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Anticonvulsant, Muscle Relaxant and *In-Vitro* Antioxidant Activities of Hydroethanol Leaf Extract of *Costus afer* Ker Gawl (Costaceae) in Mice

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ABSTRACT

Treatments for most central nervous system (CNS) disorders are not devoid of untoward effects. Recently, attention is mainly devoted to indigenous plants which are potential sources of new drugs with better efficacy and safety profile relative to conventional agents. *Costus afer* Ker Gawl (Costaceae) is a plant used in Traditional African Medicine (TAM) for the treatment of a variety of ailments, including epilepsy. This study investigated the anticonvulsant, muscle relaxant and *in-vitro* antioxidant activities of hydroethanol leaf extract of *Costus afer* in mice. The strychnine- and picrotoxin-induced convulsion (SIC and PIC) tests were used to investigate antiepileptic activity while muscle relaxant activity was evaluated using the traction and inclined screen tests. Distilled water (10 mL/kg), diazepam (3 mg/kg) and *C. afer* (25-200 mg/kg) were administered 1 h before the induction of convulsion. Animals were thereafter observed for the onset and duration of convulsion. Another set of mice were subjected to the muscle relaxant tests 1 h post-treatments and the reaction time of each mouse were subsequently observed. 2,2-Diphenyl-1-picrylhydrazyl, nitric oxide, hydrogen peroxide, lipid peroxidation and reducing power assays were used to investigate the *in-vitro* antioxidant activity. In the PIC test, *C. afer* at 50 mg/kg significantly increased the seizure latency ($p<0.05$) and decreased seizure duration ($p<0.001$). There was significant dose-dependent decrease in the post-treatment sliding latency (25-200 mg/kg) in the inclined screen test ($p<0.01$). *C. afer* elicited dose-dependent radical scavenging actions in the *in-vitro* antioxidant activity assays. Findings suggest that *C. afer* possess anticonvulsant, muscle relaxant and *in-vitro* antioxidant properties.

Keywords: *Costus afer*, Costaceae, free radical scavenger, antiepileptic action, muscle relaxant property.

Introduction

Epilepsy is ranked the fourth most common and serious brain disorder in the world, with about 50 million of the world population affected.[1,2] A significant number of persons are diagnosed yearly with epilepsy globally. About 6 in 1000 people are affected in the developed countries, while about 10 in 1000 people are victims in the developing world.[3] Although the mechanisms underlying the development of epilepsy, its progress and the intriguing nexus with other central nervous system (CNS) disorders are poorly understood,[4] oxidative stress has been identified as one of the intrinsic factors involved in the pathogenesis of CNS disorders, including neurodegenerative diseases,[5,6] which has been linked with the manifestation of epilepsy. Poor repair capacity, increased oxygen requirement, presence of lipids and massive mitochondria have been adduced as reasons for the susceptibility of the brain to the deleterious effects of reactive oxygen species.[7] Chang and Yu[8] reported that oxidative stress is a possible mechanism for the onset and development of epilepsy, while Menon *et al.*[9] demonstrated seizure-induced oxidative stress by reporting significant increase in the level of oxidative makers in epileptic patients. Antioxidant therapy in animal models, supported by clinical data, has been demonstrated to reduce oxidative stress and frequency of seizures.[10,11]

Muscle spasm is a short painful muscular contraction which may be due to epilepsy. A number of conventional anti-epileptics, muscle relaxants and antioxidant drugs are currently available, but epilepsy is still poorly managed in about 20% of affected individuals.[12] Diseases caused by oxidative damage are still on the increase and the episodes of skeletal muscle spasm continue unabated. Hence, focus remains strong on indigenous plants which are promising sources of new drugs with better efficacy and safety profile relative to conventional agents, apart from the desirable prospect of developing standardized herbal remedies for CNS disorders.

Costus afer Ker Grawl (Costaceae), Bush/Monkey sugar cane, is a perennial, herbaceous and rhizomatous plant found in Asia, Africa and Americas.[13-15] In west and tropical Africa, it grows well in moist or shady forest.[16,17] In Nigeria and other West African countries, *C. afer* is often planted in home gardens for medicinal purposes, with documentation of use in traditional medicine to treat diabetes, inflammation, joint pains,[16] measles, fever, malaria, etc. The rhizome decoction of another species (*Costus dubius*) is used to treat epilepsy.[18]

This study was carried out to investigate the anticonvulsant, muscle relaxant and *in-vitro* antioxidant activities of the hydroethanol leaf extract of *Costus afer* in mice.

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Materials and Methods

Drugs and chemicals

Diazepam (Swipha Pharmaceuticals, Lagos, Nigeria), strychnine and picrotoxin (Sigma-Aldrich, MO, USA), and ascorbic acid (Juhel Pharmaceuticals, Lagos, Nigeria).

