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Original Research

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Hexane fraction of *Costus afer* ker Gawl leaves inhibited mitochondrial membrane permeability transition, F_1F_0 adenosine triphosphatase and scavenged nitric oxide and hydrogen peroxide

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ABSTRACT

Introduction: *Costus afer* ker Gawl. is an indigenous medicinal plant used as therapy in the treatment of inflammatory disease like rheumatoid arthritis. **Objective:** This study was designed to investigate the chemoprotective potentials of the hexane fraction of *C. afer* leaves (CAHLF) on some biochemical biomarkers such as mitochondrial membrane permeability transition (MPT), F_1F_0 adenosine triphosphatase (ATPase), nitric oxide ($NO^{\bullet}$) and hydrogen peroxide (H_2O_2) activities using spectrophotometric methods. **Results:** Results showed that isolated mitochondria fractions from normal rats preloaded with 10-60 μg/ml CAHLF significantly ($P < 0.05$) inhibited Ca^{2+}-induced MPT by 85.66-90.47% respectively *in vitro*. Furthermore, *in vivo* study showed that mitochondria fractions isolated from formaldehyde-induced arthritic rats, orally treated with 50-250 mg/kg body weight CAHLF for 7 days significantly ($P < 0.05$) inhibited mitochondrial MPT by 52.22-65.0% respectively. Furthermore, there was a significant reduction ($P < 0.05$) in mitochondrial F_1F_0 ATPase activity (3.93 $\pm$ 0.01 mg/ml inorganic phosphate [Pi]) at pH 7.4 by 10 μg/ml CAHLF compared with F_1F_0 ATPase activity (6.60 $\pm$ 0.03 mg/ml Pi) by 10 μg/ml 2,4-dinitrophenol. In another study, varied concentrations of CAHLF scavenged $NO^{\bullet}$ and H_2O_2 in a concentration dependent manner with a 50% inhibitory concentration of 1.77 mg/ml and 0.33 mg/ml respectively. **Conclusion:** These data indicated that CAHLF possesses the capacity to inhibit mitochondrial MPT, F_1F_0 ATPase, $NO^{\bullet}$and H_2O_2 activities. This could further explain the medicinal potentials of CAHLF, thereby indicating that CAHLF could be a choice candidate in the drug discovery process.

KEY WORDS: *Costus afer*, hydrogen peroxide, mitochondria, nitric oxide

INTRODUCTION

Inflammation is a complex innate immune response to infection, autoimmune injury, trauma and other noxious insults [1]. Several human chronic diseases are associated with inflammation characterized by excessive generation of reactive oxygen species (ROS) [2]. In degenerating cells, mitochondria have been implicated as a major site for the production of ROS [3]. It has been reported that inflammatory responses by leukocytes to the localized site of tissue injury are often flared up by the presence of the pathogen associated molecular patterns or damage associated molecular patterns (DAMPs) [4]. In

recent times, mitochondria have emerged as a source of DAMPs and thus contribute to the DAMPs-mediated inflammatory response [5]. Hence, ROS derived from mitochondria might lead to the modification of DAMPs that can modulate immune responses and contribute to the development of inflammatory diseases [6].

Mitochondrial permeability transition has been shown to be a critical event in apoptotic and necrotic cell death that characterized many of the inflammatory disorders [7]. It refers to the massive swelling and depolarization of mitochondria that occurs under certain conditions such as Ca^{2+} overload and oxidative stress [8]. A major consequence of the membrane permeability transition (MPT) is the uncoupling of oxidative phosphorylation. This would inhibit adenosine triphosphate (ATP) synthesis and also, stimulate mitochondrial proton-translocating adenosine triphosphatase (ATPase) to actively hydrolyze ATP produced. Unrestrained, this will inevitably lead to cell death [9]. Many biological agents that target pathways in apoptosis are currently being developed [10]. This is because the biological or chemical agent that protects the mitochondrial membrane from damage would serve as an important therapeutic agent against mitochondrial mediated inflammations or diseases [11,12].

