidant studies

DPPH (1,1-diphenyl 2-picrylhydrazyl) scavenging activity assay

DPPH scavenging activities of *C. adansonii* extract/fractions were carried out following the method described by Brand-Williams et al. [17]. Varying concentrations of 1 ml CAL extract/fractions or standard ascorbic acid were added to 1 ml of 0.3 mM DPPH in methanol in test tubes. The reaction mixture was mixed and incubated for 30 minutes in the dark. Subsequently, the absorbance was read at 517 nm against a control containing 1 ml methanol.

Percentage inhibition ($I\%$) was calculated using the equation below:

$$I\% = \left[\frac{A\text{blank} - A\text{sample}}{A\text{blank}}\right] \times 100$$

Where A_{blank} = absorbance of the control

A_{sample} = absorbance of the test or standard reaction mixture.

Fifty percent inhibitory concentration (IC_{50}) was determined from the graph plot of percentage inhibition against concentrations.

Nitric oxide radical scavenging activity assay

The effects of *C. adansonii* leaves extract/fractions on nitric oxide radical were determined by using a modified method of Green et al. [18]. The reaction mixture that containing 0.1 ml of 10, 5, 2.5, 1.25, 0.625, and 0.3125 mg/ml of *C. adansonii* leaves extract/fractions or Gallic acid (standard) and 0.9 ml of 2.5 mM sodium nitroprusside in phosphate buffer saline was incubated under illumination for 150 minutes. Following the light incubation, 0.5 ml of 1% sulphanilamide in 5% phosphoric acid was added into the reaction mixtures and incubated for 10 minutes in the dark. Immediately, 0.5 ml of 0.1% N-1-naphthyl ethylenediamine dihydrochloride was added and the absorbance of the chromophores formed was determined at 546 nm. The percentage inhibition of nitric oxide radical was calculated using the formula below:

$$I\% = \left[\frac{A\text{blank} - A\text{sample}}{A\text{blank}}\right] \times 100$$

Where A_{blank} = absorbance of the control

A_{sample} = absorbance of the test or standard reaction mixture.

Half-maximal inhibitory concentration (IC_{50}) was determined from the graph plot of percentage inhibition against concentrations.

Hydrogen peroxide scavenging activity

The capacity of *C. adansonii* leaf extract/fractions to scavenge hydrogen peroxide (H_2O_2) was determined following the method described by Kumaran and Karunakaran [19]. The reaction mixture contains aliquots (0.1 ml) of different test fractions of *C. adansonii* leaf, 0.4 ml 50 mM phosphate buffer (pH 7.4) and 0.6 ml hydrogen peroxide solution (2 mM in 50 mM phosphate buffer, pH 7.4) in different test tubes, which were mixed and allowed to incubate for 10 minutes. Immediately after incubation, absorbance was read at 230 nm against a blank. The standard ascorbic acid at varying concentrations

(0.05–0.25 mg/ml) was separately mixed with 0.6 ml of 4 mM H_2O_2 solution prepared in 50 mM phosphate buffer solution. The H_2O_2 scavenging capacity was calculated using the following equation.

$$H_2O_2 \text{scavenging capacity} = \left[\frac{A\text{blank} - A\text{sample}}{A\text{blank}} \right] \times 100$$

Where A_{blank} = absorbance of the control

A_{sample} = absorbance of the test or standard reaction mixture.

Half-maximal inhibitory concentration (IC_{50}) was determined from the graph plot of percentage inhibition against concentrations.

Determination of total antioxidant capacity

The total antioxidant capacities of CAL extract/fractions were determined using a phosphomolybdate assay as described by Prieto et al. [20]. The reaction mixture containing 0.1 ml of *C. adansonii* leaf extract/fractions or ascorbic acid (standard) and 1 ml phosphomolybdate reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) was incubated in a water bath at 95°C for 90 minutes. Subsequently, the reaction mixture was allowed to cool at room temperature and the absorbance was read at 695 nm against blank. The total antioxidant activities of the extract/fractions were expressed as ascorbic acid equivalent (AAE) after extrapolation of values from the ascorbic acid standard curve.

