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CTLSR 1 (2): 1-19 (December, 2022)

Current Trends in Life Science Research

ISSN: 2814-1679

In vitro antioxidant and anti-inflammatory activities of C*ostus igneus* leaf fractions

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Abstract

Costus igneus Nak is an ethnobotanical plant used in the management of several inflammatory and oxidative stress-related disorders. There is a need to scientifically validate its use in ethnomedicine. This study investigated the antioxidant and anti-inflammatory activities of fractions from ethanol extract of *C. igneus* leaf using *in vitro* methods. Crude ethanol extract of *C. igneus* leaf was successively partitioned to obtain hexane, ethyl acetate, butanol and aqueous fractions. The fractions were screened for *in vitro* anti-inflammatory activity using erythrocyte membrane stabilization and inhibition of protein denaturation assays. *In vitro* antioxidant activity was carried out using 1-1-diphenyl, 2-picryl hydrazyl (DPPH), nitric oxide and hydrogen peroxide radical scavenging assays. Data were analyzed using GraphPad Prism 6.0 and *p* value was set at 0.05. The percentage yield of the crude ethanol extract of *C. igneus* leaf was 25.40%. The aqueous fraction (37.05%) had the highest percentage yield, followed by butanol fraction (34.33%), hexane fraction (16.58%) and the ethyl acetate fraction (12.04%). Anti-inflammatory studies showed that *C. igneus* butanol fraction (CIBF) had the highest erythrocyte membrane stabilizing effect (IC50 = 140.61 µg/mL) and highest percentage inhibition of protein denaturation (IC50 = 20.40 µg/mL) when compared to the hexane, ethyl acetate and aqueous fractions. Antioxidant study also showed that CIBF had the highest percentage inhibition of DPPH radicals (IC50 = 18.36 µg/mL), highest nitric oxide radical scavenging activity (IC50 = 67.26 µg/mL) and highest hydrogen peroxide radical scavenging activity (IC50 = 160.50 µg/mL) when compared to the hexane, ethyl acetate and aqueous fractions. The findings showed that CIBF demonstrated remarkable anti-inflammatory and antioxidant activities *in vitro*.

Key words: *Costus igneus*, Antioxidant, Anti-inflammatory, *In vitro*, Butanol fraction.

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Introduction

Inflammation and free radical generation are important biological processes which have been implicated in the pathogenesis of many diseases (Biswas, Das & Banerjee, 2017). Disease management strategies involve tackling underlying mechanisms such as inflammatory conditions and oxidative stress which are involved in the development process of the disease (Arulselvan et al., 2016). Treatment with conventional drugs have been reported to produce deleterious side effects and have not been able to eradicate all disease cases completely. Studies on the pharmacological activities of plants are therefore increasingly being explored in order to find alternatives to the use of conventional drugs for the management of diseases, as medicinal plants have been reported to have little or no toxic effects compared to most synthetic drugs (Santarelli, Neri, Carbone, Macchioni & Pittia, 2022)..

Costus igneus Nak belonging to the family *Costaceae*, has been used traditionally for the management of diseases. Phytochemical screening of aqueous and methanol extracts of *C. igneus* leaves showed that it contains flavonoids, tannins, saponins, alkaloids, glycosides, lignins, phytosterol and antioxidant components including ascorbic acid, α-tocopherol and β-carotene (Malleswari, Kausar & Bagyanarayana, 2019).

Qualitative phytochemical screening of *C. igneus* leaves revealed that it is rich in protein, iron, and antioxidant components such as ascorbic acid, α-tocopherol, β-carotene, terpenoids, steroids, and flavonoids (Shankarappa, Gopalakrishna, Jagadish & Siddalingappa, 2011). Findings from another study showed that the methanol extract of *C. igneus* leaves was found to contain the highest number of phytochemicals such as carbohydrates, triterpenoids, proteins, alkaloids, tannins, saponins, and flavonoids (Jothivel et al., 2007).

Preliminary phytochemical evaluation of *C. igneus* revealed that the leaves contain 21.2% fibers. Successive extracts gave 5.2% extractives in petroleum ether, 1.06% in cyclohexane, 1.33% in acetone, and 2.95% in ethanol. Analysis of successive extracts showed presence of steroids in all extracts. The ethanol extract also contained alkaloid. The major component of the ether fraction was bis (2'-ethylhexyl)-1, 2-benzenedicarboxylate (59.04%) apart from α-tocopherol and a steroid, ergastanol (George, Thankamma, Rema & Fernandez, 2007). The *in vitro* antioxidant and anti-inflammatory activities of *C. igneus* leaf fractions have not been exhaustively explored. This study therefore investigated the anti-inflammatory and antioxidant activities of hexane, ethyl acetate, butanol and aqueous fractions of *C. igneus* leaf crude ethanol extract using *in vitro* methods.

MATERIALS AND METHODS

Chemicals and Reagents

Chemicals and reagents used were of analytical grade.

