ANTI-INFLAMMATORY AND ANTIOXIDANT EFFECTS OF AN ETHANOLIC EXTRACT OF
THE AERIAL PARTS OF *HILLERIA LATIFOLIA* (LAM.) H. WALT. (PHYTOLACCACEAE)

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Abstract

Various parts of the perennial herb *Hilleria latifolia* (Lam.) H. Walt. (Family: Phytolaccaceae) are used in Ghanaian traditional medicine for the treatment of several inflammatory-related disorders. The present study examined the anti-inflammatory effect of an ethanolic extract of the aerial parts of *Hilleria latifolia* (HLE) in acute and chronic inflammation models. Since free radicals and reactive oxygen species are implicated in inflammatory diseases, the antioxidant potential of HLE was also investigated in *in vitro* experimental models. HLE (10-300 mg kg^{-1}, *p.o.*), either pre-emptively or curatively, significantly inhibited carrageenan-induced foot oedema in 7-day old chicks. 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.) dose-dependently reduced the oedema in both pre-emptive and curative treatments. In the Freund's adjuvant induced-arthritis model in rats, HLE as well as the positive controls, dexamethasone and methotrexate, showed significant anti-arthritic properties when applied to established adjuvant arthritis. HLE (10-300 mg kg^{-1}, *p.o.*) significantly reduced oedema in the ipsilateral paw of rats but failed to prevent systemic arthritic spread. The DMARD methotrexate (0.1-1 mg kg^{-1}, i.p.) and dexamethasone (0.3-3 mg kg^{-1}, i.p.) reduced significantly the total polyarthritic oedema as well as the spread of the arthritis from the ipsilateral to the contralateral paws of the treated animals. The extract (0.03-1.00 mg ml^{-1}) exhibited Fe^{3+} reducing activity, scavenged DPPH and prevented lipid peroxidation. These findings suggest that the extract exerts *in vivo* anti-inflammatory activity after oral administration and also has antioxidant properties which may contribute to its activity.

Keywords: Carrageenan, chicks, DPPH, Freund's adjuvant-induced arthritis, lipid peroxidation, total phenol

Introduction

Inflammatory diseases continue to be one of the main health problems of the world's population. Although several agents are known to treat these types of disorders, prolonged use is undesirable due to the severe side effects. Consequently, there is a need to develop new anti-inflammatory agents with minimum side effects (Vane and Botting, 1995; Al-Turki et al., 2010). Medicinal plant drug discovery continues to provide new and important leads against various pharmacological targets including inflammation (Braddock, 2007; Shah et al., 2011). In Ghanaian traditional medicine, various parts of several plants are used either alone or in combination therapy in the treatment of various inflammatory conditions.

Hilleria latifolia (Lam.) H. Walt. (Family: Phytolaccaceae) is a perennial herb that is common on cultivated grounds and along forest paths in Ghana. It also occurs in other parts of tropical Africa as well as South America. It is commonly known as *Avegboma, Boe* or *Kukluigbe* by the Ewes and *Anafranaku* by the Akans. In Ghanaian traditional medicine, different parts of the plant have been used in a variety of diseases. The leaves are effective against otalgia (Mshana et al., 2000), rheumatism (Dokosi, 1998; Mshana et al., 2000) and boils (Dokosi, 1998). The flowers are used for asthma (Mshana et al., 2000). *H. latifolia* is also used in Cote d'Ivoire for feverish pains and violent headache whereas the leaves are used to treat some skin diseases in Congo (Dokosi, 1998). To date, there is little scientific evidence to support the traditional use of *H. latifolia* in the treatment of inflammatory-related diseases and the possible mechanisms involved.

The present study examined the effect of the ethanolic extract of the aerial parts of *H. latifolia* on acute and chronic inflammation. Since antioxidant activity may be one of the mechanisms of anti-inflammatory action of the plant extract, this was also investigated in various *in vitro* models. This study provides justification for the use of *H. latifolia* in Ghanaian traditional medicine for the treatment of inflammatory-related disorders.

Materials and Methods
Plant material

The aerial parts of *H. latifolia* were collected from the campus of Kwame Nkrumah University of Science and Technology (KNUST), Kumasi near the Botanical Gardens (06°41′12.89″N; 01°33′59.51″W) during the month of July, 2007 and authenticated at the Department of Pharmacognosy, Faculty of Pharmacy and Pharmaceutical Sciences, College of Health Sciences, KNUST, Kumasi, Ghana. A voucher specimen (KNUST/HM1/09/L029) was kept at the herbarium of the Faculty.

Preparation of extract

The plant was room-dried for seven days and pulverised into fine powder. The powder was extracted by cold percolation with 70 % (v/v) ethanol and then concentrated into a green syrupy mass under reduced pressure at 60 °C using a

rotary evaporator. It was further dried in a hot air oven at 50 °C for a week and kept in a refrigerator for use. The yield was 19.67 %. This crude extract is subsequently referred to as HLE or extract in this study.

Animals

Cockerels (*Gallus gallus;* strain Shaver 579, Akropong Farms, Kumasi, Ghana) were obtained one day post-hatch and housed in stainless steel cages (34 cm × 57cm × 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. Overhead incandescent illumination was provided with room temperature at 29°C. Chicks were tested at 7 days of age. Sprague-Dawley rats of both sexes (120–215 g) were purchased from Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, Ghana and housed in the animal facility of the Department of Pharmacology, KNUST. The animals were housed in groups of six in stainless steel cages (34 cm × 47 cm × 18 cm) with soft wood shavings as bedding, fed with normal commercial pellet diet (GAFCO, Tema, Ghana), given water *ad libitum.* All procedures and techniques used in these studies were in accordance with accepted principles for laboratory animal use and care (EU directive of 1986: 86/609/EEC). All protocols used were approved by the Departmental Ethics Committee.

Drugs and Chemicals

The following drugs and chemicals were used: diclofenac sodium (KRKA, Slovenia); dexamethasone sodium phosphate (Pharm-Inter, Brussels, Belgium); methotrexate sodium (Dabur Pharma, New Delhi, India); carrageenan sodium salt, thiobarbituric acid (TBA), 2, 2-diphenyl-1-picrylhydrazyl (DPPH), trichloroacetic acid (TCA), Folin-Ciocalteau reagent (Sigma-Aldrich Inc., St. Louis, MO, USA); ascorbic acid, ammonium molybdate, disodium hydrogen phosphate (Na_2HPO_4), sodium dihydrogen phosphate (NaH_2PO_4), ferric chloride, linoleic acid, methanol, ethanol, *n*-propyl gallate, potassium ferricyanide, sodium bicarbonate, sodium carbonate, sulphuric acid, tannic acid (BDH, Poole, England).

Phytochemistry

The presence of saponins, alkaloids, flavonoids, steroids, terpenoids, glycosides and tannins were tested by methods described by Trease and Evans (1989).

Carrageenan-induced Oedema in Chicks

The anti-inflammatory activity of HLE was assessed using the carrageenan-induced foot oedema model in the chick (Roach and Sufka, 2003) as described earlier by Woode et al. (2009). Two sets of experiments were performed to assess the anti-inflammatory activity of HLE. The first was to study the effect of the drugs administered 1 h post carrageenan injection. The second examined the effects of the drugs given pre-emptively (30 min for i.p. route and 1 h for oral route) before the carrageenan challenge. Groups of chicks (n=6) were treated with HLE suspended in 2 % tragacanth (10-300 mg kg^{-1}, *p.o.*). Dexamethasone (0.3-3.0 mg kg^{-1}, i.p) and diclofenac (10-100 mg kg^{-1}, i.p.) were used as standards. Drug vehicle (2 % tragacanth, 10 ml kg^{-1}, *p.o.*) served as a control.