Ferric reducing antioxidant power assay

Ferric reducing antioxidant power (FRAP) assay was carried out to evaluate the antioxidant power of CAL extract/fractions according to the spectrophotometric method described by Hemalatha and Kumar [21]. A reaction mixture of 50 µl of 0.1 mg/ml *C. adansonii* leaves extract/fractions or 50 µl of ascorbic acid (standard) and 1 ml of FRAP reagent [300 mmol/l acetate buffer, pH 3.6, 10 mmol/l 2, 4, 6-tri-(2-pyridyl)-1, 3, 5-triazine, and 20 mmol/l $FeCl_3.6H_2O$ mixed in the ratio of 10:1:1, respectively] was well mixed and incubated for 10 minutes in the dark. Immediately after, the reaction mixture absorbance was read at 593 nm against a reagent blank (50 µl of distilled water). The FRAP values were expressed as ascorbic acid equivalent extrapolated from the ascorbic acid standard curve.

Determination of total phenol content

Total phenol content in CAL extract/fractions were determined according to the method described by Zovko et al. [22]. The reaction mixture containing 0.1 ml of *C. adansonii* leaves extract/fractions or Gallic acid (standard), 0.9 ml of water, and 0.2 ml of Folin-Ciocalteu's phenol reagent was mixed with a vortex mixer. After 5 minutes of standing, 1.0 ml of 7% Na_2CO_3 solution was added and the solution was made up to 2.5 ml with distilled water and incubated for 90 minutes at room temperature. Immediately after, the absorbance against a negative control containing 1 ml of distilled water was read at 750 nm.

Determination of Total flavonoid content

Total flavonoid content (TFC) in CAL extract/fractions was determined using the aluminum chloride colorimetric method as described by Zhishen et al. [23]. The reaction mixture containing 0.1 ml extract/fraction or quercetin (standard), 0.4 ml distilled water, and 0.1 ml 5% sodium nitrite was mixed and allowed to stand for 5 minutes. Subsequently, 0.1 ml of 10% aluminum chloride and 0.2 ml NaOH were added to the reaction mixture and made up to 2.5 ml with distilled water. Immediately, the absorbance was read at 510 nm against the blank. The TFCs of the *C. adansonii* leaves extract/fractions, which were expressed as mg quercetin equivalents (QEs) per gram of the plant extract, were calculated as follows:

$$\text{TFC} = q * \frac{v}{w}$$

TFC = total flavonoid content

q = Concentration of quercetin established from the standard quercetin curve

v = volume of extract/fraction (ml)

w = weight of the extract obtained.

Gas chromatography-mass spectrophotometry analysis

Gas chromatography-mass spectrophotometry (GC-MS) analysis was performed at the Department of Chemistry, University of Lagos, Akoka. The GC-MS was operated using GC-MS Agilent Technologies model with serials of 7890A GC-MS. It has a detector (MSD = 5975C) and injector (7683B series). Initial and final temperatures were 100°C for 2 minutes and 270°C at the rate of 10°C/minute, respectively. One microliter of 0.2 g/ml of *C. adansonii* leaves ethyl acetate fraction was injected. Temperature of heater was set at 250°C, pressure was set at 3.2652 psi, mode type splitless, column type (HP5MS: 30 M × 320 µM × 0.25 µM), and carrier gas (helium, 99.99% purity, flow rate = 1.4963 ml/minute;

average velocity = 45.618 cm/second). The bioactive compounds were tentatively identified by comparing the retention times and mass spectra of the authentic samples obtained by GC-MS spectra from the National Institute of Standards and Technology Version 2.0 MS database library.

Statistical analysis

Data were reported as the mean ± standard error of the mean (SEM). Statistical significance between different groups was analyzed using the one-way analysis of variance and least significant difference test as post hoc analysis using SPSS for Windows; SPSS Inc., Chicago, standard version 17.0. Fifty percent inhibitory concentration (IC_{50}) for test extract/fractions was determined from nonlinear regression using GraphPad Prism® 7.00. Graphical illustrations were plotted using Windows, Microsoft Excel® 2011 version.

