Journal of Traditional and Complementary Medicine 11 (2021) 249–258

Contents lists available at ScienceDirect

Journal of Traditional and Complementary Medicine

journal homepage: http://www.elsevier.com/locate/jtcme

Anti-inflammatory activity and mechanism of action of ethanolic leaf extract of *Morinda lucida* Benth

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ARTICLE INFO

Article history:
Received 20 December 2019
Received in revised form
15 June 2020
Accepted 9 July 2020
Available online 4 August 2020

Keywords:
Cyclooxygenase-2
Cytokines
Edema
Inflammation
Nitric oxide

ABSTRACT

Background and aim: Most developing countries resort to medicinal plants for treating diseases, but few of these have scientific backing for their use. The aim of the study was to validate traditional use of *Morinda lucida* leaves in treating inflammation and determine the mechanism of action.
Experimental procedure: Effect of hydroethanolic leaf extract of *M. lucida* (*HEML*) on localized inflammation was evaluated using rat paw edema presented by sub-planter injections of λ-carrageenan, histamine or serotonin in separate experiments. Systemic inflammation was evaluated by lipopolysaccharide (LPS)-induced hyperthermia. Antioxidant activity of HEML was also evaluated using the free-radical scavenging assay.
Results and conclusion: No mortalities were recorded in acute toxicity assay after administering 5000 mg/kg *HEML* to rats. It showed very good activity against localized and systemic inflammation in inverse dose-dependent manner and caused reduction in nitric oxide and prostaglandin E−2 levels by affecting expression of inducible nitric oxide synthase, but not cyclooxygenases-2 in LPS-activated RAW 264.7 murine macrophages. *HEML* reduced pro-inflammatory cytokines interleukin (IL)-1β and tumor necrotic factor, but elevated levels of anti-inflammatory cytokine IL-10 *in vitro*. *HEML* contains saponins, reducing sugars, polyphenols and flavonoids and showed antioxidant activity with $EC_{50} = 0.6415 \pm 0.0027$ mg/ml. In conclusion, this study provides evidence that *HEML* possesses anti-inflammatory activity, possibly through modulation of production of early/late phase inflammation mediators.

1. Introduction

Inflammation is a host defense mechanism and an important process which aids organisms to respond to harmful stimuli.[1] It is a useful process because it provides a conducive physiology to exclude invading pathogens or harmful stimulus. However, the process must be regulated since uncontrolled inflammation is critical for pathophysiological conditions like cancer.[2]

Initial stage typical inflammatory process is marked by an acute phase, where the cascade begins with primary response of immune and vascular systems right after infection or damage to tissues. This phase is rapid and persists for a short while, normally before the

Abbreviation: HEML, hydroethanolic *Morinda lucida*; CPMR, Centre for Plant Medicine Research; CPM, chlorpheniramine; SFN, sulforuphane; GRA, granisetron.

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Peer review under responsibility of The Center for Food and Biomolecules, National Taiwan University.

https://doi.org/10.1016/j.jtcme.2020.07.001

immune response becomes established. Acute inflammation also acts as a homeostatic mechanism that benefits the host in reparative process.[3] Carrageenan-induced paw-edema represents a classic model for evaluation of acute anti-inflammatory agents.[4] Its injection in rats presents a biphasic mode of inflammation which can be classified as early and late phases. Early phase lasts for about an hour and is characterized by release of pre-synthesized inflammatory mediators such as histamine, serotonin and bradykinins. Late phase begins after the first hour where early phase mediators activate processes leading to neutrophil infiltration and further release of prostaglandins formed by cyclooxygenases (COX).[4] Neutrophil-derived free radicals, nitric oxide (NO) and pro-inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF- α) are other mediators which affect the late phase of carrageenan-induced inflammation5. Persistence of the late phase mediators propagates inflammation to chronic phase, leading to inflammation-associated diseases. Induction of lipopolysaccharide (LPS), serotonin and histamine has been associated with inflammation. Lipopolysaccharides induce cellular responses that extremely stimulate innate or natural immunity.[6] Thus, stimulation of macrophages by LPS serves is a powerful means of studying inflammation that is mediated by inflammatory mediators from activated macrophages. Serotonin also regulates physiological processes such as inflammation and cell proliferation which are critically modulated by macrophages.[7] Also, histamine is an inflammatory mediator that can promote inflammatory and regulatory responses which are associated with pathological processes.[8]

Non-steroidal Anti-inflammatory Drugs (NSAIDs) represent a common class of anti-inflammatory and analgesic drugs for alleviating symptoms associated with inflammation by inhibiting COX. Unfortunately, they have adverse effects on gastrointestinal lining, coagulation of blood and renal systems due in part to inhibition of housekeeping enzyme COX-1.[9] Moreover, some newer COX-2 specific inhibitors, claimed to be devoid of these adverse effects, have not met expected outcome.[10] Thus, alternative anti-inflammatory agents with potent activity but minimal or no side-effects are needed.

In Ghana, there is increasing interest in herbs for treatment of various diseases, including inflammatory conditions. *Morinda lucida* Benth. (Rubiaceae) is one of the most popular medicinal plants widely distributed in Africa. Although it has been used in Ghanaian folk medicine in treatment of inflammation, there is little scientific data to validate this activity.

Therefore, this study was aimed at evaluating anti-inflammatory effect of hydroethanolic leaf extract of *M. lucida* (*HEML*) in Sprague-Dawley rats using carrageenan-induced paw edema and to assess the possible mechanisms involved using LPS-stimulated RAW 264.7 cells.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals and reagents

Antibodies against cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Lypopolysaccharide from *Escherichia coli* and λ-carrageenan, were obtained from Sigma-Aldrich (St. Louis, USA). All reagents used were of analytical grade and obtained from standard suppliers.

2.1.2. Ethical approval

The Scientific and Technical Committee of the Noguchi Memorial Institute for Medical Research reviewed and approved of the study (STC Paper No. 6(1)2016-17).

2.1.3. Plant collection, authentication and extract preparation

Dried leaves of *Morinda lucida* Benth were obtained from the Plant Development Department of the Centre for Plant Medicine Research (CPMR), Mampong-Akwapem, Ghana and authenticated by Mr. Heron Blagogee, Senior Botanist. A voucher specimen (No. CSRPM/8510) was deposited at CPMR herbarium. Air-dried pulverized *M. lucida* Benth, leaves 500 g, were cold-macerated in 5 L of 70% ethanol-water (v/v) for 72 h. The crude extract was filtered and concentrated using a rotary evaporator (Eyela Co. Ltd, Tokyo, Japan). Residual plant material was re-extracted thrice. Aqueous-based concentrated crude extract was freeze-dried and powdered *HEML* was kept in a desiccator until use.

