Pharmacognosy Research [Phcog Res.]
Vol 1, Issue 2, Mar-Apr, 2009 Page 80-90
[Rapid Publication]
A Supplement to Pharmacognosy Magazine – [Phcog Mag.]
Rapid publication process initiated: 20th January, 2009
Accepted: 11th February, 2009

PHCOG RES.: Research Article

Anti-inflammatory Activity of *Ixora coccinea* Methanolic Leaf Extract

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ABSTRACT

The anti-inflammatory activity of methanolic leaf extract (MLE) of *Ixora coccinea* Linn. (Rubiaceae) was investigated in this study. MLE showed dose-dependent anti-inflammatory activity in carrageenan-induced rat paw edema model ($r = 0.7$; $P<0.01$). MLE at a dose of 500, 1000, and 1500 mg/kg showed maximum inhibition of edema 36.7, 46.5, and 64.5% respectively ($P<0.01$). Oral administration of MLE of rats at a dose of 1500 mg/kg significantly inhibited peritoneal phagocytic cell infiltration (45.9%; $P<0.05$), impaired nitric oxide (NO) production in peritoneal cells (40.8%; $P<0.01$) and showed anti-histamine activity (54.9%; $P<0.01$). *In vitro* treatment of rat peritoneal cells with MLE inhibited NO production dose-dependently (82.2% at 400 µg/ml, $r = 0.99$; $P<0.05$). MLE also possessed significant, dose-dependent *in vitro* anti-oxidant activity ($r = 0.88$; $P<0.01$; IC_{50} value = 8.0 µg/ml), membrane stabilizing activity ($r = 0.81$; $P<0.01$; IC_{50} value = 6.4 ng/ml) and lipid peroxidation activity (36.7% at 250 µg/ml; $P<0.01$). Thirty-day oral treatment of rats with 1500 mg/kg did not show any adverse signs of toxicity or behavioral changes. These results suggest that anti-inflammatory activity of *I. coccinea* is mediated via inhibition NO production, phagocytic cell infiltration, anti-histamine effect, scavenging of free radicals, membrane stabilizing activity and lipid peroxidation.

KEYWORDS: anti-inflammatory activity, anti-oxidant, cell infiltration, *Ixora coccinea,* membrane stabilization, nitric oxide

INTRODUCTION

Medicinal remedies based on herbs were widely used before the advent of modern pharmacology. Presently about 80% of the world's population relies mainly on medicinal plants as a source of remedies for treatment of disease (1). In Sri Lanka, a wide variety of plants are used in both Ayurveda and traditional medicine for anti-inflammatory effects (2). *Ixora coccinea* Linn. (Rubiaceae) commonly known as *rath mal* in Sinhalese and *vedchi* in Tamil is one of these plants. It is a shrub with small obvate to oval-oblong, rounded to subcordate base leaves on branched hard heavy twigs (2). Different plant parts of *I. coccinea* are used for treatment of various disease conditions some of which are associated with inflammation. A decoction of the flowers is given for haemophytis, acute bronchitis and dysmenorrhoea (2). Further, the flowers and bark are used on reddened eyes and eruptions in children. A decoction of the root is given for dysentery, loss of appetite, fever, and gonorrhea, and as a sedative for hiccoughs and nausea. The leaves are used for dermatological disorders in traditional systems of medicine in Sri Lanka (2).

Previous studies have reported anti-inflammatory effects of aqueous leaf extract of *I. coccinea* using both acute and chronic inflammatory models (3). The aqueous leaf extract was also shown to possess anti-histamine and antinociceptive activities (4). Lupeol isolated from the petroleum ether fraction of ethanol extract of leaves was shown to have anti-inflammatory activity in carrageenan-induced rat paw edema assay (5). In this study we investigated the *in vivo* anti-inflammatory activity of methanolic leaf extract (MLE) of *I. coccinea* using the carrageenan-induced rat paw edema model and it shows potent anti-inflammatory activity. We demonstrate here for the first time, the regulatory effects of leaf extracts of *I. coccinea* on inflammatory mediators such as nitric oxide. In addition this study gives insight on the underlying

mechanism(s) of anti-inflammatory activity, the effect of MLE on immunephagocytic cell infiltration, scavenging of free radicals, lipid peroxidation and membrane stabilizing activity.

MATERIALS AND METHODS

Chemicals

Ascorbic acid, bovine serum albumin (BSA), carrageenan, 1,1-diphenyl-2-picrylhydrazil (DPPH), 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), lipopolysaccaride (LPS), $NaNO_2$, Neutral Red, N-(1-naphthyl) ethylenediamine hydrochloride, N-monomethyl-L-arginine acetate salt (NMMA; nitric oxide synthase inhibitor), sulphanilamide, thiobarbituric acid (TBA) were purchased from Sigma Aldrich (St. Louis, MO, USA). Gum acacia (GA) and histamine dihydrochloride were purchased from Fluka, (Buchs, Switzerland) and Avondale Laboratories Ltd (Banburg, U.K.) respectively. RPMI 1640 medium was obtained from GIBCO BRL, Life Technologies (Paisley, Scotland). Indomethacin, prednisolone, aspirin, chloropheniramine were purchased from the State Pharmaceutical Corporation (Colombo, Sri Lanka). Randox assay kits were purchased from Randox Laboratories Ltd. (Antrim, UK). All other chemicals and reagents were of analytical grade.

