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Research paper

Anti-inflammatory and antioxidant activities of *Costus afer* Ker Gawl. hexane leaf fraction in arthritic rat modelsGodswill Nduka Anyasor^{a,*}, Funmilayo Onajobi^a, Odutola Osilesi^a, Olugbenga Adebawo^{a,b}, Efere Martins Oboutor^c^a Department of Biochemistry, Benjamin S. Carson School of Medicine, College of Health and Medical Sciences, Babcock University, Ilisan Remo, Ogun State, P.M.B. 21244 Ikeja, Lagos, Nigeria^b Department of Biochemistry, Faculty of Basic Medical Sciences, O.A.C.H.S., Olabisi Onabanjo University, Remo Campus, Ikenne, Ogun State, Nigeria^c Department of Biochemistry, Faculty of Science, Obafemi Awolowo University, Ile-Ife, Nigeria

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Formaldehyde (CID: 712)

Arachidonic acid (CID:444899)

Diclofenac sodium (CID:5018304)

Prednisolone (CID:5755)

Hydrogen peroxide (CID:784)

Reduced glutathione (CID:124886)

ABSTRACT

Ethnopharmacological relevance: *Costus afer* Ker Gawl is an indigenous tropical African medicinal plant used as therapy in the treatment of inflammatory ailments such as rheumatoid arthritis. This study was designed to evaluate the anti-inflammatory and antioxidant activities of the hexane fraction of *C. afer* leaves (CAHLF).

Materials and methods: The anti-inflammatory effect of varying doses of CAHLF on carrageenan, arachidonic acid, and formaldehyde induced arthritis in male albino rats' models were investigated in order to study the acute inflammatory phase. Complete Freund's Adjuvant (CFA)-induced arthritis model was used to study the chronic inflammatory phase. Two known anti-inflammatory drugs, Diclofenac sodium (non-steroidal anti-inflammatory drug [NSAID]) and prednisolone (glucocorticoid [steroidal drug]) were used as standards for comparison. Various biochemical indices viz. superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), reduced glutathione (GSH) and malondialdehyde (MDA), aspartate amino transferase (AST), alanine amino transferase (ALT), alkaline phosphatase (ALP), total bilirubin (TB), total protein (TP), globulin and albumin levels were assayed using spectrophotometric methods.

Results: Control animals in which arthritis have been induced using carrageenan, arachidonic acid, formaldehyde or CFA showed significant increases ($P < 0.05$) in paw edema when compared with normal animals. Treatment of the arthritis induced rats with CAHLF significantly ($P < 0.05$) suppressed the edema. *in vivo* antioxidant study showed that CAHLF treated animals had a significantly ($P < 0.05$) elevated GSH level, SOD, CAT and GST activities while MDA levels were significantly ($P < 0.05$) reduced in the plasma, liver, kidney and brain. CAHLF treated rats had a significantly ($P < 0.05$) reduced plasma AST, ALT and ALP. Plasma TP, globulin, TB levels were reduced while albumin levels were elevated in CAHLF treated animals.

Conclusions: CAHLF possesses substantial anti-inflammatory and antioxidant activities against inflammatory diseases especially arthritis. It could be considered as a choice candidate in pharmaceutical anti-inflammatory drug development.

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Abbreviations: CAHLF, hexane fraction of *Costus afer* leaves; NSAID, non-steroidal anti-inflammatory drugs; CFA, Complete Freund's Adjuvant; ANOVA, analysis of variance; S.E.M., standard error of mean; DS, Diclofenac sodium; PN, prednisolone; NS, normal saline; COX, cyclooxygenase; LOX, lipoxygenase; PG, prostaglandin; LT, leukotriene; ROS, reactive oxygen species; NO, nitric oxide; O₂⁻, superoxide anion; *OH, hydroxyl radical; HOCl, Hypochlorous acid; H₂O₂, hydrogen peroxide; SOD, superoxide dismutase; CAT, catalase; GST, glutathione S-transferase; GSH, reduced glutathione; MDA, malondialdehyde

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1. Introduction

Chronic inflammatory autoimmune diseases such as rheumatoid arthritis (RA) affect over 21 million people worldwide. It affects three times as many women as men (Chopra and Abdel-Nasser, 2008). Although RA can affect people of all ages, it is most common in people of 30–50 years age range (Lundkvist et al., 2008). Rheumatoid arthritis is one of the leading causes of chronic morbidity in the developed world (Mody, 2009). It is characterized by the immune system attack on tissues that line the joints and destruction of synovial fluids causing pain, swelling and stiffness in the polyarticular joints leading to disability and premature mortality (Ahmed et al., 2005; Aletaha et al., 2010). Prolong arthritic disease is also associated with psychological problem such as depression (Ben et al., 2012).

There are different medical interventions adopted in the management of arthritis. However, rheumatoid arthritis patients still experience undesirable side effects especially from the use of synthetic steroidal and non-steroidal anti-inflammatory drugs (Musa et al., 2012). This is also corroborated by the WHO report which states that these drugs are often associated with drug-induced toxic effects or secondary adverse effects on long term use (Sarwar et al., 2011). The quest for an alternative source of anti-inflammatory drugs especially from plant sources with little or no side effects has recently preoccupied researchers in research institutes, academia and pharmaceutical companies (Musa et al., 2012).

Costus afer Ker Gawl is an indigenous West African plant commonly known as ginger lily or bush cane. It is a tall, perennial, and rhizomatous herb that belongs to the Family of Zingiberaceae now known as Costaceae found mostly in moist or shady forests and river banks (Aweke, 2007). In the Western part of Nigeria, it is called different names such as; “Ireke omode”, “Ireke ogun” while it is called “Okpete” or “Okpoto” in Igboland, “Kakizawa” in Hausa and “Mbritem” in Efik and Anglophone Cameroon calls it “Monkey sugar cane” (Iwu, 1993).

C. afer leaves are used as a medicinal herb, especially in treating inflammation, rheumatism, arthritis, cough, hepatic disorders, helminthic, miscarriages, epileptic attack and hemorrhoids (Iwu, 1993; Burkill, 2000; Anaga et al., 2004).