Plant material and extraction

C. afer leaves were obtained from Aiyepe Town, Odogbolu Local Government Area, Ogun State, Nigeria in August, 2018. Prof. J.D. Olowokudejo of the Department of Botany, Faculty of Science, University of Lagos, Nigeria, identified and authenticated the plant material which was assigned an institutional herbarium voucher specimen number LUH8018.

Freshly collected leaves of *C. afer* were air-dried until constant weight was obtained, after which the leaves were grinded, weighed (510 g) and macerated in 2 L of hydroethanol (1:1) for 72 h. Thereafter, decanting and sieving of the extract using muslin cloth and subsequently with Whatman filter paper was done. These processes (extraction, decanting and filtration) were repeated 2 more times with the residues obtained. Evaporation of the cumulative filtrates to dryness was achieved in a laboratory oven set at 40°C (Gallenhamp®, Leicestershire, UK) to give a dark brown solid with yield of 10.8%. The extract was dissolved in distilled water prior to oral administration to experimental animals. The doses of the extract used in this study were selected based on results of preliminary investigations.

Experimental animals

Mice (25-30 g) of either sex were procured from the Laboratory Animal Centre of the Faculty of Pharmacy, Olabisi Onabanjo University, Ago Iwoye, Ogun State, Nigeria. The animals were kept under hygienic conditions in well ventilated compartments and housing. They were maintained under standard environmental conditions with access to standard rodent feed (Livestock Feeds PLC, Ikeja, Lagos, Nigeria) and water *ad libitum*. Fourteen days period of acclimatization was observed before the commencement of the experiments. Ethical approval was obtained from the Health Research Ethics Committee (HREC) of the College of Medicine, University of Lagos, Nigeria (CMUL/HREC/12/17/330).

Preliminary phytochemical screening

The extract was screened for the presence of various phytoconstituents using established procedures.[19,20]

Fourier-transform infrared spectroscopy (FT-IR) analysis

FT-IR analysis of the extract for the presence of various functional groups was done as previously described.[21]

Strychnine-induced convulsion test

In this experiment, animals were divided into 6 groups of 5 mice each. The first group served as control and received distilled water 10 mL/kg p.o. The second group served as standard group and received diazepam 3 mg/kg p.o. The third to sixth groups received *C. afer* at 4 different doses (25, 50, 100 and 200 mg/kg p.o.), respectively. One hour later, mice in all the groups received strychnine 4 mg/kg i.p. The latency and duration of convulsion were recorded for each mouse. Animals that did not convulse after 30 min were considered protected.[22,23]

Picrotoxin-induced convulsion test

The same procedure described above in the strychnine model was followed, except that convulsion was induced with picrotoxin 5 mg/kg i.p.[24]

Traction (muscle relaxant) test

Each mouse was screened by placing their forepaws on a small twisted wire rigidly supported above a bench top. Those that were able to grasp the wire with the forepaws and place at least one hind foot on the wire within 5 sec, when allowed to hang free, were considered eligible for the test. A response longer than 5 sec was considered as failure.[25,26] Screened mice were randomly divided into 6 groups of 5 mice each. These groups were separately treated orally with distilled water (10 mL/kg), *C. afer* (25, 50, 100 and 200 mg/kg) and diazepam (5 mg/kg). Each animal was subjected to the traction test 1 h post-treatment and the reaction time for each mouse was recorded.

Inclined screen (muscle relaxant) test

This test was carried out in accordance with the method of Adebesin *et al*.[26] with little modification. Mice were grouped and treated as mentioned above in the traction test. One hour post-treatment, each mouse was subjected to the inclined screen test. Mice were in turn placed on a glass plane inclined at 35° and the time taken for each mouse to slide off the screen was recorded.

Total antioxidant capacity determination

The total antioxidant capacity (TAC) of the extract was evaluated using established procedure.[27] Three millilitre of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) and 0.3 mL of the extract (100 µg/mL) were mixed together. For 90 min, the capped tubes were incubated in a water bath containing boiling water at 95°C. The samples were allowed to cool off to room temperature and measurement at 695 nm of the absorbance of each of the aqueous solution was taken. The TAC was expressed as equivalent of ascorbic acid.

Total phenols content determination

With little modification using gallic acid as standard, the Folin-Ciocateu reagent model[28] was used to determine the total phenols content. For 15 min, the mixture of 0.1 mL of Folin-Ciocalteu reagent (0.5 N) and 0.5 mL of *C. afer* (100 µg/mL) were incubated at room temperature. Sodium carbonate solution (2.5 mL, 7.5% w/v) was thereafter added to the mixture and incubated at room temperature for another 30 min. The measurement of absorbance was done at 760 nm. Total phenols content was expressed as gallic acid equivalent.

Total flavonoids content determination

Using quercetin as standard[29] total flavonoids content of *C. afer* was evaluated with aluminium chloride. One millilitre of 100 µg/mL of the extract was added to the mixture of methanol (3 mL), aluminium chloride (0.2 mL of 10%), potassium acetate (0.2 mL of 1 M) and distilled water (5.6 mL) and this was incubated at room temperature for 30 min. The absorbance was measured at 415 nm.

