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Anti-Inflammatory Property of *Costus afer* Ker Gawl Ethanol Leaf Extract in STZ-Induced Diabetic Rats

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Abstract

Costus afer Ker Gawl is an indigenous plant, commonly called a ginger lily, spiral ginger, or bush cane, it has been reported to possess anti-inflammatory properties. The anti-inflammatory activity of *C.afer* leaf extract in streptozotocin-induced diabetic rats was investigated. Protein denaturation and erythrocyte stabilization assays were used to evaluate *in vitro* anti-inflammatory activity, and alpha-amylase and alpha-glucosidase enzyme inhibition was used to evaluate *in vitro* anti-diabetic activity. 60 male Wistar rats were used for the two inflammatory models: the 30 rats were randomly assigned into six groups (n=5) for carrageenan-induced paw edema and cotton pellet granuloma models respectively: Group I: normal, group II: control(untreated), group III: 10mg/kg *b.w* diclofenac sodium (standard), group IV, V and VI were given 50, 100, 250 mg/kg *b.w Costus afer* ethanol leaf extract (CAELE) in each of the two models. The study showed that in protein denaturation assay, CAELE and Diclofenac had 56.69% and 80.82% respectively at the highest concentration, erythrocyte stabilization had 80.40% CAELE and 94.88% Diclofenac sodium at the highest dose in a dose-dependent manner. Alpha amylase and alpha-glucosidase showed an increase in percentage inhibition activity at 65.44% and 43.72% respectively against acarbose (standard) at 56.01%. However, in the cotton pellet-induced granuloma model, the concentration exhibited high percentage inhibition (77.82%) comparable to the standard drug at 91.28%, and reduction in paw thickness was also observed in the carrageenan model in a dose-dependent manner respectively. This study showed that CAELE at different concentrations showed anti-inflammatory activity in diabetic conditions.

Keywords: *Costus afer*, Diclofenac sodium, Anti-inflammatory, Carrageenan, cotton-pellet granuloma.

1.0 INTRODUCTION

Inflammation is a fundamental physiological response that serves as a vital component in the body's defence mechanisms. Nevertheless, in cases where inflammation persists over an extended period or lacks proper regulation, it has the potential to give rise to a diverse array of incapacitating health conditions (Pahwa & Jialal, 2019). The presence of inflammation has been found to elevate the risk of developing insulin resistance and islet cell inflammation, which can subsequently impair beta-cell secretion and contribute to the development of diabetes. In subjects with diabetes and/or obesity, there is an increased risk of cardiovascular disease, which may be attributed to inflammation as suggested by Dilworth *et al*., (2021). Therefore, the potential of targeting inflammation as a therapeutic approach is emerging as a viable option in the ever-growing range of strategies for managing diabetes mellitus and its associated complications. In the context of digestion, postprandial hyperglycemia is a common occurrence. However, it can pose challenges for individuals with diabetes, especially those diagnosed with type 2 diabetes (Tsalamandris *et al*., 2019). In these instances, there is a noticeable impairment in the body's capacity to regulate blood glucose levels, resulting in prolonged increases in glucose levels after meals. Unregulated postprandial hyperglycemia has been linked to a range of health complications, such as cardiovascular disease, neuropathy, and retinopathy (Giri *et al*., 2018). This has led to the investigation of natural compounds and pharmaceutical agents that possess anti-inflammatory properties. The management of postprandial hyperglycemia is an essential aspect of diabetes control. Ensuring optimal glycemic control,

particularly postprandial, is crucial in mitigating the risk of acute and chronic complications linked to elevated blood glucose levels.

Costus afer, also known as bush cane sugar, is a plant that belongs to the Zingiberaceae family. The plant in question is an herbaceous monocot characterized by its tall, unbranched structure and a creeping rhizome (Anyasor *et al*., 2014). It is primarily distributed in specific regions of West Africa that are characterized by moist and shady forest environments (Anyanwu *et al*., 2020). The *C.afer* plant is recognized for its antioxidant, anti-inflammatory, and antidiabetic properties due to the presence of phytochemicals such as polyphenolics, saponins, alkaloids, and glycosides (Anyasor *et al*., 2014; Boison *et al*., 2019). The objective of this study was to examine the potential anti-inflammatory properties of *C. afer* ethanol leaf extract (CAELE) and compare its efficacy with the reference drug diclofenac sodium, utilizing a rat model. Gaining a comprehensive understanding of the anti-inflammatory properties exhibited by CAELE holds the potential to provide valuable insights into its therapeutic applications. Furthermore, such insights could contribute significantly to the advancement of innovative strategies aimed at effectively managing inflammatory conditions.