2.1.4. Animals/RAW 264.7 cells

Female Sprague-Dawley Rats of weight 180–200 g were acquired from Animal Experimentation Unit, CPMR. Animals were fed *ad libitum* with feed obtained from Ghana Agro Food Company (Ghana) and housed in metallic cages lined with autoclaved soft wood shavings as beddings. Animals were kept under standard laboratory conditions (temperature 28 °C±2 °C, relative humidity 60–70% and normal 12 h cycle of light and dark) and allowed access to sterilized drinking water *ad libitum* and handled in accordance with EU Directive 2010/63/EU. Murine macrophage-like RAW 264.7 cells were obtained from RIKEN BioResource Center Cell Bank (Japan).

2.2. Experimental procedure

2.2.1. Phytochemical screening/Thin Layer Chromatography

Phytochemical constituents of *HEML* were determined by standard methods.[11,12] Thin layer chromatographic (TLC) profiling was performed by spotting *HEML* on both normal (with polar matrix) and reverse phase (non-polar matrix) silica gel 60 F254 pre-coated TLC plate (Merck, Damstadt-Germany). The Normal phase TLC plate was run with chloroform/methanol (7:1 v/v) and Reverse phase TLC plate was run with methanol/water (2:1 v/v). Detection of spots was done by spraying both plates with 10% (v/v) H_2SO_4 and heating (110 °C). Retardation factor (R_f) was calculated as:

$$Rf = \frac{\text{Total distance travelled by spot}}{\text{total distance travelled by the solvent front}} \quad (1)$$

2.2.2. Determination of antioxidant property

2.2.2.1. DPPH free-radical scavenging activity. Free radical scavenging activity of *HEML* was determined as described.[11] About 100 μl of 2, 2-diphenyl-1-picryl hydrazyl (DPPH) in methanol (0.5 mM) were added to 100 μl each of varying concentrations of *HEML* and allowed to stand for 20 min at room temperature (25 °C) in the dark. Absorbance was measured at λ517nm with a microplate reader (Infinite m200pro TECAN, Austria). Butylated hydroxytoluene (BHT) was used as positive control. Percentage antioxidant activity was determined by the equation:

$$\%\ \text{Inhibition} = \frac{\text{ODB} - \text{ODS}}{\text{ODB}} \times 100 \quad (2)$$

Where: ODB = optical density of blank; ODS = optical density of test sample.

Concentration of *HEML* or BHT, which inhibited 50% of DPPH activity (EC_{50}) was determined from a plot of inhibition against log concentration. The experiment was carried out in triplicate.

2.2.3. Assessment of phytochemical content

2.2.3.1. Total phenolic content. Total polyphenolic content of *HEML*

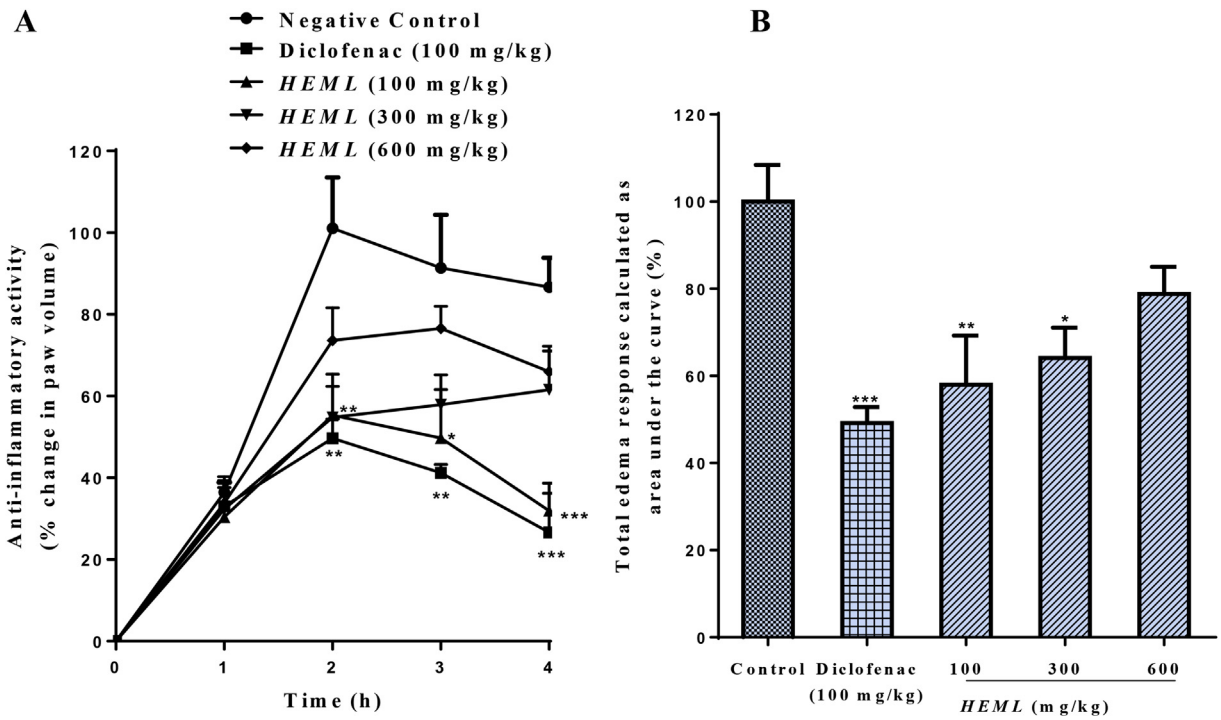

Fig. 1. Effect of HEML on carrageenan-induced paw edema in rats; (A) time-course curves and (B) areas under the curve. Data represent means ± SEM. (n = 6). Value statistically significant compared with untreated control; *p < 0.05, **p ≤ 0.01, ***p ≤ 0.001.