Plant Collection and Authentication

Fresh *Costus igneus* leaves were obtained from the Horticultural Unit of the Department of Agriculture, Babcock University, Ilishan-Remo, Ogun State. Plant authentication was done at the Forest Herbarium, Ibadan, Oyo State with voucher number: FHI. 112487. The leaves were oven-dried at 40 °C in a hot air oven (Uniscope SM9053 Oven, Surgifield Medicals England, Okehampton, UK). When completely dried, the leaves were ground into coarse powder using an electric grinder (Binatone BLG-450, Binatone Electronics International Ltd., Shanghai, China) and stored in tightly sealed glass jars.

Preparation of Extract and Fractions

One hundred grams of pulverized *C. igneus* leaves was soaked in a glass jar with 800 mL of 70% ethanol, shaken intermittently and filtered after 48 hours. The filtrate was then concentrated at 40 °C using a rotary evaporator (Buchi Rotavapor RE, Switzerland), and stored in a freezer at -4 °C as the crude ethanol extract which was further fractionated by solvent partitioning following the successive solvent partitioning method with the use of a separating funnel, as described by Sarker, Latif and Alexander (2005). Ten (10) grams of the crude ethanol extract was reconstituted in 100 mL distilled water and poured into a separating funnel. An equal volume of hexane (100 mL) was then added to the reconstituted solution in the separating funnel to get two distinct layers

with similar volumes. The mixture was shaken vigorously and then suspended to allow for the separation of the two solvents. After complete separation of the two solvent layers was observed, the lower layer (aqueous layer) was collected into a beaker while the upper layer (hexane fraction) was collected into a separate beaker. The aqueous layer was then poured into the separating funnel again and re-partitioned twice with another 50 mL each of hexane. The resulting hexane fractions were added to the previously collected hexane fraction in the beaker. The aqueous layer obtained was subsequently partitioned successively using ethyl acetate and butanol to obtain the ethyl acetate and butanol fractions respectively while the remaining portion was retained as the aqueous fraction. The fractions were then concentrated at 40 °C using a rotary evaporator. The percentage yields of the crude extract and fractions of *C. igneus* leaves were then calculated.

In Vitro Anti-Inflammatory Study of *C. igneus* Leaf Fractions

The *in vitro* anti-inflammatory activity of *C. igneus* leaf fractions was carried out using human erythrocyte membrane stabilization and inhibition of protein denaturation assays.

Membrane Stabilization Assay

The human red blood cell membrane stabilization assay was performed by adopting the method described by Oyedapo, Akinpelu, Akinwunmi, Adeyinka and Sipeolu (2010). Blood samples (10 mL) was collected through venepuncture from a relatively healthy human volunteer and transferred into sample bottles containing EDTA. The collected blood was centrifuged at 3000 g for 10 minutes. The supernatant was removed carefully and the packed red blood cell remaining was washed three times with freshly prepared normal saline. The erythrocyte suspension was prepared by diluting the washed red blood cell to 10% v/v using phosphate buffer saline (0.1 M, pH 7.4). An aliquot (100 µL) of 10% RBC was added to 100 µL of the fraction at varying concentrations (100, 200, 300, 400 and 500 µg/ mL). The resulting solution was heated at 56°C for 30 minutes, followed by centrifugation at 2500 g for 10 minutes at room temperature. The supernatant was collected and absorbance was read at 560 nm. An equal volume (100 µL) of acetyl salicylic acid (0.1 mg/mL) was used as a positive control. Percent membrane stabilization was calculated using the formula below:

$$\% \text{ Stabilization} = 100 - [\frac{A1}{A0} X\ 100]$$

Where A1 is the absorbance of the test, A0 is the absorbance of the control.

Inhibition of Protein Denaturation

Inhibition of protein denaturation was evaluated by the method described by Sakat, Juvekar and Gambhire (2010). Five hundred microliters of 1% bovine serum albumin was added to 100 µL of *C. igneus* leaf fractions at varying concentrations (100, 200, 300, 400 and 500 µg/ mL). This mixture was incubated at room temperature for 10 minutes, followed by heating at 51°C for 20 minutes. The resultant solution was allowed to cool down to room temperature and absorbance obtained was recorded at 660 nm. Varying concentrations of acetyl salicylic acid (100, 200, 300, 400 and 500 µg/ mL) served as positive control. The experiment was carried out in triplicates and percent inhibition for protein denaturation was calculated using the formula below:

$$\% \text{ Inhibition} = \frac{(A0-A1)}{A0} X\ 100$$

Where A0 is the absorbance of the control and A1 is the absorbance of the test.

In Vitro Antioxidant Study of *C. igneus* Leaf Fractions

The *in vitro* antioxidant activity of *C. igneus* leaf fractions was determined using 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay, nitric oxide radical scavenging assay and hydrogen peroxide radical scavenging assay.