Costus afer ker Gawl. of the Family Costaceae, is one of the 150 species of tall, perennial, and rhizomatous herbs [13]. It is found in moist or shady forests and river banks of tropical West Africa. It is commonly called gingerlily and in the Western part of Nigeria, it is called different names such as; "ireke omode" or "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" [14]. The leaves of *C. afer* are used locally as either prophylactic or therapeutic agent in the management of inflammatory disorders especially rheumatism and arthritis [14-16]. Researches had shown that *C. afer* possesses antioxidant, hepatoprotective and anti-inflammatory activities [13,16-18]. We have recently reported the potent anti-inflammatory fraction of *C. afer* leaves as hexane fraction (CAHLF)[19]. The hexane fraction chemical constituents- naphthalene 2,3 dimethyl, naphthalene 1,6 dimethyl, phenol 2,4-bis (1,1-dimethylethyl), phytol, 2(4H)-benzofuranone 5,6,7,7a-tetrahydro 4,4,7a-trimethyl, pentadecanoic acid, hexadecanoic acid methylester, n-hexadecanoic acid, linoleic acid, α-linolenic acid and cis-vaccenic were identified using gas chromatography/mass spectrometry analytical method [20]. Thus, this study was designed to investigate the chemoprotective effects of the CAHLF on mitochondrial MPT, F_1F_0 ATPase, nitric oxide ($NO^{\bullet}$) and hydrogen peroxide (H_2O_2) activities. This was with the rationale to proffer a scientific explanation for its folkloric use as therapy against inflammatory diseases.

MATERIALS AND METHODS

Collection of Plant Materials

C. afer was obtained from a farm land at Irolu, 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, Ilisan-Remo, Ogun State, Nigeria. A voucher sample with a number of FHI-108001 has been deposited at Forestry Herbarium Ibadan (FHI).

Plant Processing, Extraction and Partitioning

The leaves were plucked from the plant stem and air-dried under room temperature. The dried leaves were pulverized using a mechanical grinder. Pulverized leaf sample (300 g) was extracted using 1800 ml of 70% methanol at room temperature (cold extraction) with intermittent shaking for 48 h. The extract was filtered using Whatman No.1 filter paper, and the filtrate was subsequently concentrated using a rotary evaporator at 30°C (Buchi Rotavapor RE; Switzerland). The concentrate was reconstituted in distilled water in a ratio of 1:2 (concentrate: Distilled water) and further partitioned using n-hexane. The n-hexane fraction was concentrated using a rotary evaporator at 30°C and stored in the refrigerator until further use.

Animals

Twenty male albino rats (Wistar strain) weighing between 150 g and 220 g were obtained from inbred colony at the preclinical animal house, Physiology Department, the University of Ibadan, Ibadan, Nigeria. The animals were acclimatized within 2 weeks at Babcock University Animal House. Animals were maintained by following the National Institute of Health good laboratory animal care guidelines and approval was given by the Department Animal Ethics Committee.

Acute Toxicity Study

The acute toxicity study of the CAHLF was performed using the median lethal dose of fraction in albino rats following the Organization for Economic Cooperation and Development guideline 425 [21]. A male Wistar albino rat was administered 2000 mg/kg p.o. 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 50-250 mg/kg body weight (b.w) were adopted for further studies.

Formaldehyde-Induced Arthritis

Formaldehyde-induced arthritis model as described by Gupta *et al.* [22]was adopted in this study. Fifteen male rats were randomly assigned into five groups of three rats each. Formaldehyde (2% v/v) 0.02 ml, was injected in the 1st and 3rd day into the left hind paw just beneath the plantar aponeurosis to induce arthritis. Hexane fraction and normal saline were orally administered to the animals using appropriate oral dosing needles once a day for 7 days as stated below.

Group I : 1 ml of 0.9% NaCl (normal).
Group II : 1 ml of 0.9% NaCl + 0.02 ml 2% formaldehyde (control).
Group III : 50 mg/kg b.w. CAHLF + 0.02 ml 2% formaldehyde.
Group IV : 100 mg/kg b.w. CAHLF + 0.02 ml 2% formaldehyde.
Group V : 250 mg/kg b.w. CAHLF + 0.02 ml 2% formaldehyde.

The drugs were administered for 7 days. At the expiration of the treatment period, the animals were starved overnight, euthanized by cervical dislocation and sacrificed to obtained rat liver for the isolation of mitochondria fraction. The isolated mitochondria fractions were subsequently subjected to the standard protocol for mitochondrial MPT assessment. Furthermore, liver mitochondria isolated from the remaining five normal rats were incubated with 10, 20, 40 and 60 μg/ml of 0.1 mg/ml CAHLF and used for the *in vitro* mitochondrial MPT analysis.

