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Antioxidant, Anti-Inflammatory, Acute Oral Toxicity, and Qualitative Phytochemistry of The Aqueous Root Extract of *Launaea cornuta* (Hochst. Ex Oliv. & Hiern.)

Evans Kapanat Akimat[1], George Isanda Omwenga[1], Gervason Apiri Moriasi[2], and Mathew Piero Ngugi[1]

Abstract

The root and leaf extracts of *Launaea cornuta* have been locally used in traditional medicine for decades to manage inflammatory conditions and other oxidative-stress-related syndromes; however, their pharmacologic efficacy has not been scientifically investigated and validated. Accordingly, we investigated the *in vitro* antioxidant activity, anti-inflammatory (*in vitro, ex vivo,* and *in vivo*) efficacy, acute oral toxicity, and qualitative phytochemical composition of the aqueous root extract of *L. cornuta*. The ferric-reducing antioxidant power (FRAP) and the 2,2-diphenyl-2-pycrylhydrazyl (DPPH) radical scavenging test methods were used to determine the studied plant extract's antioxidant activity. Besides, the anti-inflammatory efficacy of the studied plant extract was investigated using *in vitro* (anti-proteinase and protein denaturation), *ex vivo* (membrane stabilization), and *in vivo* (carrageenan-induced paw oedema in Swiss albino mice) methods. The studied plant extract demonstrated significant *in vitro* antioxidant effects, which were evidenced by higher DPPH radical scavenging and FRAP activities, in a concentration-dependent manner ($p < 0.05$). Generally, the studied plant extract exhibited significant *in vitro, ex vivo,* and *in vivo* anti-inflammatory efficacy, respectively, and in a concentration/dose-dependent manner compared with respective controls ($p < 0.05$). Moreover, the studied plant extract did not cause any observable signs of acute oral toxicity, even at the cut-off dose of 2000 mg/Kg BW ($LD_{50} > 2000$ mg/Kg BW), and was thus considered safe. Additionally, qualitative phytochemistry revealed the presence of various antioxidant- and anti-inflammatory-associated phytochemicals, which were deemed responsible for the reported pharmacologic efficacy. Further studies to characterise bioactive molecules and their mode(s) of pharmacologic efficacy are encouraged.

Keywords

inflammation, free radicals, antioxidants, herb safety

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1 Introduction

Inflammation is a critical component of the host's response to infection and injury to maintain health. However, prolonged excessive inflammation can injuriously lead to the pathological process of certain ailments like rheumatoid arthritis, asthma, diabetes, inflammatory bowel disease, atherosclerosis, mental disorders like Parkinson's disease, among others.[1–5] Unfortunately, the conventional management of inflammation-associated pathologies is an uphill task since the available conventional drugs negatively impact our health by eliciting serious side effects.[6–11] For example, the continuous use of almost all Nonsteroidal anti-inflammatories (NSAIDs) suppresses the baseline levels of cyclooxygenase (COX-1) enzyme leading to gastric erosion and upper gastrointestinal bleeding, which later may result in death

[1] Department of Biochemistry, Microbiology, and Biotechnology, Kenyatta University, Nairobi, Kenya
[2] Department of Medical Biochemistry, Mount Kenya University, Thika, Kenya

Corresponding Author:
Evans Kapanat Akimat, Department of Biochemistry, Microbiology, and Biotechnology, Kenyatta University, PO BOX 43844-00100, Nairobi, Kenya.
Email: akimzevans@gmail.com

in some individuals.[11] In addition, these synthetic molecules are expensive and inaccessible, especially in resource-limited settings like sub-Saharan Africa, and need qualified medical personnel who are always not readily available to prescribe them.[5]

Free radicals produced during inflammation attack biomembranes and biomolecules, thereby intensifying inflammatory sequelae.[5,12] In addition, free radicals, including peroxyl and hydroxyl (OH, -OOH, and ROO-), are highly reactive molecules containing charged nitrogen and oxygen ions, which cause oxidative stress if not regulated well.[13–15] To ward off oxidative stress, antioxidants are used to scavenge free radicals, thus protecting cells/tissues against oxidative damage.[16] However, synthetic antioxidants like propyl gallate (PG), butylated hydroxytoluene (BHT), and butylated hydroxyanisole (BHA) are associated with carcinogenicity, hepatotoxicity, among other adverse effects in patients.[17–19] Moreover, they are more labile, inaccessible, and unfordable in resource-limited and remote settings, thus limiting their usefulness. Consequently, antioxidant supplements from natural sources, such as Vitamin C from herbs and fruits, offer a viable alternative for thwarting oxidative stress in the body, especially during inflammation.[5,12,20–23]

Medicinal plants are considered more beneficial than conventional drugs since they are biodegradable, natural, have minimal adverse effects, are easily accessible, and relatively affordable.[24] However, only a few plant species have been studied, and their efficacy in treating inflammatory and other oxidative stress-associated disorders validated scientifically. In addition, little is known about the toxic levels and phytochemical composition of the majority of the medicinal plant products, which has led to their slow integration into conventional medicine and acceptability.[25]

Launaea cornuta is traditionally used in Kenya by the Pokot and Embu people to treat inflammatory diseases, such as swollen testicles, earache, and joint pains/algesia.[26,27] The plant is also believed to be a rich source of ascorbic acid (vitamin C), hence considered a potent antioxidant and anti-inflammatory medicine.[28] However, despite the rich ethnomedicinal background of this plant, no focused scientific research has been conducted to confirm its anti-inflammatory effects, antioxidant activity, toxicity profile, and phytochemical composition, hence the present study.

2 Materials and Methods

2.1 Sample Collection and Preparation

Fresh roots of *L. cornuta* were collected from Irangi forest located on the eastern side of Mt. Kenya, Embu County in Kenya (S 0° 20′ 51.4716″, E 37° 27′ 4.32″). The plant was first identified by a renowned local herbalist, and then later authenticated by a taxonomist at the East African Herbaria hosted at the National Museums of Kenya [Voucher number: GM-005-2017]. Herbarium samples were deposited for future reference. The obtained plant samples were cleaned with water to remove dirt/soil particles and air-dried under shade, at 25 °C ± 1 for ten [10] days. The samples were grabbled regularly to deter moisture build-up and aid even drying. Finally, the dried roots were ground [using electric-plant mill], put in khaki envelopes, and stored at 25 °C ± 1 awaiting extraction.