Preparation of plant material and extraction

Leaves of *I. coccinea* were collected in October 2005 from Palpola area in the Kalutara district of Sri Lanka. Identification and authentication of the plant was done by Dr. S. Ranwala, Department of Botany, University of Colombo. The voucher specimen (rrk/Ic/001) was deposited at the Institute of Biochemistry, Molecular Biology and Biotechnology, University of Colombo.

The air dried leaves (100 g) were boiled in 3 L of methanol for 16 h. Methanolic leaf extract (MLE) was concentrated under reduced pressure at 40°C. Methanol was completely removed, the product was freeze dried and a brown color hygroscopic residue was obtained (yield 14.5%).

Animals

Adult albino Wistar rats (8-10 weeks, 150-200 g) were obtained from the Medical Research Institute, Colombo, Sri Lanka and kept in the animal house, Department of Zoology, University of Colombo, under standard conditions (temperature: 28±2°C; photoperiod: 12 h natural light and 12 h dark; humidity: 50±2%) with free access to food pellets (Finisher Feed, Ceylon Grain Elevators, Colombo, Sri Lanka) and tap water. Ethical clearance for this study was obtained from the Research, Ethics and Higher Degrees committee of the Institute of Biochemistry, Molecular Biology and Biotechnology, University of Colombo.

Assay for carrageenan-induced rat paw edema

MLE was resuspended in 1% gum acacia (GA) and anti-inflammatory activity of MLE was determined as described previously (6) using the rat paw-edema assay and the same doses (3). Forty eight rats were randomly divided into six groups labeled 1 to 6. Groups 1, 2 and 3 were orally administered with three different doses of MLE, 500, 1000, and 1500 mg/kg respectively (n = 8/group). Fourth group (n = 8) was treated with 1 ml of 1% GA and served as the control for groups 1 to 3. Fifth group (n = 8) was treated with indomethacin (5 mg/kg) which was used as the reference drug and the sixth group was treated with 1 ml of distilled water (DW) (n = 8) and served as the control for group 5. After 1 h, 0.1 ml of 1% carrageenan suspension in saline was injected subcutaneously into the planter surface of the left hind paw of animals under the mild ether anesthesia. The volume of the paw up to ankle joint was measured 1 h prior to the injection (V_0) and hourly up to 5 h (V_t) after the injection of carrageenan, using a plethysmometer (Panlab s.l., Barcelona, Spain). The percentage inhibition of edema was calculated using these paw volumes, with respect to their controls.

$$\% \text{ edema inhibition} = \frac{(V_t - V_0)_{control} - (V_t - V_0)_{treated}}{(V_t - V_0)_{control}} \times 100$$

Assay for infiltration of rat peritoneal cells

The effect of MLE on infiltration of rat immune cells to the peritoneal cavity was assessed by a modification (7) of a previously described method for isolation of peritoneal cells (8). Two groups of rats (n = 6/group) were orally administered with MLE (1500 mg/kg) and prednisolone (10 mg/kg) respectively. Two control groups of rats (n = 6/group) were treated with 1 ml of 1% GA and 1 ml of DW. After 1 h, carrageenan was injected into the peritoneal cavity (1 mg/ml in Phosphate Buffered Saline (PBS), pH 7.4; 5 mg/kg). Sterile PBS (40 ml) was injected 2 h later, and 5 min after this injection, 35 ml of peritoneal fluid was drained using a 18 G cannular. These procedures were performed under ether anesthesia. Peritoneal fluid was centrifuged at 150 *g* for 10 min at 4°C. The supernatant was removed and the peritoneal cells were resuspended in 1 ml of PBS. A 50 µl aliquot of the cell suspension was mixed with 10 µl of 1% Neutral Red to visualize the phagocytic cells. Total cell and phagocytic/macrophage counts were made using a haemocytometer.

Assay for nitric oxide production by peritoneal cells

Nitric oxide (NO) production by rat peritoneal cells was determined by measuring nitrite in culture supernatants using Griess reagent as described previously (8). Nitrite concentrations in cell free supernatants measured by Griess reaction according to the protocol of Steuhr and Nathan (1989) (9), serve as a reflection of NO production. The effect of MLE on NO production by rat peritoneal cells was assessed under *in vitro* and *in vivo* conditions described below, i) *In vitro*: Treatment of peritoneal cells isolated from healthy rats not exposed to MLE *in vivo*, with MLE and ii) *In vivo:* Assessing the inhibitory effects of the peritoneal cells collected from rats treated with MLE orally.

Four groups of rats (n=6/group) were orally treated with MLE (1500 mg/kg), the reference drug, prednisolone (10 mg/kg) and their respective controls (1 ml of 1% gum acacia (GA) and 1 ml of DW) as described above (7,8), and peritoneal cells that were exposed to MLE *in vivo* were obtained to assay for NO production.

In vitro treatment of MLE was performed using a modification of the previously described method (10). Peritoneal cells were collected from rats that were injected intraperitoneally with carrageenan (5 mg/kg) and then treated *in vitro* with 100, 200, and 400 µg/ml of MLE in RPMI 1640 medium supplemented with 1% BSA for 30 min at 37°C. Peritoneal cells were treated with 1 mM NMMA in RMPI 1640 medium as positive control. Cells were centrifuged at 150 *g* for 2 min and resuspended in culture medium containing 1% bovine serum albumin and cultured for 24h. The viability of cells after 30 min incubation with MLE and after 24 h in culture in culture medium was assessed by Trypan blue exclusion test (10).