Freund's Adjuvant-Induced Arthritis (AIA)
Induction of AIA

Adjuvant arthritis was induced as previously described by Pearson (1956) with modifications (Abotsi et al., 2010). Briefly, right hind paw of rats were injected intraplantar with 0.1 ml of Complete Freund's Adjuvant (CFA). The CFA was a 4 mg ml^{-1} suspension of heat killed *Mycobacterium tuberculosis* [strains C, DT and PN (mixed) obtained from the Ministry of Agriculture, Fisheries and Food, U.K] in paraffin oil. A non-arthritic control (IFA group) received only intraplantar injection of 0.1 ml Incomplete Freund's Adjuvant (IFA) (sterile paraffin oil). Nine days after inoculation, the animals were selected and distributed into eleven groups (n=6) according to the severity of arthritis, so that each group had similar disease severity at the beginning of the treatment. The vehicle-treated group (CFA group/arthritic control) received only normal saline daily; the others were given HLE (10-300 mg kg^{-1}day^{-1}, *p.o.*) or dexamethasone (0.3-3 mg kg^{-1}, i.p.; on alternate days) or methotrexate (0.1-1 mg kg^{-1}, i.p.; every 4 days) from day 9 to day 28. The extract was suspended in 2 % tragacanth mucilage whilst the reference drugs were dissolved in normal saline.

Evaluation of the Severity of Arthritis

Hind paw thickness were measured as previously described (Hoffmann et al., 1997) using digital callipers. The paw thicknesses were measured for both the ipsilateral and the contralateral hind limbs before intraplantar injection of CFA (day 0) and every other day up to the 28th day. The oedema component of inflammation was quantified by measuring the difference in paw thickness between day 0 and the various time points. The loss of body weight typical of this model of arthritis was also monitored every four days. On day 29, the severity of arthritis was graded in a blinded manner by the same person according to the method previously described (Kinne et al., 1995; Abotsi et al., 2010). The hind paw thickness and arthritic index were used as the measurement parameters of inflammation and arthritis.

Radiographic Assessment

Radiographs of the hind limbs are important and were obtained from selected animals on day 29 in a manner

similar to that described previously (Abotsi et al., 2010). Using the radiographs, the severity of bone and joint destruction was scored blindly by the same person for each hind limb, according to the method described by Hoffmann et al. (1997). The radiological score was termed the radiological index.

Effects on Some Antioxidant Enzymes

Plasma antioxidant enzymes—superoxide dismutase and catalase—which may contribute to the anti-inflammatory activity of HLE were assessed. Superoxide dismutase (SOD) enzyme activity was determined in plasma according to the method of Misra and Fridovich (1972) with modifications for a microassay. Briefly, 10 μl of diluted plasma samples were added to a 96-well plate followed by 300 μl of carbonate buffer (0.05 M, pH 10.2, 0.1 mM EDTA). Ten microlitres of 9.6 mM epinephrine (0.3 mM final concentration in well) was then added to mixture in each well. The assay plate was then immediately inserted into a BioTek absorbance microplate reader (Model: ELx808, BioTek Instruments, Inc., Vermont, USA) and the change in absorbance at 490 nm was recorded for 4 min. The enzyme activity was expressed as the amount of enzyme that inhibits the oxidation of adrenaline by 50% which is equal to 1 unit. Catalase (CAT) was measured by tracing the degradation of H_2O_2 spectrophotometrically, according to the method of Aebi (1974). The enzyme activity was expressed in units per millilitre (U/ml) using a micromolar extinction coefficient for H_2O_2 of 0.0436 cm^2 $\mu mole^{-1}$. One unit is defined as that amount of enzyme causing the decomposition of one micromole of hydrogen peroxide per minute at 25 ^{0}C and pH 7.0.

In vitro **Antioxidant Activity**
Total Phenol Assay

Total soluble phenolics present in HLE was determined by a colorimetric method using the Folin-Ciocalteau reagent (Slinkard and Singleton, 1977). Various concentrations of HLE (0.1-3.0 mg ml^{-1}) were used with tannic acid (0.01-0.3 mg ml^{-1}) as a reference standard. The total phenolics were expressed as milligrams per millilitre of tannic acid equivalents (TAEs).

Antioxidant capacity by the Phosphomolybdate method

The total antioxidant capacity assay was carried out according to the method described by Prieto et al. (1999). Various aqueous concentrations of HLE (0.1-3 mg ml^{-1}) were prepared, centrifuged and used for this test. Ascorbic acid (0.01, 0.03, 0.1, 0.3 mg ml^{-1}) was used as the standard and the total antioxidant capacity was expressed as milligrams per millilitre of ascorbic acid equivalents (AAE).

Reducing power

The antioxidant potential of the extract was determined using the Fe^{3+} reduction (reducing power test) described by Oyaizu (1986). Various concentrations of HLE (0.03-1 mg ml^{-1}) as well as the standard antioxidant *n*-propyl gallate (0.001- 0.03 mg ml^{-1}) were assessed. From the results, the concentration of the extract required to cause a 50 % decrease in the absorbance was calculated (EC50).

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

The free radical scavenging activity was determined using method as described by Blois (1958). Methanolic solutions of HLE and standard antioxidant *n*-propyl gallate were prepared, centrifuged and the supernatant used for the experiment. Results were expressed as percentages of blank and the concentration of extracts required to cause a 50 % decrease in the absorbance was calculated (EC_{50}).

Linoleic acid autoxidation

The inhibitory effect of HLE on lipid peroxidation was determined by the thiobarbituric acid method of the linoleic acid autoxidation assay as described previously by Inatani et al. (1983) but with slight modifications. Briefly, a mixture of 2 ml of HLE (0.1, 0.3, 1, 3 mg ml^{-1}) in 99.5 % ethanol, 2.05 ml of 2.51 % (v/v) linoleic acid in 99.5 % ethanol, 4 ml of phosphate buffer (0.05 M, pH 7.0), and 1.95 ml of water was placed in a vial with a screw cap and placed in an oven at 40 °C in the dark for 7 days. To 2 ml of the mixture, which was prepared above, was added 2 ml of 20 % trichloroacetic acid aq. solution and 1 ml of 0.67 % thiobarbituric acid aq. solution. This mixture was placed in a boiling water bath for 10 min and, after cooling, was centrifuged at 650 *g* for 10 min. The absorbance of the supernatant was measured at 532 nm. Ethanol was used as blank throughout the experiment whilst *n*-propyl gallate (0.003, 0.01, 0.03, 0.1 mg ml^{-1}) was used as standard. Each test was carried out in duplicates. Percentage inhibition of linoleic acid autoxidation by the test drugs was assessed, comparing the absorbance of the drug test with that of the control (mixture without any test drug). Data was presented as percentage inhibition of linoleic acid autoxidation against concentration.

Analysis of Data

For the acute inflammation experiment, 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* 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.

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

Data from the arthritis experiment was treated the same as in the acute inflammation study. Raw scores for ipsilateral and contralateral paw thicknesses were individually normalized as percentage of change from their values at day 0 and then averaged for each treatment group. Data was presented as the effect of drugs on the time course and the total oedema response of adjuvant-induced arthritis for the 28 days period. The time-course curves for paw diameters were subjected to two-way (treatment × time) repeated measures analysis of variance with Bonferroni's *post hoc* test. Total paw thickness for each treatment was calculated in arbitrary unit as the AUC and to determine the percentage inhibition for each treatment.
Differences in AUCs were analysed by one-way ANOVA followed by Student-Newman-Keuls *post hoc* test. ED_{50}, EC_{50} and IC_{50} (dose/concentration responsible for 50 % of the maximal effect) values were 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, 2003). GraphPad Prism for Windows version 5 (GraphPad Software, San Diego, CA, USA) was used for all statistical analyses and ED_{50} determinations. *P* < 0.05 was considered statistically significant.