Mitochondrial Fraction Isolation

Mitochondrial fraction from rat liver was isolated by conventional differential centrifugation in a buffer solution containing 210 mM mannitol, 70 mM sucrose, 5 mM 2-(4-[2-hydroxyethyl] piperazin-1-yl) ethanesulfonic acid (HEPES) at pH 7.4 and 1 mM ethylene glycol tretraacetic acid (EGTA). In the final wash solution, EGTA was omitted [23]. Mitochondrial protein content was determined by Folin–Ciocateau method using bovine serum albumin as standard protein [24].

Assessment of Mitochondrial Membrane Permeability Transition

Mitochondrial membrane permeability transition was assessed according to the method of Lapidus and Sokolove [25]. Change in absorbance of mitochondria was monitored at 540 nm in a double beam ultra violet/visible (UV/visible) spectrophotometer (T80 model, PG instrument). Mitochondrial fraction (0.4 mg/ml) were suspended in a medium containing 210 mM mannitol, 70 mM sucrose, 5 mM HEPES-potassium hydroxide (pH 7.4), 0.8 μM rotenone and 5 mM succinate. MPT was triggered using Ca^{2+} while spermine a polyamine served as an inhibitor of MPT.

Assessment of Mitochondrial F_0F_1 ATPase Activity at pH 7.4

Mitochondrial F_1F_0 ATPase activity was determined using a modified method described by Lardy and Wellman [26]. Each reaction vessel contained 1300 μl of 65 mM Tris-HCl (pH 7.4), 40 μl of 1 mM ATP and 250 μl of 25 mM sucrose, 200 μl of 0.5 mM KCl and 10-80 μl of 0.1 mg/ml CAHLF or standard uncoupler 10 μl of 0.1 mg/ml 2,4-dinitrophenol (DNP) and made up to a final volume of 2000 μl with distilled water. The reaction was started by the addition of 50 μl mitochondrial fraction (0.4 mg/ml) which was placed in a vortex machine for 30 min at 25°C. The reaction was stopped by the addition of 8 ml of 10% trichloroacetic acid to each test tube and then centrifuged at 3000 g. The deproteinized supernatant was kept for inorganic phosphate (P_i) determination.

Determination of P_i

The concentration of P_i released was determined according to a modified method described by Fiske and Subarrow [27]. Different concentrations of $KHPO_4$ (0.2, 0.4, 0.6 and 1.0 ml of 1.0 mg/ml) was mixed with 1.0 ml of 5% ammonium molybdate and made up to 4.0 ml with distilled water. To the reaction mixture was added 1.0 ml of 0.2% ascorbic acid. The reaction mixture was allowed to stand for 20 min before it was read in a double beam UV/visible spectrophotometer (T80 model, PG Instrument) at 660 nm. The absorbance obtained was used to plot the standard phosphate curve at $Y = 0.0712x-0.0048$, $R^2 = 0.9996$ where Y is the absorbance value and x is the concentration in mg/ml of $KHPO_4$. Similarly, 4.0 ml of the deproteinized supernatant in a test tube was added 1.0 ml of 5% ammonium molybdate and 1.0 ml of a freshly prepared solution of 0.2% ascorbic acid. The reaction mixture was allowed to stand for 20 min before it was read in a double beam UV/visible spectrophotometer at 660 nm. The concentration of P_i released was extrapolated from the equation of the standard phosphate curve. This was done in triplicate.

$NO^{\bullet}$ Radical Scavenging Activity

The $NO^{\bullet}$ radical scavenging activity of CAHLF was based on the method described by Ebrahimzadeh *et al.* [28]. Two ml of 10 mM sodium nitroprusside dissolved in 0.5 ml phosphate buffer saline pH 7.4 was added to 1 ml of 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml the CAHLF or standard ascorbic acid. The volume was made up to 1 ml using 80% methanol. The control test tubes contained 80% methanol without extract. The mixture was incubated at 25°C for 150 min. An aliquot of 0.5 ml of the solution was withdrawn and mixed with 0.5 ml Griess reagents (1.0 ml sulfanilic acid reagent [1% sulphanilamide in 2% H_3PO_4 and 0.1 ml of 0.1 % w/v N-1-Naphthylethylenediamine dihydrochloride]). The reaction mixture was incubated at room temperature for 30 min, after which absorbance of the chromophore during the diazotization of nitrite with sulfanilamide and subsequent coupling with napthylethylenediamine was read at 540 nm. Ascorbic acid served as a positive control.

