ANTI-INFLAMMATORY AND ANTIOXIDANT PROPERTIES OF THE ROOT EXTRACT OF *CARISSA EDULIS* (FORSK.) VAHL (APOCYNACEAE)

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ABSTRACT

Root extracts of Carissa edulis (Forsk.) Vahl (Apocynaceae) are used for the treatment several pathological states including inflammatory disorders. The present study was undertaken to evaluate the effect of an alcoholic extract of C. edulis (CEE) on carrageenan-induced foot oedema in chicks. Also since free radicals and reactive oxygen species are implicated in inflammatory diseases, the anti-oxidant potential of extract was investigated in in vitro experimental models. Oral administration of CEE (30-300 mg kg^{-1} p.o.) significantly inhibited carrageenan-induced foot oedemas with a maximal inhibition of 53.8±8.2%. Similarly, the NSAID diclofenac (10-100 mg kg^{-1}, i.p.) and the steroidal anti-inflammatory agent dexamethasone (0.3-3 mg kg^{-1}, i.p) reduced the total oedema with a maximal inhibition of 62.7±9.1% and 66.4±7.8% respectively. The extract also scavenged DPPH and prevented lipid peroxidation in rat brain homogenates. These results suggest that alcoholic extract of C. edulis exerts in vivo antiinflammatory activity after oral administration and also has antioxidant properties which may contribute to its activity.

INTRODUCTION

Carissa edulis (Fam. Apocyanacae) is a plant indigenous to most parts of tropical Africa, Arabia and across Asia to Indo-china. The whole plant is known to have several medicinal properties. The leaves are used to relieve toothache; the roots to treat inflammation and rheumatism (Giday *et al.*, 2003), chest pains (Bentley *et al*., 1984) and infections such as gonorrhoea, syphilis, rabies malaria and typhoid fever.

Pharmacologically, it is reported to be a diuretic (Nedi *et al.*, 2004), an antiplasmodial (Clarkson *et al.*, 2004) and a hypoglycaemic agent (El-Fiky *et al.*, 1996).

Phytochemical evaluation reveals several principles from the plant including hydroxyacetophenone (Bentley *et al.*, 1984), lignans and sesquiterpenes (Achenbach *et al.*, 1983; Achenbach *et al.*, 1985). Soluble phenolics, which are reported to be potent antioxidants by scavenging free radicals (Shahidi *et al.*, 1992), have also been identified in the plant (Reed, 1986). It has yet to be estab-

lished, however, if the reported antioxidant and the free radical scavenging actions of the plant are linked to the traditional uses of the plant as an anti-inflammatory agent.

Inflammation is characterised by release of chemicals from tissues and migrating cells (Tomlinson *et al.*, 1994) and reactive species generated at the site of inflammation that contribute immensely to tissue damage (Hischi *et al.*, 1989; Salvemini *et al.*, 1996) such that antioxidants could play a key role in resolving the inflammatory episode. To date, there is little scientific evidence to support the traditional use of *Carissa edulis* in the treatment of inflammatory-related diseases and the possible mechanisms involved.

In this study, we evaluate the root extract of *C. edulis* for possible anti-inflammatory and antioxidant activities in an acute model of inflammation.

MATERIALS AND METHODS

Plant material

The roots of *Carissa edulis* were collected from the Accra plains near Dodowa in the Republic of Ghana during the month of August, 2005 and authenticated at the Department of Pharmacognosy, Faculty of Pharmacy and Pharmaceutical Sciences, College of Health Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana.

Preparation of extract

The roots of *Carissa edulis* were chopped and sun-dried each day for a period of seven days. The dried roots were then powdered using a hammer mill. The powder was extracted with 70%^{v}/v ethanol using the Soxhlet extraction apparatus. The extract was concentrated under reduced temperature and pressure and further dried over a water bath at 50°C for three consecutive days before storage in a desiccator. This crude extract was subsequently referred to as CEE in the present study.