Previous reports had shown that the chloroform and methanol extracts from the aerial parts exhibited a significant reduction in carrageenan-induced rat paw edema, while the methanol extract of the rhizome showed significant topical anti-inflammatory activity in croton aldehyde-induced mouse ear edema (Moody and Okwagbe, 2003; Aweke, 2007). The chloroform extract of the rhizome was reported to suppress adjuvant-induced polyarthritis in rats (Iwu and Anyanwu, 1982). Investigations have also shown that *C. afer* may contain bioactive metabolites including flavonoids, phenols, anthraquinones, cardiac glycosides, terpenoids, alkaloids and tannins (Anyasor et al., 2010; Akpan et al., 2012; Ukpabi et al., 2012).

Results of the *in vitro* anti-inflammatory studies from our laboratory showed the hexane leaf fraction of *C. afer* (CAHLF) as the most active anti-inflammatory fraction and the gas chromatographic/mass spectrometric analytical data showed the presence of *n*-naphthalene 2,3 dimethyl; naphthalene, 1,6 dimethyl, phenol, 2,4-bis(1,1-dimethylethyl)-, phytol, 2(4H)-benzofuranone, 5,6,7,7-tetrahydro-4,4,7-trimethyl, pentadecanoic acid, hexadecanoic acid-methyl ester, *n*-hexadecanoic acid, linoleic acid, α -linolenic acid, and *cis*-vaccenic in CAHLF (report sent for publication). Thus, this study was designed to investigate the *in vivo* anti-inflammatory and antioxidant activities of the hexane fraction of *C. afer* leaves, with a view to gain an insight into the possible mechanism of anti-inflammatory action. To the best of our knowledge this is the first time this data is been reported.

2. Materials and methods

2.1. Collection of plant material

C. afer plant was obtained from a farm land at Irolu in Ikenne Local Government Area, Ogun State, Nigeria. The plant was identified and authenticated by Professor O.A. Denton, a crop scientist in the Department of Agronomy and Landscape Design, School of Agriculture and Industrial Technology, Babcock University, Ilesan-Remo, Ogun State, Nigeria. A voucher sample with number of FHI-108001 was deposited at Forestry Herbarium Ibadan (FHI).

2.2. Plant processing, extraction and solvent partitioning

The leaves were plucked out of the stem and air-dried under room temperature (28 ± 2 °C). The air-dried samples were pulverized using mechanical grinder. Three hundred grams powdered leaf samples were extracted using 1800 ml of 70% methanol with intermittent shaking for 48 h. The extract was filtered using Whatman No.1 filter paper and the filtrate was subsequently concentrated using rotary evaporator at 30 °C (Buchi Rotavapor RE; Switzerland). The concentrates were reconstituted with distilled water in a ratio of 1:2 (concentrate: distilled water) and partitioned using hexane solvent. The hexane fraction obtained was concentrated in a rotary evaporator at 30 °C. The fraction obtained was dissolved in 0.9% (w/v) NaCl and kept in the refrigerator at 4 °C until further use.

2.3. Animals

Male albino rats (Wistar Strain) weighing 180 ± 10.15 g were purchased from an inbred colony at the Preclinical Animal House, Physiology Department, University of Ibadan, Ibadan, Nigeria. The animals were acclimatized for two weeks at Babcock University Animal House. Animals were maintained and cared for following the National Institute of Health (NIH) animal care guidelines (NIH, 2010). The approval of Babcock University Animal Ethics Committee was obtained for the procedure adopted.

2.4. Acute toxicity study

The acute toxicity study for the hexane leaf fraction of *C. afer* leaves was performed using the median lethal dose of the fraction in albino rats following the Organization for Economic Cooperation and Development (OECD) guideline 425 (OECD, 2001). A male Wistar albino rat was orally administered with 2000 mg/kg CAHLF after fasting overnight. The animal was observed for 24 h for any clinical signs of toxicity such as change in fur color, accelerated breathing or death. The animal survived without any observable change. Subsequently, five male albino rats were selected by random sampling technique and subjected to the same protocol for 72 h and they all survived. The 2000 mg/kg fraction was considered safe and doses of 50–500 mg/kg body weight (b.w.) were adopted for further studies.

2.5. Assessment of *in vivo* anti-inflammatory activity

2.5.1. Acute study: carrageenan-induced paw edema in rats

The carrageenan-induced rat paw edema was carried out as described by Winter et al. (1962) to evaluate the acute anti-inflammatory activity of the hexane leaf fraction. This study involved seven groups of five rats each, viz. group I: rats were orally administered with 1 ml 0.9% NaCl without inducing arthritis (normal group), group II: rats were orally administered with 1 ml 0.9% NaCl with the induction of arthritis (control group), group III–IV

rats were orally administered with standard drugs 10 mg/kg Diclofenac sodium (DS) (2-[(2,6 Dichlorophenyl)amino] benzene acetic acid sodium salt) (an NSAID) and 10 mg/kg prednisolone (PN) (pregna-1,4-diene-3, 20-dione,11,17-dihydroxy-21-(phosphonoxy)) (a glucocorticoid) respectively while groups V, VI and VII were rats orally administered with 100, 250 and 500 mg/kg doses of hexane fractions of *C. afer* leaves (CAHLF) respectively. The paw edema was induced by injecting 0.1 ml of 1% (w/v) carrageenan suspension in 0.9% (w/v) saline just beneath the plantar aponeurosis tissue of the left hind paw of test groups, one hour following oral administration of test drugs. Increase in paw thickness was measured at one hour interval for six hours using a micro meter screw gauge.

The percentage inhibition of inflammation was calculated as follows:

$$\text{Percentage inhibition of inflammation} = (1 - (Vt/Vc)) \times 100$$

Vt is the average paw thickness in treated groups; Vc is the average paw thickness of the control group.

2.5.2. Acute study: arachidonic acid-induced inflammation

Arachidonic acid-induced inflammation in rats was studied as described by Di-Martino et al. (1987) and modified by Sunita et al. (2011). This study involved seven groups of six rats each viz. group I: rats were orally administered with 1 ml of 0.9% NaCl without inducing arthritis (normal group), group II: rats were orally administered with 1 ml of 0.9% NaCl and induced with arthritis (control group), groups III and IV rats were orally administered with standard drugs 10 mg/kg DS and PN respectively while groups V, VI and VII were orally administered with 50, 100 and 250 mg/kg CAHLF respectively. Paw edema was induced by a single sub-plantar injection of 0.1 ml of 2% arachidonic acid in acetone into the right hind paw of rats 30 min after oral administration of standard drugs and CAHLF. The paw edema thickness was measured every 30 min for 6 h followed by 16th, 17th and 18th h with the aid of a micrometer screw gauge to determine the linear diameter of paw edema.