Assay for nitrite

To assay the NO production by rat peritoneal cells, cells from each animal (n = 6) were plated in 96 well tissue culture plates at $1x10^6$ cells/ml in RPMI 1640 medium supplemented with 1% BSA and incubated at 37°C in CO_2 incubator (5% CO_2 ± 95% air) (Sanyo Electric Co. Ltd., Osaka, Japan). After 24 h, culture supernatant was aspirated from each well, centrifuged at 10,000 *g* for 10 min and clear supernatant was assessed for nitrite production. For quantification of nitrite, 100 µl of culture supernatant was mixed with an equal volume of Griess reagent (equal mixture of 1% Sulphanilamide in 5% phosphoric acid and 0.1% N-(1-naphthyl) ethylenediamine hydrochloride in DW), kept at room temperature (25°C) for 15 min and optical density (OD) was read at 540 nm in a ELISA plate reader (ELX 800, Bio-Tek Instruments INC, Winooski, VT, USA). The nitrite concentration was calculated using calibration curve between 0.7-100 µM $NaNO_2$.

***In vivo* assay for anti-histamine activity**

This assay was performed as described previously (11). Fur on left lateral side of the back of these rats was removed. Twenty-four h later, these rats were randomly assigned in to four groups (n = 6/group) and orally administered with MLE in 1% GA (1500 mg/kg), chlorpheniramine (0.67 mg/kg) 1 ml of 1% GA and 1 ml of distilled water. After 1 h, these rats were subcutaneously injected with 50 µl of 200 µg/ml histamine dihydrochloride in saline in to the skin where the fur had been shaved, and 2 min later the area of the wheal formed was measured. Anti-histamine activity was calculated compared to the respective controls.

Assay for *in vitro* anti-oxidant activity

In vitro anti-oxidant activity was assessed by DPPH method as described previously (12). DPPH solution (20 µg/ml) was prepared using methanol and OD value was adjusted to 0.7 at 517 nm. Trolox (25 µg/ml) solution was prepared to calculate the Trolox equivalent for MLE concentrations. A dilution series of MLE was prepared using PBS at concentrations of 10, 100, 250, 500, 1000, and 2500 µg/ml. OD_{517} value of samples were measured 5 min after mixing 300 µl of MLE dilution and 300 µl of DPPH solution. PBS was used as the control and ascorbic acid was used as the positive control. Percentage inhibition of DPPH free radical scavenging was calculated based on the control reading, which contained DPPH and PBS without any extract using the following equation:

$$\% \text{ Scavenging Activity} = [(OD_{control} - OD_{sample} / OD_{control}] \times 100$$

Anti oxidant activity of the MLE was expressed as IC_{50}. IC_{50} value was defined as the concentration (in µg/ml) of MLE that inhibit the scavenging of DPPH radicals by 50%. Trolox equivalents for MLE was also derived from a standard Trolox curve (3.3.-20 µM) (13).

Assay for membrane stabilizing activity

This assay was performed using a modification of the heat-induced hemolysis of rat erythrocytes as described previously (11). A ten fold dilution series of MLE was made using PBS for concentrations from 1 mg/ml to 0.001 µg/ml. Dilutions of aspirin was also was made for the same concentrations and used as the reference drug. One ml of PBS was used as control. Twenty µl of rat blood was added to each tube containing 1 ml of test, standard drug and control samples. All dilutions of MLE, aspirin and GA were made in triplicates. Samples were first incubated at 37°C for 15 min. The modifications included an additional centrifugation step after this initial incubation. Cell suspensions were centrifuged at 1500 *g* for 3 min, the supernatants were removed and the cells were resuspended in 1 ml of PBS. This centrifugation step removed the color

originating from the MLE itself which subsequently interference with the OD_{540} measurement. The samples were then incubated at 54°C for 25 min to initiate heat-induced hemolysis and centrifuged at 1500 *g* for 5 min. Supernatants (200 µl) were transferred into an ELISA plate and the OD value was measured at 540 nm. Percent inhibition of haemolysis was calculated with respect to the controls and IC_{50} values were derived.

Percent inhibition of haemolysis = [(OD $_{control}$-OD $_{sample}$/ OD $_{control}$] x100

***In vitro* assay for lipid peroxidation activity**

Lipid peroxidation activity was assessed using TBA reactive substances assay as described by Dorman et al. (1995) (14). The concentrations of MLE used were 15.75, 31.25, 62.5, 125, and 250 µg/ml. Ascorbic acid (100 µg/ml) was used as the positive control. OD value was measured at 540 nm and the lipid peroxidation activity was calculated with respect to the control.