2.2. Aqueous Extraction

The powdered sample (50 g) of *L. cornuta* roots was heated (60 °C) in 200 ml distilled water for about 5 min, cooled to room temperature (25 °C ± 1), decanted, filtered (Whatman No. 1 filter paper; vacuum pump), lyophilized (freeze-dried) for 48 h, and transferred to a clean-dry pre-weighed universal bottle which was then weighed. The percentage (%) yield was calculated using the formula (Eq. 1) stipulated by Truong et al.[29]: An air-tight sealed universal glass bottle containing the extract was stored in a refrigerator (4 °C) awaiting bioassays.

$$\text{Percentage Yield} = \frac{\text{Wt of the extract}}{\text{Wt of the macerated sample}} \times 100 \quad (1)$$

Where, Wt = Weight in grams

2.3 *In Vitro* Antioxidant Assays

2.3.1 In Vitro 2,2-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity. *In vitro* DPPH radical scavenging effects of the aqueous extract of *L. cornuta* were determined according to the method of Brand-Williams et al.[30] as modified by Moriasi et al.[31] Briefly, 12 mg of DPPH was accurately weighed and dissolved in 100 ml of analytical-grade methanol (Sigma-Aldrich, Germany) to obtain a 0.3 mM of DPPH solution for use. Then, 1.5 ml of six concentrations of aqueous root extract of *L. cornuta* (1000, 100, 10, 1, 0.1 and 0.01 µg/ml) were appropriately added to 1.5 ml of 0.3 mM DPPH solution, in triplicate and mixed. Also, 1.5 ml of DPPH was separately added to 1.5 ml of the six concentrations (1000, 100, 10, 1, 0.1 and 0.01 µg/ml) of ascorbic acid, which was used as the positive control. The resultant mixtures were then incubated in a dark room for 30 min and their absorbances were spectrophotometrically (Microprocessor UV-VIS Spectrophotometer; Double beam) determined at 517 nm against the negative control, which comprised distilled water (1.5 ml) and DPPH (1.5 ml). The % radical scavenging/neutralizing activity (RSA) was obtained through the formula (Eq. 2) described by Moriasi et al.[31]:

$$\%\ \text{RSA} = \left(\frac{(a-b)}{a}\right) \times 100 \quad (2)$$

Where, *b;* is the absorbance of the extract or L-Ascorbic acid, and *a;* is the absorbance of the control.

2.3.2 In Vitro ferric reducing power. The *in vitro* ferric reducing power of the aqueous extract of *L. cornuta* was determined using the protocols of Benzie and Strain[32] with slight modifications. In brief, 1.5 ml of respective concentrations of the aqueous root extract (1000, 100, 10, 1, 0.1, and 0.01 µg/ml)

and L-ascorbic acid were separately mixed with 2.5 ml of 30 mM potassium ferricyanide (2.5 ml) and 2.5 ml of phosphate buffer (pH 6.6, 200 mM). The obtained mixtures were incubated in a water bath set at 50 °C for 20 min. Then, 2.5 ml of 600 mM trichloroacetic acid was added, and the mixtures were centrifugated at 3000 rpm for 10 min. After that, 2.5 ml of the resultant supernatants, 2.5 ml of distilled water, and 0.5 ml of 6 mM $FeCl_3$ were mixed, and the absorbances of the resulting solutions were measured at 700 nm using a Microprocessor spectrophotometer, against the negative control, which comprised 1.5 ml of distilled water, 2.5 ml of phosphate buffer, 2.5 ml of potassium ferricyanide, 2.5 ml of trichloroacetic acid, and 0.5 ml of $FeCl_3$. The assay was performed in triplicate.

2.4 *Determination of in Vitro* Anti-Inflammatory Activities

2.4.1 Determination Of antiprotease activity of the studied plant extract. This experiment was performed according to the methods described by Oyedapo and Famurewa.[33] In brief,1 ml of the four concentrations of the aqueous root extract of *L. cornuta* (1000, 100, 10, and 1 µg/ml) were separately mixed with Tris-HCl buffer (20 mM, 1 ml, pH 7.4) and trypsin (0.06 ml, 10 µg/ml). The same set-up was used for the four different concentrations (1000, 100, 10, and 1 µg/ml) of Indomethacin. The obtained respective mixtures were incubated for 5 min at 37 °C, after which 1 ml of 0.8% of casein was added, and further incubated for 20 min at 37 °C. Then, 2 ml of 70% perchloric acid was added to stop the reaction. The negative control setup comprised 1 ml of normal saline (0.9% NaCl), 1 ml of Tris HCl buffer, 0.06 ml trypsin, 1 ml of 0.8% of casein, and 2 ml of 70% of perchloric acid. The solutions were centrifugated at 3500 rpm for 5 minutes, after which, the absorbances of the supernatants were measured at 210 nm using a Microprocessor spectrophotometer. This experiment was conducted in triplicate. The percentage antiproteinase activity was obtained using the formula (Eq. 3) stipulated by Oyedapo and Famurewa[33]:

$$\%\ \text{antiproteinase activity} = \left[\frac{(x-y)}{x} \times 100\right] \quad (3)$$

Where, *x:* is the absorbance of the control, and *y:* is the absorbance of the test sample

2.4.2 Inhibition Of albumin denaturation. The bovine albumin denaturation assay protocol described by Mizushima and Kobayashi[34] was adopted in this study. Briefly, 1.5 ml of the four concentrations of either the aqueous root extract of *L. cornuta* or Indomethacin (1000, 100, 10, and 1 µg/ml) were separately mixed with 1.5 ml of 1% bovine albumin. A mixture of 1 ml normal saline and 1.5 ml of 1% bovine albumin was used as the negative control. The setups were incubated at 37 °C for 20 min, in a water bath, after which the temperature was increased to 51 °C, and further incubated for 20 min. The solutions were cooled to room temperature and their optical densities were measured at 660 nm using a Microprocessor UV-VIS Spectrophotometer. The assay was conducted in triplicate and the percentage inhibition of protein denaturation was calculated using the formula (Eq. 4) described by Mizushima and Kobayashi.[34]

$$\%\ \text{inhibition denaturation} = \left[\frac{\text{AC} - \text{AS}}{\text{AC}} \times 100\right] \quad (4)$$

Where, AC: is the absorbance of the control solution, and AS: is the absorbance of the test sample

2.5 *Determination of ex Vivo* Anti-Inflammatory Efficacy of the Aqueous Root Extract of *L. cornuta*

The human erythrocyte membrane stabilization tests protocol of Oyedapo and Famurewa[33] as modified by Moriasi et al.[23] was followed in this study.