The percentage inhibition of inflammation was calculated as inhibition of paw edema thickness as stated above.

2.5.3. Acute inflammation study: formaldehyde-induced arthritis

The effects of 50–250 mg/kg body weight (b.w.) CAHLF, 10 mg/kg b.w. Diclofenac sodium and 10 mg/kg b.w. prednisolone on the acute phase of inflammation was investigated using formaldehyde induced arthritis according to the method described by Gupta et al. (2010). This study involved seven groups of six rats each viz. normal group (0.2 ml of 0.9% NaCl), control group was induced with arthritis using 2% v/v formaldehyde and orally administered with 0.2 ml 0.9% NaCl, two standard drugs treated groups (10 mg/kg Diclofenac sodium and prednisolone respectively), and three test groups administered with 50, 100 and 250 mg/kg CAHLF for 7 days. Formaldehyde (2% v/v) solution, 0.02 ml, was injected in the first and third day into the left hind paw just beneath the plantar aponeurosis to induce arthritis. The paw thickness was measured with the aid of micro meter screw gauge. This was done 30 min before induction of arthritis using formaldehyde, and every 24 h for 7 days. The percentage inhibition of inflammation was calculated as stated above.

2.5.4. Chronic inflammatory study: Complete Freund's Adjuvant-induced arthritis

Arthritis was induced in rats by injecting 0.1 ml of Complete Freund's Adjuvant (CFA) emulsion into the sub-plantar surface of right hind paw. The animals were divided into 7 groups of six rats each viz. group I rats were the normal group that was orally administered with 1 ml 0.9% NaCl but was neither induced with arthritis nor treated with any drug or CAHLF. Group II rats were

the control group induced with arthritis and received orally, 1 ml 0.9% NaCl. Groups III and IV received 10 mg/kg Diclofenac sodium and prednisolone respectively while the groups V, VI and VII received 50, 100 and 250 mg/kg CAHLF respectively. After 30 min of CFA injection into their sub-plantar region of left hind paw at day "0", saline, drug or CAHLF were orally administered to the animals once daily from 1st day and continued till the 21st day. The anti-arthritic effect of the drugs and CAHLF were evaluated by measuring the paw thickness with micro meter screw gauge to determine the linear diameter paw edema according to the method of Chaudhari et al. (2012). The percentage inhibition of inflammation was calculated as stated above.

2.6. Biochemical assays

Twenty four hours after the end of treatment periods in formaldehyde and CFA induced arthritis in acute and chronic inflammation studies respectively, the animals were euthanized using cervical dislocation and sacrificed. The blood samples were collected through cardiac puncture using 2 ml hypodermic syringes into heparinized bottles and immediately centrifuged at 3000 rpm for 10 min to obtain plasma. The liver, kidneys and brain were harvested, and subsequently homogenized to obtain a final solution of 10% w/v homogenate in 0.05 M phosphate buffer (pH 7.4). The homogenate was centrifuged at 3000 rpm for 10 min to obtain supernatant kept for antioxidant assays. The plasma samples obtained were used for the determination of aspartate amino transferase (AST), alanine amino transferase (ALT), alkaline phosphatase (ALP), total protein, total bilirubin, and antioxidant assay using spectrophotometric methods and an appropriate diagnostic kit and reagents (TECO diagnostics, Canada).

2.7. Assay for antioxidant activity (in vivo method)

The antioxidant activity in liver, kidneys and brain samples were assayed using spectrophotometric methods for the determinations of superoxide dismutase (SOD) activity (Mitra and Fridovich, 1972), catalase (CAT) activity (Sinha, 1972), glutathione S-transferase (GST) activity (Habig et al., 1974), glutathione (GSH) level was determined using Ellman's reagent (Sedlak and Lindsay, 1963; Jollow et al., 1974) and malondialdehyde level (Stocks and Dormandy, 1971).

2.8. Statistical analysis

Statistical analysis was carried out with the aid of SPSS for windows; SPSS Inc., Chicago, Standard version 17.0. Statistical comparisons were performed using one way analysis of variance (ANOVA) followed by post-hoc analysis using least significant difference test. *P* values < 0.05 indicate statistical significance. Data are expressed as means ± S.E.M.

3. Results and discussion

3.1. Effect of CAHLF on rat paw edema induced with carrageenan

The data in Fig. 1 showed that orally administered hexane leaf fraction at doses of 100, 250 and 500 mg/kg significantly (*P* < 0.05) suppressed paw edema in a dose dependent manner compared with the control group. Similar observation was recorded for the standard 10 mg/kg Diclofenac sodium and prednisolone. The percentage inhibition of carrageenan-induced paw edema by CAHLF increased with time over the 6 h period, reaching up to 86.87% at 6 h for the 500 mg/kg hexane leaf fraction treated group.

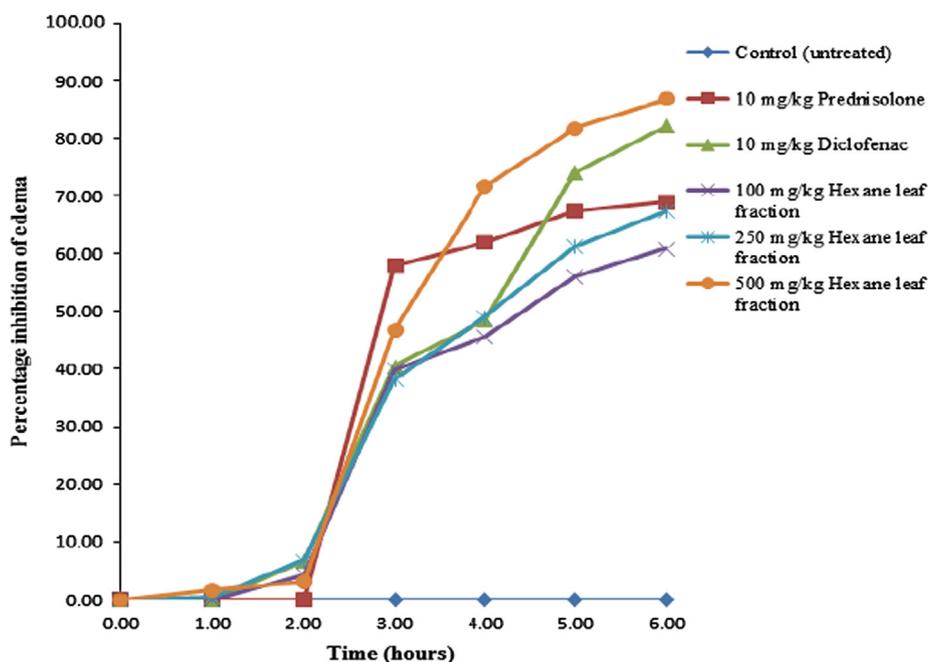


Fig. 1. Percentage inhibition of carrageenan induced-paw edema in male rats treated with 100–500 mg/kg b.w. hexane fractions of *Costus afer* leaf.