Animals

Cockerels (*Gallus gallus;* strain, Shaver 579, Akropong Farms, Kumasi, Ghana) were obtained one-day post hatch and were housed in stainless steel cages (34×57×40 cm) at a population density of 12–13 chicks per cage. Food (Chick Mash, Gafco, Tema, Ghana) and water were available *ad libitum* through 1-qt gravity-fed feeders and waterers. Room temperature was maintained at 29°C and overhead incandescent illumination was provided on a 12-h light–dark cycle. Chicks were tested at 7 days of age. Sprague-Dawley rats (200-250 g) were purchased from the Noguchi Memorial Institute for Medical Research, Accra, Ghana and housed in the animal unit of the Department of Pharmacology, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana. They were housed in groups of 5 in stainless steel cages (34´47´18 cm) with soft wood shavings as bedding and fed with normal commercial pellet diet (GAFCO, Tema, Ghana) and water *ad libitum*. The studies were conducted in accordance with internationally accepted principles for laboratory animal use and care (EEC directive of 1986: 86/609 EEC). Approval for this study was obtained from the Ethical Review Committee of the College of Health Sciences, KNUST, Kumasi, Ghana.

Effect of CEE on carrageenan-induced oedema

CEE (30, 100 and 300 mg/kg) suspended in 2% tragacanth was administered p.o. to chicks (n = 5). One hour after CEE administration oedema was induced according to the method of Roach and Sufka, (2003). Briefly, carrageenan (10 ml of a 1% solution in saline) was injected into the subplantar tissue of the right footpads of the chicks. Oedema was monitored at hourly intervals over 4 h as the percentage increase in foot volume (Fereidoni *et al.*, 2000). Dexamethasone (0.3-3.0 mg/kg, i.p) and diclofenac (10-100 mg/kg, i.p) were used as positive controls. All drugs and extract doses were administered in volumes not exceeding 100 ml kg^{-1} p.o.

Drug vehicle (2% tragacanth, 100 ml/kg p.o)

served as a negative control. Drug effects were assessed by comparing either the peak oedema response attained during the 4 h or the total oedema (monitored as area under the time course curves) response developed during the period.

Total phenol content

Total phenolics of CEE was determined using the Folin-Ciocalteau reagent (Singleton and Slinkard, 1977). Various concentrations (0.05, 0.15, 0.5, 1.5 mg/ml) of CEE (1 ml) were mixed with the Folin–Ciocalteu phenol reagent (1 ml; diluted 1:10 with distilled water). Na_2CO_3 (2% w/v, 1 ml) was added to this mixture and incubated at room temperature (28^0C) for 2 h. Absorbance was then read at 760 nm using a Cecil UV/VIS spectrophotometer (Model: CE 7200, Milton, England). Samples were centrifuged at 650 *g* for 10 minutes and the supernatant taken for measurements. Tannic acid was used as a reference standard. The total phenolics were expressed as milligrams per milliliter of tannic acid equivalents (TAEs).

Reducing power

The antioxidant potential of the extract was determined using the Fe^{3+} reduction (reducing power test) described by (Oyaizu, 1986). Briefly, 1 ml of various concentrations of CEE (0.08, 0.25, 0.83, 2.50 mg/ml) were mixed with 2.5 ml of 0.2M sodium phosphate buffer (pH 6.6) and 2.5 ml 1% potassium ferricyanide solution $[K_3Fe(CN)_6]$ in a test tube. The mixtures were incubated at 50 °C for 20 min. Trichloroacetic acid (10%; 1.5 ml) was then added to the mixtures and centrifuged at 650 *g* for 10 minutes. Two and a half milliliters of the supernatant was mixed with 2.5ml distilled water and 0.5 ml of 0.1% ferric chloride solution and the absorbance read at 700 nm. For blank samples, 1 ml distilled water was added to 2.5ml sodium phosphate buffer and 2.5 ml potassium ferricyanide and the mixture processed as above. Propyl gallate was used as standard antioxidant. All measurements were done in triplicate. Results were then expressed as percentages of blank and the concentration of extracts required to cause a 50% decrease in the absorbance was calculated (IC_{50}).

Scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH)

The test is based on decolourisation of the purple coloured methanolic solution of DPPH free-radical to yellow by free-radical scavengers. One millilitre methanolic solution of CEE (0.1, 0.3, 1.0 and 3.0 mg/ml) were added to 3 ml methanolic solution of DPPH (20mg/l) in a test tube. The reaction mixtures were kept at 25˚C for 1 h. The absorbance of the residual DPPH was then determined at 517nm using a Cecil UV/VIS spectrophotometer (Model: CE 7200, Milton, England). The scavenging action of CEE (0.1, 0.3, 1.0 and 3.0 mg/ml in methanol) was compared to the standard, *n*-propyl gallate (0.01, 0.03, 0.1, 0.3 mg/ml in methanol). One millilitre methanol (99.8%) added to 3.0 ml DPPH solution, incubated at 25˚C for 1 h served as control and methanol (99.8%) was used as blank. The absorbance decreases with increasing free radical scavenging activity. Results were expressed as percentages of blank and the concentration of extracts required to cause a 50% decrease in the absorbance was calculated (IC_{50}).

Lipid peroxidation

For lipid peroxidation determination, rats weighing 200-250 g were euthanized and whole brain (except cerebellum) was dissected out and homogenized (100 mg/ml) in ice-cold phosphate buffer (0.1 M, pH 7.4) and used as a source of polyunsaturated fatty acids. The extract (1-30 mg/ml) and n-propyl gallate (0.01-0.3 mg/ml), were dissolved in methanol. The extent of lipid peroxidation in brain tissue was assayed by measuring the thiobarbituric acid-reactive substances (TBARS) using the standard method (Ohkawa *et al.*, 1979), with modifications. Briefly, 2.5 ml of brain homogenate was mixed with 1 ml of phosphate buffer and 0.5 ml of test drug. Lipid peroxidation was then initiated by the addition of 0.5 ml of 0.1 mM ascorbic acid and 0.5 ml of 5 mM fer-

ric chloride. The mixture was incubated in a shaking water bath at 37°C for one hour after which 0.1 ml of the reaction mixture was transferred into a test tube containing 1.5 ml of 10% trichloroacetic acid (TCA). The tubes were allowed to stand for ten minutes before centrifugation at 1150 *g* for 10 minutes.

The supernatants were separated and mixed in a tube containing 1.5 ml of 0.67% thiobarbituric acid (TBA) in 20% acetic acid. The reaction mixtures were heated in a hot water bath at 85°C for one hour, allowed to cool and absorbance determined at 535 nm in a Cecil UV/VIS spectrophotometer (Model: CE 7200, Milton, England). Phosphate buffer was used as blank in the experiments. Test results were determined in quadruplicates.

Percentage inhibition of lipid peroxidation by the test drugs was assessed, comparing the absorbance of the drug test with that of the control (homogenate mixture without any drug).

Data was presented as percentage inhibition of lipid peroxidation against concentration.

Data analysis.

Raw scores for right foot volumes were individually normalized as percentage of change from their values at time 0 then averaged for each treatment group. The time-course curves for foot volume were subjected to two-way (*treatment* × *time*) repeated measures analysis of variance with Bonferroni's *post hoc t* test. Total foot volume for each treatment was calculated in arbitrary unit as the area under the curve (AUC) and to determine the percentage inhibition for each treatment, the following equation was used.

$$\%\ inhibition\ of\ edema = \left(\frac{AUC_{control} - AUC_{treatment}}{AUC_{control}} \right) \times 100$$

Differences in AUCs were analyzed by ANOVA followed by Student-Newman-Keuls' *post hoc* test. Doses and concentration responsible for 50% of the maximal effect (ED_{50} and IC_{50}) for each drug was determined by using an iterative computer least squares method, with the following nonlinear regression (three-parameter logistic) equation

$$Y = \frac{a + (b - a)}{\left(1 + 10^{(LogED_{50} - X)}\right)}$$

Where, X is the logarithm of dose and Y is the response. Y starts at a (the bottom) and goes to b (the top) with a sigmoid shape.

The fitted midpoints (ED_{50}s) of the curves were compared statistically using F test (Miller, 2003; Motulsky and Christopoulos *et al.*, 2003). GraphPad Prism for Windows version 4.03 (GraphPad Software, San Diego, CA, USA) was used for all statistical analyses and ED_{50} determinations. $P < 0.05$ was considered statistically significant.