2.5.1 Preparation Of the human Red blood cells (HRBCs). The blood sample, from which the HRBCs were derived, was acquired from the National blood bank at the Kenyatta National Hospital, Nairobi, Kenya. About 10 ml of blood and 10 ml of Alseiver's solution (aseptic; anticoagulant) were mixed and then centrifugated at 3000 rpm for 10 min. The obtained packed RBCs were rinsed three times using normal saline (0.9% NaCl, pH 7.0). After that, a 10% (v/v) erythrocyte suspension was prepared in normal saline and used for hypotonicity-induced and heat-induced haemolysis assay for HRBC stability.

2.5.2 Hypotonicity-induced HRBC haemolysis. In this experiment, 1 ml of either the studied plant extract or Indomethacin, at various concentrations (1000, 100, 10, and 1 µg/ml) were separately dissolved in 1 ml of a phosphate buffer (150 mM, pH 7.4), and mixed with 0.5 ml of the HRBC suspension and 2 ml of hyposaline (0.36% NaCl). Besides, the negative control setup comprised 1 ml of normal saline, 1 ml of phosphate buffer (150 mM, pH 7.4), and mixed with 0.5 ml of the HRBC suspension and 2 ml of hyposaline (0.36% NaCl). The setups were then incubated at 37 °C for 30 min and centrifugated at 3000 rpm using a Labtech centrifuge for 5 min. The supernatants were aspirated and their absorbances were read at 560 nm using a Microprocessor Ultraviolet-Visible spectrophotometer. This experiment was conducted in triplicate. Then, the percentage membrane protection/stabilization of HRBCs was calculated using the formula (Eq. 5) described by Oyedapo and Famurewa.[33]

$$\%\ \text{HRBC membrane stabilization} = 100 - \left[\frac{AbS}{AbC} \times 100\right] \quad (5)$$

Where, *AbS*: is the absorbance of the test sample, and *AbC*: is the absorbance of the control.

2.5.3 Heat-induced Haemolysis. In this assay, 1 ml aliquots of various concentrations (1000, 100, 10, and 1 µg/ml) of either the aqueous root extract of *L. cornuta* or Indomethacin were separately mixed with 1 ml of the HRBC suspension, in triplicate, and incubated at 56 °C for 30 min in a water bath. The solutions were then cooled to room temperature and centrifugated at 2500 rpm for 5 min. After that, the respective absorbance values were read at 560 nm using a Microprocessor Ultraviolet-Visible spectrophotometer. The negative control setup comprised 1 ml of normal saline and 1 ml of the HRBC suspension. The percentage protection or stabilization was calculated according to the formula (Eq. 5) of Oyedapo and Famurewa,[33] described in section 2.5.2.

2.6 *Investigation of in vivo* anti-inflammatory efficacy of the aqueous root extract of *L. cornuta*

2.6.1 Experimental Animals. Swiss-Albino mice (male and female aged 4-5 weeks and weighing 25 ± 2 g) were were obtained from the animal breeding unit of the Kenya Medical Research Institute, Nairobi, and kept in propylene cages, furnished with softwood shavings as bedding material. The mice were housed in the animal handling laboratory facility, under standard conditions (Temperature: 25 ± 2 °C; Relative humidity: 55-65%; 12 h of dark and 12 h of light cycle, and 360 lux lighting). The animals received water and standard rodent feed *ad-libitum*. They were acclimatised to the laboratory conditions for five days before the study.

2.6.2 Induction Of inflammation. Carrageenan (1%; 100 µl) was injected into the subplantar region of the right hind paw of the experimental mice to induce oedema/swelling (inflammation) according to the protocol described by Rahman et al.[35]

2.6.3 Experimental Design and bioassay. We adopted a completely controlled randomized experimental study design, from which we derived an experimental design for this study. Briefly, Swiss albino mice were randomly put into seven groups, each consisting of 5 mice (2 males and 3 females). At the onset, group I and II (normal and negative control groups) received normal saline 0.9% NaCl (10 ml/Kg BW; *p.o*) group III (positive control animals) received Dexamethasone (4.00 mg/Kg BW; *p.o*), while groups IV to VII received the aqueous root extract of *L. cornuta* at doses of 31.25, 62.50, 125.00, and 250.00 mg/Kg BW *p.o*, respectively. After half an hour, oedema was induced in all mice except in the normal control group, as described in section 2.6.2. Table 1 summarizes the experimental design adopted in this study.

The change in paw size was measured immediately after inducing inflammation (0 h) and periodically after 1 h, 2 h, 3 h, and 4 h, respectively, using a vernier calipers. The percentage (%) reduction in paw size (percentage inhibition of oedema) was calculated using the formula (Eq. 6) of Rahman et al.[35]:

Table 1. Experimental Design for *in Vivo* Anti-Inflammatory Assay

Experimental group	Treatment
I	Normal saline (10 ml/Kg BW) *p.o.*
II	Normal saline (10 ml/Kg BW; *p.o*) + Carrageenan (100 µl; *s.p*)
III	Dexamethasone (4.00 mg/Kg BW; *p.o*) + Carrageenan (100 µl; *s.p*)
IV	Extract (31.25 mg/Kg BW; *p.o*) + Carrageenan (100 µl; *s.p*)
V	Extract (62.50 mg/Kg BW; *p.o*) + Carrageenan (100 µl; *s.p*)
VI	Extract (125.00 mg/Kg BW; *p.o*) + Carrageenan (100 µl; *s.p*)
VII	Extract (250.00 mg/Kg BW; *p.o*) + Carrageenan (100 µl; *s.p*)

Extract: aqueous root extract of *L. cornuta*; *p.o* = *per os*/oral administration; *s.p* = subplantar injection; n = 5 mice per treatment group.

$$\%\,\text{Inhibition of oedema (inflammation)} = \left[\frac{Vc - Vt}{Vc}\right] \times 100 \quad (6)$$

Where *Vc* represents the mean paw size of the negative control mice, and *Vt* represents the mean paw size/volume in the extract treated and Indomethacin treated groups of mice.