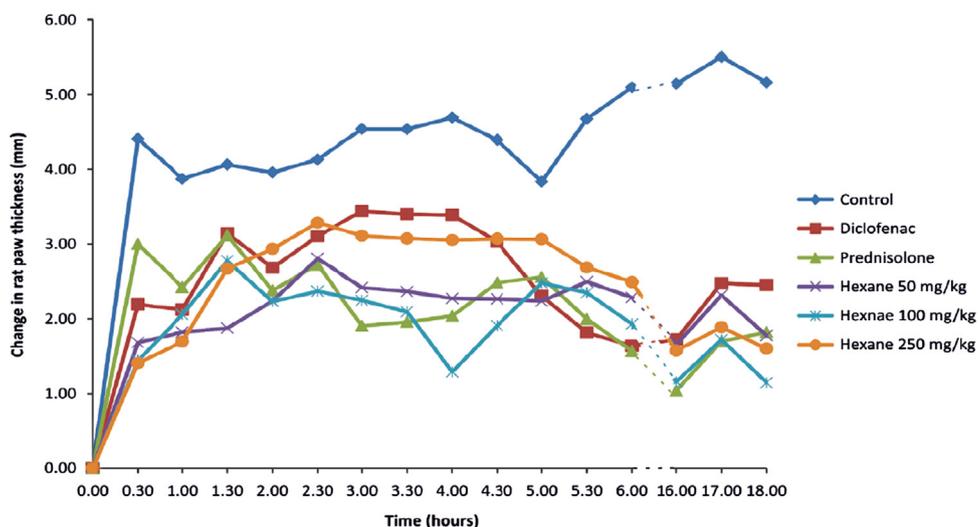


Fig. 2. Change in rat paw thickness by different doses of hexane fractions of *Costus afer* leaf in arachidonic acid induced paw edema.

This suggests that the CAHLF may possess anti-inflammatory activity *in vivo*. Previous studies on other plant extracts have also reported similar findings (Srikanth et al., 2013). Carrageenan induced paw edema is believed to be a biphasic mechanism. The early phase of 1–2 h is mainly mediated by histamine, serotonin and increased synthesis of prostaglandins (PGs) in the localized damaged tissue (Chavan et al., 2011). The late phase 3rd h is sustained by prostaglandin release and is mediated by bradykinin, leukotrienes (LT), polymorphonuclear cells and prostaglandins produced by tissue macrophages (Chaudhari et al., 2012). The late phase is believed to be sensitive to most of the clinically effective anti-inflammatory drugs (Pandurangana et al., 2009). In this study, CAHLF suppressed carrageenan induced paw edema at the 3rd h (late phase). CAHLF suppressed carrageenan induced paw edema slightly after 1 h (early phase) and appreciably after the 2nd h (late phase) which suggest that the mechanism of action of this fraction may be related to histamine and prostaglandin biosynthesis inhibitions.

3.2. Effect of CAHLF on rat paw edema induced with arachidonic acid

The results in Fig. 2 showed that administration of 0.1 ml of 2% arachidonic acid into the sub-plantar aponeurosis of rats increased paw thickness all through an 18 h period in control group compared with the normal control group. Orally administered CAHLF at doses of 50–250 mg/kg, 10 mg/kg Diclofenac and prednisolone significantly ($P < 0.05$) suppressed the edema when compared with the control group. This also indicates that the mechanism of action of CAHLF may be related to inhibition PGs and histamine biosynthesis and probably leukotriene production. The arachidonic acid metabolites (prostaglandins and leukotrienes) formed via cyclooxygenase (COX) and lipoxygenase (LOX) pathways respectively, represent two important classes of inflammatory mediators (Sunita et al., 2011). Previous findings have also shown that Diclofenac and prednisolone inhibited COX and LOX enzymes (Rolf et al., 1990; Elenkov and Chrousos, 2002; Wise et al., 2008). Prednisolone has also been reported to inhibit

histamine while contrary opinion still exist with regards to Diclofenac inhibition of histamine synthesis and release (Xue et al., 2009; Asero and Tedeschi, 2010).

3.3. Effect of CAHLF on rat paw edema induced with formaldehyde

The results in Fig. 3 showed that oral administered hexane leaf fraction at doses of 50–250 mg/kg to rats induced with arthritis using 2% v/v formaldehyde led to a significantly ($P < 0.05$) reduced paw edema thickness when compared to the control group. Administration of standard 10 mg/kg Diclofenac sodium and prednisolone also led to a reduced paw thickness compared with the control group. The highest dose of 250 mg/kg hexane leaf fraction treated group suppressed paw edema thickness starting from the 2nd day to the 7th day while the other treatment groups showed a suppression of paw edema from the 3rd day to the 7th day. This also suggests that the CAHLF may possess anti-arthritic activity comparable to the standard anti-inflammatory drugs. Inflammation induced by formaldehyde is also biphasic, and measures pain of both neurogenic (first phase) and inflammatory (second phase) origins (Bulus and Abdul, 2007). The first phase is as a result of direct stimulation of nociceptor and it measures centrally mediated effects and is insensitive to anti-inflammatory agents while the second phase is dependent on peripheral inflammation and changes in central procession due to chemical mediators released from damaged cells that stimulate nociception (Bulus and Abdul, 2007). These statements may account for the observed CAHLF suppression of the formaldehyde-induced paw edema after the 2nd day of treatment. Previous studies have also shown that anti-inflammatory agents are capable of blocking the second phase of formaldehyde-induced arthritis model more prominently than the first phase (Gupta et al., 2010; Sandeep et al., 2010). An early neurogenic phase has been reported to be mediated by substance P and bradykinin followed by a tissue mediated response where histamine, 5-HT (serotonin), PGs and bradykinin are known to be involved (Wheeler-Aceto and Cowan, 1991). The initial phase of the edema is due to the release of histamine and 5-HT and the edema is maintained during the plateau phase by kinin like substance and the second accelerating phase of swelling was due to the release of PGs like substances (Chauhan et al., 1998). Hence, it is speculated that CAHLF may be inhibiting the chemical mediators of inflammation.