Phytochemical analysis and determination of metal ions

Qualitative analysis for tannins, phlobatannins, saponin, flavonoids steriods, terpenoids (Salkowski test) and cardiac glycosides (Keller-Killani test) were carried out as described previously (15). Metal ions (K, Ca, Mg, Na, Fe, Zn, Cr, Ni, Mn, Cu, Cd, Hg) were quantified as previously described (16). Air-dried plant materials were dried in an electric oven at 105°C until constant weight was reached. Half a gram was digested with 10 ml of concentrated H_2So_4 and 7.5 ml of conc. HNO_3 for 2 h at 450°C on an electric digester. Solution was brought to room temperature and a few drops of H_2O_2 were added until it become colorless. The colorless solution was filtered and diluted up to 50.00 ml. Absorbance was measured using Atomic Absorption Spectrophotometer (GBC Scientific Equipment, USA) and concentrations of metal ions were calculated using respective calibration curves.

Evaluation of toxicity

Rats (n = 6/group) were treated either with 1500 mg/kg/day of MLE or 1 ml of 1% GA daily for 30 consecutive days. After the oral treatment, rats were observed for overt clinical signs of acute toxicity or stress during the period of treatment. Rats were weighed prior to the start of the experiment and on day 1 of post-treatment. On day 1 of post-treatment, 1 ml of blood was obtained from the tail under mild ether anesthesia and serum was separated. The red blood cell (RBC) and white blood cell (WBC) counts were made using fresh blood as described previously (4). Serum concentrations of albumin, creatinine, alanine aminotransferase (ALT) and aspartate aminotransferase (AST), formally referred to as glutamic pyruvic transaminase (SGPT) and glutamic oxaloacetic transaminase (SGOT) respectively, and urea were determined using Randox assay kits. Na and K were determined using Atomic Absorption Spectrophotometer (GBC Scientific Equipment, USA).

Statistical analysis

Data are presented as the mean ± standard error of mean (SEM). Statistical analyses used one-way analysis of variance (ANOVA) to account for the different treatment doses and were complemented with unpaired *t*-test. Differences were considered statistically significant at $P < 0.05$. Statistical analysis was performed using SPSS version 15.0.

RESULTS

Inhibition of carrageenan-induced paw edema by MLE

As shown in Fig. 1, compared with the 1% GA control, treatment with MLE at 1500, 1000 and 500 mg/kg showed significant inhibition of paw edema in a dose-dependent manner ($r = 0.7$; $P < 0.01$). Maximum inhibition of paw edema was recorded as 64.5 ± 3.2%, 46.5 ± 3.7% and 36.7 ± 5.5% respectively at 2 h (mean ± SEM) ($P < 0.01$). Inhibition of paw edema was significant up to 5^{th} h at 1500 mg/kg ($P < 0.05$) and up to 4^{th} h in the other two doses ($P < 0.05$). The reference drug, indomethacin showed significant inhibition (59.7 ± 7.1%) up to 2 h ($P < 0.01$). Although the inhibition declined, it was significant up to the 5^{th} h ($P < 0.05$). In contrast to the two lower doses of MLE (1000 and 500 mg/kg), the highest dose of MLE (1500 mg/kg) showed a different pattern of inhibition which was similar to that of indomethacin, especially with respect to the anti-inflammatory activity up to 2 h.

Infiltration of rat peritoneal cells

Infiltration of total peritoneal cells was significantly inhibited (47.7 ± 3.8%) by MLE compared to the GA control ($P < 0.05$) (Fig. 2). Of the two reference drugs used, indomethacin was not effective (18.8 ± 10.4% inhibition) where as prednisolone significantly inhibited infiltration of peritoneal cells (91.3 ± 4.61%; $P < 0.05$). Differential counts of peritoneal cells of the control rats showed 54.9 ± 1.2% of phogocytic cells. Compared to the control rats, significant reductions were observed in the phagocytic cell counts in both MLE and prednisolone treated rats (50.5 ± 9.4% and 91.2 ± 1.8% reduction; $P < 0.05$ and $P < 0.01$ respectively). .

Inhibition of nitric oxide production

Peritoneal cells obtained from rats given oral treatment of MLE showed significant inhibition of their NO production

(40.8 ± 4.8%; P < 0.001) (Fig. 3A). Oral treatment of rats with prednisolone, the reference drug, also inhibited the NO production significantly (63.5 ± 4.8%; P < 0.001).

Assay for *in vitro* cytotoxicity of the MLE using the Trypan blue test showed comparable viable cells counts in MLE concentrations less than 800 µg/ml (85.6 ± 0.58% in 400 µg/ml) to that of normal culture medium (87.1 ± 0.65%.) These viable cell counts obtained after 30 min incubations were also reflected in the cell counts taken after 24 h in culture (78.6 ± 0.39% and 79.6 ± 1.04 respectively) indicating that MLE concentrations of 400 µg/ml or lower were not cytotoxic to peritoneal cells and are suitable for the *in vitro* treatment to assay for NO inhibitory activity.

Peritoneal cells obtained from carrageenan-treated rats had produced 50.1 µM of NO *in vitro* where as the cell free medium showed very low levels of NO (Fig. 3B) indicating minimal background levels of nitrite in culture supernatants. *In vitro* MLE treatment of peritoneal cells demonstrated a dose-dependent inhibition of NO production (r = 0.99; P < 0.05). Maximum inhibition by MLE was observed at 400 µg/ml (82.2 ± 1.2%; P < 0.01). NMMA, which is a nitric oxide synthase inhibitor also showed a comparable inhibition of NO production (70.1 ± 2.6%; P < 0.01).