Results
Phytochemistry

Preliminary phytochemical screening of HLE revealed the presence of saponins, tannins, glycosides, steroids, terpenoids as well as little amounts of flavonoids and alkaloids.

Carrageenan-induced Oedema in Chicks

Administration of carrageenan (10 µl, 2 % suspension) induced moderate inflammation resulting in foot oedema in the 7-day old chicks peaking at 2-3 h as described by Roach and Sufka (2003)(Figure 1). Figures 1 (a, c, e) and 2 (a, c, e) show the time course curves for effects of HLE, diclofenac and dexamethasone on carrageenan-induced oedema. Two-way ANOVA (treatment × time) revealed a significant effect of drug treatment for HLE (pre-emptive: $F_{3, 120}$= 28.59, *P*<0.0001; curative: $F_{4, 25}$= 2.87, *P*=0.044), diclofenac (pre-emptive: $F_{3, 120}$= 27.60, *P*<0.0001; curative: $F_{3, 20}$=4.96, *P*=0.0098) and dexamethasone (pre-emptive: $F_{3, 120}$=34.14, *P*<0.0001; curative: $F_{3, 20}$=3.74, *P*<0.028). Total oedema produced by each treatment is expressed in arbitrary units as AUC of the time-course curves.
HLE (30-300 mg kg⁻¹, *p.o.*) significantly reduced foot oedema with maximal inhibition of 38.11± 5.55 % and 30.91±4.66 % for pre-emptive (Figure 1b) and curative (Figure 2b) treatments respectively. Similarly, the NSAID diclofenac (10-100 mg kg⁻¹, i.p.) dose dependently reduced the oedema by 59.33±10.82 % and 42.87±7.46 % respectively for pre-emptive (Figure 1d) and curative treatments (Figure 2d). Dexamethasone (0.3-3 mg kg⁻¹, i.p.), a steroidal anti-inflammatory agent inhibited the oedema with maximal effect of 42.77±7.64 % (pre-emptive; Figure 1f) and 36.60±6.76 % (curative; Figure 2f). Dose-response curves for the inhibition of foot oedema are shown in Figure 4. HLE displayed a U-shaped dose response relationship with approximate ED_{50} values: 59.70 and 151.01 mg kg⁻¹ (pre-emptive treatment); 72.95 and 213.80 mg kg⁻¹ (curative treatment). By comparing the ED_{50} values from the curves, HLE was significantly less potent than diclofenac (ED_{50} pre-emptive: 11.58±17.83 mg kg⁻¹, curative: 25.65±32.48 mg kg⁻¹) and dexamethasone (ED_{50} pre-emptive: 0.77±0.85 mg kg⁻¹, curative: 2.93±1.02 mg kg⁻¹).