Results

In vitro anti-inflammatory assays

Data in Figure 1 and Table 1 showed that CALM and its fractions, as well as diclofenac, strongly inhibited heat-induced BSA denaturation in a concentration-dependent manner. CALE exhibited the highest inhibitory activity (IC_{50} = 0.04 ± 0.00 mg/ml) against heat-induced BSA denaturation, higher than that of the standard diclofenac (IC_{50} = 0.12 ± 0.03 mg/ml), while CALH showed the lowest activity (IC_{50} = 0.14 ± 0.08 mg/ml).

Data in Figure 2 and Table 1 showed that CALM and most of its fractions, as well as diclofenac strongly inhibited the hemolysis of human erythrocytes by hypotonic solution in a concentration-dependent manner, with CALE (IC_{50} = 0.15 ± 0.00 mg/ml) showing the highest stabilizing effect on the erythrocyte membrane, while CALH (IC_{50} = 1.00 ± 0.11 mg/ml) had the lowest stabilizing effect.

In vitro antioxidant assays

Data in Figure 3 and Table 2 showed CALM and its fractions; all scavenged DPPH in a concentration-dependent manner. CALE exhibited the highest DPPH scavenging activity with IC_{50} = 0.32 ± 0.01 mg/ml, while the aqueous fraction had the lowest DPPH scavenging activity with IC_{50} = 1.46 ± 0.04 mg/ml. Nitric oxide scavenging activity was highest for CALE and lowest for CALA. This Table also showed the hydrogen peroxide scavenging activity of CALM and its fractions. CALB demonstrated the highest hydrogen peroxide scavenging activity (IC_{50} = 0.11 ± 0.00 mg/ml), while CALA had the least activity (IC_{50} = 0.30 ± 0.00 mg/ml).

Data in Table 3 showed that CALM and its fraction exhibiting varying degree of antioxidant activity, with CALB displaying a significantly ($p < 0.05$) high FRAP (7.6 ± 0.20 mg AAE/g); total antioxidant

Table 1. Fifty percent inhibitory concentration (IC_{50}) of *C. adansonii* leaf extract/fractions on BSA denaturation.

Test samples	IC_{50} (mg/ml)	
	BSA denaturation assay	Stabilization of HRBC assay
Methanol leaf extract (CALM)	0.12 ± 0.01^{bc}	0.19 ± 0.01^{f}
Hexane leaf fraction (CALH)	0.14 ± 0.08^{d}	1.00 ± 0.11^{i}
Ethyl acetate leaf fraction (CALE)	0.04 ± 0.00^{a}	0.15 ± 0.00^{e}
Butanol leaf fraction (CALB)	0.12 ± 0.04^{b}	0.35 ± 0.07^{g}
Aqueous leaf fraction (CALA)	0.09 ± 0.01^{b}	0.32 ± 0.01^{g}
Diclofenac sodium	0.12 ± 0.03^{b}	0.83 ± 0.0^{2}h

Different superscript letter across column indicates significantly different at $p < 0.05$.

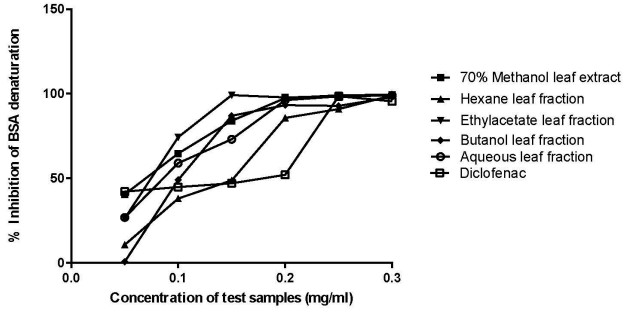

Figure 1. Effect of *C. adansonii* leaf methanol extract and fractions on BSA denaturation.