Anti-oxidant activity of MLE

Percentage scavenging of MLE increased dose-dependently upto 100 µg/ml (r = 0.88; P < 0.01) and thereafter reached a plateau (Table 1). The IC_{50} values for MLE and ascorbic acid were found to be 8.0 µg/ml and 4.9 µg/ml respectively. The corresponding Trolox equivalents for MLE and ascorbic acid were 19.3 and 13.4 µM respectively.

Membrane stabilizing activity

In the modified assay in which the MLE dilutions were removed by centrifugation following the initial incubation at 37°C, so that the color of the MLE would not interfere with the final OD_{540} reading, a very clear pattern of increase in inhibition of haemolysis was observed with the increasing MLE concentrations (Fig. 4). With the increasing MLE concentrations, the inhibition of haemolysis increased dose dependently (r = 0.81; P < 0.01) up to 100 µg/ml and thereafter reached a plateau indicating constant higher inhibitions maintained at high MLE concentrations (P < 0.01). The IC_{50} values for membrane stabilizing activity for MLE and aspirin were 6.4 ng/ml and 0.25 ng/ml respectively.

Assay for anti-histamine effect

MLE had induced a significant anti-histamine effect (54.9 ± 6.5%; P < 0.01), which was comparable to that of the reference drug, chloropheniramine (60.0 ± 5.6%; P < 0.01).

***In vitro* lipid peroxidation activity of MLE**

The lipid peroxidation activity (36.7 ± 1.8%) detected at 250 µg/ml of MLE decreased to 15.3 ± 2.8% at 125 µg/ml (P < 0.01). The standard drug, ascorbic acid showed a 52.1 ± 2.2% activity at 100 µg/ml (P < 0.01).

Toxicity

Treatment with 1500 mg/kg/day of MLE for 30 days failed to produce any overt clinical signs of toxicity or stress. MLE treatment did not significantly alter the body weights (control vs MLE: 284.8 ± 3.9 versus 283.8 ± 4.0 g), serum levels of creatinine (control vs MLE; 2.41 ± 0.03 mg/dl vs 2.30 ± 0.02) and albumin: 3.31 ± 0.53 g/dl vs 3.93 ± 0.05 g/dl, Na (control vs MLE; 7256.57 ± 143.7 ppm vs 7125.43 ± 27.4 ppm), K (control vs MLE; 296.14 ± 13.41 ppm versus 284.43 ± 4.11 ppm) or hematological parameters (RBC count: control vs MLE; 3.78 ± 0.14 x 10^6 vs 3.98 ± 0.22 x 10^6; WBC count: 10.0 ± 0.51 x 10^3 vs 9.9 ± 0.24 x 10^3). Serum levels of AST and ALT were elevated following MLE treatment (AST: control vs MLE; 16.07 ± 0.17 U/l vs 22.65 ± 1.35 U/l; ALT: 14.08 ± 0.54 vs 18.40 ± 0.51 U/l; urea: 42.21 ± 1.46 mg/dl vs 48.51 ± 0.57 mg/dl) (P < 0.01), however, these levels were within the normal range of in-house animals (13).

Analysis for phytochemicals and metal ions

Qualitative phytochemical screening showed the presence of alkaloids, saponins, tannins, phlobatanins, steroids, flavonoids, terpenoids and cardiac glycosides. The metal ion content in *I. coccinea* leaves were K: 20.634 ± 4.472 mg/g, Ca: 16.139 ± 1.996 mg/g; Mg: 1.731 ± 0.808 mg/g; Na: 0.530 ± 0.028 mg/g; Fe: 0.293

Table 1. Anti-oxidant activity of methanolic leaf extract of Ixora coccinea.

Concentration (µg/ml)	Anti-oxidant activity*			
	Methanolic Leaf Extract		Vitamin C	
	Percentage Scavenged	Trolox equivalent (µM)	Percentage Scavenged	Trolox equivalent (µM)
1.3	8.3 ± 0.32	2.5 ± 0.02	35.8 ± 2.42	10.0 ± 0.72
2.5	22.6 ± 0.91	6.4 ± 0.08	44.7 ± 2.15	12.4 ± 1.10
5.0	41.8 ± 1.05	17.2 ± 0.40	55.6 ± 1.26	20.4 ± 0.90
10	56.7 ± 2.36	20.8 ± 0.68	62.2 ± 6.29	22.0 ± 0.86
50	65.7 ± 1.95	23.2 ± 1.00	90.1 ± 0.48	29.2 ± 1.10
100	78.2 ± 2.19	26.4 ± 0.72	91.5 ± 0.74	29.6 ± 0.82
250	78.3 ± 1.06	26.4 ± 0.40	92.1 ± 0.59	30.0 ± 0.91
500	77.9 ± 0.98	26.4 ± 0.20	92.3 ± 0.75	30.0 ± 0.85

**** Anti-oxidant activity of MLE of Ixora coccinea was expressed as percent scavenging of DPPH radicals and the Trolox equivalents in µM compared to PBS control. Values represent mean ± SEM.***