2.7 *Acute Oral Toxicity Study*

The acute oral toxicity effects of the studied plant extracts were investigated according to the Up-and-Down-Procedure (UDP) described by the Organisation for Economic Development and Corporation (OECD).[36] Swiss albino mice were randomly allotted into four groups comprising five animals and fasted for 4 h before experimentation. Normal control group (Group I) mice were orally administered with normal saline (10 ml/Kg BW) while experimental groups (Groups II-IV) were orally administered with the aqueous root extract of *L. cornuta* at doses of 175 mg/Kg BW, 550 mg/Kg BW, and 2000 mg/Kg BW respectively, in a stepwise manner according to the guidelines of the OECD.[36] Wellness parameters were keenly observed after 30 min, 1 h, 2 h, 4 h, 6 h, 12 h, 24 h, 48 h, seven days, and 14 days for any abnormal behaviour or signs of toxicity. The monitored parameters included the appearance of skin fur, salivation, mucous membrane, lethargy, eyes, convulsions, diarrhoea, coma, tremors, sleep, body weight, and mortality.[36]

2.8 *Qualitative Phytochemical Analysis*

The aqueous root extract of *L. cornuta* was analysed as per the standard protocols described by Harborne[37] and modified by Moriasi et al.[22] The phytochemicals screened were phenols, tannins, alkaloids, flavonoids, steroids, terpenoids, cardiac glycosides, anthraquinones, saponins and coumarins.

2.9 Data Management and Statistical Analysis

Antioxidant and anti-inflammatory effects data were tabulated in an Excel spreadsheet (Microsoft office 2019) and exported to Minitab software version 20 for analysis. The data were descriptively analysed, and results were expressed as mean $\bar{x} \pm$ SEM (standard error of the mean). Unpaired student t-test and One-Way Analysis of Variance (ANOVA) with Fisher's LSD were used appropriately for pairwise comparisons and separation of means. $P < 0.05$ was considered statistically significant.

2.10 Ethical Considerations

Ethical approval for this study was granted by the National Commission of Science, Technology, and Innovation, Kenya (NACOSTI/P/21/9936).

3 Results

3.1 Percentage Yield of Aqueous Root Extract of *L. cornuta*

After the extraction process, a light brown powdered extract, with a yield of 13% was obtained.

3.2 *In Vitro* Antioxidant Activities of *L. cornuta* Extract

3.2.1 DPPH Radical scavenging activity. In the present study, the standard (L-Ascorbic Acid) exhibited a significantly higher DPPH radical scavenging activity compared to the aqueous root extract of *L. cornuta* at all the tested concentrations ($p < 0.05$; Table 2). Further, the percentage DPPH radical scavenging effects of the studied plant extract and L-ascorbic acid were concentration-dependent (Table 2). However, there was no significant difference in DPPH radical scavenging activity among concentrations of 0.1 to 10 µg/ml and between 10 and 100 µg/ml of the aqueous root extract of *L. cornuta* respectively ($p > 0.05$; Table 2).

Table 2. *In Vitro* DPPH Radical Scavenging Activity of the Aqueous Root Extract of *L. cornuta*

	Percentage free radical scavenging activity (% RSA) ($\bar{x} \pm$ SEM)	
Concentration (µg/ml)	The aqueous root extract of *L. cornuta*	Standard (Ascorbic Acid)
0.01	53.30 ± 0.55^{bD}	60.52 ± 0.38^{aF}
0.1	60.28 ± 0.91^{bC}	71.49 ± 0.88^{aE}
1	61.05 ± 0.50^{bC}	79.32 ± 2.50^{aD}
10	62.28 ± 1.26^{bBC}	99.01 ± 0.010^{aC}
100	66.72 ± 1.78^{bB}	99.08 ± 0.017^{aB}
1000	83.84 ± 0.75^{bA}	99.11 ± 0.01^{aA}

Values are expressed as $\bar{x} \pm SEM$; Values with different lower-case superscript letters within the same row (Unpaired student t-test) and different upper-case superscript letters within the same column (One-Way ANOVA with Fisher's LSD *post hoc*) are significantly different ($p < 0.05$).

Table 3. Ferric Antioxidant Reducing Power of Aqueous Root Extract of *L. cornuta*

	Absorbance ($\bar{x} \pm$ SEM)	
Concentration (µg/ml)	The aqueous root extract of *L. cornuta*	Standard (Ascorbic Acid)
0.01	0.56 ± 0.06^{bB}	2.31 ± 0.08^{aE}
0.1	0.66 ± 0.01^{bAB}	2.48 ± 0.02^{aD}
1	0.68 ± 0.00^{bA}	2.54 ± 0.01^{aC}
10	0.70 ± 0.01^{bA}	2.61 ± 0.04^{aB}
100	0.72 ± 0.01^{bA}	2.65 ± 0.02^{aA}
1000	0.74 ± 0.01^{bA}	2.65 ± 0.06^{aA}

Values are expressed as $\bar{x} \pm SEM$; Values with different lower-case superscript letters within the same row (Unpaired student *t*-test) and different upper-case superscript letters within the same column (One-Way ANOVA with Fisher's LSD *post hoc*) are significantly different ($p < 0.05$).