2.0 MATERIALS AND METHODS

2.1 Location of Study

The research was carried out at Babcock University, Biochemistry Laboratory, Illishan-Remo Ogun state.

2.2 Ethical Approval

Ethical approval was obtained from Babcock University Health Research Ethics Committee (BUHREC) with number BUHREC/333/22.

2.3 Chemicals and Reagents

Ethanol, Dimethyl sulphoxide, Bovine serum albumin, and streptozotocin were procured from Sigma Chemical Co. (St.Louis, MO, USA), and Diclofenac sodium was purchased from Hovid Pharmaceuticals. All other chemicals were of analytical grade.

2.4 Preparation of Plant Material

Freshly collected *C. afer* leaves were obtained from a farm in Irolu, Ikenne LGA of Ogun State Nigeria. The leaves were washed and dried in a hot air oven at 40°C for 4 days and then pulverized using an electric blender. The pulverized leaves were soaked in 95% ethanol for 72 hours after which it was sieved using a cloth bag and then filtered with filter paper. Evaporation was done using a rotatory evaporator and the crude extract was left to become paste in the hot air oven at 40°C for 12 hours.

2.5 *IN VITRO* INFLAMMATORY ASSAY

2.5.1 Inhibition of Protein Denaturation Assay

Anti-denaturation of bovine serum albumin (BSA) was done using the method described by Ogunbiyi *et al*., 2021 with little modifications. Briefly, the assay mixture contained a solution of BSA (45 µL, 0.5% w/v) and 5 µL of *C. afer* ethanol leaf extract (CAELE) in different concentrations of 100-500 µg/mL respectively. The mixture was incubated at 37°C for 20 mins in a water bath (Uniscope SM801A; Surgifield Medicals) and the temperature was increased to 57°C and left for 3 mins. After cooling, 2.5 ml of phosphate buffer saline (pH 6.3) was added to each mixture and absorbance was measured at 416 nm using a spectrophotometer. 45 µL distilled water and 5µL of test solution (i.e. BSA and *C.afer* ethanol extract) were used as a test control. The experiment was carried out in triplicates and percentage inhibition for protein denaturation was calculated.

2.5.2 Human red blood cell (HRBC) membrane stabilization assay

The effect of CAELE extract on hypotonicity-induced hemolysis of human red blood cell membrane (HRBC) was carried out in according to the protocol described by Ogunbiyi *et al*., 2021 with modifications. Fresh whole human blood (5ml) was obtained using a 5ml syringe and transferred into an ethylenediamine tetra-acetic acid (EDTA) bottle and centrifuged at 3000rpm for 10 mins, the supernatant was carefully removed and the packed red volume (PCV) cells were washed in freshly prepared normal saline (0.9% w/v NaCl) and centrifuged, the process was repeated until the supernatant became clear. 10% HRBC membrane was then resuspended in 0.9% w/v NaCl as stock. The assay mixture contained 1 ml of sodium phosphate buffer (0.15M, 7.4 pH), 2 ml hyposaline (0.36% w/v NaCl), 0.5 ml HRBC suspension (10% w/v) with 0.5 ml diclofenac sodium (standard drug) or CAELE extract in various concentrations were placed in test tubes. For the control, distilled water was used in place of NaCl (0.36%, w/v) to induce 100% hemolysis. The hemolysis produced in the presence of distilled water was taken as 100%. The different test tubes were incubated at 56°C in a water bath (Uniscope SM801A; Surgifield Medicals) for 30 min and then centrifuged at 5000 rpm. The estimation of haemoglobin content in each tube was measured spectrophotometrically at 560 nm. The experiment was done in triplicates for all the test samples.

$$\%\ Erythrocyte\ stabilization = 100 - (OD\ of\ extract \div OD\ of\ control) \times 100$$

2.6 *IN VITRO* ANTI-DIABETES ASSAYS

2.6.1 Alpha-amylase Enzyme Inhibition Assay

α-amylase (0.5 mg/ml) was mixed with the sample at various concentrations (100-500 µg/ml), 1% of starch solution and 100 µl of 0.2 mm of phosphate buffer (pH -6.9) was then added. The reaction was carried out at 37°C for 5 min and terminated by the addition of 2 ml

of 3, 5-dinitro salicylic acid reagent. The reaction mixture was heated for 15 min at 100°C and diluted with 10 ml of distilled water in an ice bath. The α-amylase activity was determined by measuring colour intensity at 540 nm in a spectrophotometer (Upreti *et al*., 2019).