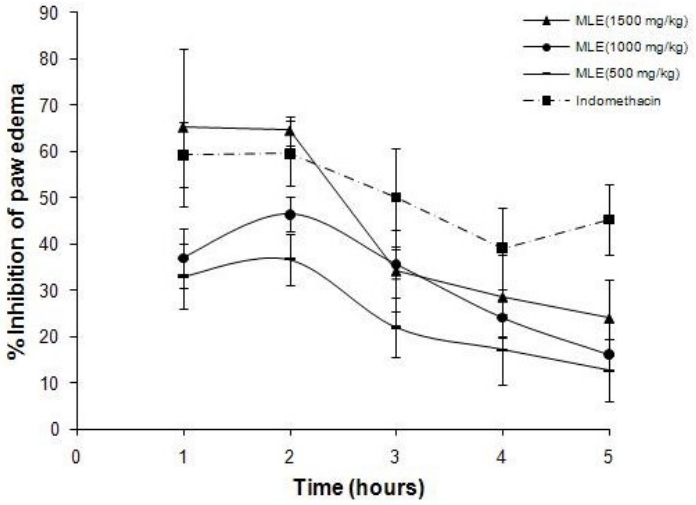

Figure 1: Inhibition of carrageenan-induced rat paw edema following oral treatment with MLE of Ixora coccinea. MLE was administered orally at 500, 1000 and 1500 mg/kg. Indomethacin (5 mg/kg) was used as the reference drug. One ml each of gum acacia (1%) and distilled water served as controls for MLE and indomethacin respectively. Paw volumes were measured using a plethysmometer and percentage inhibition of edema was calculated. Values represent mean ± SEM. Significant inhibition of edema ($P < 0.05$) upto 5^{th} h compared with control for 1500 mg/kg dose and indomethacin. Significant inhibition of edema ($P < 0.05$) upto 4^{th} h compared with control for 1000 mg/kg and 500 mg/kg doses.

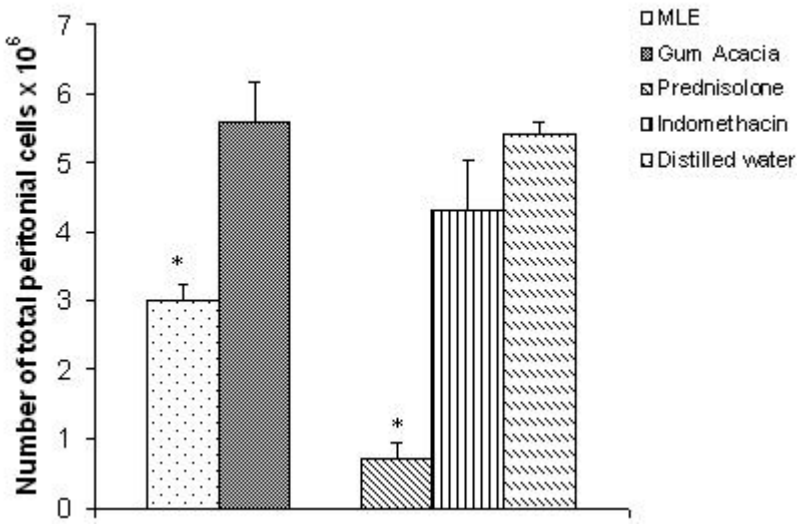

Figure 2: Effect of MLE of Ixora coccinea on rat peritoneal cell infiltration. The total number of peritoneal cells was assessed following oral treatment of MLE (1500 mg/kg) and induction of inflammation by carrageenan. Prednisolone (10 mg/kg) was used as reference drug. Control groups for MLE and prednisolone were orally administrated with 1ml of 1% GA and 1 ml of distilled water respectively. Values represent mean ± SEM. * P < 0.05 compared to control group.

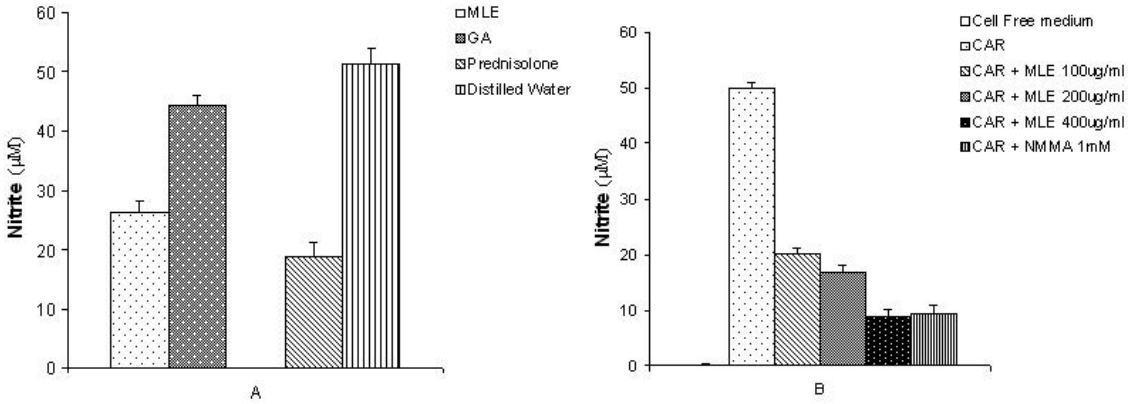

Figure 3. Effect of MLE of Ixora coccinea on NO production by rat peritoneal cells. A. Rats were orally treated with MLE (1500 mg/kg), prednisolone (10 mg/kg) and control groups were treated with 1 ml of 1% GA and 1 ml of distilled water. Following this in vivo MLE treatment, rats were injected with carageenan and the production of NO was determined. B. Peritoneal cells were obtained from rats injected with carrageenan and treated in vitro with MLE (100, 200, and 400 µg/ml) and NMMA (1 mM) which is a specific nitric oxide synthase inhibitor. Cell free medium shows minimal nitrite levels. Values represent mean ± SEM.* P < 0.05 compared to control group.