3.2.2 Ferric Antioxidant reducing power. In this study, L-ascorbic acid exhibited significantly higher *in vitro* ferric ion-reducing efficacy than the aqueous root extract of *L. cornuta* at all concentrations ($p < 0.05$; Table 3). No significant differences in *in vitro* ferric ion reduction efficacy of the studied plant extract were observed among concentrations of between 0.1 µg/ml and 1000 µg/ml ($p > 0.05$; Table 3).

3.3 *In Vitro* Anti-Inflammatory Activity of the Aqueous Root Extract of *L. cornuta*

3.3.1 Antiprotease Activity. The aqueous root extract of *L. cornuta* exhibited significantly higher antiprotease activity at concentrations of 100 µg/ml and 10 µg/ml than Indomethacin ($p < 0.05$; Table 4). However, at concentrations of 1 µg/ml and 1000 µg/ml, no significant differences in antiprotease effects were observed between the studied extract and Indomethacin (standard drug) ($p > 0.05$; Table 4). Generally, the studied plant extract and indomethacin exhibited a concentration-dependent increase in antiprotease activity (Table 4).

Table 4. Percentage Antiprotease Activity of Aqueous Root Extract of *L. cornuta*

	% Antiprotease activity ($\bar{x} \pm$ SEM)	
Concentration (µg/ml)	Aqueous root extract of *L. cornuta*	Standard (Indomethacin)
1	10.69 ± 0.42^{aC}	10.40 ± 0.20^{aC}
10	15.23 ± 0.18^{aB}	14.56 ± 0.08^{bB}
100	20.27 ± 0.39^{aA}	14.91 ± 0.31^{bB}
1000	21.42 ± 0.47^{aA}	20.98 ± 0.41^{aA}

Values are expressed as $\bar{x} \pm SEM$; Values with different lower-case superscript letters within a row (Unpaired student t-test) and different upper-case superscript letters within a column (One-Way ANOVA with Fisher's LSD *post hoc*) are significantly different ($p < 0.05$).

Table 5. Percentage Inhibition of Protein Denaturation by the Aqueous Root Extract of *L. cornuta*.

	% Inhibition of protein denaturation ($\bar{x} \pm$ SEM)	
Concentration (µg/ml)	Aqueous root extract of *L. cornuta*	Standard (Indomethacin)
1	76.22 ± 0.55^{aD}	40.31 ± 0.13^{bD}
10	88.43 ± 0.21^{aC}	76.28 ± 0.24^{bC}
100	90.66 ± 0.36^{aB}	80.67 ± 0.41^{bB}
1000	93.11 ± 0.15^{aA}	91.91 ± 0.28^{bA}

Values are expressed as $\bar{x} \pm SEM$; Values with different lower-case superscript letters within a row (Unpaired student t-test) and different upper-case superscript letters within a column (One-Way ANOVA with Fisher's LSD *post hoc*) are significantly different ($p < 0.05$).

3.3.2 Albumin Denaturation. The aqueous root extract of *L. cornuta* produced significantly higher percentage inhibition of albumin denaturation than the standard (indomethacin) at all the tested concentrations ($p < 0.05$; Table 5). Generally, the percentage inhibitions of albumin denaturation caused by the studied extract and Indomethacin were concentration-dependent ($p < 0.05$; Table 5).

3.4 *Ex Vivo* Anti-Inflammatory Activity of the Aqueous Root Extract of *L. cornuta*

3.4.1 Hypotonicity-induced HRBC haemolysis. The results showed that, at all the studied concentrations, *L. cornuta* extract and standard drug (Indomethacin) significantly protected the human red blood cell membranes against hypotonicity-induced cell lysis ($p < 0.05$; Table 6). Furthermore, the extract exhibited varying degrees of membrane stabilization ranging from 13.18% to 82.19% in a concentration-dependent manner (Table 6). Notably, the percentage inhibitions of hypotonicity-induced erythrocyte haemolysis caused by the aqueous root extract of *L. cornuta* at concentrations of between 10 µg/ml and 1000 µg/ml, were significantly higher than those produced by indomethacin, at the similar concentrations ($p < 0.05$; Table 6).

Table 6. Percentage HRBC Membrane Stabilization Effect of the Aqueous Root Extract of *L. cornuta* Following Hypotonicity-Induced Haemolysis.

	% Membrane stabilization effect ($\bar{x} \pm$ SEM)	
Concentration (µg/ml)	Aqueous root extract of *L. cornuta*	Standard (indomethacin)
1	13.18 ± 1.15^{bD}	35.91 ± 0.26^{aD}
10	50.22 ± 1.89^{aC}	43.90 ± 0.73^{bC}
100	71.40 ± 0.23^{aB}	56.68 ± 0.52^{bB}
1000	82.19 ± 1.06^{aA}	74.95 ± 0.38^{bA}

Values are expressed as $\bar{x} \pm SEM$; Values with different lower-case superscript letters within the same row (Unpaired student t-test) and different upper-case superscript letters within the same column (One-Way ANOVA with Fisher's LSD *post hoc*) are significantly different ($p < 0.05$).

Table 7. Percentage HRBC Membrane Stabilization Effect of the Aqueous Root Extract of *L. cornuta* Following Heat-Induced Haemolysis

	% Membrane stabilization effect ($\bar{x} \pm$ SEM)	
Concentration (µg/ml)	Aqueous root extract of *L. cornuta*	Standard (Indomethacin)
1	76.65 ± 0.69^{aB}	60.58 ± 0.093^{bD}
10	81.16 ± 0.37^{aA}	68.44 ± 0.45^{bC}
100	81.79 ± 0.55^{aA}	75.83 ± 0.17^{bB}
1000	82.24 ± 0.43^{bA}	85.91 ± 0.46^{aA}

Values are expressed as $\bar{x} \pm SEM$; Values with different lower-case superscript letters within a row (Unpaired student t-test) and different upper-case superscript letters within a column (One-Way ANOVA with Fisher's LSD *post hoc*) are significantly different ($p < 0.05$).