DPPH Radical Scavenging Assay

The effect of the *C. igneus* leaf fractions on DPPH radical was performed using the method described by McCune and Johns (2002). DPPH solution (0.3 mM) was prepared by dissolving 11 mg of DPPH in 100 mL of methanol. The reaction mixture (3.0 mL) consisted of 1.0 mL of DPPH in methanol, 1.0 mL of *C. igneus* leaf fractions (100, 200, 300, 400 and 500 µg/mL) and 1.0 mL of methanol. This was then incubated for 10 minutes in the dark after which the absorbance was measured

at 517 nm. Ascorbic acid (100, 200, 300, 400 and 500 µg/mL) was used as standard. The percentage antioxidant potential was calculated using the formula:

$$\% \text{ antioxidant potential} = \frac{(A0-A1)}{A0} X\ 100$$

Where A0 is the absorbance of the control and A1 is the absorbance of the test.

Nitric Oxide Radical (NO) Scavenging Assay

The effect of the *C. igneus* leaf fractions on nitric oxide radical was performed following the method described by Green et al. (1982). An aliquot of 3.0 mL of 10 mM sodium nitroprusside in phosphate buffer was added to 2.0 mL of *C. igneus* leaf fractions and reference compound (gallic acid) in different concentrations (100, 200, 300, 400, 500 µg/mL). The resulting solutions were incubated at 25 °C for 60 minutes. A similar procedure was repeated with methanol as blank which served as control. An aliquot of 5.0 mL of the incubated sample was taken out and 5.0 mL of Griess reagent (1% sulphanilamide, 0.1% naphthyethylene diamine dihydrochloride in 2% H_3PO_3) was added to it. The absorbance of the chromophore (purple azo dye) formed during the diazotisation of nitrite ions with sulphanilamide and subsequent coupling with naphthylethylene-diaminedihydrochloride was measured at 540 nm. Percentage inhibition of the nitrite oxide generated was calculated using the formula:

$$\% \text{ inhibition of nitric oxide radical} = \frac{(A0-A1)}{A0} X\ 100$$

Where A0 is the absorbance of the control and A1 is the absorbance of the test.

Hydrogen Peroxide Radical Scavenging (H_2O_2) Assay

The effect of *C. igneus* leaf fractions on hydrogen peroxide radical was performed according to the method described by Ruch, Cheng and Klaunig (1989). A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (50 mM, pH 7.4). An aliquot of 0.5 mL *C. igneus* leaf fractions (50, 100, 200, 400, 500 µg/mL) was added to 0.5 mL hydrogen peroxide in phosphate buffer and the reaction mixture was vortexed. After 10 minutes of reaction time, the absorbance was measured at 230 nm against a blank solution containing phosphate buffer without hydrogen peroxide. Ascorbic acid (50, 100, 200, 400, 500 µg/mL) was used as standard. The percentage hydrogen peroxide radical scavenging activity was calculated using the formula:

$$\% \ H_2O_2 \text{ radical scavenging activity} = \frac{(A0-A1)}{A0} X\ 100$$

Where A0 is the absorbance of the control and A1 is the absorbance of the test.

RESULTS

Percentage Yield of Different Fractions of *Costus igneus* Ethanol Extract

Table 1 shows that the percentage yield of the crude 70% ethanol extract of *C. igneus* leaf in 100 g dry sample was 25.40%. The aqueous fraction (37.05%) had the highest percentage yield, while the ethyl acetate fraction (12.04%) had the lowest percentage yield.

Table 1: Percentage yield of 70% ethanol extract and different fractions of *C. igneus* leaf in dry sample

Crude Extract/ Fraction	**% Yield**
Crude Extract	
70% Ethanol	25.40
Fractions	
Aqueous	37.05
Butanol	34.33
Hexane	16.58
Ethyl acetate	12.04

***In vitro* Anti-inflammatory Activity of *C. igneus* Leaf Fractions**

Stabilization of Erythrocyte Membrane against Heat-induced Haemolysis by *C. igneus* Leaf Fractions

Figure 1 and Table 2 show that diclofenac sodium (IC50 = 140.42 µg/mL) had the best percentage stabilization of erythrocyte membrane against heat-induced haemolysis, followed by the butanol fraction, ethyl acetate fraction, aqueous fraction and hexane fraction, in a concentration dependent manner.