In-vitro antioxidant activity

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

Following established procedure,[30,31] the DPPH free radical scavenging activity of the extract was investigated. Different concentrations (20, 40, 60, 80, and 100 µg/mL) of the extract (0.5 mL) in ethanol (95%) were mixed with the reagent solution (2.0 mL; 0.004 g of DPPH in 100 mL methanol). The control had only DPPH solution in place of the sample, while methanol was used as the blank. After intense shaking, the mixture was left at room temperature. The absorbance was measured at 517 nm after 30 min. Ascorbic acid was used as standard.

The scavenging activity was calculated using the expression:

$$\text{Inhibition (\%)} = \frac{[A_x - A_y]}{A_x} \times 100$$

Where A_x is the absorption of the control and A_y is the absorption of the extract or standard.

Nitric oxide scavenging assay

Different concentrations (20, 40, 60, 80, and 100 µg/mL) of the extract (4 mL) were placed in different test tubes and sodium nitroprusside (1 mL; 5 mM in phosphate buffered saline) solution was added into the test tubes. The mixtures were incubated at room temperature for 2 h. Two millilitres of the sample was taken from the mixture and mixed with 1.2 mL of Griess reagent (1% sulphanilamide, 0.1% naphthylethylenediaminedihydrochloride in 2% H_3PO_4). The absorbance was measured at 550 nm.[32] Ascorbic acid was used as standard.

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$$\text{Inhibition (\%)} = \frac{[A_x - A_y]}{A_x} \times 100$$

Where A_x is the absorbance of the control and A_y is the absorbance of the extract or standard.

Reducing power assay

Varying extract concentrations (20, 40, 60, 80, and 100 µg/mL) were mixed with sodium phosphate buffer (2.5 mL of 200 mmol/L; pH 6.6) and potassium ferricyanide (2.5 mL of 1%). Incubation was done at 50°C for 20 min. Trichloroacetic acid (2.5 mL of 10% w/v) was later added to the mixture which was centrifuged at 650 rpm for 10 min. The upper layer was mixed with deionized water (2 mL) and ferric chloride (1 mL of 0.1%). The absorbance was measured at 517 nm.[30] Ascorbic acid was used as standard.

Lipid peroxidation assay

The lipid peroxidation activity was investigated in accordance with the method of Buege and Aust.[33] Fresh rat liver was cut and homogenized to obtain 10% homogenate in cold 150 mM KCl-Tris-HCl buffer. The mixture contained liver homogenate, Tris-HCl buffer (20 mM; pH 7.0), $FeCl_2$ (2 mM), ascorbic acid (10 mM), and extract (0.5 mL) at various concentrations (20, 40, 60, 80, and 100 µg/mL) in a final volume of 1 mL. The mixture was incubated at room temperature for 1 h. Lipid peroxidation was measured as malondialdehyde (MDA) equivalent. The mixture was later mixed with thiobarbituric acid (TBA) - trichloroacetic acid (TCA) reagent (2 mL) and boiled on water bath for 15 min. Upon cooling, the precipitate was removed by centrifugation. Malondialdehyde absorbance was determined spectrophotometrically at 535 nm. Ascorbic acid was used as standard.

Hydrogen peroxide scavenging assay

The hydrogen peroxide scavenging activity of the extract was determined using the procedure of Ruch *et al*.[34] Varying concentrations of the extract (20, 40, 60, 80, and 100 µg/mL) were mixed with hydrogen peroxide solution (0.6 mL, 40 mM; pH 7.4 buffer). Hydrogen peroxide absorbance was measured at 230 nm for 10 min. Ascorbic acid was used as standard.

$$\text{Inhibition (\%)} = \frac{[A_x - A_y]}{A_x} \times 100$$

Where A_x is the absorbance of the control and A_y is the absorbance of the extract or standard.

Statistical analysis

The data generated in this study were expressed as mean ± standard error of mean (S.E.M.). One-way ANOVA (followed by Dunnett's and Tukey's multiple comparison tests) using GraphPad Prism 6 Software (GraphPad Software Inc., CA, USA) was used for data analysis. Results were considered significant at $p < 0.05$.

Results and Discussion

The central nervous system is a complex, sophisticated entity that regulates and coordinates body activities. Disorders of this system can lead to some neurological abnormalities which are manifested as seizures, insomnia, muscle spasm, neurodegenerative diseases, and are managed largely by orthodox medicines. As a result of paradigm shift, the use of ethnobotanicals in treating some of these disorders is gaining more acceptance. Accordingly, this study was conducted to investigate the anticonvulsant, muscle relaxant and *in-vitro* antioxidant activities of the hydroethanol leaf extract of *Costus afer* sequel to the claims by traditional medicine practitioners that the plant can be used to manage convulsion, muscle spasm and oxidative stress related diseases.