3.4.2 Heat-induced HRBC Haemolysis

The aqueous root extract of *L. cornuta* exhibited significantly higher membrane stabilization efficacy in the heat-induced erythrocyte haemolysis than the standard drug (Indomethacin) at concentrations of between 1 to 100 µg/ml ($p < 0.05$; Table 7). However, at a 1000 µg/ml concentration, the extract showed a significantly lower percentage membrane stabilization effect compared to that of the standard drug ($p < 0.05$; Table 7).

3.5 *In Vivo* Anti-Inflammatory Activity of Aqueous Root Extract of *L. cornuta*

In this study, the aqueous root extract of *L. cornuta* significantly reduced carrageenan-induced paw oedema (inflammation) in a dose- and time-dependent manner (Table 8). Generally, the anti-inflammatory effects of the positive control (Dexamethasone) were significantly higher than the effects of the studied plant extract at doses of 31.25 mg/Kg BW, 62.5 mg/Kg BW, and 125.0 mg/Kg BW, at each hour ($p < 0.05$; Table 8). Notably, the aqueous root extract of *L. cornuta,* at a dose of 250 mg/Kg BW showed significantly higher reductions of carrageenan-induced paw oedema than those recorded for the positive control (Dexamethasone) at each hour ($p < 0.05$; Table 8).

3.6 *Acute Toxicity of L. cornuta* Extracts

The aqueous root extract of *L. cornuta* did not exhibit any observable signs of acute oral toxicity in experimental mice at the tested doses (175 mg/Kg BW, 550 mg/Kg BW, and 2000 mg/Kg BW, respectively), throughout the 14-day experiment period. In addition, the behavioural and wellness parameters, such as the appearance of skin fur, salivation, mucous membrane, lethargy, eyes, convulsions, diarrhoea, coma, tremors, sleep, and body weight were normal throughout the experimental period, and no mortality was recorded. Therefore, the LC_{50} of the extract was considered to be >2000 mg/Kg BW according to the OECD guidelines.[36]

Table 8. *In Vivo* Anti-Inflammatory Activity of Aqueous Root Extract of *L. cornuta* in Carrageenan-Induced paw Oedema in Mice.

	Percentage change of oedema (% reduction of oedema)			
Treatment	first Hour	second Hour	third Hour	4thHour
normal control	100.19 ± 0.10^{bA} (-0.19)	99.39 ± 0.18^{bA} (0.61)	99.60 ± 0.10^{bA} (0.40)	99.91 ± 0.062^{bA} (0.09)
negative control	123.33 ± 0.14^{aC} (-23.33)	124.50 ± 0.22^{aBC} (-24.50)	125.02 ± 0.32^{aAB} (-25.02)	126.61 ± 0.17^{aA} (-26.61)
Positive control	92.54 ± 0.22^{dA} (7.47)	83.79 ± 0.21^{fB} (16.21)	74.22 ± 0.20^{eC} (25.78)	67.32 ± 0.21^{eD} (32.68)
LCR (31.25 mg/Kg BW)	97.42 ± 0.19^{cA} (2.58)	95.48 ± 0.14^{cB} (4.52)	93.35 ± 0.18^{cC} (6.65)	87.38 ± 0.18^{cD} (12.62)
LCR (62.50 mg/Kg BW)	92.37 ± 0.24^{dA} (7.63)	90.28 ± 0.21^{dB} (9.72)	89.63 ± 0.17^{dB} (10.37)	81.40 ± 0.18^{dC} (18.60)
LCR (125.00 mg/Kg BW)	89.19 ± 0.11^{eA} (10.81)	86.19 ± 0.10^{eB} (13.81)	74.81 ± 0.66^{eC} (25.19)	67.36 ± 0.13^{eD} (32.64)
LCR (250.00 mg/Kg BW)	84.73 ± 0.10^{fA} (15.27)	80.75 ± 0.10^{gB} (19.25)	68.93 ± 0.17^{fC} (31.07)	61.18 ± 0.13^{fD} (38.82)

Values are expressed as $\bar{x} \pm SEM$; Values with different lower case superscript letters within the same column and those with different uppercase superscript letters within the same row are significantly different ($p < 0.05$; One-Way ANOVA with Fisher's LSD *post hoc* test).
LCR = Aqueous root extract of *L. cornuta*.

3.7 Qualitative Phytochemical Composition of the Aqueous Root Extract of L. cornuta

In the present study, all the tested phytochemicals were detected in the aqueous root extract of *L. cornuta* except saponins and coumarins, which were absent (Table 9).

4 Discussion

Medicinal plants/herbs have been used since time immemorial to cure and manage various diseases as they are considered a healthy source of medicines.[38] It is now known that the medicinal benefit of plants is conferred by some phytoactive ingredients that influence physiological activity in our body's processes.[39] Among the many substances produced by plants are coumarins, alkaloids, flavonoids, glycosides, tannins, and phenolic compounds responsible for pharmacological bioactivity.[22,40]

Table 9. Qualitative Phytochemical Composition of the Aqueous Root Extract of *L. cornuta*

Phytochemical	Observation
Phenols	+
Tannins	+
Alkaloids	+
Flavonoids	+
Steroids	+
Terpenoids	+
Cardiac glycosides	+
Anthraquinones	+
Saponins	−
Coumarins	−

(+) implies the presence of respective phytochemicals while (−) indicates their absence.

Inflammation produces free radicals, which exacerbate inflammatory processes by attacking cellular components as witnessed in diseases like arthritis and arteriosclerosis, among others.[5,41] Furthermore, oxidative stress leads to the destruction of biomolecules in cells.[42,43] Antioxidants exert their quenching effects by either preventing the synthesis of free radicals, or by inactivating them. Endogenous body systems produce antioxidants to counteract the effects of radicals produced during inflammation; however, the concentration of free radicals produced may be too much for the body's antioxidants to avert, hence the need to supplement antioxidants in the diet to aid in fighting oxidative stress.[44,45]

The DPPH radical scavenging[30] and the ferric reducing antioxidant power (FRAP)[32] assays were employed in determining the *in vitro* antioxidant activity of aqueous root extract of *L. cornuta*. Based on the obtained results, *L. cornuta* aqueous extract was a powerful free radical neutralizer and can be a good antioxidant in treating chronic inflammatory conditions. Research has shown that polyphenols like flavonoids, phenols, and tannins have profound antioxidant activity.[31,42,46] These polyphenolics may have provided electrons required to stabilize or scavenge free radicals in these assays,[47] depicting its antioxidant efficacy. Furthermore, hydroxyl groups and conjugated structures, which scavenge free radicals through hydrogenation by donating hydrogen atoms, make polyphenols excellent antioxidants.[48,49] Therefore, the fact that the aqueous root extract of *L. cornuta* possesses antioxidant efficacy may explain its anti-inflammatory effects in managing various inflammatory conditions in traditional medicine.