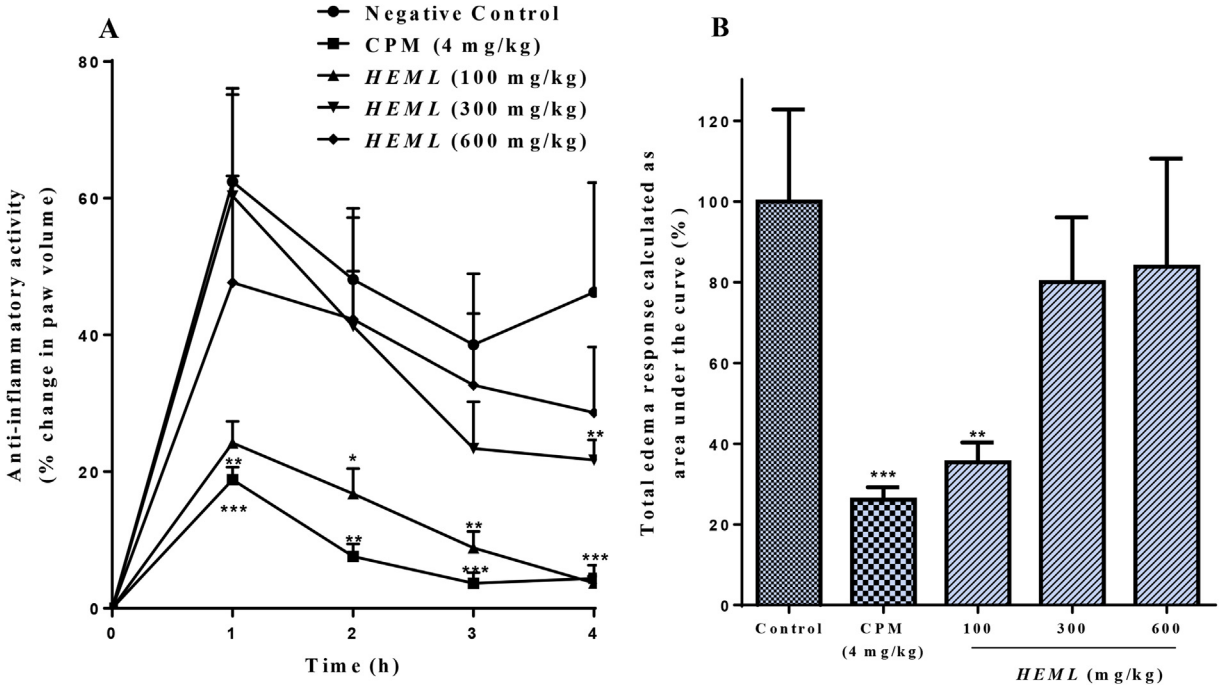

Fig. 2. Effects of pretreatment with HEML on suppression of histamine-induced paw edema in rats with time post-histamine challenge; (A) time-course curves and (B) areas under the curve. Data represents means ± SEM. (n = 6). Value statistically significant compared with untreated; **p ≤ 0.01, ***p ≤ 0.001.

was determined using Folin Ciocalteau method.[13] Briefly, a mixture of 10 μl of 5 mg/ml of *HEML* suspended in distilled water were mixed with 0.79 μl of distilled water and 50 μl of Folin Ciocalteau reagent. After 8min of incubation, 150 μl of 0.09 M Na_2CO_3 was added and incubated further for 2 h at 25 °C. Absorbance of the mixture was read at λ750nm. Results were expressed in milligram of Gallic acid per 100 mg of dry sample (mg GAE/100 mg sample).

2.2.3.2. Quantification of flavonoids. Flavonoid content of *HEML* was estimated by colorimetry.[13] About 100 μl *HEML* (5 mg/ml in methanol) was added to 100 μl of 2% $AlCl_3$ (w/v) and incubated for 20 min at 25 °C. The absorbance of the mixture was read at λ415nm.

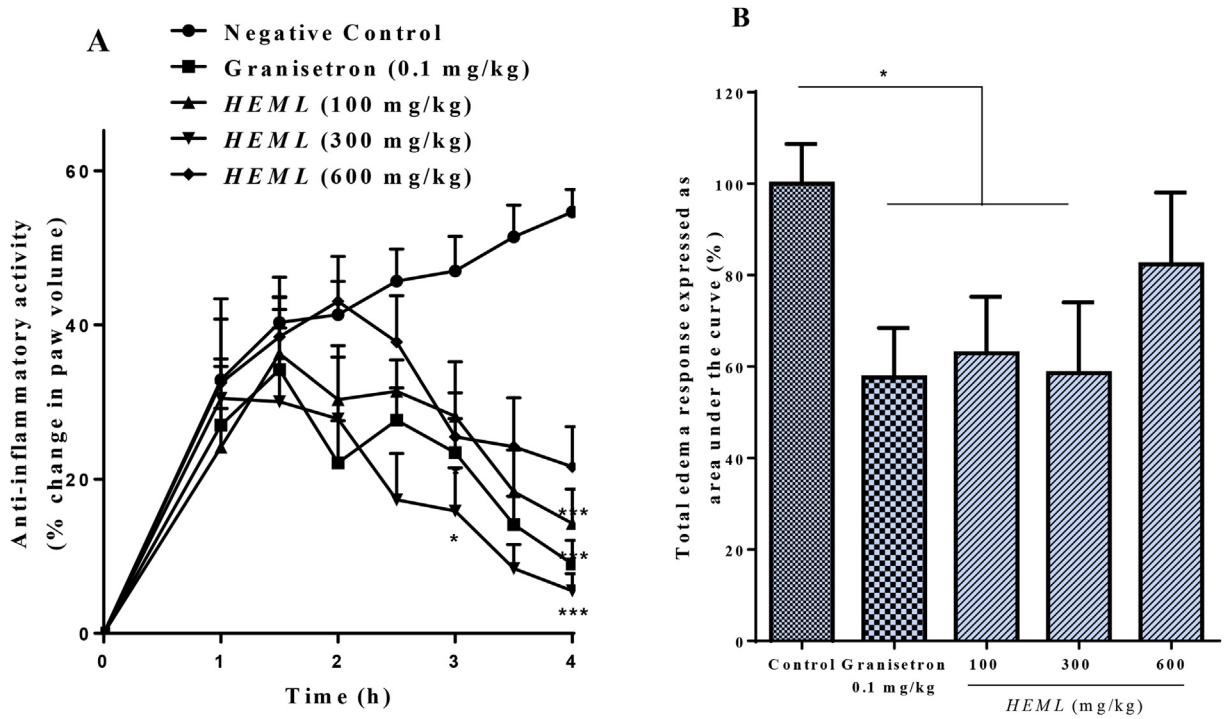

Fig. 3. Effects of pretreatment with HEML on suppression of serotonin-induced paw edema in rats with time post-serotonin challenge; (A) time-course curves and (B) area under the curve. Data represents means ± SEM. (n = 6). Value statistically significant compared with untreated; **p ≤ 0.01, ***p ≤ 0.001.