RESULTS

Carrageenan-induced oedema

Carrageenan (10 ml, 1% suspension) induced a time-dependent oedema response in 7-day old chicks that peaked at 2-3 h, CEE, dexamethasone and diclofenac significantly suppressed the time-course the oedema respetively. Two-way ANOVA (*treatment ´ time*) revealed a significant effect of drug treatments on the oedema (CEE: $F_{3,64}$=4.76, P<0.05;, diclofenac: $F_{3,64}$=16.20, P<0.001; and dexamethasone: $F_{3,64}$=5.45, P<0.01

CEE (30-300 mg kg^{-1}, *p.o*) dose-dependently and significantly reduced the total foot oedema response attained over 4 h with a maximal inhibition of 53.8±8.2% (Fig 1b). Similarly, the NSAID diclofenac (10-100 mg kg^{-1}, i.p.) and the steroidal anti-inflammatory agent dexamethasone (0.3-3 mg kg^{-1}, i.p) reduced the the total oedema oedema by 62.7±9.1% (Fig 2b) and 66.4±7.8% (Fig 3b) respectively.

Dose-response curves for the inhibition of foot edema are shown in Fig 4. CEE (ED_{50} 135.5±40.9 mg kg^{-1}) exhibited similar potency to diclofenac (ED_{50} 45.5±13.0 mg kg^{-1}; $F_{1,27}$= 0.03, P=0.87) but was significantly less potent than dexamethasone (ED_{50} 1.2±0.3 mg kg^{-1}; $F_{1,25}$=67.0, P<0.001).

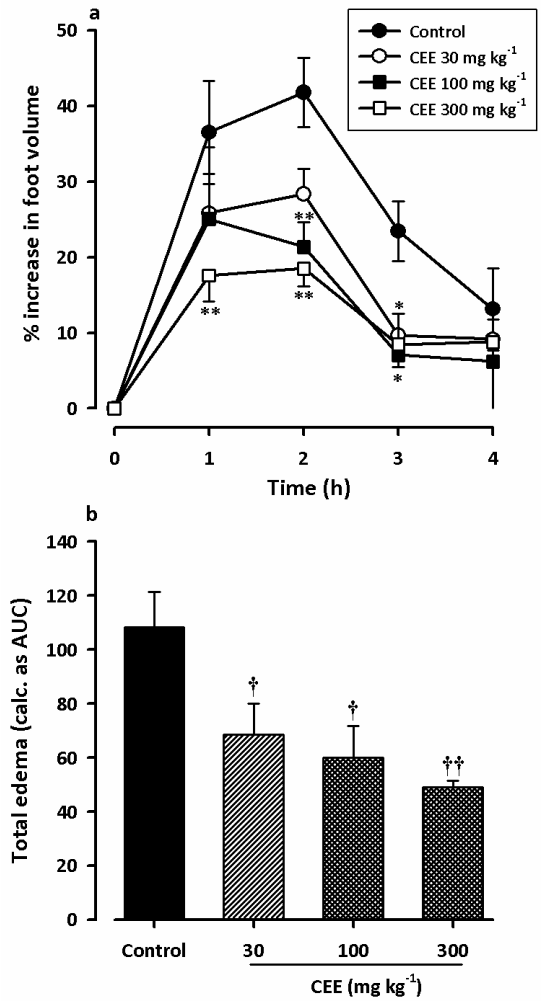

Fig. 1: Effect of CEE (30-300 mg kg^{-1}; *p.o.*) on time course curve(a) and the total edema response in carrageenan-induced foot edema in chicks (b). Values are means ± s.e.m. (n=5). ** $P < 0.01$; *$P < 0.05$ compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test). $^{\dagger\dagger}P<0.01$; $^{\dagger}P<0.05$ compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's *post hoc* test).