Leukocyte proteinases cause tissue damage when secreted in excessive amounts;[50] thus, inhibition of proteinases can provide significant protection against harmful inflammatory response.[29] The denaturation mechanism of serine proteinases may be associated with the alteration of hydrogen and Van der Waals bonds

in their structure, which disrupt the tertiary, secondary, or quaternary structures of proteins and nucleic acids leading to loss of bioactivity. Denaturation occurs following subjection of proteins to pH extremes (strong acids and bases), high temperatures, organic solvents, or highly concentrated inorganic salts.[51]

As in the case of chronic inflammation, most biological proteins lose their biological function when denatured, leading to a myriad of disease conditions associated with inflammation.[52] When proteins are heated, they undergo denaturation, leading to exposure of antigens that resemble the antigens produced during chronic inflammation like in rheumatoid arthritis.[53] The NSAIDs, apart from suppressing prostaglandins synthesis, have been found to protect proteins from denaturation.[24]

The low trypsin inhibitory activity of the aqueous root extract of *L. cornuta* and standard drug (Indomethacin), observed herein, indicates low efficacy against proteinase-induced tissue damage, such as during haemolysis of the lysosomes.[23] This means that the potency of the extract might be through the alteration of other pathways of inflammation. Besides, the phytocompounds present in the *L. cornuta* extracts could prevent albumin (protein) from getting denatured by reacting with the amino acids exposed due to heat. Thus, interaction with amino acids like lysine and threonine could prevent protein precipitation.[53] Therefore, it can be deduced that, the prevention of protein denaturation may be one mechanism by which the aqueous root extract of *L. cornuta* exerts its anti-inflammatory efficacy against the various conditions it is traditionally used to treat.

We adopted the hypotonicity- and heat-induced haemolysis of the human erythrocyte membrane techniques[23] to evaluate the *ex vivo* anti-inflammatory efficacy of the aqueous root extract of *L. cornuta*. The membrane stabilization process involves maintaining the integrity of biological membranes upon exposure to high temperatures or hypotonic medium.[48,54] Human red blood cells' lysis mimics, to a great extent, the lysis of the lysosomal membrane due to inflammation.[23,48] The lysosomal enzymes (e.g bactericidal protease) released following the rapture of lysosomal membranes upon stimulation by an inflammatory event are key pathological drivers of various disorders, such as diabetes mellitus.[15,52] Therefore, stabilising the lysosomal membranes of activated neutrophils prevents the secretion of proteases and bacterial enzymes that would aggravate the inflammatory process.[46] Additionally, various commonly used NSAIDs work by inhibiting the activity of these enzymes or preventing lysis of the lysosomal membranes to block release of the proteases.[55,56]

We noted that the aqueous root extract of *L. cornuta* exerted significant membrane stabilization activity in hypotonic and heat induced HRBC haemolysis. Our findings corroborate those observed by Leelaprakash et al.[51] Our study suggests that the aqueous root extract of *L. cornuta* possesses phytochemicals that stabilise erythrocyte membranes and prevent lysis upon exposure to osmotic or heat stress. Some bioactive phytochemicals bind to erythrocytes, thereby causing alterations of ionic charges on cell surfaces, which may hinder interaction with lysis-causing agents or literary repel like-charges that would cause haemolysis. Flavonoids and tannins have been demonstrated to stabilize lysosomal membranes, and the erythrocyte membrane, by binding negatively charged ions, thereby averting haemolysis.[48] Therefore, the studied plant extract's efficacy could be based on the inhibition of tissue-damaging enzymes' release by stabilizing lysosomal membranes and inhibiting protein denaturation, as observed by Govindappa et al.[52]

Previous reports demonstrate that the primary cause of biological membrane lysis is oxidative stress, which impairs the ability of HRBCs to endure heat and osmotic stress.[23] The existence of antioxidant phytochemicals like tannins and flavonoids in *L. cornuta* extract may neutralise reactive oxygen/nitrogen species, thereby averting oxidative damage. Decoctions from different parts of this plant (*L. cornuta*) are reportedly used to treat swollen testicles, joint pains, and wounds.[27] The stabilization/protection of the membranes of lysosomes could be the probable mechanism of action of *L. cornuta* extracts when used to decrease inflammation during wound healing since it aids the release of less tissue-damaging enzymes to the inflamed site.[26,27]

The Carrageenan-induced paw oedema/swelling technique described by Boukhatem et al.[57] and Rahman et al.[35] was adopted to determine the *in vivo* anti-inflammatory activity of the studied plant extract. Carrageenan, a polysaccharide extracted from algae (*Chondrus crispus*),[58,59] induces inflammation in a highly sensitive and reproducible way.[60,61] Upon introducing carrageenan into mice's hind paw, it acts as a thermal and chemical stimulus, thus provoking the mice's immune defence mechanisms to respond, with the aim of destroying and clearing the stimulus.[35,57,59] In the process, complex inflammatory mechanisms evoke the development of oedema, which is the physical manifestation of inflammation.[62]

The development of the swelling (oedema) in the sub plantar tissue of the mice after injection of carrageenan is caused by the release of serotonin, prostaglandin, bradykinin, histamine, among other pro-inflammatory mediators.[58,63] Furthermore, the swelling is known to occur in two phases (biphasic); the first phase (0-1 h) of the swelling is due to the production of hydroxytryptamine, histamine, bradykinin, and serotonin, while the second phase (1-4 h) is contributed by the release of prostaglandin-like mediators, free radicals, and Cox-2 activation.[54,64–66]