H_2O_2 Scavenging Activity

The capacity of the CAHLF to scavenge H_2O_2 was determined according to the method adopted by Karunakaran and Kumaran [29]using ascorbic acid as standard. A solution of 4 mM H_2O_2 was prepared in phosphate buffer (0.1 M pH 7.4). The hexane fraction (4 ml) was prepared in methanol at varying concentrations (0.025-0.5 mg/ml) and mixed with 0.6 ml of 4 mM H_2O_2 solution prepared in phosphate buffer solution. The absorbance of H_2O_2 at 230 nm was determined after 10 min against a blank solution containing the hexane fraction without H_2O_2. The result obtained was compared with standard ascorbic acid.

The percentage inhibition was calculated using the formula

$$\%\text{inhibition} = \left[\frac{(A_{control} - A_{sample})}{A_{control}}\right] \times 100$$

Where,

$A_{control}$ = Absorbance of control

A_{sample} = Absorbance of the test fraction/standard.

The 50% inhibitory concentration (IC_{50}) was extrapolated from a linear regression plot of the percentage inhibition against concentration of the test fraction/standard. The assay was performed in triplicates.

Statistical Analysis

Statistical analysis was carried out with the aid of SPSS for Windows; SPSS Inc., Chicago, Standard version 17.0 to determine the difference between mean using analysis of variance. $P < 0.05$ was considered significant. IC_{50} was extrapolated from the linear regression equation. All studies were conducted in triplicate and reported as mean ± standard error of the mean.

RESULTS AND DISCUSSION

As shown in Figure 1, the data indicated that the isolated mitochondria fraction were intact in the absence of Ca^{2+} ion in a normal respiration sucrose phosphate buffer. However, mitochondrial MPT was induced, when mitochondria fractions were incubated with Ca^{2+} ion, while spermine inhibited the Ca^{2+} induced mitochondrial MPT. It has been reported that an elevated intra cytosolic Ca^{2+} promotes mitochondrial MPT, while spermine inhibits MPT pore formation [25,30].

The data in Figure 2 showed that the incubation of mitochondria fractions with 10, 20, 40 and 60 μg/ml CAHLF and Ca^{2+} *in vitro* for 12 min significantly ($P < 0.05$) inhibited MPT by 85.66%, 86.93%, 87.47% and 90.47%, respectively. This suggests that the CAHLF possesses the capacity to protect the mitochondria from stimuli that could trigger the induction of a mega pore or permeability transition, which could eventually lead to rupture of the outer mitochondrial membrane with the release of intra-mitochondrial components. These released intra-mitochondrial components could activate the caspase-dependent or independent cascade pathway, collapse the electrochemical gradient and generate ROS with the resultant death of the cell [31,32].

In vivo study showed that varying doses of 50, 100 and 250 mg/kg b.w. CAHLF significantly ($P < 0.05$) inhibited mitochondrial MPT by 52.22%, 61.11% and 65.0% respectively in a formaldehyde-induced arthritic rats compared with mitochondrial MPT of untreated arthritic rats [Figure 3]. This further strengthens the observation in vitro that CAHLF may be conferring protection

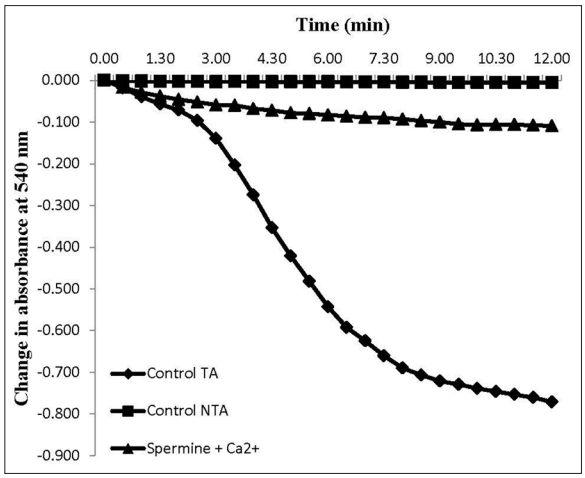

Figure 1: Effect of the presence and absence of triggering agent Ca^{2+} on mitochondrial membrane permeability transition pore energized sodium succinate and inhibited by spermine monitored as swelling at Δ540 nm for 12 min