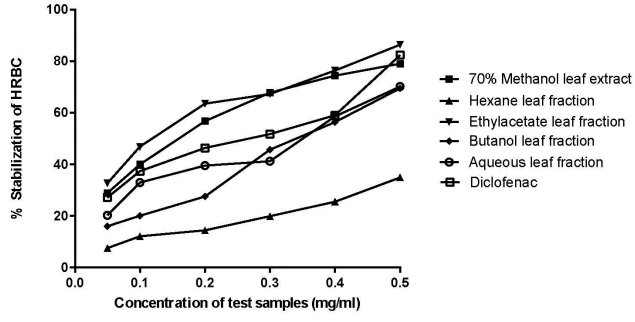

Figure 2. Effect of *C. adansonii* leaf methanol extract and its fractions on hypotonicity-induced HRBC.

capacity (TAC) (44.19 ± 0.54 mg AAE/g), TFC (17.27 ± 1.29 mg QE/g), and total phenol (TP) content [12.31 ± 0.90 mg gallic acid equivalent (GAE)/g], while CALH showed the significantly ($p < 0.05$) low FRAP (0.10 ± 0.06 mg AAE/g), TAC (3.17 ± 0.03 mg AAE/g), TF (0.66 ± 0.17 mg QE/g), and TP (0.09 ± 0.02 mg GAE/g). More so, the antioxidant activity of CALB was followed by CALE exhibiting FRAP (4.50 ± 0.10 mg AAE/g), TAC (18.10 ± 2.60 mg AAE/g), and TF (9.28 ± 0.42 mg QE/g).

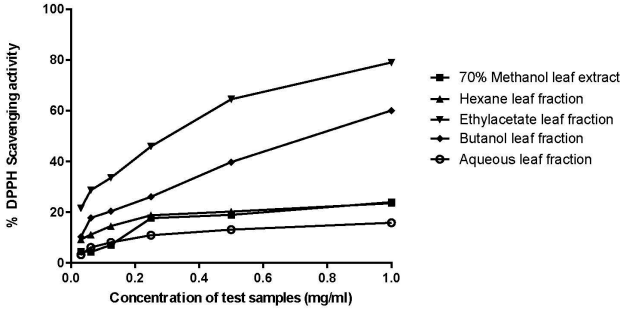

Figure 3. DPPH scavenging activities of *C. adansonii* leaf methanol extract and its fractions.

Table 2. Fifty percent inhibitory concentrations (IC_{50}) of *C. adansonii* leaf methanol extract and fractions for different antioxidant activities.

	IC_{50} (mg/ml)		
Test samples	**DPPH scavenging activity**	**Nitric oxide scavenging activity**	**H_2O_2 scavenging activity**
Methanol extract	0.78 ± 0.01^{c}	0.66 ± 0.01^{h}	0.18 ± 0.01^{m}
Hexane fraction	0.98 ± 0.01^{d}	-------	0.14 ± 0.00^{l}
Ethyl acetate fraction	0.32 ± 0.01^{b}	0.25 ± 0.00^{f}	0.22 ± 0.01^{n}
Butanol fraction	0.77 ± 0.04^{c}	-------	0.11 ± 0.00^{j}
Aqueous fraction	1.46 ± 0.04^{e}	1.13 ± 0.02^{i}	0.30 ± 0.00^{o}
Ascorbic acid	0.08 ± 0.03^{a}	-------	0.12 ± 0.00^{k}
Gallic acid	-------	0.38 ± 0.04^{g}	-------

Different superscript letter across column indicates significantly different at $p < 0.05$.

In vivo anti-inflammatory assays

Data in Figure 4 showed that rat paw edema, as measured by the paw thickness, increased dramatically in the control untreated rats and was still increasing for 6 hours after the induction of arthritis using 2% v/v formaldehyde. The arthritic rats treated with varying doses of CALE and standard diclofenac sodium also initially had an increase in paw edema but this began to decrease after 4 hours of arthritis induction. By 6 hours, the paw thickness in animals treated with 100, 200, and 300 mg/kg b.w. CALE and 10 mg/kg diclofenac sodium had decreased by 16.5%, 9.79%, 27.02%, and 51%, respectively.

Data in Figure 5 showed that serum AST and ALT values were low in normal, non-arthritic rats. Induction with arthritis raised the values tremendously (7–8 folds). However, administration of 100–300 mg/kg of CALE to the arthritic rats appreciably decreased the serum AST and ALT in a dose-dependent manner. 10 mg/kg b.w. diclofenac sodium treatment also led to a reduction in the serum AST (19.0 ± 0.00 U/l) and ALT (30.0 ± 0.01 U/l) with values comparable to AST (19.0 ± 0.00 U/l) and ALT (29.5 ± 0.01 U/l) of rats treated with 300 mg/kg b.w. CALE.