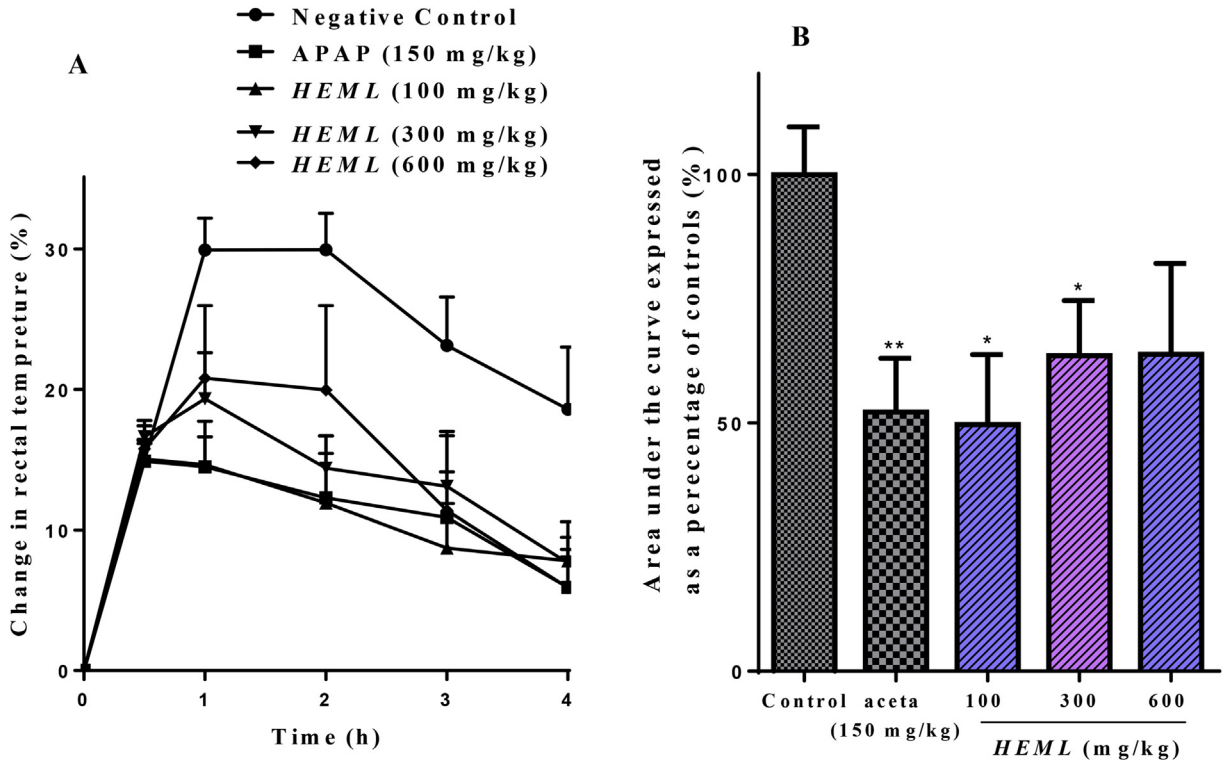

Fig. 4. Effect of HEML (or SFN) on LPS-induced fever in rats; (A) time-course curves and (B) areas under the curve in arbitrary units. APAP (aceta), acetaminophen (150 mg/kg). Data represents means ± SEM (n = 6). Values statistically significant compared with untreated; *p < 0.05, **p ≤ 0.01.

Total flavonoid content was expressed as milligram of quercetin per 100 mg dry sample (mg QE/100 mg sample).

2.2.4. Determination of median lethal dose (LD_{50})

A single dose of 5000 mg/kg *HEML* was administered orally to six (6) female Sprague Dawley rats (n = 6). They were observed