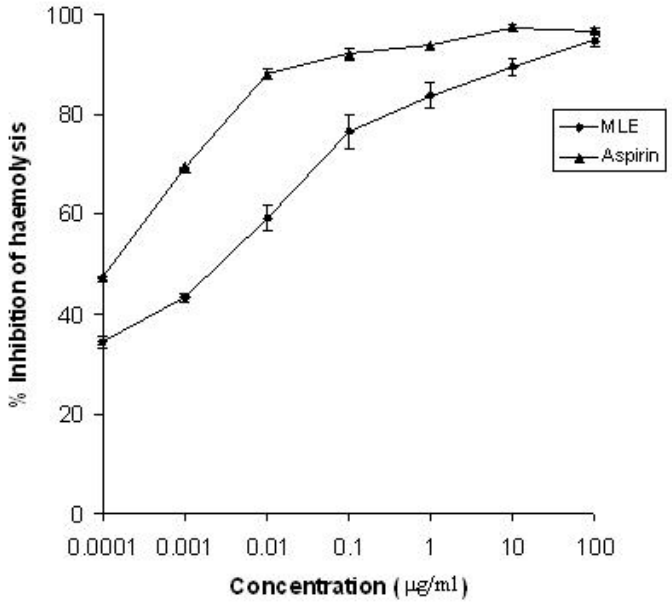

Figure 4. Membrane stabilizing activity of MLE of Ixora coccinea on rat erythrocytes. Rat erythrocytes were treated with 0.1 ng/ml – 100 µg/ml of MLE of I. coccinea and aspirin as the reference drug in this heat-induced hemolysis assay. Color of the MLE was removed by centrifugation after the initial incubation at 37°C, thereafter incubated at 54°C to induce hemolysis and OD was measured at 540 nm. Inhibition of hemolysis was calculated with respect to the PBS control. Values represent mean ± SEM.

± 0.034 mg/g; Zn: 0.019 ± 0.016 mg/g and Cr/Ni/Mn/Cu/Cd/Hg < 0.0001 mg/g.

DISCUSSION

This study examined the anti-inflammatory activity of MLE of *I. coccinea* using the carrageenan-induced rat paw edema assay and the results show a dose-dependent *in vivo* anti-inflammatory activity. These results are consistent with our previous findings using aqueous leaf extracts of *I. coccinea* (3). This study also demonstrated for the first time the inhibitory effects of leaf extracts of *I. coccinea* on nitric oxide which is a key inflammatory mediator and other activities such as infiltration of phagocytic cells, membrane stabilization and scavenging of free radicals.

The paw edema assay for anti-inflammatory activity showed some contrasting differences with MLE especially during the early phase of the assay compared to the previously described findings with the aqueous leaf extract of *I. coccinea* (3). High concentration of MLE (1500 mg/kg) showed the significantly higher inhibition of paw edema one hour after carrageenan injection compared to the lower concentrations of the MLE (500 and 1000 mg/kg). This may be due to the presence of different anti-inflammatory components in the two extracts prepared using methanol and water having different polarities. Further studies are required to separate the anti-inflammatory active components from these solvent extracts.

The anti-inflammatory effects of the MLE reached its higher levels during the period between 1-2 hours. Cyclooxygenase (COX) inhibitors have been shown to inhibit the edema during this phase (17) and it is possible that inhibition of arachidonic acid metabolites is involved in the anti-inflammatory activity of MLE. The fact that the strong inhibition by MLE during 1-2 hours overlaps that by indomethacin supports this notion. Further, as shown previously (3) and in this study, *I. coccinea* contains flavonoids and tannins. Flavonoids and tannins have been shown to impair cyclooxygenase/lipoxygenase activities that would reduce the levels of prostaglandins and other arachidonic acid metabolites (18- 20).

Migration or the infiltration of immune cells to the site of inflammation is an important processe that take place in an inflammatory response. Experimental systems such as carrageenan-induced pleurisy in rats have been used to study cell migration process and the inhibitory effects of drugs on cell migration (21). In the present study, we used a recently developed *in vivo* assay, ie., carrageenan-induced infiltration of rat peritoneal cells (7) to assess the inhibitory effects of MLE on immune cell infiltration. Our findings show

significant inhibition of rat peritoneal cell infiltration which indicate that MLE of *I. coccinea* has potent leukocyte infiltration/migration inhibitory activity.