GC-MS analysis

Findings in Figure 6 and Table 4 showed that the GC-MS analysis of CALE revealed the presence of 19 bioactive compounds, which have been previously reported to have anti-inflammatory and/or antioxidant activity. Most of these compounds are long chain fatty acids (saturated and unsaturated) and their esters.

Discussion

The adverse side effects of non-steroidal and steroidal anti-inflammatory drugs have made the search for an alternative source of the drug from medicinal plants a paramount interest to drug

Table 3. Ferric reducing antioxidant power (FRAP), TAC, TFC, and total phenol content of *C. adansonii* leaf methanol extract and fractions.

Test samples	**FRAP (mg AAE/g)**	**TAC (mg AAE/g)**	**TFC (mg QE/g)**	**Total phenol content (mg GAE/g)**
Methanol extract (CALM)	4.38 ± 0.10^{b}	12.19 ± 0.50^{f}	8.97 ± 1.19^{j}	4.96 ± 0.02^{p}
Hexane fraction (CALH)	0.10 ± 0.06^{a}	3.17 ± 0.03^{e}	0.66 ± 0.17^{i}	0.09 ± 0.02^{m}
Ethyl acetate fraction (CALE)	4.50 ± 0.10^{b}	18.10 ± 2.60^{g}	9.28 ± 0.42^{Jk}	3.2 ± 0.02^{n}
Butanol fraction (CALB)	7.60 ± 0.20^{d}	44.19 ± 0.54^{h}	17.27 ± 1.29^{l}	12.31 ± 0.90^{q}
Aqueous fraction(CALA)	4.68 ± 0.10^{bc}	10.03 ± 0.10^{f}	8.20 ± 0.48^{j}	4.54 ± 0.14^{o}

Different superscript letter across column indicates significantly different at $p < 0.05$.

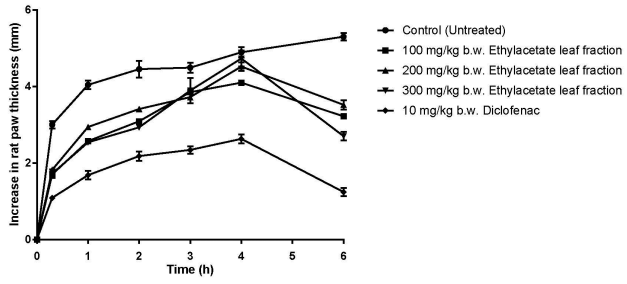

Figure 4. Effect of varying doses ethyl acetate fraction of *C. adansonii* leaves on paw thickness of rats induced with arthritis.

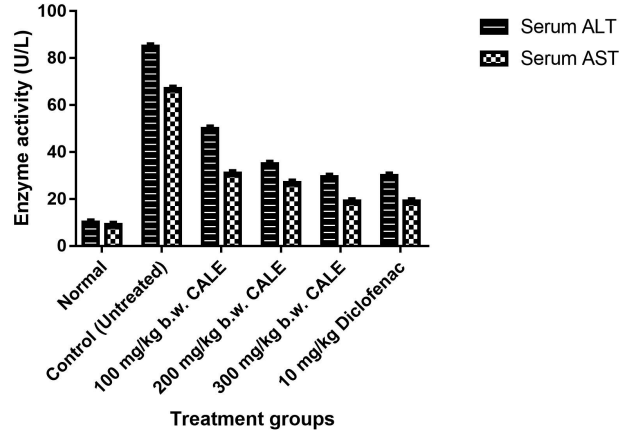

Figure 5. Effect of ethyl acetate fractions of *C. adansonii* leaves on serum ALT and AST of rats induced with arthritis.