2.6.2 Inhibition of α-glucosidases Enzyme

Alpha-glucosidase activity was evaluated using the method of SLDV *et al*., 2017. The inhibitory activity was determined by incubating 1 ml of starch solution (2% w/v maltose) with 0.2 M tris buffer (pH 8) and various concentrations of the sample. The reaction mixture was incubated at 37°C for 10 min. The reaction was initiated by adding 1 ml of the α-glucosidase enzyme (1 U/ml) to it and incubation was done at 35°C for 40 min. The reaction was terminated by the addition of 2 ml of HCl. The intensity of the colour was measured at 540 nm in a spectrophotometer.

The result was expressed as % inhibition using the formula:

$$\%\ Inhibitory\ Activity = \frac{Ac - As}{Ac} \times 100$$

Where Ac is the absorbance of the control and As is the absorbance of the sample.

2.7 *IN VIVO* ASSAYS

Experimental animals

Sixty (60) male Wistar rats weighing between 150- 250g were used for the experiments. The rats were kept in plastic cages with wooden shavings to prevent coprophagy. The rats were given water *ad libitum* and given standard feed. They were kept under standard conditions of temperature (varying between 26-30°C) and humidity (30-70%). the animals went through acclimatization for two weeks. Animal Handling and treatment were done using the ethical guidelines for the use of animals in research.

Treatment Groups for Animals

The animals were divided into two major divisions for acute and sub-acute inflammation and in each division, there were 30 rats with five in a group (n=5).

Group 1: Normal
Group 2: Induced untreated rats
Group 3: 10 mg/kg Diclofenac-sodium
Group 4, 5 and 6: 50, 100 and 250 mg/kg of CAELE respectively

Experimental procedure for inducing diabetes

The rats belonging to groups 2-6 of divisions one and two were induced using 60mg/kg *b.w* of streptozotocin (STZ). The rats were starved 12 hours before induction, their food was withdrawn and they were only given water, upon 12 hours of starvation they were administered freshly prepared STZ intraperitoneally. Three days after induction, their blood glucose was measured using a glucometer.

2.7.1 Acute: Carrageenan model

The carrageenan model was done as described by Olajide & Anyasor, 2021. A solution of carrageenan was used to induce acute swelling in the rats. The rats were divided into 6 groups, grp 1: positive control (un-induced), grp 2: negative control (induced), grp 3: 10 mg/kg *b.w* diclofenac sodium, and grps 4-6: 50, 100, 250 mg/kg *b.w*. of the CAELE extract. Carrageenan (0.1 mL of 1% prepared as a suspension in distilled water) was injected into the sub-plantar tissue of the left hind paw of the rat 30 minutes after treatment. The volume of the resulting paw edema was measured at zero (0), half an hour, and a 1-hour interval for 6 hours using a micrometre screw gauge. The percentage inhibition at each time interval was calculated as follows:

$\%inhibition = [(C_t - C_0)\ control - (C_t - C_0)\ treated \div (C_t - C_0)\ control] \times 100$

C0 = paw size before carrageenan injection
Ct = paw size after carrageenan injection

2.7.2 Subacute: cotton pellet model

The anti-granulomatous activity of the *C.afer* was evaluated with a cotton pellet inflammatory model as described by Olajide & Anyasor, 2021. The rats were divided into six groups with five animals (n=5) in a group: Group 1: normal, Group 2: untreated, Group 3: 10 mg/kg Diclofenac sodium, Groups 4, 5 and 6: 50, 100, and 250 mg/kg *b.w* of *C.afer* ethanol leaf extract. After 30 mins of administering treatment, autoclaved cotton pellets **(**50mg±1.0mg) were aseptically implanted into the dorsal region of each rat subcutaneously while the rats were anaesthetized with 75 mg/kg *b.w*. of ketamine. The rats were treated daily for 7 days. On the 8th day, they were anaesthetized again, the cotton pellets were surgically removed and extraneous tissue was removed, the cotton pellets were dried in a hot air oven at 60 °C overnight. The weights of the dried pellets were recorded and the percentage inhibition of granuloma tissue development was determined.