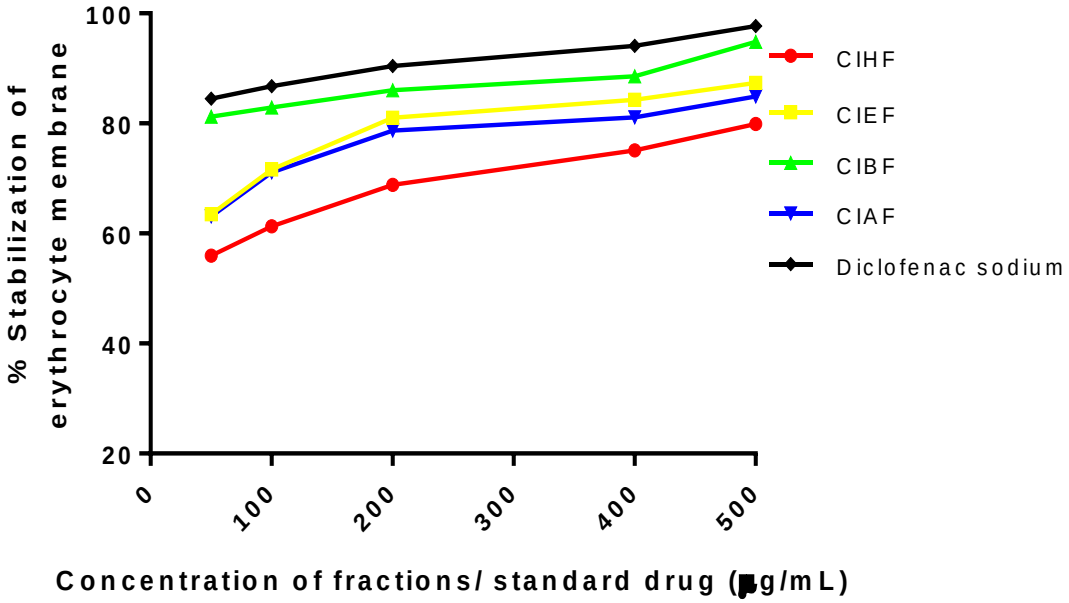

Figure 1: Percentage stabilization of erythrocyte membrane by Diclofenac sodium and different fractions of *C. igneus* leaf at varying concentrations

CIHF indicates hexane fraction of *C. igneus* leaf; CIEF indicates ethyl acetate fraction of *C. igneus*; leaf; CIBF indicates *C. igneus* leaf butanol fraction; CIAF indicates aqueous fraction of *C. igneus* leaf

Table 2: Fifty percent inhibitory concentrations (IC50) of Diclofenac sodium and different fractions of *C. igneus* leaf for stabilization of erythrocyte membrane

Fractions of *C. igneus* leaf	**IC50 (µg/mL)**
Diclofenac sodium	140.42
Butanol	140.61
Ethyl acetate	220.29
Aqueous	240.10

Hexane	250.80

Inhibition of Protein Denaturation by *C. igneus* Leaf Fractions

Figure 2 and Table 3 show that diclofenac sodium (IC50 = 15.14 µg/mL) had the best percentage inhibition of protein denaturation, followed by the butanol fraction, ethyl acetate fraction, aqueous fraction and hexane fraction, in a concentration dependent manner.

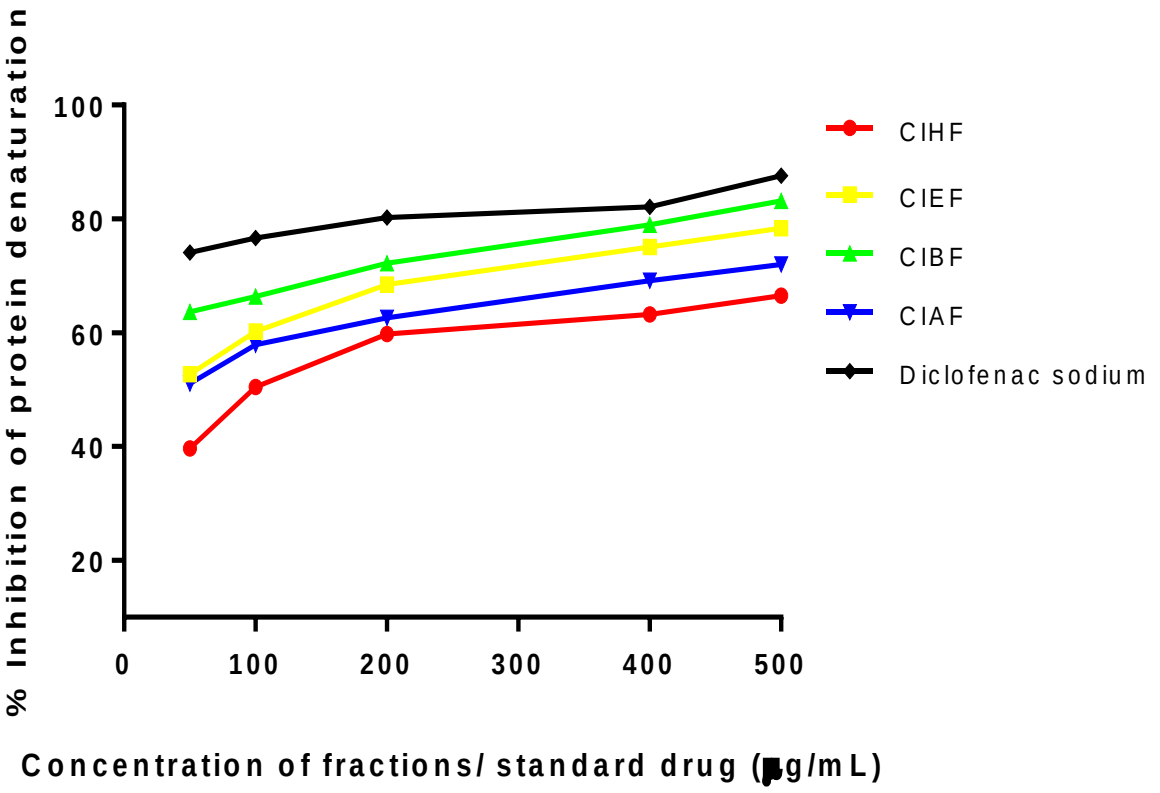

Figure 2: Percentage inhibition of protein denaturation by Diclofenac sodium and different fractions of *C. igneus* leaf at varying concentrations