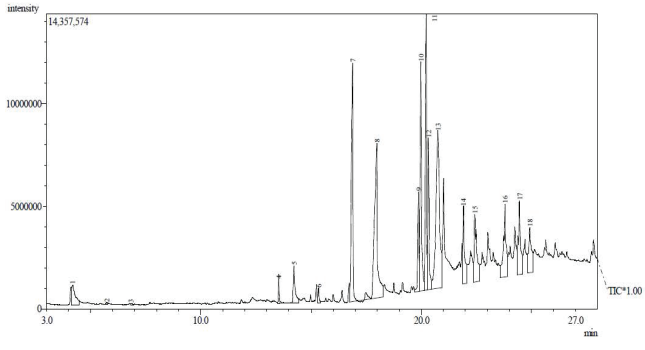

Figure 6. Chromatogram of ethyl acetate fraction of *C. adansonii* leaves.

discovery scientists. In this present study, *C. adansonii* leaf extract and fractions were evaluated to determine which test sample possesses the most active anti-inflammatory activity. *In vitro* anti-inflammatory study indicated that CALE had the most effective inhibitory action against heat-induced protein denaturation when compared with the other test samples. In addition, CALE exhibited the most effective stabilization action against hypotonicity-induced HRBC hemolysis when compared with the other test samples. This suggested that CALE could possess anti-inflammatory property. Previous studies reported that protein denaturation is a common occurrence during inflammation episodes [14,32]. Protein denaturation may involve an alteration in amino acids electrostatic, hydrogen, hydrophobic, and disulfide bondings. This indicates that drugs possessing the capacity to prevent these structural and chemical changes in protein could serve as a potential anti-inflammatory agent. In addition, many anti-inflammatory drugs have been demonstrated to stabilize heat-induced denaturation of the protein [33]. Furthermore, HRBC membrane stabilization has been strongly linked with anti-inflammatory activity in many plant extracts [34–36].

GC-MS analysis identified the bioactive compounds in CALE previously reported to possess anti-inflammatory activity as 5-methoxymethy1-[1,3,4] thiadiazole-2-ylamine, thiazoline-4-one, 5-ethyl-2-imino, hexadecanoic acid, 15-methyl, tetradecanoic acid, hexadecanoic acid, octadecanoic acid, pentadecanoic acid, 14-methyl, decanoic acid, methyl ester, hexadecadienoic acid, methyl ester, octadecadiennoic acid, methyl ester, octadecatrienoic acid, phytol (2hexadecen-1-ol,3,7,11,15-tetramethyl), piperidinone, stearic acid hydrazide, hexadecanoyl, and oleic acid [24–31].

Investigation of the antioxidant activity of *C. adansonii* leaf extract and fractions for the most effective activity showed that CALE had the most effective DPPH and nitric oxide scavenging activities, while CALB had high FRAP, TAC, TPC, and TFC when compared with other test samples. CALE also exhibited appreciable levels of FRAP, TAC, and TC following CALB. In overall, CALE was generally selected for its antioxidant properties. GC-MS data identified the bioactive compounds in CALE previously known to exhibit antioxidant activity as tetradecanoic acid, hexadecanoic acid, pentadecanoic acid, pentadecanoic acid, 14-methyl, piperidinone, docosenoic, and oleic acid identified in CALE using GC-MS analytical method and previously shown to possess antioxidant activities [26,27,30]. These bioactive compounds in CALE could readily donate proton [H^+] to the unstable reactive compounds. It has been reported that plant extracts with free radical scavenging capacity donate hydrogen ion(s) to stabilize the tissue-damaging ROS and also

Table 4. Compounds with anti-inflammatory and/or antioxidant activities identified in *C. adansonii* leaf ethylacetate fraction.