The anti-inflammatory activity in the paw edema assay showed that MLE has significant inhibitory activity up to the 4^{th} hour. Previous studies have shown that mobilization of phagocytic cells, neutrophils, and monocytes/macrophages to the site of inflammation is a characteristic feature of this late phase (17) apart from other events linked to the release of oxygen free radicals (22 and 23) and nitric oxide (24). This inhibitory effect of MLE on cell migration and mobilization *in vivo* as shown in the infiltration of rat peritoneal cell assay suggest that similar inhibitory effect of MLE on immune cell migration could have contributed to the curtailment of inflammation *in vivo*, in the rat paw edema assay. Further, prednisolone, the reference drug used showed a significantly higher inhibition of peritoneal cell infiltration suggesting that cell infiltration being inhibited by phospholipase 2 activity at an earlier step of processing of arachidonate metabolites or by arachidonate cyclooxygenase (COX) inhibitors. Recent studies have shown either a low (25) or no effect (26, 27) of COX inhibitors on migration of leukocytes in different experimental models. It is possible that MLE of *I. coccinea* may inhibit cell infiltration by affecting the arachidonate pathway at steps other than the COX pathway or affecting other activities such as those of leucocyte chemotactic factors and monocyte specific chemotactic factors (28).

The present study also showed a strong *in vivo* anti-histamine activity of the MLE which was detected within 2 min of histamine injection which is consistent with the previous findings on aqueous leaf extracts of *I. coccinea* (3). The activity of inflammatory mediators such as histamine, serotonin, and arachidonic acid metabolites has been highlighted during this early phase (17). Histamine released from mast cells is known to stimulate endothelial cells to increase vascular permeability (29). It is possible that this anti-histamine activity of the MLE could at least in part contributed to the impairment of carrageenan-induced microvascular leakage in the paw edema assay reflecting on the anti-inflammatory mechanisms operating during the early phase. Triterpenes from angiosperms are known to impair histamine release from mast cells and exert anti-inflammatory activity (30) and phytochemical analysis of *I. coccinea* leaves performed in this study as well as in a previous study (3) has shown the presence of triterpenoids. MLE also showed marked and dose-dependent anti-oxidant activity. Carrageenan-induced paw edema is sensitive to anti-oxidants (22). This is likely to be another mechanism by which MLE mediates impairment of late phase of the anti-inflammatory response. The anti-oxidant activity of the MLE could be due to the presence of flavonoids and phenols (18, 19). A recent study has shown anti-oxidant activity of flowers of *I. coccinea* and suggests that the anti-oxidant activity involves the presence of hydrophilic phenolics (31). In addition, MLE also showed membrane stabilizing activity in the heat-induced hemolysis of rat erythrocytes *in vitro*. Although the previous study had not shown significant membrane stabilizing activity in ALE of *I. coccinea* (11), the present study showed very high activity in MLE. This indicates that the MLE could stabilize the lysosomal membranes to inhibit the release of proteolytic enzymes; lysosomes play a major role in the inflammatory reaction (32, 33). There is a close similarity between erythrocytes and lysosomal membrane system (32).

An important finding of this study is that MLE showed a significant inhibitory effect on NO production by rat peritoneal cells. This was shown by inhibitory effect on NO production of peritoneal cells following *in vivo* treatment of rats with MLE as well as direct effect on peritoneal cells, following *in vitro* treatment with MLE. Nitric oxide is an important mediator in an inflammatory response (24). Several compounds such as sesquiterpene lactone from *Artemesia princes* Pampan (Asteraceae), flavin 3,3′-digallate which is a polyphenol from Black tea and resveratrol which is a naturally occurring flavinoid from grapes and grapefruits have been shown to inhibit the inducible nitric oxiside synthase (iNOS) expression (34). These results emphasis the importance of conducting further studies to elucidate the specific mechanisms of NO inhibitory activity of MLE of *I. coccinea.* This study also showed that the lipid peroxidation activity of MLE decreased proportionately with MLE concentration. This has some implications on the activity of MLE *in vivo*. The interaction of NO with the super oxide anion gives rise to peroxynitrite which is a highly potent oxidant that damages the tissue in inflammation. Previous studies have shown that peroxynitrite-dependent lipid peroxidation can be regulated by high concentrations of NO (35). Dose-dependent inhibition of NO production by MLE observed in the present study suggests that the peroxynitrite-dependent lipid peroxidation could also be reduced by MLE.

Thirty day (chronic) treatment of MLE was well tolerated, there were no deaths and it did not induce any unacceptable side effects or any overt signs of clinical toxicity. The biochemical analysis also did not indicate any hemotoxicity, nephrotoxicity or hepatotoxicity. The analysis of metal ions

in leaves of *I. coccinea* indicated the absence of toxic, heavy metal such as Cr, Cd, and Hg.

In conclusion, this study has shown promising anti-inflammatory activity in the methanolic leaf extracts of *I. coccinea.* Compared to the previous study on aqueous leaf extracts of *I. coccinea* (3), the present study has demonstrated specific mechanisms of the anti-inflammatory activity mediated by inhibition of peritoneal immune cell infiltration and NO production and also membrane stabilizing activity and anti-oxidant activity. Findings of the present study also corroborate the use of *I. coccinea* in traditional medicinal practice in Sri Lanka for treatment of diseases associated with inflammation.

ACKNOWLEDGEMENTS

Authors wish to thank J.R.A.C. Jayakody, Department of Zoology, Faculty of Science, University of Colombo for technical assistance. Authors thank E. H. Karunanayake and K. H. Tennekoon for valuable advice during this study and critically reviewing the manuscript. Financial assistance from grant No 2005/NSF/HS/15 of the National Science Foundation and grant No 05-52 of the National Research Council, Sri Lanka are acknowledged.

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