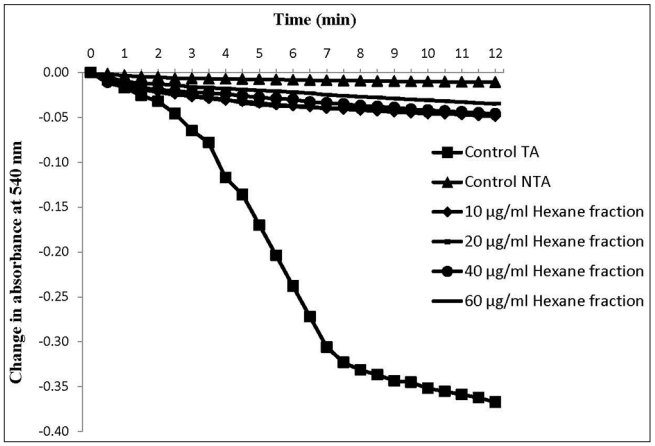

Figure 2: Inhibitory effect of 10, 20, 40, 60 µg/ml of the hexane fraction of *Costus afer* leaf on Ca^{2+}- induced mitochondrial membrane permeability transition pore energized by succinate in 12 min at Δ540 nm

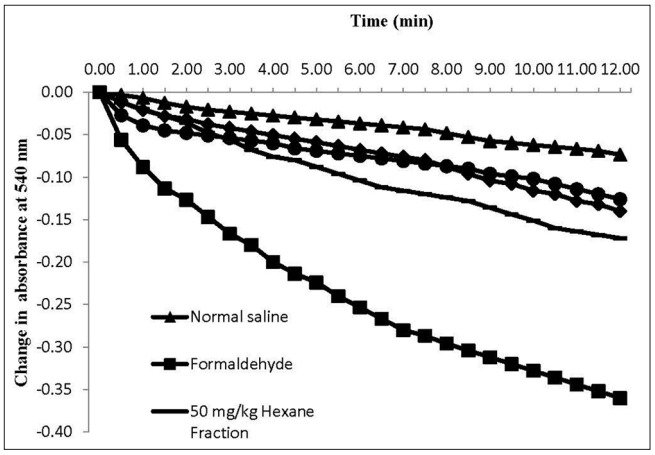

Figure 3: Inhibitory effect of 50-250 mg/kg b.w. hexane fraction of *Costus afer* leaf on Ca^{2+}-induced mitochondrial membrane permeability transition pore energized by succinate in 12 min at Δ540 nm *in vivo*

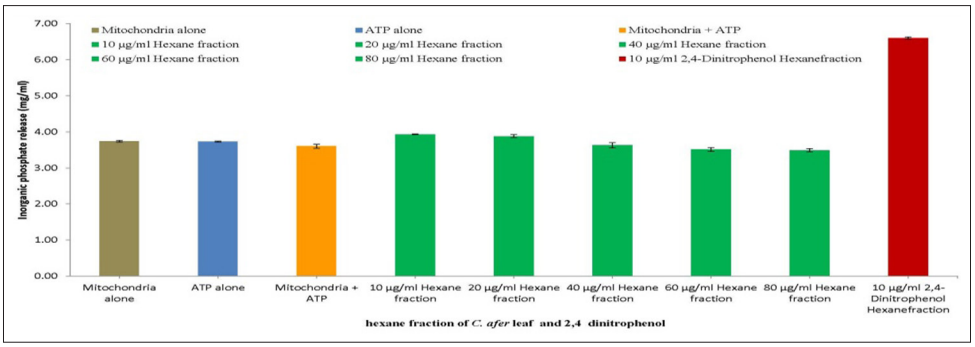

Figure 4: Effect of the hexane fraction of *Costus afer* leaf and 2,4-dinitrophenol on mitochondrial adenosine triphosphatase activity

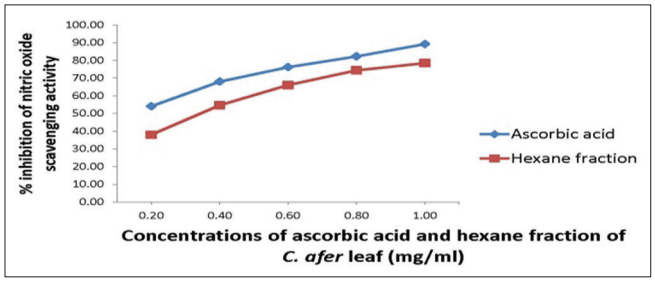

Figure 5: Nitric oxide scavenging activity of hexane leaf fraction and ascorbic acid