3.4. Effect of CAHLF on rat paw edema induced with Complete Freund's Adjuvant

Data in Fig. 4 showed that orally administered hexane leaf fraction at doses of 50–250 mg/kg, 10 mg/kg Diclofenac sodium and 10 mg/kg prednisolone to the CFA infected groups had a significantly ($P < 0.05$) reduced paw thickness compared to the control group. This suggests that CAHLF was effective as an anti-arthritic agent comparable with those of standard drugs. The CFA is composed of inactivated and dried mycobacterium tuberculosis and is effective in stimulating inflammatory response in cell mediated immunity and may lead to the potentiation of certain immunoglobulins production (Kaithwas and Majumdar, 2010). Adjuvant induced arthritis in rat mimics many of the clinical and pathological features of human rheumatoid arthritis, such as paw swelling, joint erosions and ankylosis and it is the most commonly used models for rheumatoid arthritis study (Hong-Meixu et al., 2007).

3.5. Effects of CAHLF on antioxidant system in arthritic rats

Results in Tables 1 and 2 showed a significantly elevated ($P < 0.05$) superoxide dismutase (SOD), catalase (CAT), glutathione *S*-transferase (GST) and reduced glutathione (GSH) in liver, kidney, brain and plasma by 100 and 250 mg/kg hexane leaf fractions compared with control groups in 7 and 21 days treatments. CAHLF treated arthritic rats had a significantly ($P < 0.05$) reduced malondialdehyde (MDA) levels in the brain, liver and kidney compared with control group.

This suggests that CAHLF treated arthritic rats were better protected against the insurgence of free radicals. CAHLF might also contain phytochemicals which could be playing a contributory antioxidant role either by augmenting or complementing the endogenous antioxidant defense system, thereby reducing the oxidative stress status of the rats. Several lines of evidence had implicated oxidative stress in the pathogenesis of rheumatoid arthritis especially in the proliferation of destructive synovitis (Maurice et al., 1997; Tak et al., 2000). In many joint diseases, pro-inflammatory factors such as cytokines and prostaglandins, together with ROS and nitric oxide (NO) are released at sites of inflammation (Sakurai et al., 1995). These factors are associated with very low SOD activity during inflammation (Afonso et al., 2007). This may account for the reduced SOD activity in the control group compared with the CAHLF treated groups. SOD activity is a key component of the cellular antioxidant system that protects cells and the extracellular matrix from the harmful effects

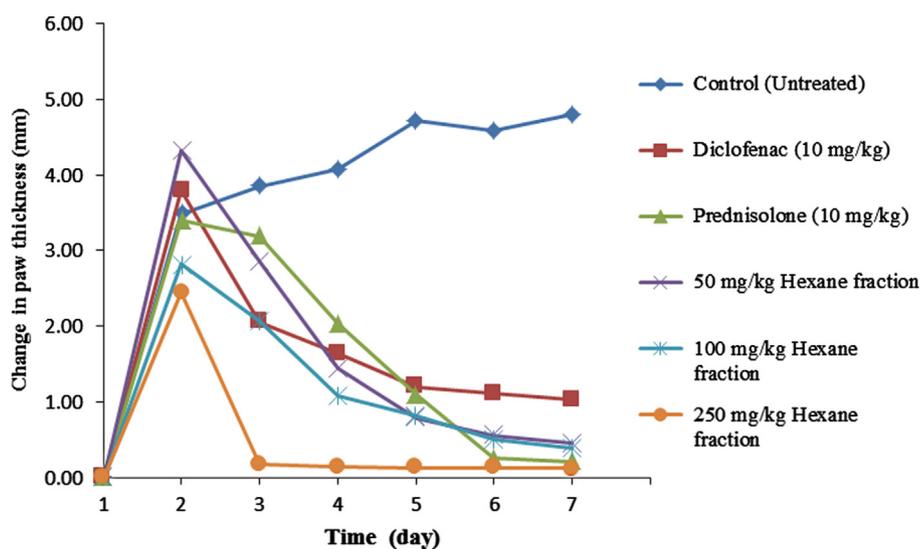


Fig. 3. Change in paw thickness by different doses of hexane fraction of *Costus afer* leaf in rat induced with arthritis using formaldehyde.

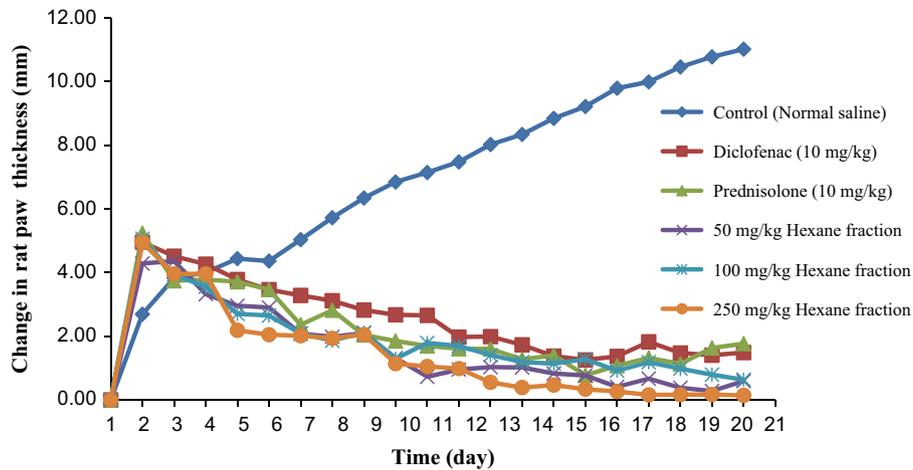


Fig. 4. Change in paw thickness in Complete Freund's Adjuvant induced-paw edema in male rats by different doses of hexane fractions of *Costus afer* leaf.

Table 1

Effects of varying doses of CAHLF on liver, kidney and brain antioxidants in rats induced with arthritis using formaldehyde for 7 days ($n=6$ rats per group).