$\%inhibition = [(weight\ of\ pelle(control) - weight\ of\ pellet\ (treated)) \div weight\ of\ pellet(control)] \times 100$

2.8 Statistical analysis

Data obtained from this study were analyzed using Analysis of Variance (ANOVA) and expressed as mean ± SEM and $p<0.05$ is taken as significance level.

3.0 RESULTS

3.1 *IN VITRO* ANTI-INFLAMMATORY ASSAYS

3.1.1 Effect of CAELE on the erythrocyte membrane stabilization

Figure 1 showed that CAELE and diclofenac sodium inhibited heat-induced protein denaturation in a concentration-dependent manner and exhibited the highest inhibition at the maximum concentration 500 mg/kg 56.69% and 80.82% respectively with significant ($p<0.05$) difference across the groups. The reference

drug (diclofenac sodium) had higher anti-denaturation potential better than ethanol extract of *C. afer* leaf.

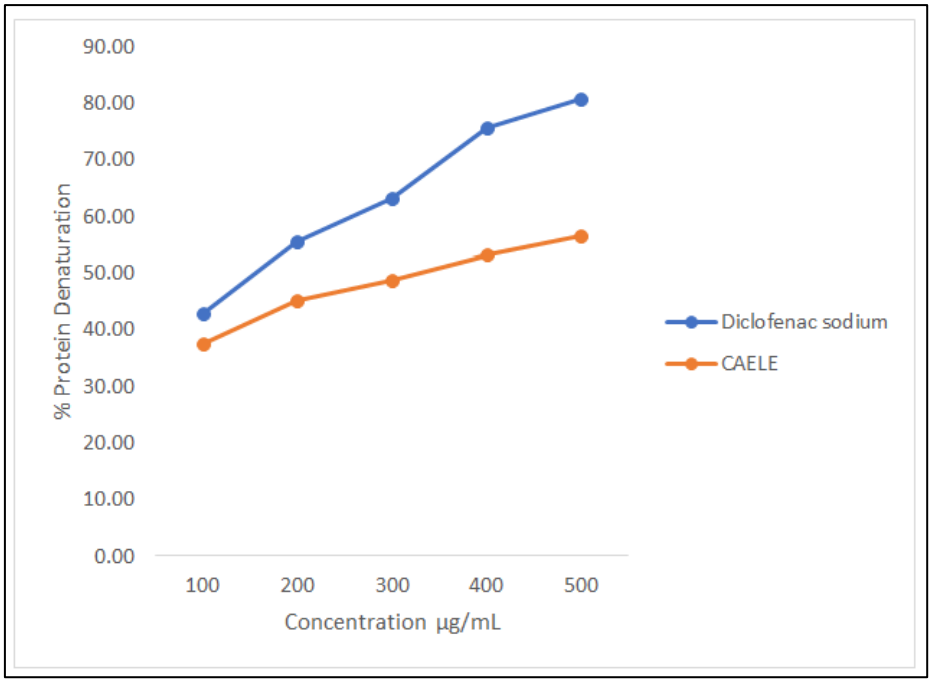

Figure 1: Showed the % protein denaturation between CAELE and the standard drug with significant ($p<0.05$) difference

3.1.2 Effect of CAELE on erythrocyte membrane stabilization

Data from Figure 2 showed that diclofenac sodium and *C. afer* ethanol leaf extract stabilized HRBC membrane against hemolysis induced by hypotonic solution in a concentration dependent manner. CAELE stabilized erythrocyte membrane against hypotonic-induced hemolysis at 80.40% compared with diclofenac sodium at 94.88% at the highest concentration.