Adjuvant – Induced Arthritis

Intraplantar injection of CFA into the right foot pad of rats induced an inflammatory response characterized by paw swelling in both the ipsilateral and the contralateral paw. The response on the injected paw was biphasic. It consisted of an acute phase (days 0-10 post CFA inoculation) characterized by unilateral inflammatory oedema of the ipsilateral paw peaking around days 4-6 and a subsequent polyarthritic/chronic phase (10-28 post CFA inoculation) characterised by inflammatory oedema of the contralateral paw. Throughout the 28-day experiment, there was no significant change in the paw volume of the non-inflamed control groups injected with IFA.
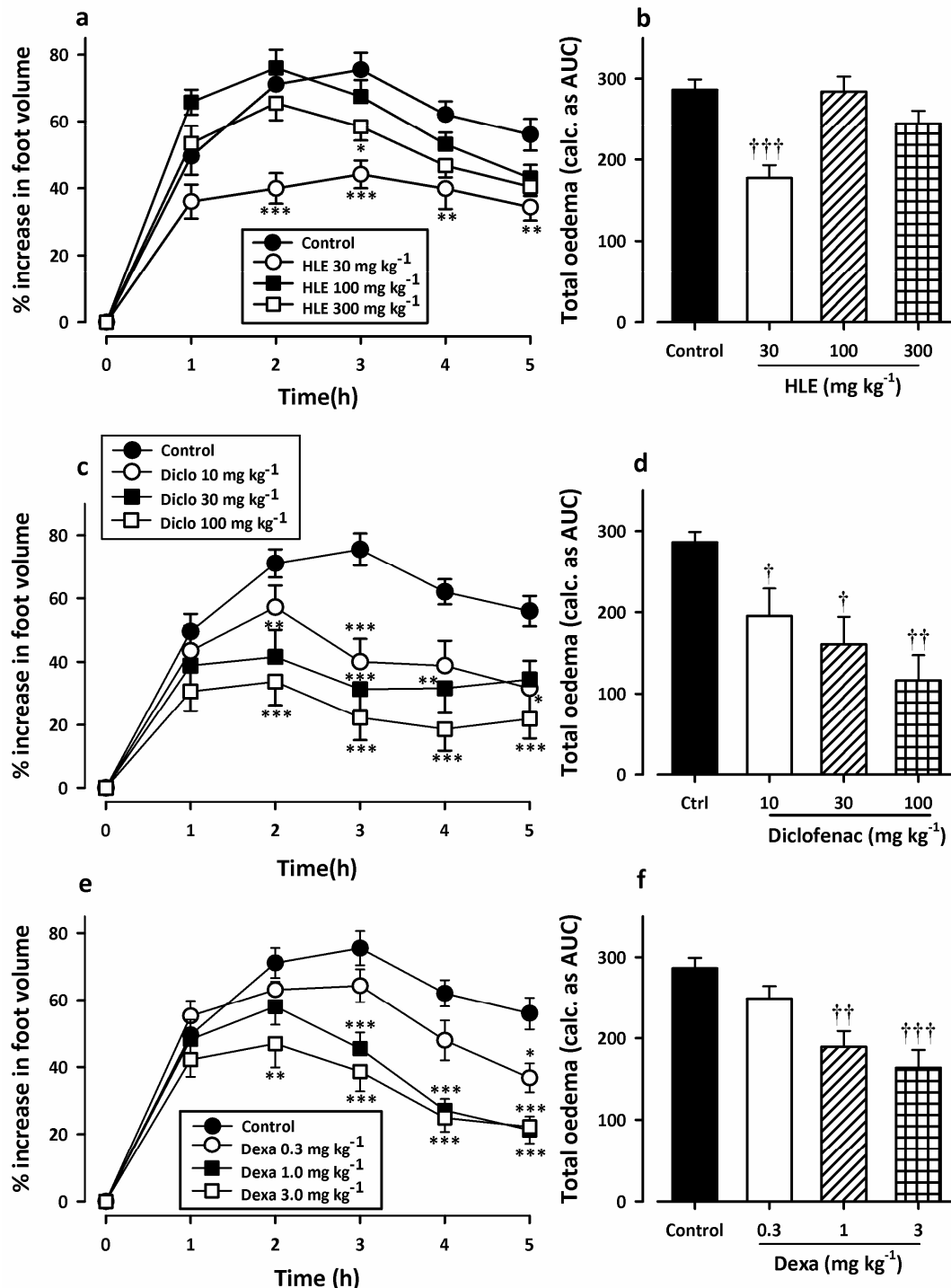
Figure 1: Effect of HLE (30-300 mg kg^{-1}; *p.o.*), diclofenac (10-100 mg kg^{-1}, i.p.) and dexamethasone (0.3-3 mg kg^{-1}, i.p.) on time-course curves (a, c and e respectively) and the total oedema response (b, d and f respectively) in the pre-emptive protocol of carrageenan-induced foot oedema in chicks. Values are means ± S.E.M. (n=6). *$P<0.05$; **$P<0.01$; ***$P<0.001$ compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test). †$P<0.05$; ††$P<0.01$; †††$P<0.001$ compared to vehicle-treated group (One-way ANOVA followed by Newman-Keuls *post hoc* test).
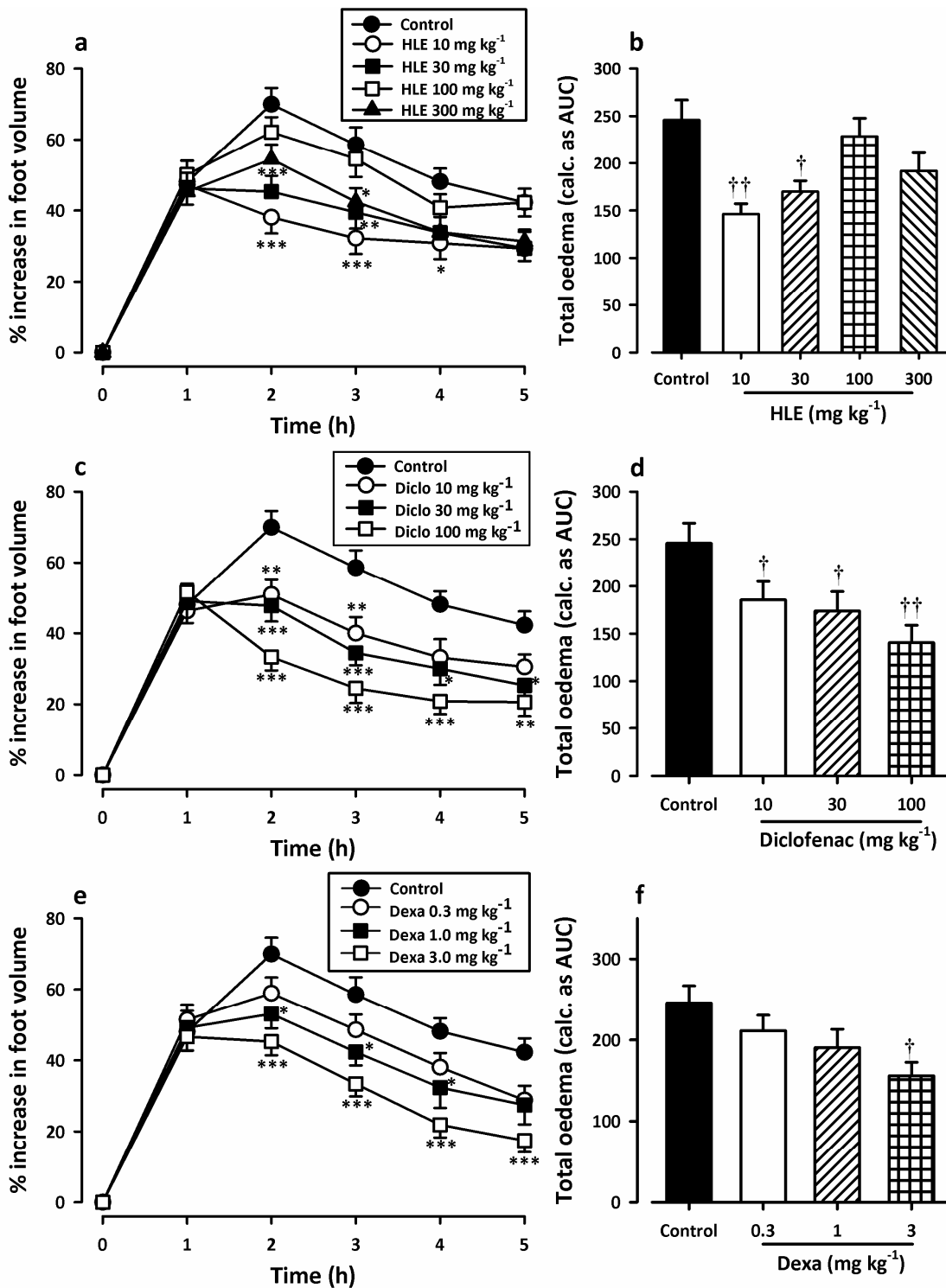
Figure 2: Effect of HLE (10-300 mg kg^{-1}; *p.o.*), diclofenac (10-100 mg kg^{-1}, i.p.) and dexamethasone (0.3-3 mg kg^{-1}, i.p.) on time-course curves(**a**, **c** and **e** respectively) and the total oedema response (**b**, **d** and **f** respectively) in the curative protocol of carrageenan-induced foot oedema in chicks. Values are means ± S.E.M. (n=6). *$P<0.05$; **$P<0.01$; ***$P<0.001$ compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test). †$P<0.05$; ††$P<0.01$; †††$P<0.001$ compared to vehicle-treated group (One-way ANOVA followed by Newman-Keuls *post hoc* test).

HLE, dexamethasone and methotrexate significantly suppressed the time-course of ipslateral and contralateral paw oedema in rats. Two-way ANOVA (*treatment × time*) revealed a significant effect of drug treatments on the paw oedema (HLE: $F_{5,30}$=78.44, P<0.0001; dexamethasone: $F_{4,25}$=144.88, P<0.0001 and methotrexate: $F_{4,25}$=80.05, P<0.0001; Figure 3a, c, e).

HLE (10-300 mg kg^{-1}, *p.o*) significantly reduced the total ipslateral paw oedema response over the 19 days of treatment with a maximal inhibition of 32.64± 2.74 % (Figure 3b). Similarly, the DMARD methotrexate (0.1-1 mg kg^{-1}, i.p.) and dexamethasone (0.3-3 mg kg^{-1}, i.p) profoundly reduced the total ipslateral paw oedema by 57.30±4.96 % (Figure 3f) and 64.51±2.30 % (Figure 3d) respectively. HLE (10-300 mg kg^{-1}) could not significantly reduce ($F_{4,25}$=0.74, P=0.57) the extent of spread of oedema from the ipsilateral to the contralateral paw (Figure 3a, b). However, dexamethasone ($F_{4,25}$= 9.23, P=0.0001) and methotrexate ($F_{4,25}$=7.04, P=0.0006) significantly prevented the spread of the arthritis from the ipsilateral to the contralateral paws of the treated animals (Figure 3c, d, e, f). Dose-response curves for the inhibition of paw oedema are shown in Figure 4c. HLE displayed a bell-shaped dose response relationship with approximate ED_{50} values: 19.91 and 47.64 mg kg^{-1}. HLE was the least potent compared to methotrexate and dexamethasone. Dexamethasone was the most potent.

The body weight changes of rats in each treatment group over the 28 days period is shown in Table 2. Rats in the IFA control group gained the most weight (43.75±8.31 %). HLE 10 mg kg^{-1} showed weight gain comparable to the CFA group. However, there was a general dose-dependent reduction in weight gain in HLE treated group. Dexamethasone (0.3-3 mg kg^{-1}) caused significant ($F_{3, 20}$=12.70, P<0.0001) weight loss compared to the CFA group. Except for the dose of 0.3 mg kg^{-1}, rats in the methotrexate treated group generally gained weight comparable to that of the CFA group.