Parameters	Tissues	Normal	Control	10 mg/kg Diclofenac	10 mg/kg Prednisolone	50 mg/kg CAHLF	100 mg/kg CAHLF	250 mg/kg CAHLF
SOD (Units/mg protein)	Plasma	0.67 ± 0.01	0.21 ± 0.02 ^a	0.28 ± 0.01 ^b	0.31 ± 0.02 ^{bc}	0.26 ± 0.03 ^b	0.34 ± 0.04 ^c	0.43 ± 0.01 ^d
	Liver	0.45 ± 0.03	0.32 ± 0.04 ^a	0.46 ± 0.07 ^d	0.42 ± 0.07 ^c	0.44 ± 0.05 ^d	0.48 ± 0.08 ^d	0.40 ± 0.02 ^b
	Kidney	1.06 ± 0.05	0.76 ± 0.08 ^a	1.02 ± 0.03 ^c	1.02 ± 0.02 ^c	0.92 ± 0.07 ^b	0.87 ± 0.07 ^b	1.01 ± 0.06 ^{cd}
	Brain	1.02 ± 0.01	0.71 ± 0.09 ^a	1.26 ± 0.04 ^d	1.03 ± 0.03 ^c	1.10 ± 0.03	0.88 ± 0.02 ^b	0.97 ± 0.02 ^{bc}
CAT (Units/mg protein)	Plasma	0.52 ± 0.01	0.21 ± 0.01 ^a	0.34 ± 0.02 ^b	0.41 ± 0.01 ^d	0.37 ± 0.01 ^c	0.41 ± 0.02 ^d	0.42 ± 0.02 ^d
	Liver*	0.55 ± 0.07	0.31 ± 0.03 ^a	0.38 ± 0.03 ^b	0.39 ± 0.02 ^b	0.46 ± 0.04 ^c	0.42 ± 0.04 ^{bc}	0.52 ± 0.05 ^d
	Kidney*	1.04 ± 0.04	0.63 ± 0.03 ^a	1.15 ± 0.20 ^d	1.19 ± 0.05 ^d	0.86 ± 0.07 ^c	0.87 ± 0.06 ^c	0.78 ± 0.03 ^b
GSH (Units/mg protein)	Plasma	2.29 ± 0.14	1.88 ± 0.08 ^a	2.48 ± 0.14 ^{bc}	2.43 ± 0.05 ^{bc}	2.59 ± 0.08 ^c	2.30 ± 0.11 ^b	2.04 ± 0.16 ^b
	Liver	0.42 ± 0.03	0.22 ± 0.02 ^a	0.31 ± 0.01 ^b	0.30 ± 0.02 ^b	0.35 ± 0.02 ^b	0.34 ± 0.01 ^b	0.40 ± 0.02 ^c
	Kidney	1.31 ± 0.16	0.93 ± 0.03 ^a	1.36 ± 0.04 ^e	1.08 ± 0.03 ^b	1.29 ± 0.06 ^d	1.16 ± 0.05 ^c	1.14 ± 0.06 ^f
GST (μmol/min/mg protein)	Plasma	1.10 ± 0.05	0.97 ± 0.01 ^a	1.04 ± 0.02 ^b	1.07 ± 0.01 ^b	1.14 ± 0.03 ^c	1.28 ± 0.03 ^d	1.31 ± 0.09 ^d
	Liver	0.002 ± 0.00	0.001 ± 0.00 ^a	0.002 ± 0.00 ^b	0.003 ± 0.00 ^c	0.002 ± 0.00 ^b	0.002 ± 0.00 ^b	0.003 ± 0.00 ^c
	Kidney	0.003 ± 0.00	0.001 ± 0.00 ^a	0.001 ± 0.00 ^b	0.001 ± 0.00 ^b	0.002 ± 0.00 ^c	0.002 ± 0.00 ^c	0.003 ± 0.00 ^e
MDA (× 10 ⁻⁶ nmol/mg protein)	Plasma	0.002 ± 0.00	0.001 ± 0.00 ^a	0.002 ± 0.00 ^b	0.002 ± 0.00 ^b	0.002 ± 0.00 ^b	0.003 ± 0.00 ^c	0.004 ± 0.00 ^d
	Liver	0.92 ± 0.09	1.10 ± 0.09 ^d	0.22 ± 0.01 ^a	0.27 ± 0.08 ^a	0.46 ± 0.09 ^{ab}	0.50 ± 0.05 ^c	0.40 ± 0.07 ^{ab}
	Kidney	0.79 ± 0.10	1.58 ± 0.05 ^c	0.80 ± 0.05 ^{ab}	0.87 ± 0.20 ^{ab}	0.56 ± 0.20 ^a	0.48 ± 0.60 ^a	0.55 ± 0.20 ^a
Brain	2.18 ± 0.23	3.12 ± 0.25 ^e	2.09 ± 0.35 ^c	1.55 ± 0.14 ^{ab}	1.38 ± 0.29 ^a	2.20 ± 0.22 ^d	1.23 ± 0.17 ^a	

Different superscripts across row indicates significantly different at $P < 0.01$.

* Indicates superscripts across row are significantly different at $P < 0.05$.

Table 2

Effects of CAHLF, DS and PN on liver, kidney and brain antioxidants in rats induced with arthritis using Complete Freund's Adjuvant for 21 days ($n=6$ rats per group).