Strychnine- and picrotoxin-induced convulsion tests are two widely used animal models to identify the antiepileptic-like activity of drug substances. Strychnine is a neurotoxin/chemical convulsant which blocks both glycine and acetylcholine receptors.[35] It binds to the glycine receptor, thereby preventing the inhibitory effects of glycine on the postsynaptic neuron in the spinal cord. The extract did not elicit any significant change ($p > 0.05$) in seizure latency and duration compared with the control in the strychnine model. However, diazepam produced significant increase ($p < 0.001$) in seizure latency with no significant change ($p > 0.05$) in seizure duration (Figure 1). The lack of significant ameliorative effects of *C. afer* in respect of the onset and duration of convulsion in this model suggest a lack of interaction with glycine receptors. A similar effect was obtained with diazepam in the strychnine-induced convulsion test, except for the significant increase in the onset of convulsion. Diazepam is known to elicit its anxiolytic, sedative-hypnotic, anticonvulsant, and muscle relaxant effects via positive allosteric modulation of the $GABA_A$-receptor,[26] gamma-aminobutyric acid (GABA) being a major inhibitory neurotransmitter in the CNS.[35]

In the picrotoxin model, *C. afer* at 50 mg/kg significantly increased ($p < 0.05$) the seizure latency and decreased ($p < 0.001$) the seizure duration. However, diazepam only significantly decreased ($p < 0.05$) the seizure duration. *C. afer* at doses of 25 and 200 mg/kg also significantly decreased ($p < 0.01$) the seizure duration (Figure 2). The mechanism of epileptogenic action of picrotoxin (a $GABA_A$-receptor antagonist) has been generally reported to be by inhibiting gamma-aminobutyric acid (GABA) neurotransmission and blocking chloride-ion channels linked to $GABA_A$ receptors.[36] Augmentation of GABAergic neurotransmission has been reported to prevent, block or attenuate seizures, while its antagonism enhances and facilitates seizures.[37] Enhancement of GABAergic neuron and interaction with $GABA_A$ receptor by the extract may therefore be suggested as the possible mechanism of antiepileptic action of *C. afer*.

Muscle relaxant-like actions of drug substances are widely investigated using traction and inclined screen tests.[26] In the traction test, mice with proper muscular coordination are those that are able to grasp the horizontally hanged twisted wire with their forepaws and place at least one hind foot on the wire within 5 sec when allowed to hang freely. This capability could be altered in animals with relaxed muscles. An increase in the reaction time of mice in the traction test suggests muscle relaxant activity.[26] *C. afer* (25-200 mg/kg) elicited non-significant ($p > 0.05$) increase in the reaction time of mice at all the doses used in this study compared to control, suggesting a trend towards inherent muscle relaxant effect. Diazepam, an established muscle relaxant, produced significant increase ($p < 0.01$) in the reaction time of mice compared to the control (Figure 3).

Possession of muscle relaxant property by the extract was however established in the inclined screen test. Adebesin *et al*.[26] reported that reduction in the post-treatment sliding latency relative to the corresponding pre-treatment sliding latency suggest muscle relaxant activity. *C. afer* (25-200 mg/kg) produced significant dose-dependent decrease ($p < 0.01$, 0.001) in the post-treatment sliding latency compared to the corresponding pre-treatment latency values.

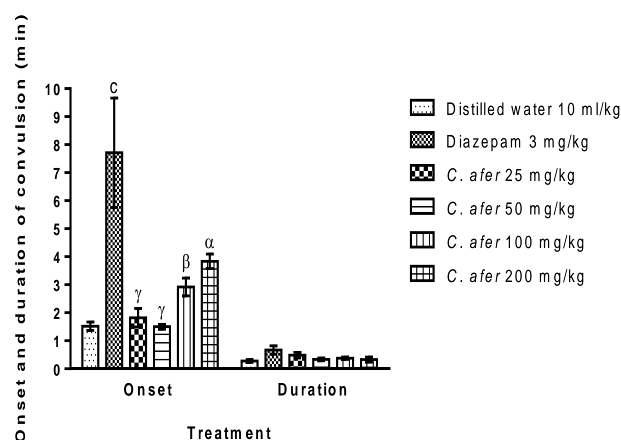

Figure 1: Effect of *C. afer* in strychnine-induced convulsion test in mice. Values are mean ± S.E.M. (n = 5). $^c p < 0.001$ vs. distilled water; $^\alpha p < 0.05$, $^\beta p < 0.01$, $^\gamma p < 0.001$ vs. diazepam (one-way ANOVA followed by Tukey's multiple comparison test).