The results from the assay of superoxide dismutase and catalase are shown in Table 3. There was a decrease in SOD and CAT activity levels in the CFA-treated group compared to the IFA-treated group. Except for HLE at doses 10 and 300 mg kg^{-1}, all drug treatments did not significantly affect plasma SOD levels compared to the CFA group. In the case of catalase, all the drug treatments failed to reverse ($F_{11,24}$=1.32; P=0.276) the fall in the enzyme levels induced by the adjuvant arthritis.

Table 1: Arthritic and radiological indices of rats in the adjuvant-induced arthritis.

Groups	Arthritic Index	Radiological Index
IFA	0***	0***
CFA	6.67±0.42	3.33±0.21
H. latifolia extract		
10 mg kg^{-1}	2.67±0.33***	1.50±0.22***
30 mg kg^{-1}	6.18±0.65	3.00±0.26
100 mg kg^{-1}	4.50±1.03*	2.50±0.22*
300 mg kg^{-1}	4.00±0.93**	1.83±0.31***
Dexamethasone		
0.3 mg kg^{-1}	1.83±0.67***	0.17±0.17***
1 mg kg^{-1}	1.33±0.21***	0***
3 mg kg^{-1}	0.83±0.66***	0***
Methotrexate		
0.1 mg kg^{-1}	2.40±0.51***	0.33±0.21***
0.3 mg kg^{-1}	1.67±0.33***	0.20±0.20***
1 mg kg^{-1}	1.50±0.22***	0***

*P<0.05; **P<0.01; ***P<0.001 compared to CFA group (One-way ANOVA followed by Newman-Keuls *post hoc* test).

From the arthritic indices (Table 1), HLE (10-300 mg kg^{-1}), dexamethasone (0.3-3 mg kg^{-1}) and methotrexate (0.1-1 mg kg^{-1}) showed significant ($F_{11, 59}$=15.58, P<0.0001) dose-dependent clinical improvement in arthritis. HLE reduced the arthritic index by a maximum of 60.00 % whilst dexamethasone and methotrexate similarly inhibited by 87.50 % and 77.50 % respectively. The CFA group developed the severest arthritis showing immense erythema and swelling in both the ipsilateral and contralateral paws. The IFA groups showed no sign of erythema or swelling. As regards the effectiveness of the doses of HLE used, 30 mg kg^{-1} was the least effective (Table 1).

From radiographic score of the hind limbs (Table 1), the CFA group demonstrated the most severe bone destruction displaying reduced bone density and focal areas of excessive bone resorption. The bones were intact in the IFA/non-arthritic control which recorded the lowest radiological index. HLE at doses 10 and 300 mg kg^{-1} suppressed the pathological changes

in bone with maximal inhibition of radiological index of 54.95 %, compared with that of the CFA group. Similarly, dexamethasone (0.3-3 mg kg^{-1}) and methotrexate (0.1-1 mg kg^{-1}) almost totally prevented bone destruction in AIA radiographically (Table 1); both reducing the radiological index by maxima of 100 %.
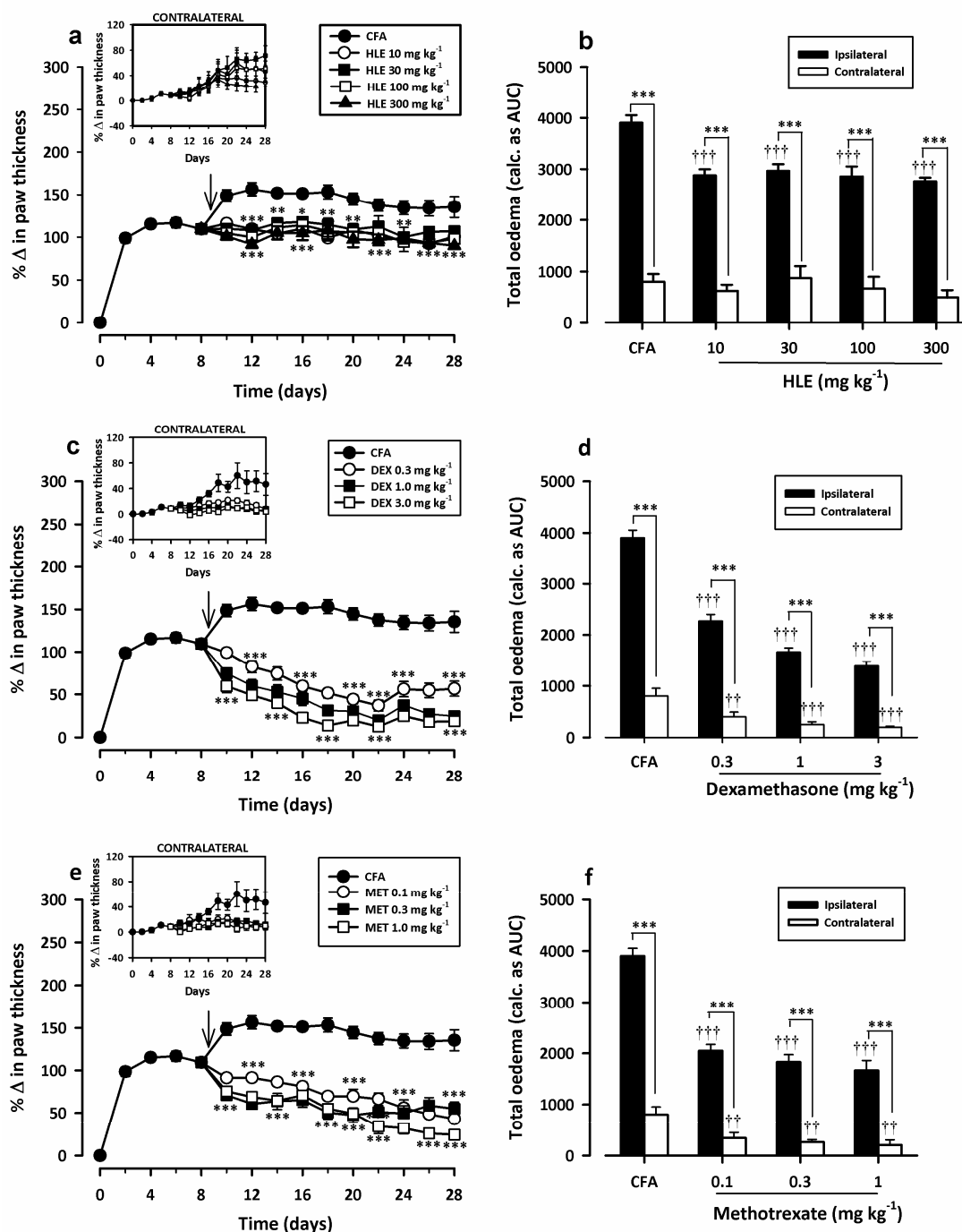