Parameters	Tissues	Normal	control	10 mg/kg Diclofenac	10 mg/kg Prednisolone	50 mg/kg CAHLF	100 mg/kg CAHLF	250 mg/kg CAHLF
SOD (Units/mg protein)	Plasma	0.56 ± 0.02	0.37 ± 0.03 ^a	0.54 ± 0.02 ^b	0.61 ± 0.05 ^c	0.63 ± 0.03 ^c	0.81 ± 0.07 ^d	0.85 ± 0.03 ^d
	Liver	0.78 ± 0.09	0.54 ± 0.03 ^a	0.65 ± 0.10 ^b	0.64 ± 0.07 ^b	0.65 ± 0.13 ^b	0.81 ± 0.06 ^c	0.97 ± 0.07 ^{d*}
	Kidney	1.04 ± 0.03	0.76 ± 0.03 ^a	1.02 ± 0.07 ^{e*}	1.02 ± 0.01 ^{e*}	0.92 ± 0.08 ^c	0.82 ± 0.06 ^b	0.97 ± 0.02 ^d
	Brain	0.98 ± 0.05	0.76 ± 0.04 ^a	0.96 ± 0.03 ^b	1.01 ± 0.02 ^c	0.99 ± 0.03 ^c	1.10 ± 0.04 ^{d*}	0.97 ± 0.01 ^{d*}
CAT (Units/mg protein)	Plasma	0.53 ± 0.06	0.21 ± 0.02 ^a	0.32 ± 0.02 ^b	0.47 ± 0.02 ^d	0.41 ± 0.03 ^c	0.33 ± 0.01 ^b	0.34 ± 0.02 ^b
	Liver*	1.44 ± 0.05	0.41 ± 0.03 ^a	0.70 ± 0.05 ^b	0.49 ± 0.01 ^b	0.60 ± 0.04 ^c	0.68 ± 0.04 ^{bc}	0.77 ± 0.05 ^d
	Kidney*	1.05 ± 0.03	0.77 ± 0.05 ^a	1.09 ± 0.02 ^d	1.11 ± 0.19 ^d	0.91 ± 0.12 ^c	0.92 ± 0.02 ^c	0.99 ± 0.09 ^b
GSH (Units/mg protein)	Brain*	2.27 ± 0.09	1.77 ± 0.08 ^a	2.34 ± 0.06 ^c	2.30 ± 0.05 ^c	2.37 ± 0.11 ^d	2.18 ± 0.05 ^b	2.12 ± 0.05 ^b
	Plasma	1.56 ± 0.06	1.20 ± 0.04 ^a	1.34 ± 0.05 ^b	1.32 ± 0.02 ^b	1.39 ± 0.04 ^b	1.38 ± 0.03 ^b	1.42 ± 0.04 ^d
	Liver	1.40 ± 0.02	1.25 ± 0.02 ^a	1.38 ± 0.02 ^b	1.33 ± 0.02 ^b	1.35 ± 0.02 ^b	1.39 ± 0.05 ^b	1.36 ± 0.10 ^b
GST (μmol/min/mg protein)	Kidney	1.49 ± 0.04	1.40 ± 0.07	1.58 ± 0.04	1.49 ± 0.08	1.48 ± 0.07	1.42 ± 0.05	1.51 ± 0.03
	Plasma	0.004 ± 0.00	0.001 ± 0.00 ^a	0.002 ± 0.00 ^b	0.002 ± 0.00 ^b	0.002 ± 0.00 ^b	0.001 ± 0.00 ^a	0.002 ± 0.01 ^b
	Liver	0.004 ± 0.00	0.001 ± 0.00 ^a	0.003 ± 0.00 ^c	0.002 ± 0.00 ^b	0.002 ± 0.00 ^b	0.003 ± 0.00 ^d	0.003 ± 0.00 ^d
MDA (× 10 ⁻⁶ nmol/mg protein)	Kidney	0.017 ± 0.00	0.010 ± 0.00	0.016 ± 0.00	0.017 ± 0.00	0.016 ± 0.00	0.015 ± 0.00	0.016 ± 0.00
	Liver	0.50 ± 0.09	0.80 ± 0.02 ^c	0.46 ± 0.07 ^{ab}	0.29 ± 0.05 ^a	0.33 ± 0.08 ^a	0.40 ± 0.04 ^{ab}	0.41 ± 0.17 ^{ab}
	Kidney	0.62 ± 0.11	0.94 ± 0.09 ^c	0.44 ± 0.19 ^a	0.48 ± 0.03 ^{ab}	0.38 ± 0.06 ^a	0.78 ± 0.08 ^{bc}	0.41 ± 0.07 ^a
Brain	2.19 ± 0.23	2.79 ± 0.80 ^b	2.30 ± 0.30 ^a	2.40 ± 0.50 ^a	1.96 ± 0.70 ^a	2.25 ± 0.30 ^a	2.40 ± 0.50 ^a	

Different superscripts across row indicates significantly different at $P < 0.01$.

* Indicates superscripts across row are significantly different at $P < 0.05$.

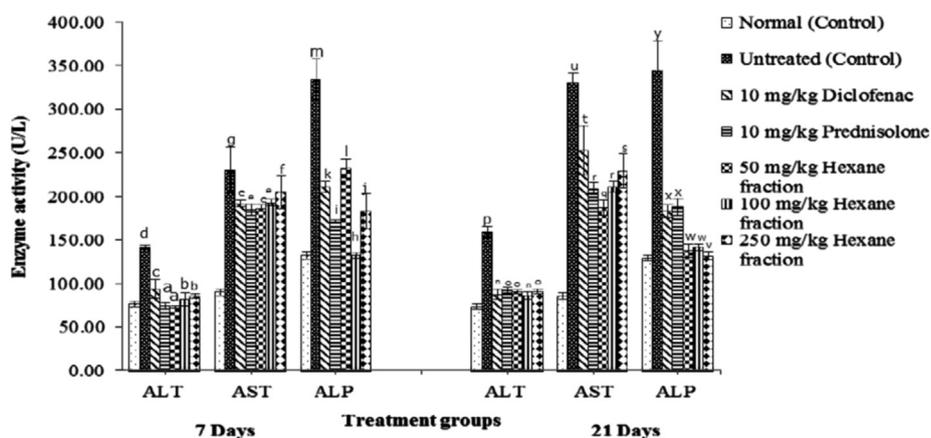


Fig. 5. Effects of different doses of CAHLF on plasma ALT, AST and ALP activities in rats induced arthritis with formaldehyde and Complete Freund's Adjuvant. Different superscripts indicates significantly different at $P < 0.05$.

Table 3

Effects of CAHLF on plasma total bilirubin, protein, globulin and albumin concentrations in rats induced with arthritis using formaldehyde and Complete Freund's Adjuvant.