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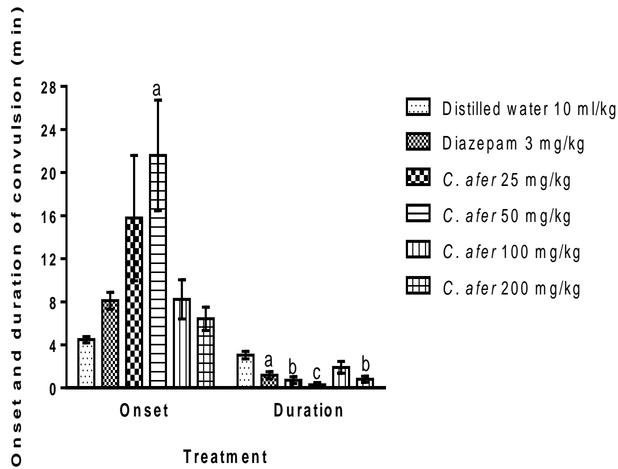


Figure 2: Effect of *C. afer* in picrotoxin-induced convulsion test in mice. Values are mean ± S.E.M. (n = 5). $^{a}p < 0.05$, $^{b}p < 0.01$, $^{c}p < 0.001$ vs. distilled water (one-way ANOVA followed by Dunnett's multiple comparison test).

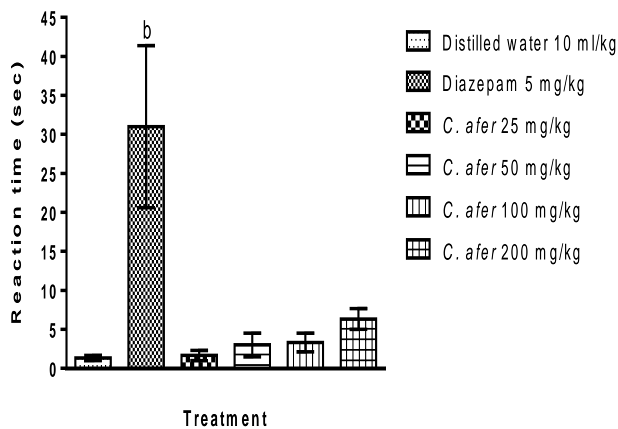


Figure 3: Effect of *C. afer* on muscle relaxation in traction test in mice. Values are mean ± S.E.M. (n=5). $^{b}p<0.01$ vs. distilled water (one-way ANOVA followed by Dunnett's multiple comparison test).

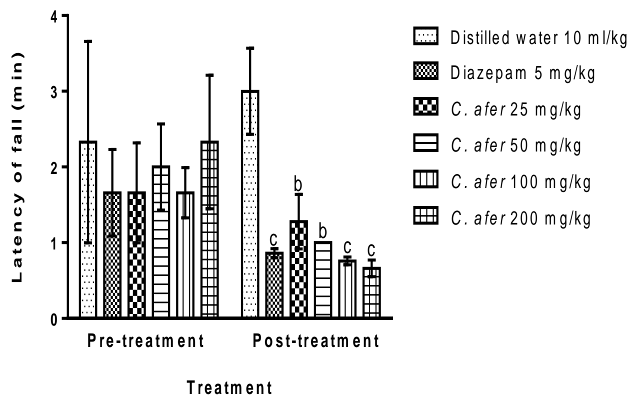
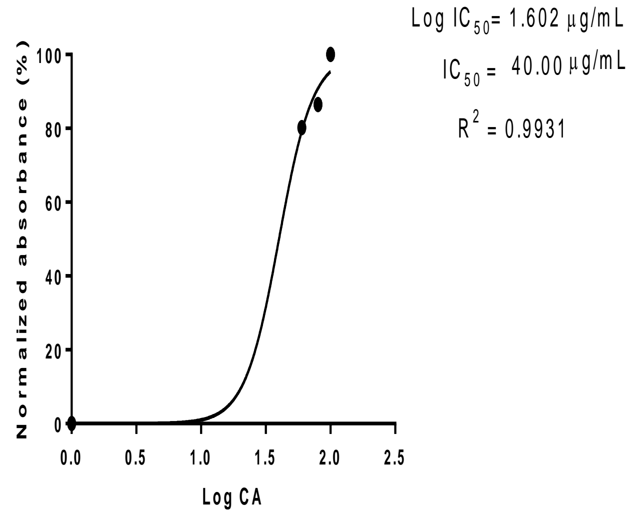


Figure 4: Effect of *C. afer* on muscle relaxation in inclined screen test in mice. Values are mean ± S.E.M. (n = 5). $^{b}p < 0.01$, $^{c}p < 0.001$ vs. corresponding pre-treatment latency (one-way ANOVA followed by Dunnett's multiple comparison test).



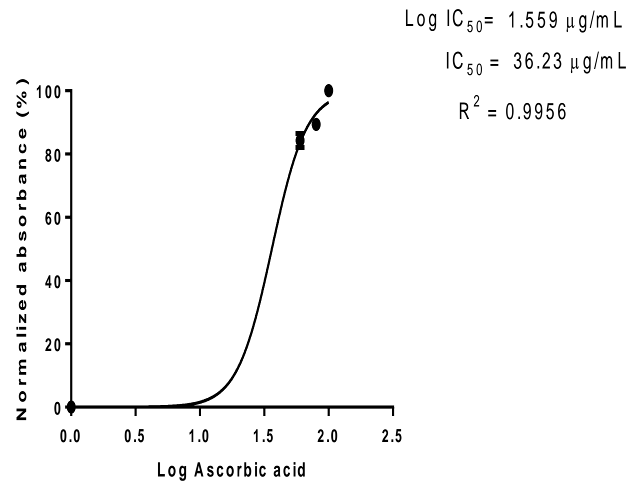


Figure 5: DPPH radical scavenging activity of *C. afer* (top) and ascorbic acid (bottom).