CIHF indicates hexane fraction of *C. igneus* leaf; CIEF indicates ethyl acetate fraction of *C. igneus* leaf; CIBF indicates *C. igneus* leaf butanol fraction; CIAF indicates aqueous fraction of *C. igneus* leaf

Table 3: Fifty percent inhibitory concentrations (IC50) of Diclofenac sodium and different fractions of *C. igneus* leaf at for inhibition of protein denaturation

Fractions of *C. igneus* leaf	**IC50 (µg/mL)**
Diclofenac sodium	15.14
Butanol	20.40
Ethyl acetate	24.64
Aqueous	28.90
Hexane	38.43

In vitro Antioxidant Activity of *C. igneus* Leaf Fractions

1,1-Diphenyl 2-picryl hydrazyl (DPPH) Radical Scavenging Activity of *C. igneus* Leaf Fractions

Figure 3 and Table 4 show that ascorbic acid (IC50 = 18.16 µg/mL) had the best percentage inhibition of DPPH radicals, followed by the butanol fraction, ethyl acetate fraction, aqueous fraction and hexane fraction, in a concentration dependent manner.

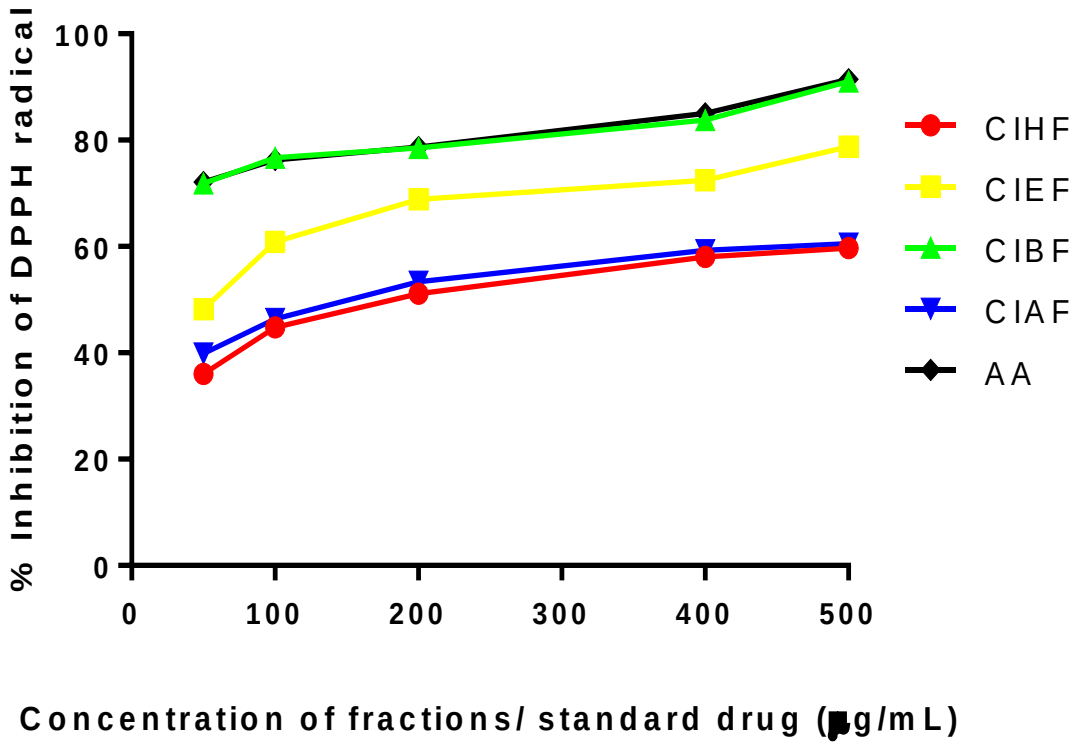

Figure 3: 1,1-diphenyl 2-picryl hydrazyl (DPPH) radical scavenging activity of ascorbic acid and different fractions of *C. igneus* leaf at varying concentrations
CIHF indicates hexane fraction of *C. igneus* leaf; CIEF indicates ethyl acetate fraction of *C. igneus* leaf; CIBF indicates *C. igneus* leaf butanol fraction; CIAF indicates aqueous fraction of *C. igneus* leaf; AA indicates ascorbic acid

Table 4: Fifty percent inhibitory concentrations (IC50) of ascorbic acid and different fractions of *C. igneus* leaf on 1,1-diphenyl 2-picryl hydrazyl (DPPH) radicals

Fraction (50 – 500 µg/mL)	IC50 (µg/mL)
Ascorbic acid	18.16
butanol	18.36
Ethyl acetate	31.25
Aqueous	34.72
Hexane	37.51

Nitric Oxide Radical Scavenging Activity of *C. igneus* Leaf Fractions

Figure 4 and Table 5 show that gallic acid (IC50 = 65.47 µg/mL) had the best nitric oxide radical scavenging activity, followed by the butanol fraction, aqueous fraction, ethyl acetate fraction and hexane fraction, in a concentration dependent manner.