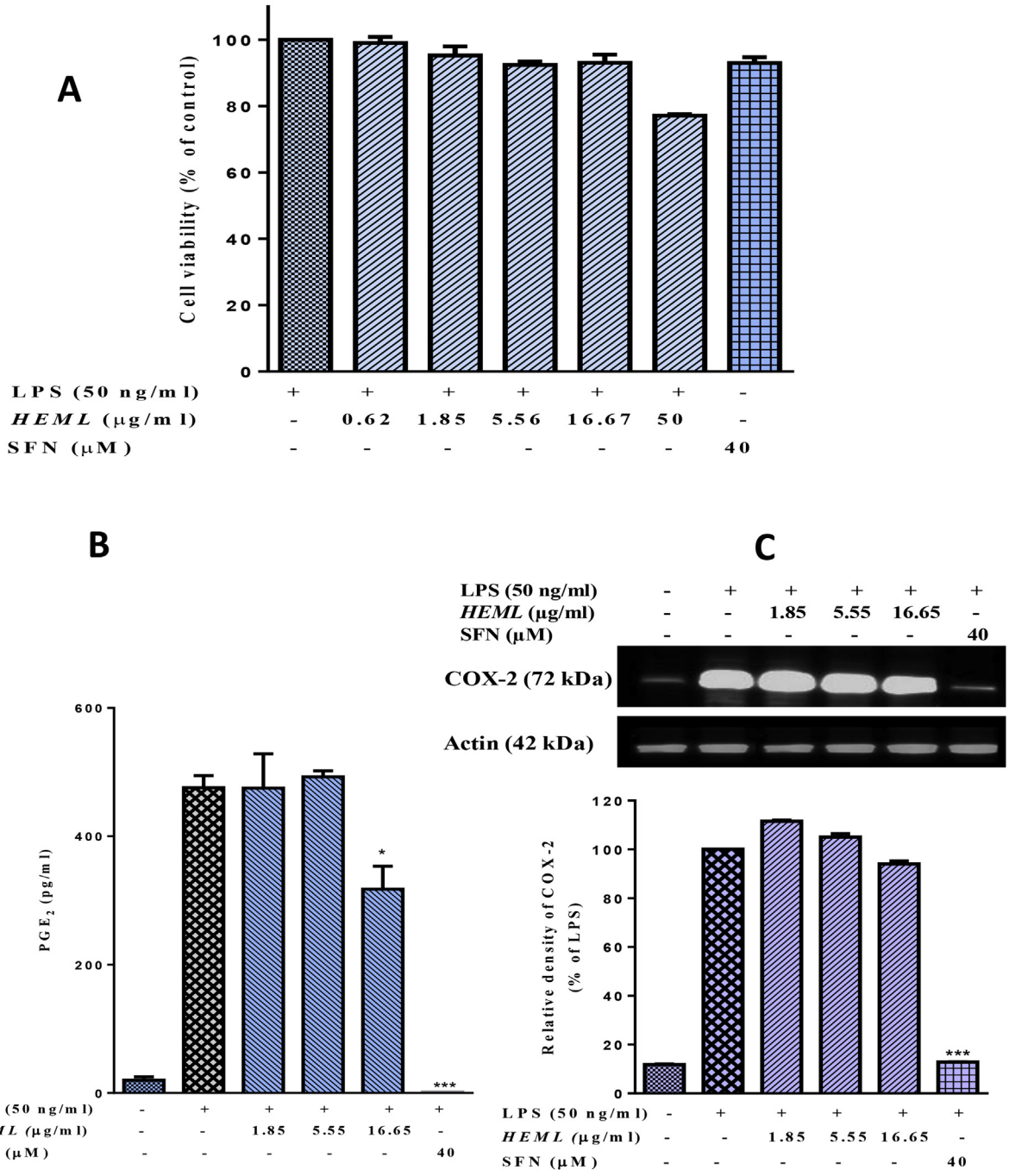

Fig. 5. Effect of HEML on cell viability of LPS-activated RAW 264 cells (A), PGE_2 release (B) COX-2 expression and (C) LPS-induced RAW 264.7 cells. Data represents means ± SEM (n = 3). Value statistically significant compared with control (LPS treated); *p < 0.05, ***p ≤ 0.001.

over 48 h for number of deaths and general behavior. Surviving animals were further observed for additional 12 days for signs of toxicity such as abnormalities in feeding and breathing, locomotion, lachrymation, and pilo-erection.

2.2.5. Carrageenan-induced paw edema test

Effect of *HEML* on carrageenan-induced paw edema was evaluated as described[14] with slight modifications. Briefly, rats were fasted overnight and grouped into 5 different metallic cages at 6 rats/group. Initial paw volume of the right hind limbs of the animals was taken at baseline using plethysmometer. Sterile carrageenan (0.1 ml), at 1% (w/v) in normal saline, was injected (s.c) into subplanter tissue of the right hind paw of each rat. Paw volumes of injected rats were taken after 30min to ascertain the levels of edema after which drug/extract was administered. Paw edema was monitored at hourly intervals post-oral drug/extract administration for 4 h. Animal groups received either diclofenac (100 mg/kg), positive control or *HEML* (100, 300 and 600 mg/kg), while negative control animals received the vehicle (sterilized water). Percentage change in paw edema was calculated as follows:

$$\%\ \text{Change in paw edema} = \frac{VT - V0}{V0} \times 100\% \quad (3)$$

Where: VT = Paw volume at time t; V0 = Paw volume at baseline.