Diazepam produced a significant ($p < 0.001$) reduction in the post-treatment sliding latency relative to the pre-treatment latency value, thus confirming its well-known muscle relaxant activity (Figure 4). Oxidative stress is an imbalance between the destructive reactive oxygen species and protective/defensive antioxidant mechanisms.[38] Rice-Evans[39] reported medicinal plants as natural source of antioxidants. These natural antioxidants protect and reduce the vulnerability of the body to certain diseases such as cardiovascular diseases and cancer.[40] DPPH, NO, lipid peroxidation, H_2O_2 and reducing power assays are widely used *in-vitro* tests to investigate the antioxidant properties of natural products.[41] *C. afer* extract in a concentration-dependent fashion scavenged generated radicals in the DPPH (IC_{50} = 40.00 µg/mL vs. 36.23 µg/mL for ascorbic acid; Figure 5), NO (IC_{50} = 13.50 µg/mL vs. 21.41 µg/mL for ascorbic acid; Figure 6), and H_2O_2 (IC_{50} = 30.66 µg/mL vs. 18.06 µg/mL for ascorbic acid; Figure 7) assays. The extract also elicited concentration-dependent reducing power (IC_{50} = 36.58 µg/mL vs. 37.20 µg/mL for ascorbic acid; Figure 8) and anti-lipid peroxidation (IC_{50} = 42.11 µg/mL vs. 35.23 µg/mL for ascorbic acid; Figure 9) effects. In respect of the NO assay, the IC_{50} value for the extract was lower than that of ascorbic acid, while values were comparable in the reducing power assay. For the DPPH, H_2O_2, and lipid peroxidation assays, the established

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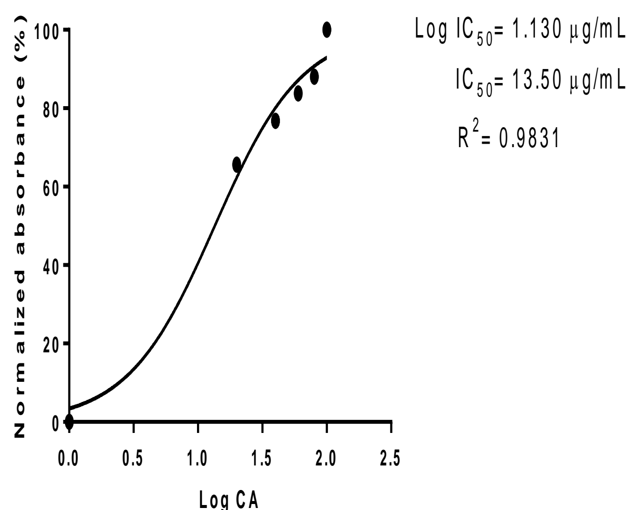
antioxidant used as standard (ascorbic acid) had lower IC_{50} values relative to *C. afer*. The *in-vitro* antioxidant activities demonstrated by the extract in this study indicate potential therapeutic usefulness in diseases linked to oxidative stress. This finding is supported by the values obtained with the extract in respect of total antioxidant capacity, total phenols and total flavonoids contents (31.78 ± 0.24 mg/100 g, 17.60 ± 0.97 mg/100 g and 25.34 ± 0.64 mg/100 g equivalent of standards, respectively). In respect of the FT-IR analysis, the extract showed a broad band at 3355.71 cm^{-1} corresponding to OH stretching in alcohols. The peak at 2890.26 is due to aliphatic (C-H) stretching. The peak at 1795.60 is due to carbonyl stretching, while the peak at 1631.53 is due to C=C absorption. The 1400.59 is due to C-H bending. The band at 1075.86 could be attributed to C-O stretching vibration. The 1030.08 indicated the presence of C-O stretching absorption; ethers (Figure 10). Qualitative phytochemical screening of the extract revealed the presence of flavonoids, steroids, glycosides, phenols, alkaloids, terpenoids and tannins. Some of these chemical constituents have been reported to be responsible for anticonvulsant, muscle relaxant and antioxidant activities of medicinal plants.[42-44]