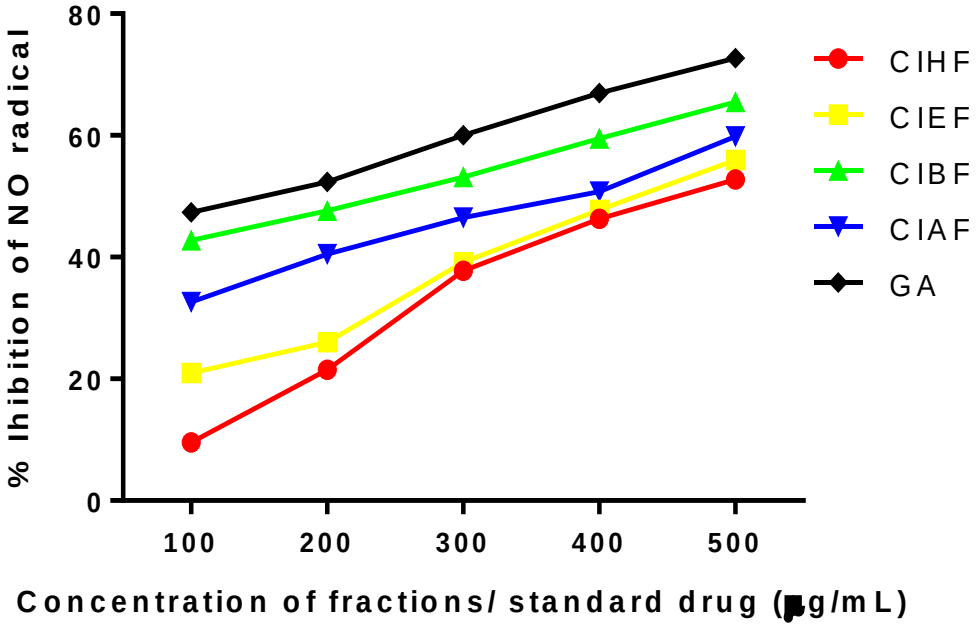

Figure 4: Nitric oxide (NO) radical scavenging activity of gallic acid and different fractions of *C. igneus* leaf at varying concentrations

CIHF indicates hexane fraction of *C. igneus* leaf; CIEF indicates ethyl acetate fraction of *C. igneus* leaf; CIBF indicates *C. igneus* leaf butanol fraction; CIAF indicates aqueous fraction of *C. igneus* leaf; GA indicates gallic acid

Table 5: Fifty percent inhibitory concentrations (IC50) of gallic acid and different fractions of *C. igneus* leaf on nitric oxide (NO) radicals

Fraction (100 – 500 µg/mL)	IC50 (µg/mL)
Gallic acid	65.47
Butanol	67.26
Aqueous	95.41
Ethyl acetate	166.60
Hexane	212.90

Hydrogen Peroxide Radical Scavenging Activity of *C. igneus* Leaf Fractions

Figure 5 and Table 6 show that ascorbic acid had the best (IC50 = 154.40 µg/mL) hydrogen peroxide radical scavenging activity, followed by the butanol fraction, ethyl acetate fraction, aqueous fraction and hexane fraction, in a concentration dependent manner.