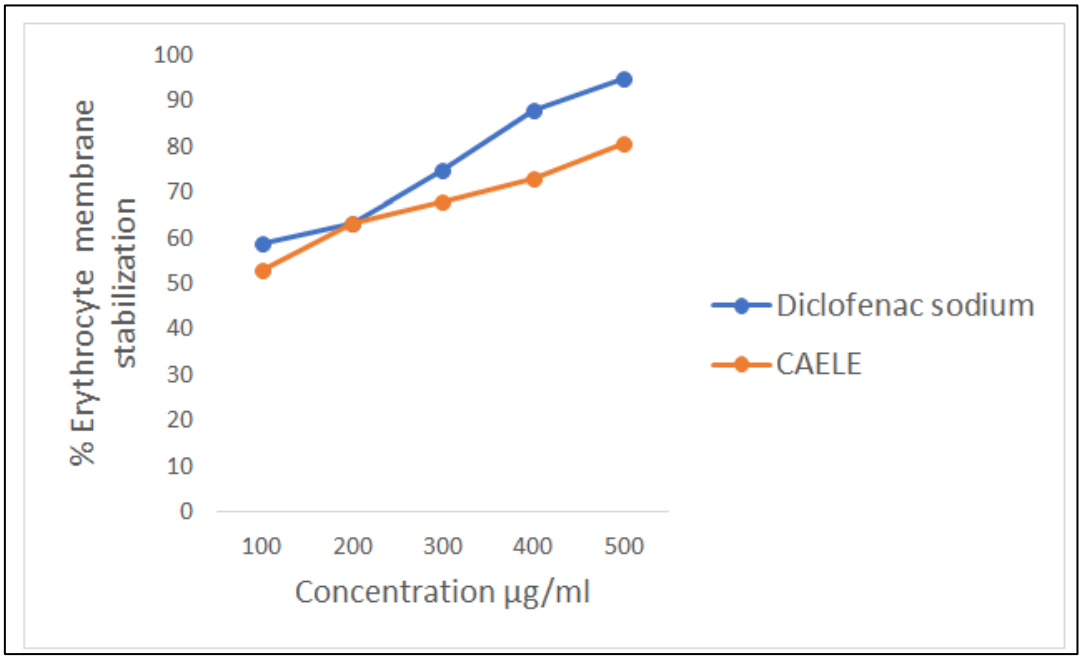

Figure 2: This figure showed % erythrocyte membrane stabilization at significant ($p<0.001$) difference between the standard drug and the extract. CAELE: *Costus afer* ethanol leaf extract

3.2 *IN VITRO* ANTIDIABETIC ASSAY

3.2.1 Effect of CAELE on alpha-amylase enzyme inhibition

Figure 3 and 4 depicts the effect of CAELE on the in vitro antidiabetic enzymes. The extract demonstrated increase in the % inhibition of alpha amylase enzyme and alpha glucosidase enzyme respectively. At the highest dose, in Fig 3, the extract exhibited 65.44% inhibition compared with the reference drug at 56.01% for α-amylase. In Fig 4, α-glucosidase exhibited %inhibition of alpha-glucosidase enzyme at 43.72%.

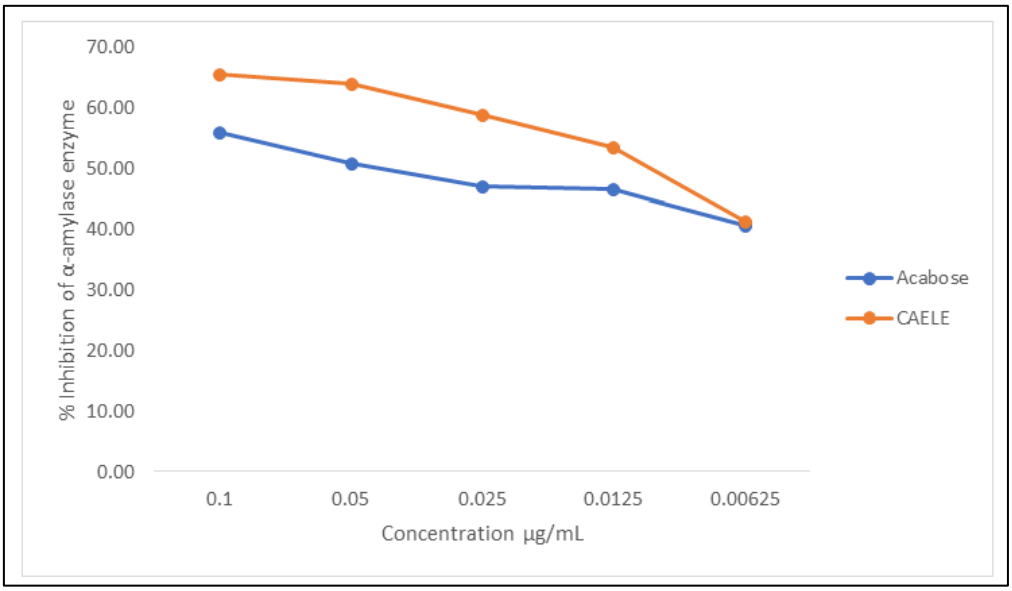

Figure 3: Showed the effect of CAELE on α-amylase enzyme inhibition enzyme compared with acarbose (reference drug)