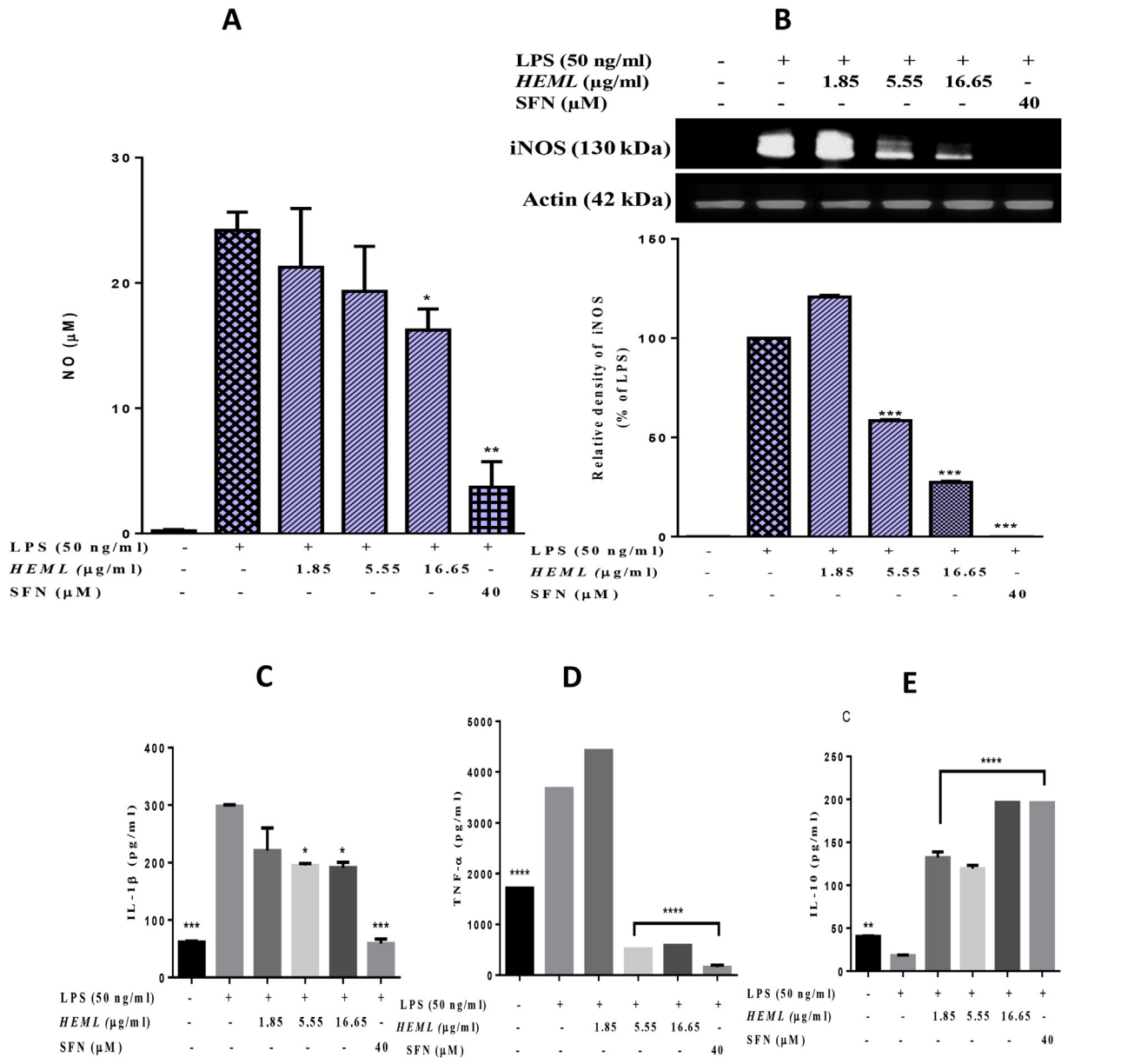

Fig. 6. Effect of HEML on NO production (A) iNOS expression (B) and cytokine levels, IL-1β (C), TNF-α (D) and IL-10 (E) in LPS-induced RAW 264.7 cells. Data represents means ± SEM (n = 3). Value statistically significant compared with control (LPS treated); *p < 0.05, **p ≤ 0.01, ***p ≤ 0.001.

2.2.6. Anti-histaminic and anti-serotogenic activity

Anti-histaminic and anti-serotogenic effects of *HEML* were assessed in histamine/serotonin-induced paw edema assay using histamine or serotonin as phlogistic agent as described[15] with slight modifications. Negative control rats received distilled water (1 ml) orally, while positive controls animal received chlorpheniramine (CPM, 4 mg/kg) or granisetron (0.1 mg/kg) in water and test animals received *HEML* (100, 300 and 600 mg/kg) 1 h prior to induction of edema for 4 h. Paw edema was calculated using equation (3) above.

2.2.7. Determination of anti-pyretic activity

Hyperthermia was induced in the animals using LPS from *E. coli* cell wall as previously described.[16] The anti-pyretic effect of *HEML* was assessed as described[17] with slight modifications. Briefly, female SDRs were fasted overnight. Rectal temperature of animals was taken in a temperature-controlled room at 25±2 °C prior to injection of LPS (1.29 x 10^9 cells). Rectal temperature of test animals was taken 30min post-LPS injection and animals with differences in temperature were randomly selected into five different groups of 6 animals. Test groups were treated orally with *HEML* (100, 300 and 600 mg/kg) or acetaminophen (positive control, 150 mg/kg) suspended in sterile water. Normal control group received only sterile water. Rectal temperatures of rats were monitored at 1 h intervals for 4 h post-*HEML*/acetaminophen administration. Percentage change in temperature was calculated as follows:

$$\%\ \text{Change in Temperature} = \frac{Tt - T0}{T0} \times 100 \qquad (4)$$

Where: T_0 = baseline temperature; Tt is the temperature at each hour.

2.2.8. Cell culture/survival assay

RAW 264.7 cells, were cultured in a humidified incubator at 37 °C in 5% CO_2 using Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin solution. Subsequent experiments with RAW 264.7 cells were carried out under serum-free conditions. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay[18] with slight modifications. RAW 264.7 cells (2 x 10^4 cells/well) were seeded in 96-well micro-titer plates and incubated for 24 h, after which cells were starved by culturing in serum-free medium for another 2.5 h. Cells were then treated with 50, 16.67, 5.56, 1.85 and 0.62 μg/ml of *HEML* or sulforuphane (SFN), 40 μM as positive control and incubated for 30min. Cells were stimulated with 50 ng/ml LPS in phosphate buffer saline (PBS) and incubated for 12 h. To each well, 20 μl of MTT solution in PBS was added and further incubated for 4 h. Formazan formed was solubilized by the addition of 150 μl of acidified isopropanol (0.04 N HCl-isopropanol) in 1% v/v Triton-X 100 and incubated overnight in the dark at 25 °C. The concentration of formazan formed was measured at λ570nm using a microplate reader (Infinite 200pro TECAN, Grodig, Austria). Percentage cell survival was determined using the formula:

$$\%\ \text{Cell survival} = \frac{A1 - A0}{A2 - A0} \times 100 \quad (5)$$

Where A_1 = absorbance of test and positive control (treated cells), A_0 = absorbance of blank (mixture without cells) and A_2 = absorbance of negative control (untreated cells).

2.2.9. Measurement of nitric oxide, prostaglandin E–2 and cytokines levels

Concentration of NO in culture supernatant was determined by measuring the concentration of nitrite produced using the Griess reaction.[19] The RAW 264.7 cells (1 x 10^6 cells/well) were seeded in 48-well plates and left overnight. Cells were starved for 2.5 h by replacing culture medium with serum-free medium. Cells were treated with *HEML* (0.62–50 μg/ml) or SFN (40 μM) for 30min, stimulated with 50 ng/ml of LPS and incubated for further 12 h. Equal volumes of cell culture supernatant (100 μl) were mixed with the Griess reagent (1% w/v sulfanilamide in 5% v/v phosphoric acid and 0.1% w/v naphthylethylenediamine dihydrochloride in water) in a 96-well plate and incubated for 10min. The absorbance was read at λ550nm. Concentration of nitrite generated in cell culture supernatant was estimated by extrapolation from a sodium nitrite standard curve.

Cell culture process was repeated for prostaglandin E–2 (PGE_2), pro-inflammatory (IL-1β and TNF-α) and anti-inflammatory (IL-10) cytokines using ELISA test kits (R & D Systems, USA) for quantification.