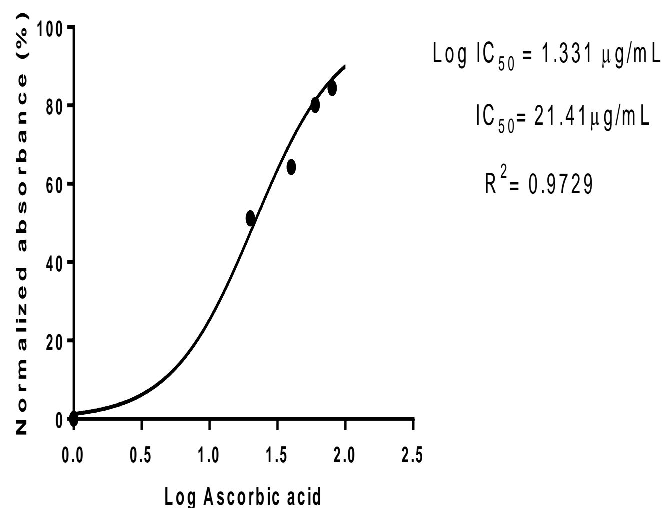
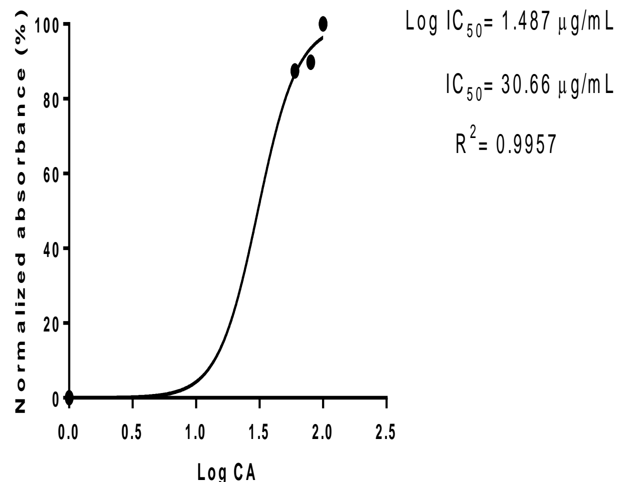


Figure 6: NO radical scavenging activity of *C. afer* (top) and ascorbic acid (bottom).



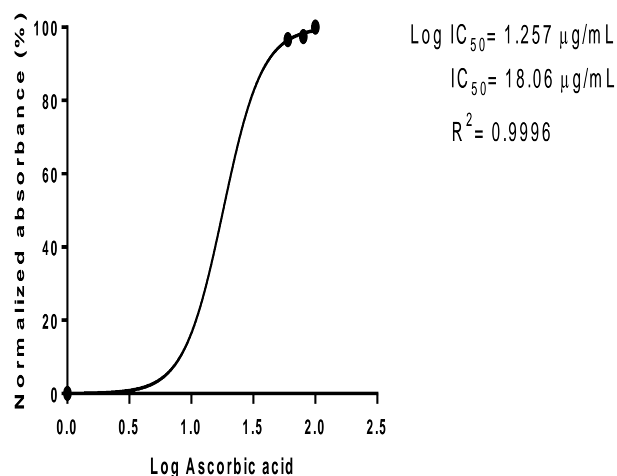


Figure 7: H_2O_2 radical scavenging activity of *C. afer* (top) and ascorbic acid (bottom).

Conclusion

The findings in this study suggest that the hydroethanol leaf extract of *Costus afer* possess anticonvulsant, muscle relaxant and antioxidant activities. The anticonvulsant and muscle relaxant activities of the extract are possibly mediated via positive modulation of the $GABA_A$ receptor-chloride channel complex. The outcome of this study presents the extract as a candidate for standardized herbal remedy development for the treatment of convulsion, muscle spasm and diseases linked to oxidative stress.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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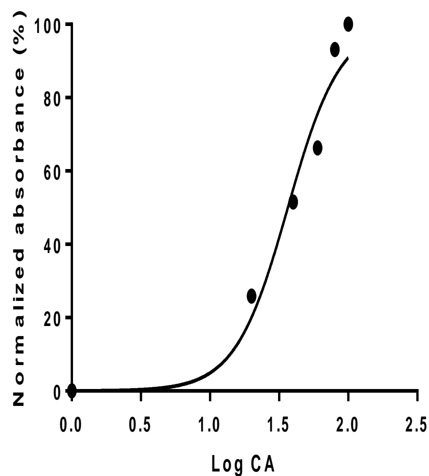