Figure 3: Effect of HLE (10 - 300 mg kg^{-1}; *p.o.*), dexamethasone (0.3 - 3 mg kg^{-1}; i.p.) and methotrexate (0.1 - 1 mg kg^{-1}; i.p.) on time course curve (a, c and e respectively) and the total oedema response (b, d and f respectively) in adjuvant-induced arthritis in rats. The total oedema was calculated as AUCs over the 19 d period of drug treatment. Values are means ± S.E.M. (n = 5). *$P<0.05$; **$P<0.01$; ***$P<0.001$ compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test). †$P<0.05$; ††$P<0.01$; †††$P<0.001$ compared to vehicle-treated group (One-way ANOVA followed by Newman-Keuls *post hoc* test).
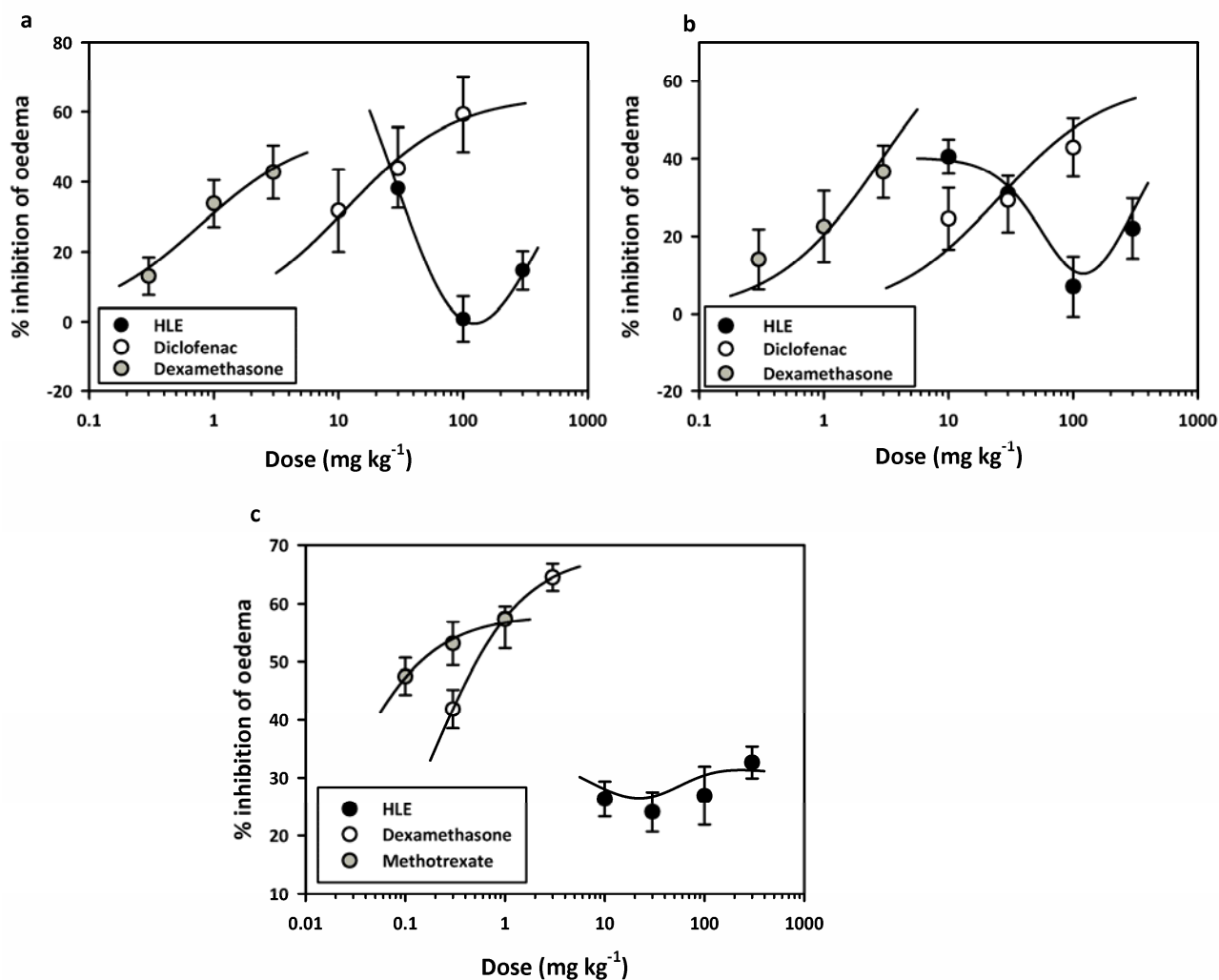
Figure 4: Dose response curves for HLE, diclofenac and dexamethasone in the pre-emptive (a) and curative (b) protocols of carrageenan-induced foot oedema in the chick and in Freund's adjuvant-induced arthritis (c) as well as for methotrexate in Freund's adjuvant-induced arthritis (c). Each point represents the mean±SEM (n=5-6).

Total Phenol Content

The total phenol content determination showed a concentration-dependent increase ($F_{3,6}$=427.9, P<0.0001) in the total phenolics in HLE when expressed in tannic acid equivalents. The total phenol content was estimated to be 29.40±1.09 mg tannic acid equivalent/g of HLE.

Total Antioxidant Capacity

HLE (0.1-3 mg ml^{-1}) showed a concentration-dependent increase ($F_{3,8}$=44.86, P<0.0001) in total antioxidant capacity when expressed as ascorbic acid equivalents. The total antioxidant capacity of the HLE was estimated to be 55.16±13.60 mg ascorbic acid equivalent/g of HLE.

Reducing Power

HLE (0.03-1 mg ml^{-1}) and n-propyl gallate (0.001-0.03 mg ml^{-1}) exerted a concentration-dependent Fe^{3+} reducing activity with EC$_{50}$ values (in mg ml^{-1}) of 2.071±0.782 and 0.1071±0.049 respectively (Figure 5). The n-propyl gallate was however more potent, exhibiting a 19-fold reducing power compared to the extract ($F_{1,20}$=7.76, P=0.0114).

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

The DPPH assay determines the ability of an agent to scavenge free radicals. HLE (0.1-1 mg ml^{-1}) showed a concentration-dependent scavenging activity in a similar manner to n-propyl gallate (Figure 5). The EC$_{50}$ values (in mg ml^{-1}) of 0.2269±0.037 and 0.00323±0.001 for HLE and n-propyl gallate respectively, suggests that HLE has lesser ability to scavenge free radicals compared to n-propyl gallate ($F_{1,14}$=114.7, P<0.0001).

Table 2: Body weights changes in adjuvant-induced arthritis in rats

Treatment	Body weight changes (%)				
	Day 0	Day 8	Day 16	Day 24	Day 28
IFA	0	17.97±3.44***	37.92±7.45***	40.60±8.49***	43.75±8.31***
CFA	0	-0.12±1.62	5.87±2.60	11.15±2.22	14.35±1.76
HLE					
10 mg kg^{-1}	0	1.75±1.56	8.20±1.68	14.52±2.93	17.37±4.39
30 mg kg^{-1}	0	1.03±1.22	1.15±1.38	4.50±2.37	5.10±2.70
100 mg kg^{-1}	0	-0.68±3.43	-3.27±4.18	1.58±6.49	0.73±6.68
300 mg kg^{-1}	0	-0.50±1.94	-1.82±1.89	0.33±0.93	-2.87±1.68**
DEX					
0.3 mg kg^{-1}	0	-1.25±2.06	-6.70±2.22**	-6.27±2.33***	-5.38±1.97***
1.0 mg kg^{-1}	0	0.13±0.75	-5.95±2.18***	-6.58±2.19***	-6.08±2.42***
3.0 mg kg^{-1}	0	-1.87±2.15	-9.13±1.62**	-11.85±1.25***	-13.47±1.60***
MET					
0.1 mg kg^{-1}	0	3.55±2.80	6.45±3.82	8.30±3.69	9.03±3.84
0.3 mg kg^{-1}	0	-10.37±9.33	-9.19±4.72	-0.31±3.41	0.95±3.74
1.0 mg kg^{-1}	0	1.68±0.93	8.78±2.84	12.12±3.54	13.73±2.86