2.2.10. Western blot analysis

Western blot analysis was performed as previously described.[20] Cultured cells (RAW 264.7, 1 x10^6 cells) starved in serum-free medium were incubated with *HEML* at 0.62–50 μg/ml and SFN at 40 μM and for 30min before exposure to 50 ng/ml of LPS overnight. The negative control was incubated with 50 ng/ml LPS. Cells were removed from medium, washed with PBS, harvested and lysed using RIPA buffer. Cell lysate was centrifuged at 10,000 rpm for 10 min at 4 °C and the supernatant was collected. Protein concentration in the supernatant was determined by Bradford's method[21] using bovine serum albumin as protein standard. Supernatant obtained from the lysate was denatured at 70 °C for 10min in SDS sample buffer. Equal amounts of protein (40 μg/ml) were loaded and run using SDS-PAGE (Invitrogen NuPAGE 12% Bis-Trisgel). Proteins were electrophoretically blotted unto a polyvinylidene difluoride membrane (Immobilon P; Millipore, USA). The membrane was incubated overnight at 4 °C with specific primary antibody; anti-goat iNOS/COX-2 at 1:200 dilutions with buffer after blotting. It was further incubated for an hour with horseradish peroxidase (HRP) at 1:2000 dilutions before substrate solution was added. A camera system was used for detection of bound antibodies. Intensities of bands were quantified using Image J software and normalized to that of internal control (actin bands).

2.2.11. Statistical analysis

Significant variations between all treatment and untreated groups were analyzed using One-Way Analysis of variance and student t-test with GraphPad prism 6, considering $p \leq 0.05$ as significant.

3. Results

3.1. Extract yield

Solvent extraction and freeze-drying 500 g of air-dried pulverized *M. lucida* leaves yielded 46.04 g of powdered *HEML* extract, representing a yield of 9.21% (w/w).

3.2. HEML chromatographic profiling

Phytochemical screening of *HEML* showed the presence of saponins, reducing sugars, polyphenols and flavonoids, with absence of polyuronides, cyanogenic glycosides, alkaloids, anthraquinosides, triterpenes and phytosterols. Thin Layer Chromatography profiles of *HEML* on normal (Plate A) and reversed (Plate B) phase silica gels, in two different solvent systems, exhibited the presence of 8 spots with R_f values 0.92, 0.87, 0.77, 0.75, 0.74, 0.66, 0.40 and 0.26 on plate A, and 7 spots with R_f values of 0.87, 0.78, 0.71, 0.62, 0.20, 0.15 and 0.09 on plate B (Fig. A1).

3.3. Antioxidant activity

Antioxidant activities of *HEML* and BHT (positive control), measured as DPPH radical scavenging activity and expressed as EC_{50}, were 0.6415 ± 0.0027 and 0.017 ± 0.0015 mg/ml, respectively (Fig. B1).

3.4. Total phenolic and flavonoid contents

Total polyphenolics and flavonoids present were 2.23 mg GAE/100 mg and 0.15 mg QE/100 mg of *HEML*, respectively.

3.5. Median lethal dose (LD_{50}) of HEML

A single oral dose of 5000 mg/kg *HEML* administered to the Sprague- Dawley rats did not cause any mortality after 48 h and 12 days. There were no observable signs of toxicity since experimental animals showed normal locomotion, breathing, gaiting, lachrymation and no signs of pilo-erection. Median lethal dose of *HEML* was >5000 mg/kg.

3.6. Effects of HEML on carrageenan-induced rat paw edema

There was 100% increase in paw volume in carrageenan-challenged normal control animals by 2 h with about 10% dip by the 4th hour (Fig. 1A). Also, there were lower paw volumes with the different doses of *HEML* at 2 h (30–55%) relative to the normal control and no significant changes in paw volume of the 300 and 600 mg/kg *HEML* treatment groups by the 4th hour but a further

decline (20%) in respect of the 100 mg/kg *HEML* group. Positive control, diclofenac, showed a trend similar to *HEML* dose at 100 mg/kg, albeit with slightly higher reductions in paw volume (Fig. 1A). These results are depicted as areas under the curves (AUC) over the 4 h period (Fig. 1B), which showed diclofenac with a 50% significant reduction ($p < 0.001$) in total paw volume followed by *HEML* at 100 mg/kg (40%; $p < 0.01$) and 300 mg/kg (35%; $p < 0.05$). Reduction in paw volume seen with *HEML* at 600 mg/kg (20%) was not significant ($p > 0.05$) compared to the normal control.

3.7. Effects of HEML histamine-induced paw edema

Pre-treatment of animals with *HEML* or chlorpheniramine (CPM) as positive control followed by histamine challenge 1 h later, showed an increase (62%) in paw volume of negative control animals followed by reduction in paw volume to 50% by 4 h post-histamine challenge (Fig. 2A). There was no reduction in paw volume of the negative control animals 1 h after treatment with 300 mg/kg *HEML*, but there was significant reduction (37%) at 4 h post-histamine challenge relative to normal control at 1 h (62%). *HEML* dose 600 mg/kg caused 15% reduction in paw volume of negative control at 1 h followed by further decline of about 10% by the 4th hour. However, the increase (62%) in paw volume of control animals was significantly reduced to 22% by *HEML* at 100 mg/kg and to 18% by CPM at 1 h post-histamine challenge, which reduced further to about 5% in both cases by the 4th hour (Fig. 2A). These changes were reflected in the AUC in which there were significant reductions in total paw volume as seen with *HEML* at 100 mg/kg (65%; $p < 0.01$) and CPM (75%; $p < 0.001$) relative to negative control. No significant reductions in total paw volume were seen with *HEML* pretreatment at 300 and 600 mg/kg over the period after histamine challenge (Fig. 2B).

3.8. Serotonin-induced paw edema in rats

Pre-treatment of animals with *HEML* or granisetron (GRA) as positive control followed by serotonin challenge 1 h later, showed an increase (25%) in paw volume of serotonin-challenged negative control animals within 1 h followed by a gradual increase in paw volume to 55% by 4 h post-serotonin challenge. Increase in paw volume of animals pretreated with *HEML* at 600 mg/kg peaked at 2 h (42%) post-serotonin challenge followed by a significant decline to about 22% at 4 h. All other test animal treatment groups showed increases in paw volume (22–35%); 1.5 h post-serotonin challenge, followed by significant declines to 5–15% by the 4th hour (Fig. 3A). These results are depicted in the AUC in which there were significant reductions in total paw volume seen with *HEML* at 100 and 300 mg/kg (40–42%; $p < 0.05$) and GRA (42%; $p < 0.05$) relative to the negative control. No significant reduction ($p > 0.05$) in paw volume was observed with *HEML* pretreatment at 600 mg/kg over the period after serotonin challenge (Fig. 3B).