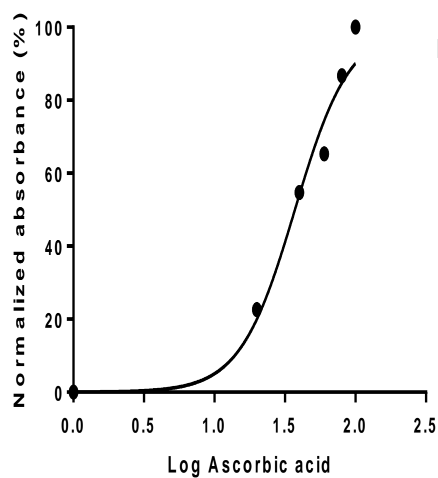
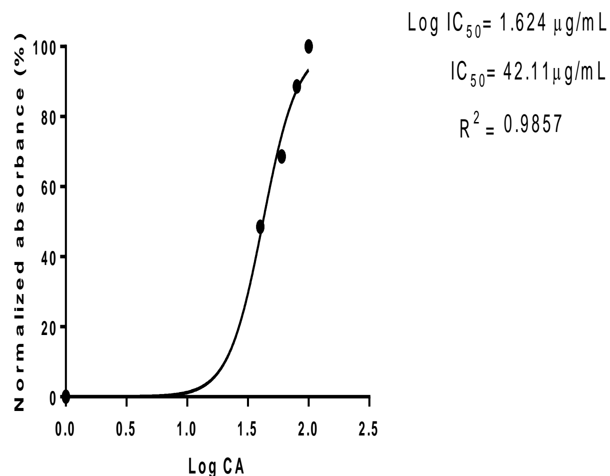


Figure 8: Reducing power of *C. afer* (top) and ascorbic acid (bottom).



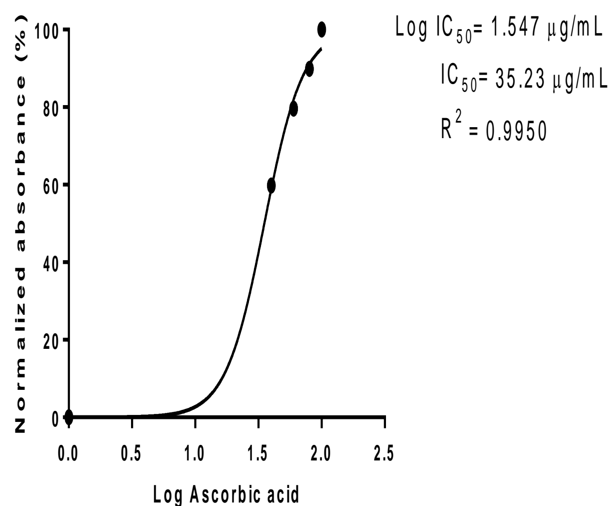


Figure 9: Anti-lipid peroxidation effects of *C. afer* (top) and ascorbic acid (bottom).

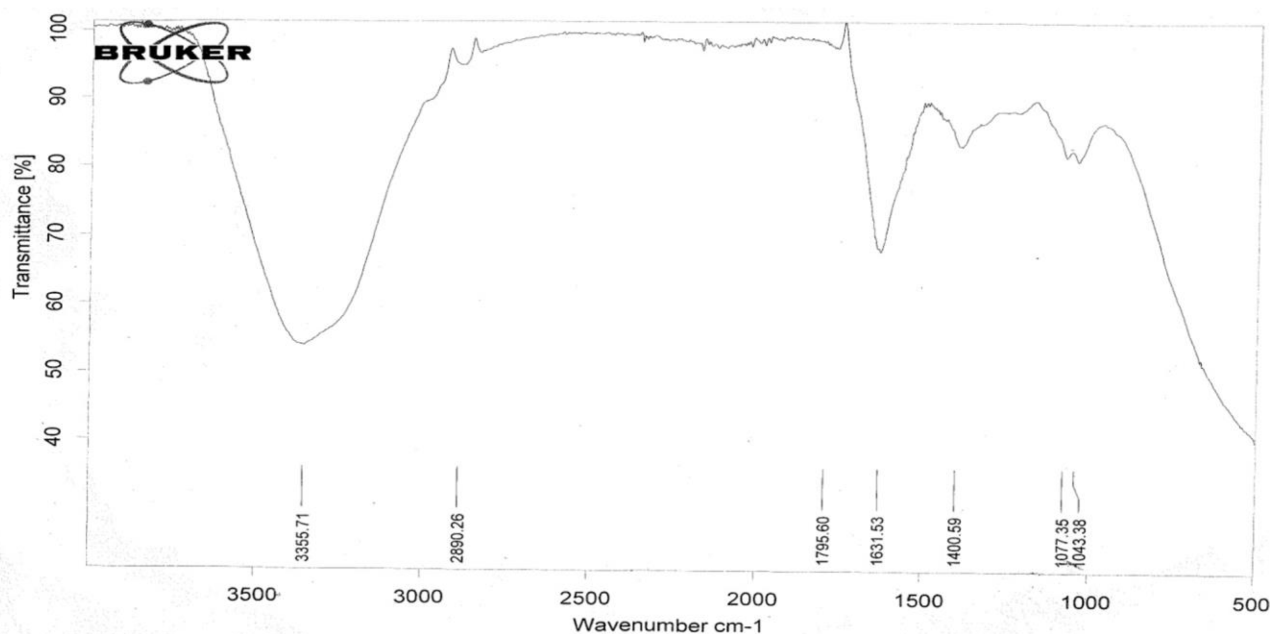


Figure 10: FT-IR spectrum of *C. afer*.

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