Treatment groups	Total bilirubin (mg/dl)	Total protein (g/dl)	Globulin (g/dl)	Albumin (g/dl)
7 Days				
Normal	1.42 ± 0.18	6.45 ± 0.19	3.30 ± 0.24	3.05 ± 0.08
Control (0.9% NaCl)	2.60 ± 0.37 ^c	8.23 ± 0.46 ^{d*}	4.53 ± 0.65 ^d	2.83 ± 0.10 ^a
10 mg/kg Diclofenac	2.20 ± 0.45 ^b	7.23 ± 0.23 ^b	3.60 ± 0.20 ^a	3.63 ± 0.05 ^d
10 mg/kg Prednisolone	1.03 ± 0.16 ^a	6.98 ± 0.10 ^a	3.32 ± 0.12 ^a	3.67 ± 0.05 ^d
50 mg/kg CAHLF	2.35 ± 0.19 ^b	7.43 ± 0.10 ^b	4.22 ± 0.13 ^c	3.22 ± 0.04 ^b
100 mg/kg CAHLF	2.18 ± 0.34 ^b	7.33 ± 0.22 ^b	3.82 ± 0.19 ^b	3.52 ± 0.04 ^c
250 mg/kg CAHLF	1.15 ± 0.24 ^a	6.90 ± 0.14 ^a	3.82 ± 0.19 ^b	3.08 ± 0.13 ^b
21 Days				
Normal	1.28 ± 0.12	6.40 ± 0.30	3.40 ± 0.30	3.00 ± 0.00
Control (0.9% NaCl)	3.12 ± 0.17 ^d	9.78 ± 0.40 ^{d*}	5.78 ± 0.39 ^d	2.00 ± 0.23 ^a
10 mg/kg Diclofenac	2.48 ± 0.31 ^c	8.23 ± 0.12 ^c	4.47 ± 0.12 ^c	3.77 ± 0.08 ^d
10 mg/kg Prednisolone	2.17 ± 0.61 ^b	8.32 ± 0.13 ^c	4.57 ± 0.20 ^c	3.77 ± 0.10 ^d
50 mg/kg CAHLF	2.35 ± 0.20 ^b	7.85 ± 0.18 ^b	4.47 ± 0.25 ^c	3.30 ± 0.09 ^b
100 mg/kg CAHLF	2.28 ± 0.23 ^b	7.55 ± 0.22 ^{ab}	4.03 ± 0.20 ^b	3.52 ± 0.04 ^c
250 mg/kg CAHLF	1.58 ± 0.21 ^a	7.27 ± 0.20 ^a	3.73 ± 0.15 ^a	3.53 ± 0.10 ^c

Different superscripts across row indicates significantly different at $P < 0.01$.

* Indicates superscripts across row are significantly different at $P < 0.05$.

of superoxide anion ($O_2^{\bullet-}$) and its derivatives such as hydroxyl radical ($\bullet OH$) (Afonso et al., 2007). SOD is known to convert $O_2^{\bullet-}$ to H_2O_2 (Afonso et al., 2007). CAT is known to detoxify the H_2O_2 generated into water and oxygen. In this present study, the elevation of tissue CAT activity may confer protection against ROS mediated inflammation in the CAHLF treated groups whereas the reduction of tissue CAT enzyme activity in control group would invariably heighten the oxidative stress in tissues. This is because the H_2O_2 generated could be converted to HOCl, an inflammatory agent which could also react with $O_2^{\bullet-}$ to form $\bullet OH$ which may react and damage at a diffusion controlled rate with almost all molecules in living cells (Hitchon and Ei-Gabalawy, 2004). Oxidation of proteins, lipids, DNA and polysaccharides has been shown to be increased in rheumatoid arthritis patients (Hitchon and Ei-Gabalawy, 2004). Glutathione S-transferase (GST) catalyzes the initial reaction involving the conjugation of reactive electrophilic constituent with reduced glutathione (GSH) thereby removing reactive electrophiles and hence protecting vital nucleophilic groups in macromolecules such as proteins and nucleic acids (Singh and Gupta, 2011). Previous studies have also shown that GSH could serve as a sulfhydryl buffer to protect the thio ($-SH$) groups of macromolecules especially, protein from the damaging effects of ROS (Singh et al., 2011). The decreased MDA concentrations in CAHLF treated groups also suggested that CAHLF could be protecting tissue macromolecules from the deleterious effect of ROS mediated lipid peroxidation (Andy et al., 2004).

3.6. Effects of CAHLF on plasma ALT, AST and ALP activities in arthritic rats

Data in Fig. 5 showed that doses of CAHLF at 50, 100 and 250 mg/kg treated groups had a significantly reduced ($P < 0.05$) plasma ALT, AST and ALP when compared with the control groups for 7 and 21 days. This suggests that the CAHLF may possess anti-inflammatory activity against tissue damage. It has been reported that most bioactive compounds with antioxidant activity may also protect tissues against ROS induced tissue damage (Adewusi and Afolayan, 2010). Assessment of the plasma levels of AST, ALT and ALP has been reported to be a good and simple tool to measure the anti-inflammatory activity of target compounds (Kataoka et al., 2002; Edoardo et al., 2005). AST, ALT and ALP are enzymes present in higher concentrations in hepatocytes and some other tissues. The presence of tissue damage causes these enzymes to leak into the blood stream in conformity with the extent of tissue damage (Sacher and Mepherston, 1991; Giannini et al., 2005).

3.7. Effects of CAHLF on plasma TB, TB, globulin and albumin in arthritic rats

Data in Table 3 showed that 50, 100 and 250 mg/kg CAHLF had a significantly ($P < 0.05$) reduced plasma total bilirubin, total protein and globulin concentrations compared to the control

groups in the 7 and 21 days treatments. Conversely, the plasma albumin concentrations in CAHLF treated groups were significantly elevated ($P < 0.05$) when compared with control group in the 7 and 21 days treatment. This also suggests that CAHLF may possess hepatoprotective potential. The measurements of plasma bilirubin, protein, globulin and albumin concentrations are acceptable biomarkers for the assessment of the functional state of the liver and could also serve as an indicator of pathogenicity or inflammation (Tetik et al., 2010). Elevated plasma bilirubin concentrations have been reported in rheumatoid arthritis patients as there was a positive correlation between the activation of rheumatic disease and the expansion of hepatobiliary dysfunction (Niino-Nanke et al., 1998). The ability of the host to produce antibodies (immunoglobulins) during inflammation may be reflected in the increased concentration of plasma globulin which would in turn elevate plasma total protein concentration as observed in the control group when compared with the treated groups. Elevated level of total protein has been reported to be found in the plasma of rheumatoid arthritis patients (Alturfan et al., 2007; Tetik et al., 2010).

Furthermore, it has been reported that a decline the concentration of plasma albumin serves as an indicator of liver inflammation. The liver is the site of albumin synthesis and damage to the liver tissue would invariably affect the plasma albumin concentration (Philip, 1994). This further suggests that CAHLF possesses anti-inflammatory activity against tissue damage.

4. Conclusion

The CAHLF exhibited a substantial anti-inflammatory activity *in vivo* which could be possibly through inhibition of prostaglandin synthesis and scavenging of perpetuating ROS generated during inflammation. Thus, CAHLF could be considered as an important source of anti-inflammatory compounds for pharmaceutical drug development. Further studies are required to gain complete knowledge of the CAHLF anti-inflammatory mechanism of action.

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