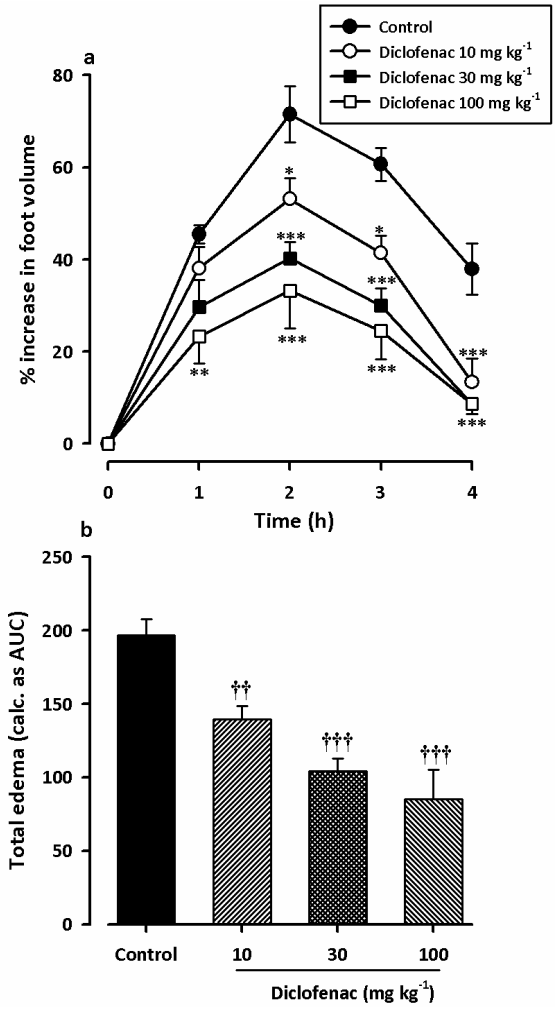

Fig. 2: Effect of diclofenac (10-100 mg kg^{-1}; *i.p.*) on time course curve (a)and the total edema response in carrageenan-induced foot edema in chicks (b). Values are means ± s.e.m. (n=5). ***$P < 0.001$; ** $P < 0.01$; *$P < 0.05$ compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test). $^{\dagger\dagger\dagger}P<0.001$; $^{\dagger\dagger}P<0.001$ compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's *post hoc* test.

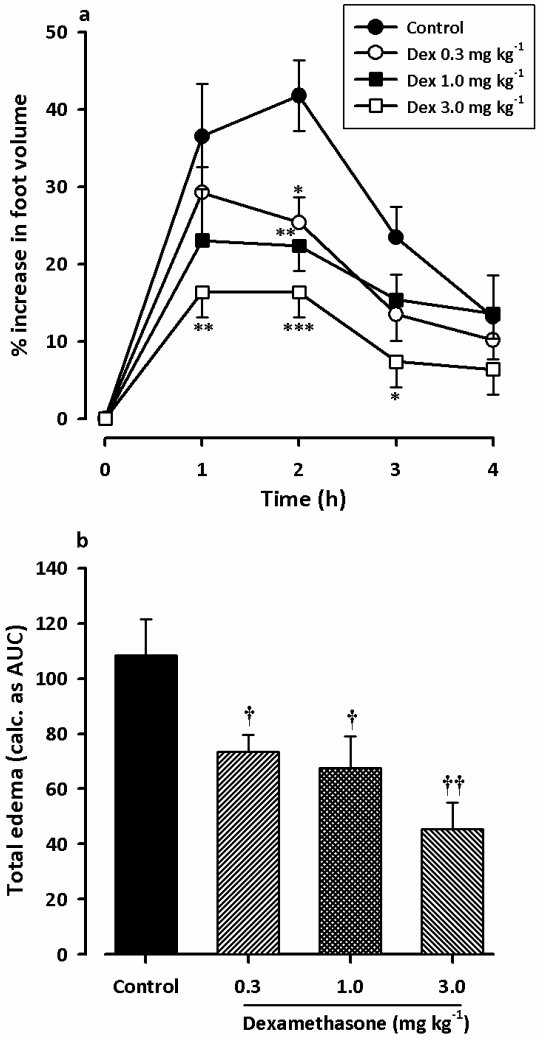

Fig. 3: Effect of dexamethasone (0.3-3 mg kg^{-1}; *i.p.*) on time course curve (a) and the total edema response in carrageenan induced paw edema in chicks (b). Values are means ± s.e.m. (n=5). ***$P < 0.001$; ** $P < 0.01$; *$P < 0.05$ compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test). $^{\dagger\dagger}P<0.001$; $^{\dagger}P<0.05$ compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's *post hoc* test).