Values are mean±SEM (n=5). **$P<0.01$; ***$P<0.001$ compared to CFA group (Two-way ANOVA followed byBonferroni's *post hoc* test). DEX: dexamethasone. MET: Methotrexate

Linoleic Acid Autoxidation

The result of the linoleic acid autoxidation determination (Figure 5) showed a concentration-dependent inhibitory activity by both HLE and the standard, *n*-propyl gallate with IC$_{50}$ values (in mg ml^{-1}) of 0.1122±0.010 and 0.03657±0.007 respectively. *N*-propyl gallate was more potent when compared to FEE ($F_{1,14}$=27.29, P=0.0001).
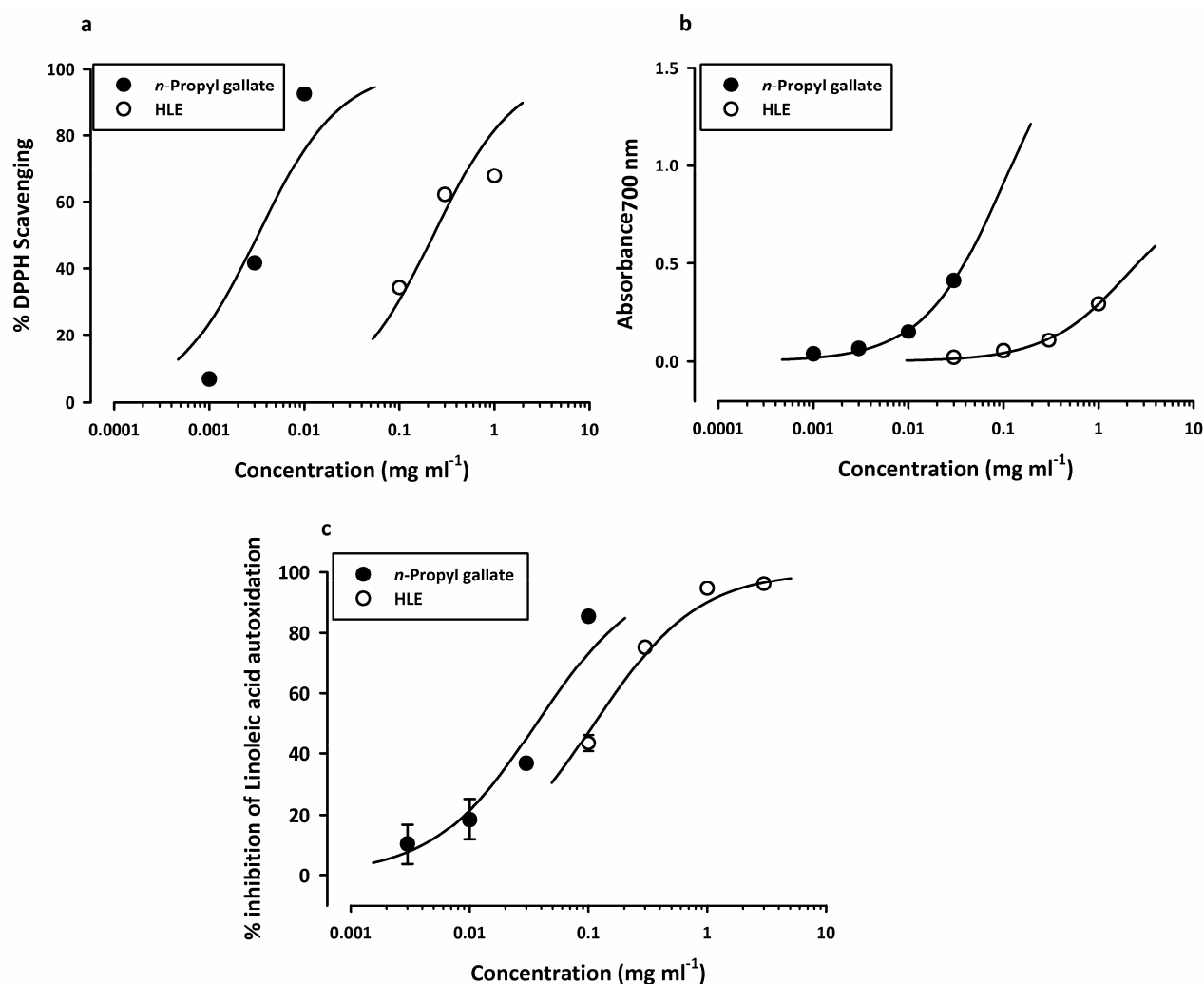
Figure 5: Free radical scavenging ability of HLE (0.1-1 mg ml^{-1}) compared to *n*-propyl gallate (0.001-0.01 mg ml^{-1}) in the DPPH radical assay(a), reducing power of HLE (0.03-1 mg ml^{-1}) compared to *n*-propyl gallate (0.001-0.03 mg ml^{-1})(b), and Percentage inhibition of lipid peroxidation (linoleic acid autoxidation) by HLE (0.1-3 mg ml^{-1}) compared to *n*-propyl gallate (0.003-0.1 mg ml^{-1})(c). Values are means ± S.E.M.

Discussion

      The present study established the anti-inflammatory activity of the aerial parts of *H. latifolia* in the acute and chronic inflammatory animal models used. It also evaluated the *in vitro* antioxidant properties of HLE, since it may be one of the mechanisms of its anti-inflammatory action. HLE exhibited antioxidant activity in all the five assays models used: total phenol assay, reducing power test, total antioxidant capacity, DPPH scavenging activity and lipid peroxidation assay.

      Carrageenan-induced oedema test (Winter et al., 1962) is a classical model of acute inflammation that has been extensively used to screen new anti-inflammatory drugs (Di Rosa and Willoughby, 1971). In this study chicks were used instead of the commonly used rodents. Carrageenan-induced oedema has been validated in chicks (Roach and Sufka, 2003), and is much more economical than rodent models. Furthermore, chicks are easier to handle. The inflammatory response induced by carrageenan is characterized by a biphasic response (Vinegar et al., 1969) with marked oedema formation resulting from the rapid production of several inflammatory mediators such as histamine, serotonin and bradykinin (first-phase), which is subsequently sustained by the release of prostaglandins and nitric oxide (second-phase) with peak at 3 h, produced by inducible isoforms of COX (COX-2) and nitric oxide synthase (iNOS), respectively (Seibert et al., 1994; Thomazzi et al., 2010). The second (late) phase is sensitive to most clinically effective anti-inflammatory drugs (Vinegar et al., 1969; Di Rosa et al., 1971). HLE clearly suppressed inflammation induced by carrageenan in both pre-emptive and curative protocols of the anti-inflammatory activity assessment. The finding justifies the use of the extract traditionally in the treatment of inflammatory conditions. Although the actual mechanism of action of HLE in inflammation is unknown, the fact that it inhibited both early and late phases of oedema suggests that it could be acting through the inhibition of the release

and/or action of those inflammatory mediators involved in carrageenan-induced oedema which include cytoplasmic enzymes, histamine, serotonin, bradykinin, prostaglandins and other cyclooxygenase products. The exact mechanism, however, needs to be established.

Table 3: Effect of various treatments on the plasma levels of antioxidant enzymes SOD and CAT in adjuvant-induced arthritis in rats.