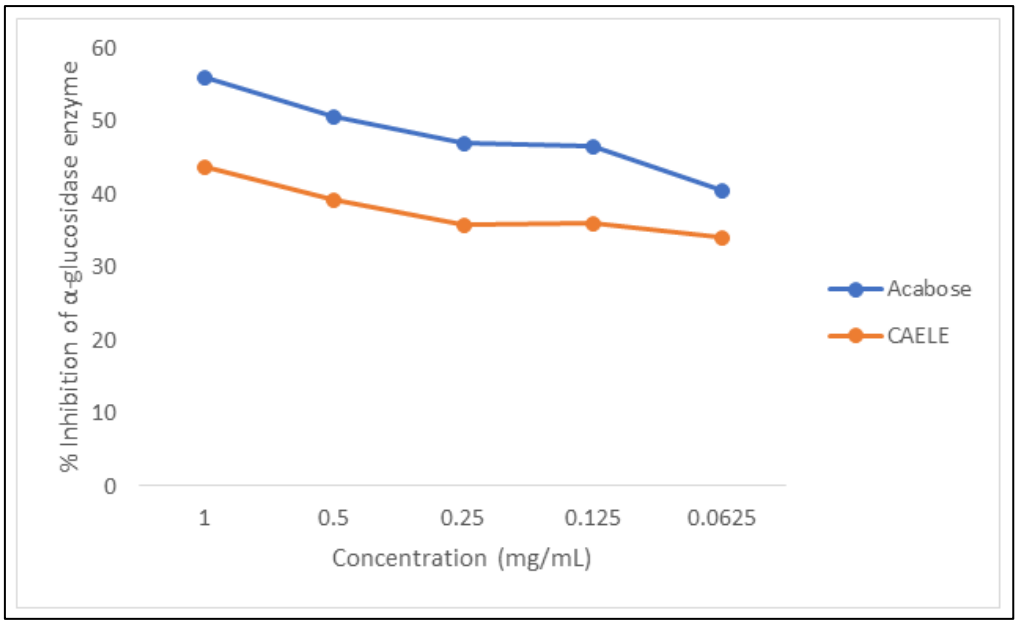

Figure 4: Data expressed the effect of CAELE on the percentage α-glucosidase enzyme inhibition and that of the reference drug (acarbose). The significant level is set at P<0,05

3.3 IN VIVO ANTI-INFLAMMATORY MODELS

3.3.1 Anti-inflammatory effect of CAELE on cotton pellet-induced granuloma.

The data in Figure 5 showed that animals treated with 10 mg/kg b.w. diclofenac sodium possess 8.60 ± 3.59 mg (91.27%) and CAELE at 50 mg/kg b.w. 69.27 ± 2.66 mg (29.73%), 100 mg/kg b.w. 49.78 ± 2.47 mg (49.49%) and 250 mg/kg b.w. 21.86 ± 5.70 mg (77.82%) had significant ($p<0.05$) difference in inhibition of coton pellets-induced granuloma when compared with control untreated group at 98.56 ± 11.14 (0%). However, animals treated with diclofenac sodium possess significantly ($p<0.05$) high percentage inhibition compared with CAELE treated at different doses.

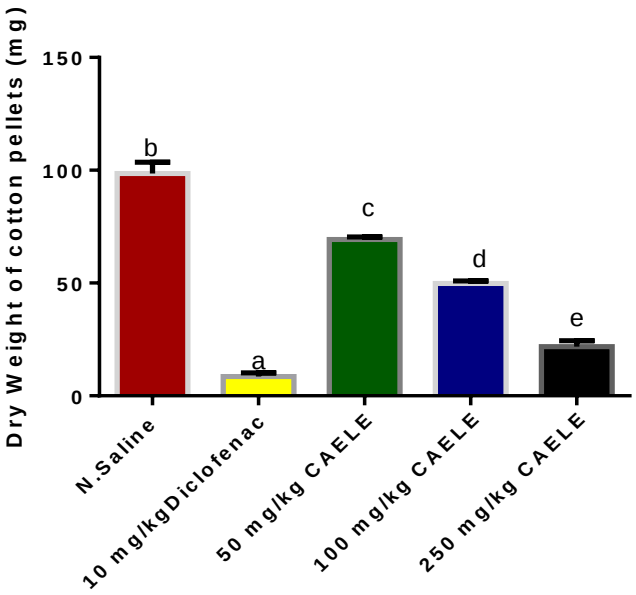

Figure 5: Effect of CAELE on cotton pellet-induced granuloma. Different letters indicate significance (p<0.001), CAELE = *Costus afer* ethanol leaf extract, N. saline= normal saline (not induced and not treated but given normal saline)