We observed that the standard drug (Dexamethasone) inhibited inflammation at all hours (1-4 h: both phases of inflammation). Dexamethasone is a steroidal (synthetic glucocorticoid) anti-inflammatory agent, which inhibits phospholipase A_2 enzyme, whose function is to cleave phospholipids to liberate arachidonic acid, hence hindering prostaglandins synthesis. In addition, Dexamethasone inhibits the synthesis of proteins (expression of inflammatory mediators) that would initiate and maintain the inflammatory process.[67,68] Similarly, the aqueous root extract of *L. cornuta* generally reduced swelling at all hours (first through to the fourth hour; both phases) and at all the administered doses. The same trend was observed by Ilavarasan et al.[42], Fathiazad et al.[66], and Demsie and Yimer,[69] in similar investigations. Moreover, the aqueous

root extract of *L. cornuta* exhibited significantly higher inhibition of oedema at the last hour (fourth hour). Demsie and Yimer[69] attested that the late phase is more sensitive to conventionally used agents, which supports our findings. The observed extract's potency against inflammation, in both phases (early and late phases), may be attributed to the existence of bioactive constituents in *L. cornuta* root extract that are capable of attenuating secretion of amines (histamine) and peptides (kinins) in the first phase, as well as eicosanoids and nitrogen/oxygen species which are released in the second phase of inflammation. It was also observed that at a dose of 250 mg/Kg BW, the aqueous root extract of *L. cornuta* registered greater potency than Dexamethasone, perhaps due to higher concentrations of bioactive substances present at this dose level.

Natural products from plants are regarded, especially in developing countries, as safe and are consumed with no regard to their potential toxic effects; hence assessing their safety is crucial.[70] Notably, most herbs used in traditional medicine have not been investigated and validated scientifically as medicines against the diseases they are claimed to cure, and their safety profiles are unknown.[71] For instance, *L. cornuta* is used in Kenyan communities to treat ailments such as arthritis for ages; however, its safety and toxicologic profile data are lacking. Therefore, we evaluated the acute oral toxicity effects of the aqueous root extract of *L. cornuta* in experimental mice to appraise its safety. Research reports indicate that mice, just like humans, are sensitive to toxic compounds in plant extracts since their biological activities and genetics resemble those of humans to a greater degree.[72,73]

We observed no mortality, abnormal behaviour, or any acute oral toxicity associated signs in experimental animals that were administered with the aqueous root extract of *L. cornuta,* even at the limit dose of 2000 mg/Kg BW. According to the OECD guidelines,[36] a plant extract or chemical that do not elicit any observable signs of acute oral toxicity even at a dose of 2000 mg/Kg BW is deemed safe. Therefore, the aqueous root extract of *L. cornuta* was safe as its LD_{50} was greater than 2000 mg/Kg BW; however, further toxicological investigations are necessary to exhaustively establish its safety and toxicity profile. Besides, research shows that orally administered chemical substances or drugs with $LD_{50} > 1000$ mg/Kg BW are considered practically non-toxic and safe.[71] Elsewhere, similar results were obtained for single-oral dose (2000 mg/Kg BW) administration of *C. fistula* extracts in mice.[70]

Qualitative phytochemical screening of the aqueous root extract of *L. cornuta* revealed the presence of steroids, phenols, tannins, flavonoids, alkaloids, and anthraquinone glycosides. Previous research shows that flavonoids have anti-inflammatory activity.[5,74,75] Further research indicates that flavonoids inhibit prostaglandin synthetase (endoperoxidase), thereby exerting their anti-inflammatory effects.[65] Elsewhere, Ilavarasan et al.[42] demonstrated that flavonoids are potent agents with anti-arthritic activity. Besides, the antioxidant potency of flavonoids is probably responsible for quenching excess free radicals generated during the process of inflammation to thwart oxidative damage to cellular components.[5]

Furthermore, terpenoids inhibit crucial steps in the nuclear factor – kappa B (NF-kB) signalling pathway, thereby down-regulating the production of some pro-inflammatory mediators like cytokines. Furthermore, steroids have been reported to inhibit the secretion of anti-inflammatory mediators.[56] Therefore, these compounds may have played a critical role, either solely, or synergistically, in conferring the antioxidant and anti-inflammatory efficacy of the aqueous root extract of *L. cornuta.* Therefore, the findings of this study partly validate the healing claims against inflammatory-associated disease, as reported in traditional medicine.

5. Conclusion and Recommendations

Based on this study's findings, the aqueous root extract of *L. cornuta* is a potent scavenger of the DPPH radical and reducer of the ferric ion *in vitro,* which indicates its potent antioxidant activity. Besides, the aqueous root extract of *L. cornuta* exhibits remarkable *in vitro, ex vivo,* and *in vivo* anti-inflammatory efficacy. The studied plant extract contains various antioxidant and anti-inflammatory-associated phytochemicals, which may be responsible for its pharmacologic efficacy. Furthermore, the studied plant extract does not cause any observable acute oral toxicity effects in experimental mice. Further research aimed at isolating specific active compounds responsible for anti-inflammatory and antioxidant activities from the studied plant extract, determining their mode(s) of action, and fully establishing their toxicity profiles and safety are encouraged.

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Author Contributions

Evans Akimat conceived the research idea, performed the experiments, and drafted the manuscript under the supervision of George Omwenga and Mathew Ngugi. Gervason Moriasi designed the experiments, donated research materials, assisted in data collection, analysis, and interpretation of the findings. All authors reviewed the draft manuscript and approved the final version for publication.

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ORCID iDs

Evans Kapanat Akimat https://orcid.org/0000-0001-9290-9956
Gervason Apiri Moriasi https://orcid.org/0000-0001-5604-9987
Mathew Piero Ngugi https://orcid.org/0000-0002-0226-9092

Source of Experimental Animals

Swiss-Albino mice (4-5 weeks old, 25 ± 2 g BW) were obtained from the Kenya Medical Research Institute Nairobi.

Data Availability

All data is included in the manuscript, and any additional information is available from the authors upon request.

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