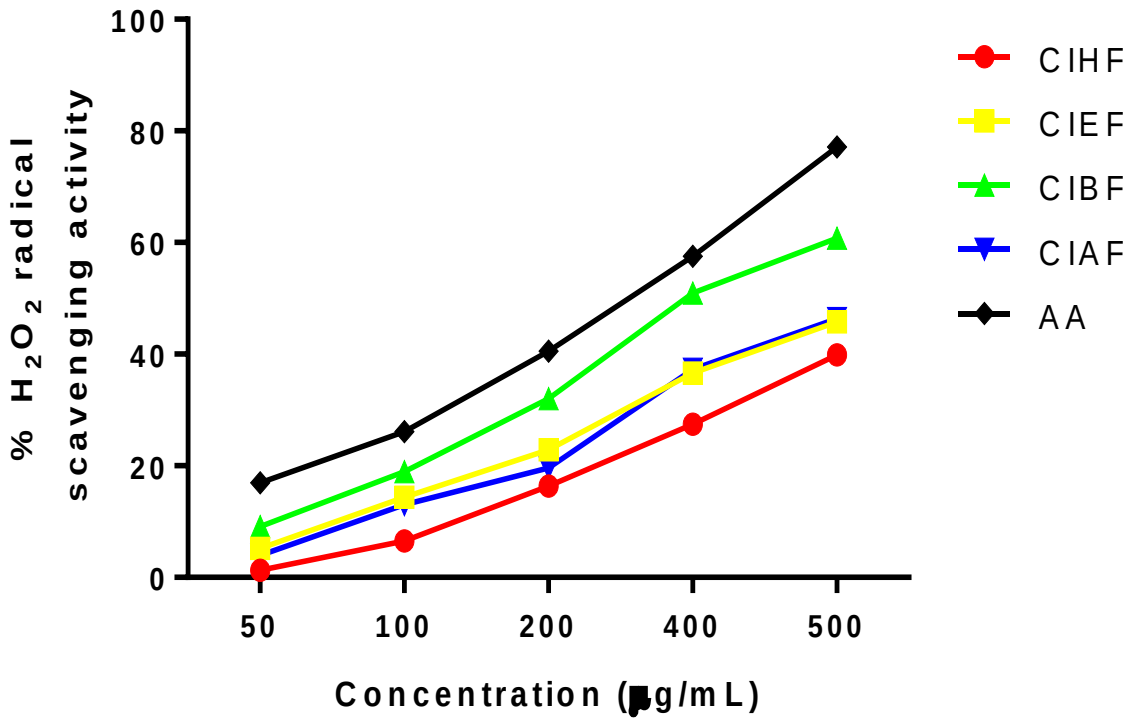

Figure 5: Hydrogen peroxide (H_2O_2) radical scavenging activity of ascorbic acid and different fractions of *C. igneus* leaf at varying concentrations

CIHF indicates hexane fraction of *C. igneus* leaf; CIEF indicates ethyl acetate fraction of *C. igneus* leaf; CIBF indicates *C. igneus* leaf butanol fraction; CIAF indicates aqueous fraction of *C. igneus* leaf; AA indicates ascorbic acid

Table 6: Fifty percent inhibitory concentrations (IC50) of ascorbic acid and different fractions of *C. igneus* leaf on hydrogen peroxide (H_2O_2) radicals

Fraction (50 – 500 µg/mL)	**IC50 (µg/mL)**
Ascorbic acid	154.40
butanol	160.50
Ethyl acetate	170.00
Aqueous	172.10
Hexane	227.80

DISCUSSION

The leaves of *C. igneus* were extracted with 70% ethanol and sequentially fractionated into hexane, ethyl acetate, butanol and aqueous fractions respectively. This separated the bioactive components of *C. igneus* leaf based on their polarity with the hexane fraction containing the least polar compounds and the aqueous fraction containing the most polar compounds. The aqueous fraction had the highest yield, followed by the butanol, hexane and ethyl acetate fractions, respectively. This finding is similar to a previous study that reported solvents with high polarity index have higher extraction yields (Aladhreai & Jamal, 2019). This could be due to the presence of compounds in the ethanol extract of *C. igneus* leaf that are mostly soluble in solvents with high polarity index such as water and butanol.

In vitro investigation of the anti-inflammatory activity of *C. igneus* leaf fractions showed that the *C. igneus* butanol fraction (CIBF) had higher stabilizing effect on heat-induced haemolysis of red blood cell membrane. Erythrocyte membrane stabilization by drug or plant extracts is a key indicator of anti-inflammation property *in vitro*. The erythrocyte membrane is assumed to be similar in resemblance to the lysosomal membrane, hence human red blood cells (HRBC) membrane stabilization could be inferred from lysosomal membrane stabilization which is a widely accepted postulation used for explanation of *in vitro* anti-inflammatory mechanism of action (Anyasor, Okanlawon & Ogunbiyi, 2019; Paul et al., 2021). During inflammation, lysosomal membrane lysis occurs with a release of their component degradative enzymes which produce diverse disorders. Non-steroidal anti-inflammatory drugs (NSAIDs) act by either inhibiting the release of lysosomal enzymes or by stabilizing the lysosomal membranes (Zhang, Qiu, Chen, Liu, Chang & Wang, 2020). When red blood cells are exposed to injurious substances such as heat,

membrane lysis occurs, followed by haemolysis and haemoglobin oxidation leading to the release of pro-inflammatory markers (Pan, Yuan, Farouk, Qin & Bao, 2021). Findings from this study correlate with previous report on the anti-inflammatory activity of methanol extract of garden egg, which identified membrane stabilization as the mechanism by which it exerts its anti-inflammatory activity (Anosike, Obidoa & Ezeanyika, 2012). Another study also reported a similar finding on the anti-inflammatory activity of ethanol root extract of Choi (*Piper chaba*) which was exerted through inhibition of heat-induced haemolysis (Yesmin et al., 2020). The haemolytic effect of heat may be associated with denaturation of membrane proteins on exposure to heat which results in the rupturing of its membrane (Halabi et al., 2019). Membrane stabilization by CIBF helped to maintain the integrity of the membrane and prevented leakage of the cell components during the period of heat exposure.