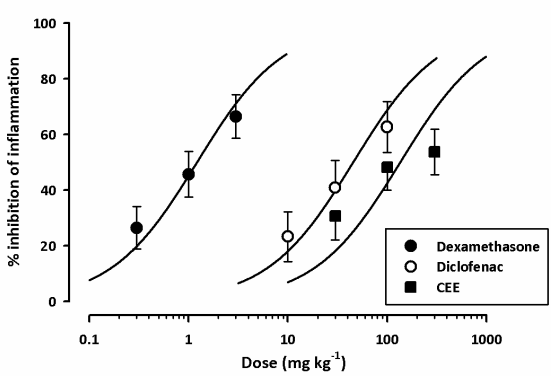

Fig. 4: Dose response curves for dexamethasone (0.3-3.0 mg kg^{-1} i.p), diclofenac (10-100 mg kg^{-1} i.p) and CEE (30-300 mg kg^{-1} *p.o.*) on carrageenan induced foot edema in chicks.

Total phenols

The result of the total phenol content determination showed a concentration dependent increase in the total phenolics in *C. edulis* (Fig 5a) when expressed in tannic acid equivalents.

Reducing power

We observed a concentration-dependent reducing activity by both *Carissa edulis* extract and n-propyl gallate with IC_{50} values (in mgml^{-1}) of 1.35±0.15 and 0.03±0.01 respectively (fig 5b; table 1). The n-propyl gallate however exhibited a 40-fold potency in reducing power compared to the extract (table 1).

DPPH scavenging

The DPPH assay determines the ability of an agent to scavenge free radicals. *Carissa edulis* extract showed a concentration-dependent scavenging activity in a similar manner to n-propyl gallate (fig. 6a). The IC_{50} values (in mg ml^{-1}) of 0.21±0.03 and 0.002±0.0004 for CEE and, *n*-propyl gallate respectively (Table 1) suggests that CEE has lesser ability to scavenge free radicals compared to n-propyl gallate.

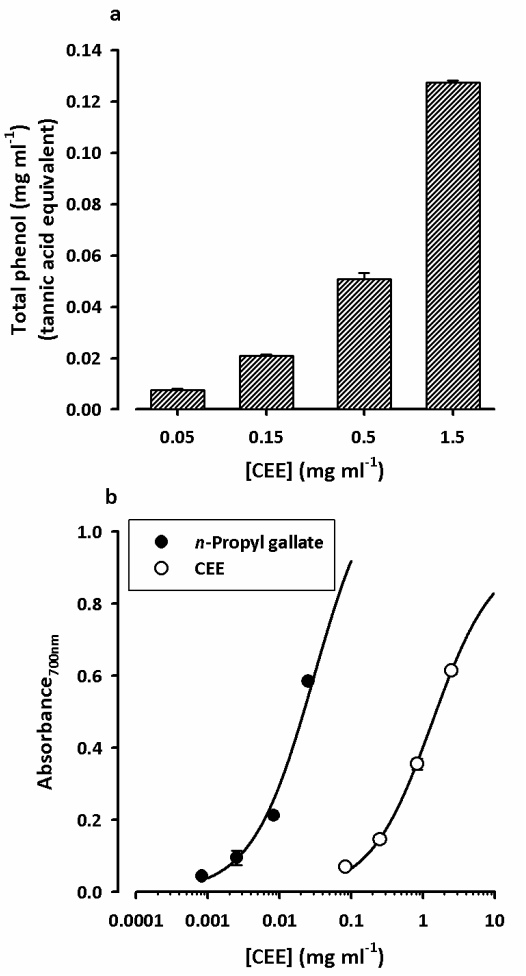

Fig. 5: Total phenols present in various concentrations of CEE (0.05-1.5 mg ml^{-1}), expressed as tannic acid equivalent (a) and reducing power of CEE (0.1-3 mg ml^{-1}) compared to *n*-propyl gallate (0.001-0.03 mg ml^{-1}) (b). Values are means ± s.e.m. (n = 3).