Treatment	SOD (U/ml)	CAT(U/ml)
IFA	21.34±3.50*	122.40±8.48
CFA	11.10±0.98	90.09±4.34
HLE		
10 mg kg^{-1}	28.40±1.18*	77.42±2.71
30 mg kg^{-1}	7.66±1.73	80.46±19.12
100 mg kg^{-1}	11.73±1.34	86.29±22.38
300 mg kg^{-1}	16.02±6.04	66.22±21.10
Dexamethasone		
0.3 mg kg^{-1}	9.54±0.80	84.79±18.69
1.0 mg kg^{-1}	8.17±1.78	97.44±33.00
3.0 mg kg^{-1}	8.75±1.18	96.47±9.57
Methotrexate		
0.1 mg kg^{-1}	8.61±0.30	108.61±8.59
0.3 mg kg^{-1}	11.32±0.80	102.3±16.16
1.0 mg kg^{-1}	7.28±0.88	51.54±13.01

Values are mean±SEM (n=3). *P<0.05 compared to CFA group (One-way ANOVA followed by Newman-Keuls *post hoc* test). SOD: Superoxide dismutase; CAT: Catalase.

Adjuvant-induced arthritis (AIA) is one of the most widely used animal models to study the effect of anti-rheumatic agents and represents a systemic inflammatory disease, with bone and cartilage changes similar to those observed in rheumatoid arthritis, but with an accelerated time span (Osterman et al., 1994). The common pathological features of adjuvant arthritis in rat and rheumatoid arthritis in humans are joint swelling associated with cellular and pannus invasion of the joint space, release of lysosomal constituents into the joint space, and bone resorption (Osterman et al., 1994). The induction of adjuvant arthritis is thought to be due to bacterial peptidoglycan and muramyl dipeptide and occurs through cell mediated-autoimmunity by structural mimicry between mycobacteria and cartilage proteoglycans in rats (van Eden et al., 1985; Vijayalakshmi et al., 1997). In this study, oral administration of HLE caused clinical improvement of arthritis and a significant reduction in inflammation shown as decrease in paw thickness. The action of HLE on arthritis seems to be largely anti-inflammatory (i.e. reduction in oedema, erythema, pain, etc, as seen in the acute model) rather than an immunologic effect on the course of the disease. This argument is supported by the fact that HLE did not significantly prevent the systemic spread of the adjuvant arthritis—a process that is largely immunologic (Donaldson et al., 1995). Nevertheless, further studies are needed to establish these assertions.

Radiographs are necessary to determine true remission of disease and for accurate evaluation of disease status (Kitamura et al., 2007). The extract, as evidenced from radiological indices (Table 1), at doses of 10 mg kg^{-1} and 300 mg kg^{-1} protected against bone loss due to reduced bone formation and increased resorption which are the causes of bone loss in

adjuvant-induced arthritis in rats (Aota et al., 1996; Findlay and Haynes, 2005). It is doubtful if this effect is mediated by an immunologic protection of the bones. It is most likely due to protection offered to the bone as a result of the anti-inflammatory effect of HLE, which was most seen at the two doses (i.e. 10 mg kg^{-1} and 300 mg kg^{-1}). Further studies on the effect of HLE on the bone will be necessary to establish exact mechanisms.

Changes in weight reflect arthritic disease progression and general health status, while exaggerated weight loss above that observed in the arthritic control group may be indicative of toxicity (Schopf et al., 2006). Generally, the extract could not protect against arthritic-induced weight loss. While the least dose of the extract (10 mg kg^{-1}) permitted slight weight gain beyond the arthritic control (CFA) group, higher doses (30-300 mg kg^{-1}) resulted in weight loss. This could be attributed to the fact that aside 10 mg kg^{-1} of HLE, the higher doses could not produce much clinical improvement in arthritis to allow significant weight gain. It is also possible that weight loses are due to HLE toxicity at high doses. Dexamethasone and methotrexate, which are well known to cause weight loss (Orzechowski et al., 2000; Lucas et al., 2003; Kolli et al., 2007), also acted similarly to HLE and were unable to offer protection against arthritis-induced weight loss.

HLE exhibited a non-monotonic dose-response pattern in both carrageenan-induced oedema test and the adjuvant-induced arthritis (Figure 4). The exact biochemical mechanism underlying this pharmacological inversion is not yet clear, and remains to be established. The anti-inflammatory action of HLE can be attributed to one of its chemical constituents. Indeed, HLE has been shown by phytochemical analysis to contain alkaloids, flavonoids, tannins, saponins, phytosterols and terpenoids and one of them may be responsible for the anti-inflammatory effect especially as a lot of these secondary plant metabolites identified have been shown to exhibit anti-inflammatory properties (Whitehouse et al., 1994; Guardia et al., 2001; Barbosa-Filho et al., 2006).

Activated neutrophils and macrophages are implicated in the release of free radicals and reactive oxygen species (ROS) in inflammation (Conner and Grisham, 1996). Since free radicals and ROS are important mediators that provoke or sustain inflammatory processes, their neutralization by antioxidants and radical scavengers can attenuate inflammation (Winrow et al., 1993; Conforti et al., 2008). Indeed, several anti-inflammatory agents have been shown to have antioxidant and/or radical scavenging mechanisms as part of their activity (Geronikaki and Gavalas, 2006). Consequently, one of the possible mechanisms by which HLE exerts anti-inflammatory activity is through the suppression of the effect of free radicals and ROS during inflammation. This can be achieved through the direct action of the extract as an antioxidant, or indirectly by boosting the levels of *in vivo* antioxidant enzymes including superoxide dismutase (SOD) and catalase (CAT). The impact of HLE on antioxidant enzymes SOD and CAT was investigated during the adjuvant arthritis experiment while the direct effect of HLE as an antioxidant was assessed with *in vitro* models.

Evidence suggests oxidative stress is elevated in arthritis (Bae et al., 2003; Mahajan and Tandon, 2004). This initially triggers feedback increases in activity of antioxidant enzymes but is usually overwhelmed, resulting ultimately in decreased activity of antioxidant enzymes including SOD and CAT (Vijayalakshmi et al., 1997; Kumar et al., 2002; Jung et al., 2005; Narendhirakannan et al., 2005; He et al., 2006). This was observed in this study—the arthritic control (CFA) group showed significant decrease in the antioxidant enzymes SOD and CAT compared to the non-arthritic control (IFA) group. HLE at doses 10 mg kg^{-1} and 300 mg kg^{-1} caused an increase in SOD. This could be attributed to transcriptional activation of the enzyme or amelioration of oxidative stress by HLE at these doses. HLE, however, did not affect the decreased levels of catalase induced by the arthritis. Dexamethasone and methotrexate did not also significantly reverse the arthritis-induced reduction in the levels of SOD and catalase.

From the powerful activity shown by HLE *in vitro* with reference to free radical scavenging, reducing capacity and inhibition of lipid peroxidation, it is clear that HLE is an antioxidant. The mechanism of antioxidant activity of HLE can be stipulated from above findings as the reduction of free radicals as well as scavenging of reactive oxygen species and other free radicals. The observed *in vitro* activities suggest that HLE could exert protective effects also *in vivo* against oxidative and free radical injuries occurring in inflammatory disorders.

The total phenol test confirmed the presence of appreciable amounts of phenolics in HLE. The antioxidant activity of phenolic compounds is well known and is mainly due to their redox properties, which play an important role in neutralizing free radicals, quenching singlet and triplet oxygen species, or decomposing peroxides (Rice-Evans et al., 1996). Though the exact phytochemical constituents of HLE which showed *in vitro* antioxidant activity in this study is still unclear, phenolic compounds may be partly responsible.

Conclusion

It is clear from the study that HLE has anti-inflammatory activity in both acute and chronic inflammation models. HLE also has antioxidant activity, which may contribute to its anti-inflammatory activity.

Acknowledgements

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