3.3.2 Mean change in paw thickness in carrageenan-induced paw edema

Data in Table 1 shows the changes in paw thickness of carrageenan-induced rats at hourly intervals, from 0 hour to the 6th hour. The table showed that there was a reduction in paw after 3 hours of treatment in both diclofenac sodium and CAELE treated groups and no significant difference when compared with the untreated group. The data is expressed in mean ± SD.

Table 1:

	0 hour (mm)	30 mins (mm)	1 hour (mm)	2 hours (mm)	3 hours (mm)	4 hours (mm)	5 hours (mm)	6 hours (mm)
Normal	**0.00±0.00**	**0.00±0.00**	**0.00±0.00**	**0.00±0.00**	**0.00±0.00**	**0.00±0.00**	**0.00±0.00**	**0.00±0.00**
Control (Untreated)	**0.00±0.00**	**1.84±0.72**	**1.93±0.74**	**2.28±0.46**	**2.53±0.66**	**2.41±1.28**	**2.19±1.22**	**2.33±0.95**
Diclofenac Sodium (10mg/kg)	**0.00±0.00**	**1.57±0.08**	**2.41±0.02**	**3.27±0.33**	**3.50±0.26**	**3.04±0.11**	**2.25±0.02**	**1.93±0.31**
CAELE (50mg/kg)	**0.00±0.00**	**1.98±0.33**	**2.18±0.33**	**3.06±0.63**	**2.60±0.69**	**2.48±0.12**	**2.69±0.47**	**2.10±0.68**
CAELE (100mg/kg)	**0.00±0.00**	**2.24±0.35**	**2.00±0.81**	**2.19±1.06**	**2.73±1.03**	**2.50±0.57**	**2.64±1.48**	**2.15±1.38**
CAELE (250mg/kg)	**0.00±0.00**	**3.32±0.30**	**3.11±0.40**	**3.23±0.84**	**4.14±0.36**	**3.25±0.14**	**2.91±0.23**	**2.56±0.44**

DISCUSSION

Naturally occurring compounds serve as a remarkable repository of pharmacologically active compounds (Daniyan *et al*., 2023). The results of this study indicate that CAELE possesses a significant ability to inhibit heat-induced protein denaturation in a concentration-dependent manner. Protein denaturation is a crucial event in several pathological conditions, and agents that can prevent or mitigate this process have potential therapeutic relevance (Adeoye *et al*., 2022). Diclofenac sodium, a reference drug with known anti-inflammatory properties, exhibited a higher anti-denaturation potential compared to CAELE. This finding suggests that diclofenac sodium may have a more potent effect in preserving protein structure and function under heat-induced stress. The higher inhibition by diclofenac sodium may be attributed to its well-established anti-inflammatory and anti-denaturation properties (Alfaro & Davis, 2023).

Distinctively, data obtained from this study showed the concentration-dependent effects of *C. afer* ethanol leaf extract (CAELE) and diclofenac sodium on the stabilization of human red blood cell (HRBC)

membranes against hypotonic-induced hemolysis. Both CAELE and diclofenac sodium demonstrated the ability to protect HRBC membranes, with diclofenac sodium displaying a slightly higher degree of stabilization at the highest concentration. The results of this study are significant in the context of cellular membrane protection, particularly in conditions involving hypotonic stress (Dias & Nylandsted, 2021). Hypotonic-induced hemolysis, which results in the rupture of red blood cells, is a physiological phenomenon that has implications for various clinical conditions (Adetunji *et al*., 2022). Understanding the concentration-dependent effects of different agents on HRBC membrane stability is essential for potential therapeutic applications (Farooq *et al*., 2022). The finding that CAELE stabilized HRBC membranes against hypotonic-induced hemolysis at 80.40% at the highest concentration indicates its potential as a protective agent. This suggests that CAELE can preserve HRBC membrane integrity in hypotonic conditions, which can be particularly valuable in medical contexts where preventing hemolysis is crucial. Notably, diclofenac sodium exhibited a slightly higher degree of HRBC membrane stabilization at 94.88% at the maximum concentration. This observation underscores the potential advantages of diclofenac sodium in protecting HRBC membranes under hypotonic stress. The superiority of diclofenac sodium may be attributed to its established mechanism of action and pharmacological characteristics (Kołodziejska & Kołodziejczyk, 2018).