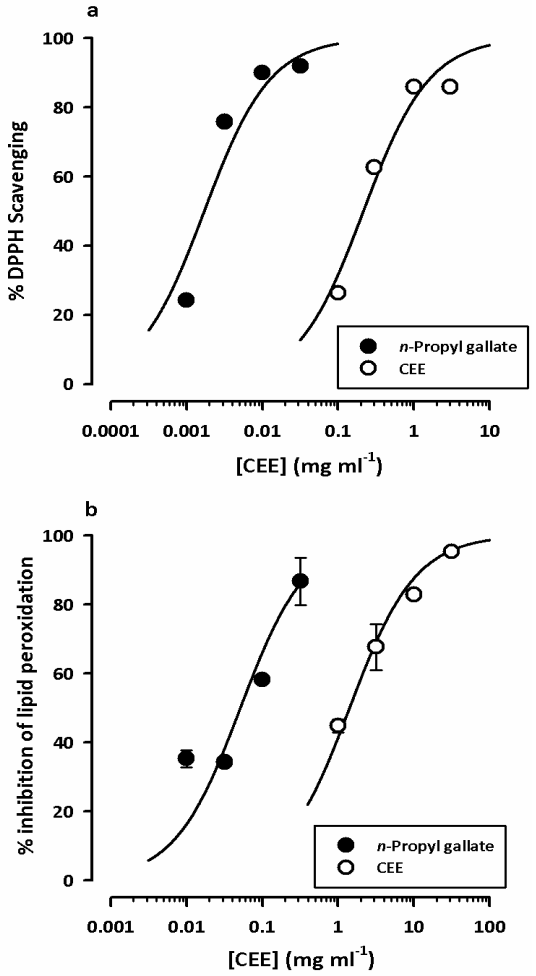

Fig. 6: Free radical scavenging ability of CEE (0.1-3 mg ml^{-1}) compared to *n*-propyl gallate (0.01-0.3 mg ml^{-1}) and in the DPPH radical assay (a) and percentage inhibition of lipid peroxidation by CEE (1-30 mg ml^{-1}) compared to *n*-propyl gallate (0.03-0.3 mg ml^{-1}) (b). Values are means ± s.e.m.(n = 3).

Table 1: IC_{50} values for *C. edulis* extract and n-propyl gallate in the reducing power, DPPH scavenging and lipid peroxidation assays

Drug	IC_{50} (mg ml^{-1})		
	Reducing Power	*DPPH Scavenging*	*Lipid Peroxidation*
C. edulis extract	1.35±0.15***	0.21±0.03***	1.42±0.15***
n-Propyl gallate	0.03±0.01	0.002±0.0004	0.05±0.01

Lipid peroxidation

The result of the lipid peroxidation determination (Fig. 6b) showed a concentration-dependent inhibitory activity by both *Carissa edulis* extract and the standard, n-propyl gallate with IC_{50} values (in $mgml^{-1}$) of 1.42±0.15 and 0.05±0.01 respectively (Table 1).

DISCUSSION

The purpose of this study was to evaluate the anti-inflammatory and antioxidant properties of *Carissa edulis* with the aim of establishing scientific basis of the traditional medicinal uses of *C. edulis* in inflammatory conditions.

Carrageenan-induced acute footpad edema in laboratory animals was first introduced by Winter *et al* (1962).It has been generally used to screen new anti-inflammatory drugs (Di Rosa and Willoughby, 1971). According to Vinegar *et al*. (1987) the development of the carrageenan-induced paw edema derives from the release of cytoplasmic enzymes and serotonin from mast cells and the increase of prostaglandin in the inflammatory area. In particular, the initial phase of inflammation (edema, 0-2) has been attributed to the release of histamine, and kinins, followed by a late phase (2.5-6h) mainly sustained by prostaglandin release and more recently has been attributed to the induction of cyclooxygenase-2 in the tissue (Di Rosa, 1972). We have shown that the root extract of *Carissa edulis* profoundly inhibits acute oedema induced by carageenan in the chick foot. The extent of inhibition was, however, less than the standard anti-inflammatory drugs diclofenac and dexamethasone.