S/N	Mass peak	Retention time	Library ID	Biological activity reported
1	34	6.833	5-methoxymethy 1-{1,3,4} thiadiazole-2-ylamine	Anti-inflammatory [24]
2	34	6.833	Thiazoline-4-one, 5-ethyl-2-imino	Anti-inflammatory [25]
3	74	13.542	Tridecanoic acid. Methyl eater	Anti-oxidant [26]
4	74	13.542	Hexadecanoic acid, 15-methyl	Anti-inflammatory [27]
5	79	14.242	Tetradecanoic acid (Myristic acid)	Anti-inflammatory, Anti-oxidant [27]
6	79	14.242	Hexadecanoic acid (Palmitic acid)	Anti-inflammatory, Anti-oxidant [26]
7	79	14.24	Pentadecanoic acid	Anti-oxidant [27]
8	79	14.242	Octadecanoic acid (Stearic acid)	Anti-inflammatory [28]
9	116	16.875	Pentadecanoic acid, 14-methyl	Anti-oxidant, Anti-inflammatory [27]
10	116	16.875	Decanoic acid, methyl ester	Anti-inflammatory [27]
11	124	19.867	Hexadecadienoic acid, methyl ester	Anti-inflammatory [27]
12	164	19.983	Octadecadiennoic acid, methyl ester	Anti-inflammatory [27]
13	164	19.983	Octadecatrienoic acid (Linolenic acid)	Anti-inflammatory [27]
14	91	20.225	Phytol(2hexadecen-1-ol,3,7,11, 15-tetramethyl)	Anti-inflammatory [29]
15	111	21.908	Piperidinone	Anti-oxidant, Anti-inflammatory [30]
16	111	21.908	Stearic acid hydrazide	Anti-inflammatory [31] (Khan *et al*. 2011)
17	111	21.908	Hexadecanoyl	Anti-inflammatory [31]
18	112	23.800	Docosenoic	Anti-oxidant [27]
19	142	24.917	Oleic acid (9-Octadecenoic acid)	Anti-inflammatory, Anti-oxidant [30]

terminate free radical initiated deleterious chain reactions [37].

Furthermore, previous studies had shown that during inflammatory responses, ROS are generated and propagated through the release of interleukin-I, tumor necrosis factor-α, and interferon-γ, which stimulate the recruitment of additional neutrophils and macrophages to sustain the inflammatory processes [38–40]. Hence, antioxidant compound with the capacity to scavenge ROS could attenuate the inflammatory mediators [41,42]. Furthermore, phenolic and flavonoid compounds have been reported to exert a profound stabilizing effect on inflammatory cells [15]. This could further explain the *in vitro* anti-inflammatory activities exhibited by CALE.

The animal study was designed to ascertain the potential anti-inflammatory activities of CALE. Findings from the animal study showed that CALE suppressed the formaldehyde-induced rat paw edema. This indicates that CALE possesses the anti-inflammatory property and it equally supports the observations made using the *in vitro* systems. Formaldehyde-induced inflammation model has been documented as a biphasic process that accesses the pain of both neurogenic (first phase) and inflammatory (second phase) events [43]. Gupta et al. [44] explained that most anti-inflammatory agents possess the capacity to inhibit the inflammatory phase more prominently than the neurogenic phase. In this present study, a plateau phase at 4 hours was followed by a substantial reduction of the formaldehyde-induced paw edema at the sixth hour in rats treated with CALE and diclofenac. More so, the second phase is reported to be mediated by kinin-like and prostaglandin-like substances that could promote the stimulation of pro-inflammatory mediators like cytokines [45]. Based on this premise, it is suggested that CALE may be inhibiting the inflammatory mediators of the second phase. In addition, the antioxidant compounds in CALE may be neutralizing ROS, thereby limiting the stimulation of pro-inflammatory mediators.

Furthermore, CALE treated animals induced with arthritis had reduced tissue damage biomarkers such as AST and ALT activities when compared with untreated arthritic animals. This further supports the statement that CALE

exhibited an anti-inflammatory effect *in vivo.* Measurement of serum AST and ALT are known indicators used for the assessment of the extent of tissue damage [46]. ALT and AST are normally located within the cytoplasm of hepatocytes and other tissues. During tissue damage, these cytoplasmic enzymes seep into the bloodstream [47]. In addition, the level of serum ALT and AST in 300 mg/kg b.w. CALE treated animals was comparable to those of the standard diclofenac-treated group. This suggests that CALE contains bioactive compounds that could be an effective therapeutic agent in the treatment of inflammation.

Conclusion

Findings from this study indicated that CALE fraction possesses bioactive compounds with the anti-inflammatory property. The possible CALE anti-inflammatory mechanism could be through suppression of pro-inflammatory mediators and interferences with inflammation-induced ROS generation process. Thus, CALE fraction could be harnessed as a source of lead drug candidates, as GC-MS identified 19 bioactive compounds. In addition, further study is still required to ascertain the full mechanism of CALE anti-inflammatory action.

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Disclosure Statement

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