Another important mechanism that has been recognized as an important marker of inflammation is protein denaturation. The process of denaturation disrupts the native quaternary, tertiary and secondary structures of proteins leading to a loss of function (Mandal & Molla, 2020). Membrane proteins are largely responsible for the physical properties of the cell membrane and may contribute to regulation of the volume and water content of cells by controlling the influx and efflux of sodium and potassium ions which help to maintain cellular homeostasis (Kapoor et al., 2022). It has been reported that protein denaturation leads to production of autoantigens during inflammatory conditions and these antigens are associated with hypersensitive reactions which are linked to certain disease conditions (Aidoo, Konja, Henneh & Ekor, 2021). Any medicinal plant that has the potential of preventing protein denaturation could be identified as containing anti-inflammatory agent. It was observed in the present study that CIBF showed remarkably highest inhibitory effect on Bovine serum albumin (BSA) denaturation, in a concentration-dependent manner, when compared with the other fractions. However, Diclofenac sodium, which was used as standard drug, had the highest percentage inhibition of BSA denaturation in a dose-dependent manner. This was further buttressed by the IC50 of Diclofenac sodium (15.14 µg/mL) which was found to be slightly lower than that of CIBF (20.40 µg/mL). CIBF may therefore be considered a potential source of anti-inflammatory agent(s) capable of preventing the denaturation of proteins in the body system. A similar report was given by Yesmin et al. (2020) who indicated that ethanol extract of *Piper chaba* exhibited anti-inflammatory activity through inhibition of protein denaturation.

The *in vitro* antioxidant properties of *C. igneus* fractions were evaluated by investigating their free radical scavenging activity on DPPH, nitric oxide and hydrogen peroxide radicals. DPPH has been identified as the most popular free radical for investigation of *in vitro* antioxidant (Romulo, 2020). Upon reaction with antioxidants, DPPH radicals accept an electron donated by an antioxidant compound and the solution loses its color from purple to pale yellow (Baschieri & Amorati, 2021). In the present study, a pale yellow colouration was observed in the reaction of DPPH with ascorbic acid and *C. igneus* leaf butanol fraction which also exhibited the highest DPPH radical scavenging activities when compared with the other fractions. This may be as a result of donation of electrons to DPPH radicals by antioxidant compounds present in CIBF and hence supporting its antioxidant effect.

Nitric oxide radical scavenging activity was also exhibited by *C. igneus* leaf fractions as a mechanism of *in vitro* antioxidant activity, with the butanol fraction having the highest activity when

compared with the other fractions. Nitric oxide is involved in a variety of biological functions, including neurotransmission, vascular homeostasis, antimicrobial, and antitumor activities. Despite these beneficial effects, excess concentration of nitric oxide is implicated in the cytotoxic effects observed in various disease conditions (Król & Kepinska, 2020; Bourgognona et al., 2021; Onder, Nahar, Cinar & Sarker, 2022). This is due to the fact that nitric oxide radicals contribute to oxidative damage when they react with superoxide to form peroxynitrite anion, which is an oxidant that can decompose to produce hydroxyl radicals (Radi, 2018). *C. igneus* butanol fraction demonstrated significantly highest nitric oxide radical scavenging activity, when compared to the other factions which may be via competing with oxygen to react with nitric oxide and thus inhibit the generation of nitrite and peroxy nitrite anions.

Another important antioxidant defense mechanism of plants is via hydrogen peroxide radical scavenging activity. Hydrogen peroxide on its own is not very reactive, but can sometimes become toxic as it may give rise to hydroxyl radicals (Ueno, Nakanishi & Matsumoto, 2021). Thus, the elimination of hydrogen peroxide is very important for antioxidant defense in cell or food systems. The *C. igneus* leaf butanol fraction had the highest hydrogen peroxide radical scavenging activity when compared with the other fractions and this also confirms its potential antioxidant activity.

The antioxidant activity of plant products is mainly attributed to their redox properties, which can play an important role in adsorption and neutralization of free radicals, quenching of singlet and triplet oxygen molecules, or through decomposition of peroxides (Demirci-Çekiç, et al., 2022). The present study suggest that *C. igneus* fractions have moderate to potent antioxidant activity. Previous studies have also reported the antioxidant activity of some plant extracts and fractions as involving scavenging of oxygen radicals (Boutoub et al., 2020; Iqbal et al., 2021; Kiselova-Kaneva, Galunska, Nikolova, Dincheva & Badjakov, 2022).

The consistently highest *in vitro* anti-inflammatory and antioxidant activities exhibited by *C. igneus* butanol fraction implicated it as the most active fraction having the highest *in vitro* anti-inflammatory and antioxidant activities when compared with the other fractions.

CONCLUSION

The present study revealed that the butanol fraction of *C. igneus* leaf ethanol extract exhibited anti-inflammatory and antioxidant activities which were more substantial than the hexane, ethyl acetate and aqueous fractions. Further research is recommended for assessment of the *in vitro* anti-inflammatory and antioxidant activities of *C. igneus* butanol fraction.

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