Although the actual mechanism of action of CEE in inflammation is not known, it is possible that the extract inhibits the release of those inflammatory mediators involved in carrageenan-induced edema which include cytoplasmic enzymes, serotonin from mast cells, bradykinin, prostaglandins and other cyclooxygenase products (Di Rosa and Willoughby, 1971).

Furthermore, a large pool of evidence implicates free radicals in the inflammatory process (Vinegar *et al.*, 1987; Hischi *et al.*, 1989; Salvemini *et al.*, 1996), we investigated the ability of CEE to act as an antioxidant. Our data indicates that the anti-inflammatory activity of CEE may be linked to its antioxidant properties. The mechanisms proposed, through which ROS and other free radicals mediate cellular damage include initiation of lipid peroxidation, the inactivation of a variety of enzymes and depletion of glutathione (Cuzzocrea *et al.*, 2001). Some plant antioxidants are also known to inhibit arachidonic acid metabolism during the stage of the enzymatic peroxidation reaction (Nakadate *et al.*, 1984). It is most probable that CEE may also have a similar effect.

We evaluated the antioxidant activity of CEE by four different assays: the total phenol content, reducing power, DPPH scavenging ability and lipid peroxidation inhibition.

Phenolic antioxidants are potent free radical terminators (Shahidi *et al.*, 1992). The high potential of phenolic compounds to scavenge radicals may be explained by their phenolic hydroxyl groups (Sawa *et al.*, 1999). Detection of phenols in CEE therefore strongly suggested a possible antioxidant activity.

The reductive capacity of CEE, in terms of Fe^{3+}-Fe^{2+} transformation, is a significant indicator of its antioxidant activity. The result clearly shows that CEE has significant, concentration-dependent reducing activity but with less potency compared to standard, n-propyl gallate.

DPPH (1, 1-diphenyl-2-picrylhydrazyl) is a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule The reduction in DPPH radical was observed as decrease in its absorbance at 517 nm induced by n-propyl gallate and CEE acting as an antioxidant. Both CEE and n-propyl gallate showed a concentration dependent free radical scavenging activity.

The inhibition of lipid peroxidation by CEE could be attributed to scavenging of OH or superoxide radicals, by changing the Fe^{3+}/Fe^{2+} state, by reducing the rate of conversion of ferrous to ferric or by chelating the iron itself.

From the powerful activity shown by CEE in reference to free radical scavenging, reducing capacity and inhibition of lipid peroxidation, it is clear that CEE is an antioxidant that can be classified as preventive, scavenging or chain-breaking. The mechanism of antioxidant activity of CEE can be stipulated from above findings as the reduction of free radicals as well as scavenging of reactive oxygen species and other free radicals. Several compounds have been isolated from the plant including 2-hydroxyacetophenone (Bentley et al., 1984), soluble phenolics, insoluble proanthocyanidins (Reed, 1986), lignans, predominantly (−)-nortrachelogenin, carinol and carissanol (Achenbach *et al.*, 1983) and sesquiterpenes (Achenbach *et al.*, 1985). Also, carissone, cryptomeridol, β-eudesmol, sesquiterpenes of the eudesmane type and germacrane derivatives have been isolated (Achenbach *et al.*, 1985). Most of these components have been shown to have antioxidant property: 2-hydroxyacetophenone (Litwinienko and Dabrowska, 2001); (−)-nortrachelogenin (Tiwari *et al.*, 2001); soluble phenolics (Rice-Evans *et al.*, 1996; Hagerman *et al.*, 1998) proanthocyanidins (Rice-Evans *et al.*, 1996; Bagchi *et al.*, 1998). Moreover some sesquiterpenes of the eudesmane type have been shown to inhibit NF-kB (Campagnuolo *et al.*, 2005), a major mediator of inflammation.

CONCLUSION

Results of the present work clearly demonstrate significant anti-inflammatory and anti-oxidant activities of *C. edulis* extract. The anti-inflammatory activity of *C. edulis* extract may be related to antioxidant activity of *C. edulis* extract shown. The anti-inflammatory and anti-oxidant activity found in the present studies therefore, supports the use of *C. edulis* extract in the treatment of inflammation conditions in traditional medicine.

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