Interestingly, plant-based natural products are well-reputed as regulators of postprandial hyperglycemia (Adeoye *et al*., 2022). The data presented in Figures 3 and 4 illustrates the impact of CAELE on in vitro antidiabetic enzymes, specifically alpha-amylase and alpha-glucosidase. The results indicate an increase in the percentage inhibition of these enzymes, suggesting potential antidiabetic properties of CAELE. At the highest dose tested, CAELE demonstrated a higher inhibition of alpha-amylase compared to the reference drug, while the inhibition of alpha-glucosidase was observed at a slightly lower percentage. According to Figure 3, the CAELE exhibited a 65.44% inhibition of alpha-amylase at the highest dose. This finding indicates that CAELE can inhibit the activity of alpha-amylase, an enzyme responsible for the breakdown of complex carbohydrates into simpler sugars. Inhibition of alpha-amylase can be a valuable attribute in managing blood sugar levels, particularly in individuals with diabetes (Oyedemi *et al*., 2017). In Figure 4, CAELE displayed a 43.72% inhibition of alpha-glucosidase. Alpha-glucosidase is another crucial enzyme involved in the digestion of carbohydrates, playing a role in the absorption of glucose. Inhibition of alpha-glucosidase can lead to a slower release of glucose into the bloodstream, which is beneficial in controlling postprandial (after-meal) blood glucose spikes, commonly observed in diabetic individuals (Dirir *et al*., 2022). It is noteworthy that in Figure 3, the reference drug demonstrated a slightly lower percentage of inhibition (56.01%) of alpha-amylase compared to CAELE, indicating that the extract may have a more potent inhibitory effect on this enzyme.

The data presented in Figure 5 illustrates the effects of *C. afer* ethanol leaf extract (CAELE) on cotton pellet-induced granuloma relative to diclofenac sodium. The results indicate significant differences in the inhibition of granuloma formation compared to the untreated control group. Additionally, diclofenac sodium was found to possess a significantly higher percentage of inhibition when compared to CAELE at various doses. The control group, which received no treatment, had a granuloma weight of 98.56 ± 11.14 mg (0% inhibition), indicating the baseline granuloma formation in untreated animals. Statistical analysis demonstrated that the differences in granuloma inhibition between the control group and the CAELE-treated group were significant ($p < 0.05$), indicating the efficacy of CAELE in inhibiting granuloma formation. Importantly, this showed that CAELE likely possess bioactive compounds which can potentially modulate the proliferative phase of inflammation. Furthermore, when comparing the effects of diclofenac sodium and CAELE, it is evident that diclofenac sodium had a significantly higher percentage of inhibition in inhibiting cotton pellet-induced granuloma when compared to CAELE at different doses. This suggests that diclofenac sodium may have a more potent anti-inflammatory effect in this specific experimental model.

Moreover, the data presented in Table 1 depicts the changes in paw thickness in carrageenan-induced rats at hourly intervals, ranging from the initial 0 hours to the 6th hour. Our findings revealed a reduction in paw thickness observed after 3 hours of treatment in both the diclofenac sodium and CAELE-treated groups. Importantly, this reduction in paw thickness was not found to be significantly different when compared to the untreated group. This data suggests that at the 3-hour time point, both diclofenac sodium and CAELE had a similar effect in reducing paw swelling as the untreated group. This indicates that, at this specific time point, neither diclofenac sodium nor CAELE provided a significant additional reduction in paw thickness compared to the untreated control group. The findings in this table provide important insights into the time-dependent changes in paw thickness in the context of carrageenan-induced inflammation in the rat model. The observed reduction in paw thickness after 3 hours in the treated groups indicates the potential anti-inflammatory effects of both diclofenac sodium and CAELE. However, the lack of a significant difference compared to the untreated group at this specific time point suggests that the effects of these treatments may take some time to manifest or that their effectiveness in reducing paw thickness might not be significantly different from the natural course of inflammation in the untreated animals.

CONCLUSION AND RECOMMENDATION

The ethanol extract of *Costus afer* leaf possesses anti-inflammatory activity even in diabetic conditions. However, further studies could be done on its effect on specific biomarkers.

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