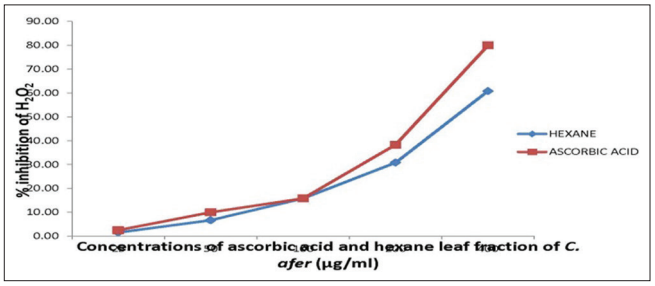

Figure 6: Hydrogen peroxide scavenging activity of hexane lead fraction and ascorbic acid

against mitochondria damage induced by noxious signals. However, the percentage inhibitions of mitochondrial MPT analysis in vivo were reduced when compared with those of the in vitro study. This reduction could be explained as the hexane fraction might have undergone hepatic first pass metabolism, which may account for the reduced MPT inhibition observed in vivo [33]. The capacity of CAHLF to inhibit the formation of MPT pore might be attributed to the bioactive compounds present in the CAHLF [20]. These compounds are known to specifically possess anti-inflammatory, antioxidant and cation chelating potentials which may be stabilizing mitochondrial membranes [34-36].

Further investigation of the effect of varying concentrations of 10, 20, 30, 40, 60 and 80 μg/ml CAHLF on mitochondrial F_1F_0 ATPase activity at physiological pH revealed that the incubated mitochondria released 3.93 ± 0.01, 3.88 ± 0.04, 3.63 ± 0.07, 3.51 ± 0.05 and 3.49 ± 0.04 mg/ml P_i respectively while 10 μg/ml DNP induced the liberation of 6.60 ± 0.03 mg/ml P_i [Figure 4]. The concentration of P_i released by CAHLF treated mitochondria was found not to be significantly different ($P > 0.05$) from control tests without treatment (mitochondria alone, ATP alone, mitochondria + ATP alone)[Figure 4]. This indicates that CAHLF might not have a stimulatory effect on mitochondrial F_1F_0 ATPase activity. In a damaged cell undergoing apoptosis or inflammation, a collapse in mitochondrial electrochemical gradient usually results in the hydrolysis of ATP to adenosine diphosphate and P_i by ATPase [37,38]. The depletion of intracellular ATP by mitochondrial F_1F_0 ATPase leads to alteration of ionic homeostasis and prolong MPT pore opening; this could lead to irreversible cellular damage, necrosis and inflammation [39]. The preservation of the constituents of mitochondrial electron transport chain is paramount in maintaining the bioenergetics status of the mitochondrion and indeed cell homeostasis [11].

Figure 5 shows different concentrations of CAHLF (0.2-1.0 mg/ml) scavenged NO• radical in a concentration-dependent manner with an IC50 of 1.77 mg/ml. Hexane leaf fraction at 1.0 mg/ml inhibited NO• by 78.48%, while 0.2 mg/ml inhibited NO• by 38.03%. Furthermore, the standard ascorbic acid also scavenged NO• in a concentration-dependent with an IC50 of 0.77 mg/ml. The ascorbic acid at 1.0 mg/ml inhibited NO• by 89.24% ± 0.00, while 0.2 mg/ml ascorbic acid inhibited NO• by 54.09%.

Furthermore, CAHLF at 25, 50, 100, 200, and 400 μg/ml had a concentration-dependent inhibition of H_2O_2 by 1.5%, 6.67%, 15.83%, 30.83%, and 60.83% respectively with an IC_{50} of 0.33 mg/ml. Similar concentrations of standard ascorbic acid also had a concentration-dependent inhibition of H_2O_2 by 2.5%, 10%, 15.83%, 38.33%, and 80.0% with an IC_{50} of 0.26 mg/ml [Figure 6]. This indicated that CAHLF possesses the capacity to scavenge NO• and H_2O_2.

NO• and H_2O_2 are also part of the pro-inflammatory mediators released at the site of inflammation especially during edema formation in rheumatoid arthritis and can be cytotoxic to DNA, protein, lipids and carbohydrates, leading to impaired cellular function and enhanced inflammatory response [40]. It has been reported that the inhibitors of NO• and H_2O_2 activities could also reduce the development of arthritis [41,42]. This further supports the previous report that CAHLF may possess antioxidant and anti-inflammatory activities [19].

Therefore, data from this study suggested that the CAHLF inhibited mitochondrial membrane permeability transition pore formation, F_1F_0 ATPase activity and scavenged $NO^{\bullet}$ and H_2O_2 radicals. This may further account for the purported therapeutic and preventive potentials of *C. afer* leaves used in folk medicines against inflammatory diseases, especially rheumatoid arthritis.

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