3.9. LPS-induced fever in rats

Injection of LPS from *E. coli* (i.p) caused a rise in rectal temperature of normal control animals by 30% within 1 h followed by a decline (18%) by the 4th hour (Fig. 4A). Results on the 300 and 600 mg/kg *HEML* treatment groups followed a similar trend with 20–25% increases in rectal temperature at 1 h followed by declines to 5.9–8.2% by the 4th hour. The 100 mg/kg *HEML* and acetaminophen groups showed a peak increase of 15% at 30min followed by a decline to 5.9–7.7% by the 4th hour (Fig. 4A). These results are depicted in the AUC in which there were significant ($p < 0.05$-0.01) reductions in rectal temperatures with respect to acetaminophen and the 100 and 300 mg/kg *HEML* (38–52%) relative to normal control with insignificant reductions in case of the 600 mg/kg *HEML* group (Fig. 4B).

3.10. LPS-activated RAW 264.7 cell viability

Cell viability studies using RAW 264.7 cells in MTT assay showed that there were no significant differences ($p > 0.05$) in cell viability between cells treated with only LPS or SFN and cells treated with LPS plus *HEML* at concentrations of 0.62–16.67 μg/ml (Fig. 5A). However, at *HEML* concentration of 50 μg/ml, there was 22% reduction in cell viability although this was insignificant.

3.11. PGE-2 levels and COX-2 expression in RAW 264.7 cells

At the concentration of 16.65 μg/ml, *HEML* markedly inhibited (33.4%) LPS-activated release of PGE-2 by cells (Fig. 5B) but did not suppress the expression of COX-2 (Fig. 5C). On the other hand, *HEML* at 1.85 and 5.55 μg/ml neither affected levels of PGE-2 release nor suppressed COX-2 expression. The positive control, SFN, on the other hand, almost completely inhibited PGE-2 release (98%) and strongly suppressed COX-2 expression (84%) to the same level as cells without LPS and *HEML*/SFN (unstimulated cells).

3.12. Nitric oxide levels and iNOS expression in RAW 264.7 cells

In the presence of LPS, *HEML* caused concentration dependent reduction (16–36%) in NO production by RAW 264.7 cells compared to cells with LPS only, whereas SFN caused an 84% reduction (Fig. 6A). Cells in the absence of LPS and *HEML*/SFN produced negligible amounts of NO. Expression of iNOS in the RAW 264.7 cells was increased 100% after LPS exposure, but this was inhibited in cells pretreated with *HEML* at 5.55 μg/ml (40%) and 16.65 μg/ml (70%) or 40 μM SFN (100%). On the other hand, at the concentration of 1.85 μg/ml, *HEML* showed 20% increase in iNOS expression (Fig. 6B).

3.13. Cytokine expressions by RAW 264.7 cells

Cells treated with LPS alone without *HEML*/SFN (stimulated cells) expressed 2–4.8-fold increase in IL-1β and TNF-α levels but 60% reduction in IL-10 levels compared to unstimulated cells. At the three concentrations used, *HEML* reduced IL-1β of LPS-stimulated cells by 19–33% whilst markedly reducing TNF-α by about 87% at *HEML* concentrations of 5.55 and 16.65 μg/ml but increasing it by 15% at 1.85 μg/ml of *HEML* (Fig. 6C and D). SFN markedly reduced levels of IL-1β and TNF-α to unstimulated cell levels and below, respectively. *HEML* caused about 7 to 10-fold increase in IL-10 levels whilst SFN caused a 10-fold increase relative to LPS-stimulated cell control level, which was 50% lower than the unstimulated cell level (Fig. 6E). The changes caused by *HEML* in all the cytokines measured were not concentration-dependent.

4. Discussion

Although macrophages typically promote inflammation at sites of inflammation or infection, it is possible to induce macrophages to differentiate into subsets that become more immunomodulatory in response to external stimuli.[22,23] Macrophages that are pro-inflammatory belong to the M1 macrophage subset and have killer activity, while those that are anti-inflammatory belong to M2 subset that promote repair of damaged tissue.[24–26] In this study we have shown using macrophages that the anti-inflammatory effect of HEML may be due to inhibition of pro-inflammatory mediators such as serotonin and histamine, inhibition of pro-inflammatory cytokines (IL-1β and TNF-α), inflammatory enzyme expressions

(iNOS and COX-2) and their products (NO and PGE_2). We also established that *HEML* attenuated systemic inflammation (with fever) induced by LPS in rats and upregulated the anti-inflammatory cytokine, IL-10. *HEML* showed strong antioxidant property compared to the positive control.

Results from acute toxicity study indicate that *HEML* has low oral toxicity, since administration of 5000 mg/kg single oral dose did not cause death or signs of toxicity in the animals even 48 h post-administration.

Carrageenan-induced paw edema is a classical animal model used in studying acute inflammation, such that mediators involved in the inflammatory process are used to evaluate the anti-edematous effect of many natural products.[27] *HEML* exhibited an inverse dose-dependent anti-inflammatory effect on carrageenan-induced paw edema in rats.[28] The reduction in action of carrageenan observed in this study after higher dose treatments may be due to the presence of antagonistic component(s) of *HEML* that enhances the inflammatory process resulting in increase in level of edema. Thus, at lower doses of *HEML* this antagonistic effect may be negligible and so the anti-inflammatory effect is observed.

Polyphenols, flavonoids and triterpenes are few phytochemicals known to possess antioxidant and anti-inflammatory effects in animal models.[29] *HEML* is a crude extract and contains phytochemicals such as saponins, reducing sugars, polyphenols and flavonoids. Hence, antioxidant and anti-inflammatory activities of *HEML* could be related to the presence of these phytochemicals especially polyphenols and flavonoids. Flavonoids have been shown to possess strong anti-inflammatory activity which is desirable for the management of chronic inflammatory diseases.[30,31]

Expression of iNOS can result in excess generation of NO from L-arginine. Beyond physiologic concentrations, NO exerts detrimental effects by causing cell injury through generation of reactive free radicals like peroxynitrite and acts as mediator of inflammatory diseases, even though it could be a beneficial antimicrobial at normal physiologic concentrations.[32] The study showed that *HEML* suppressed LPS-induced production of iNOS and its product NO *in vitro*.

Inducible enzyme, COX-2, is known to catalyze production of prostaglandins (PGs) from arachidonic acid.[4] Inflammatory cells such as macrophages, endothelial cells and fibroblasts tend to express COX-2 when activated. However, over-expression of COX-2 has been associated with inflammation and cancer[33] due to increased PGE_2 production. According to a previous report[34], there is a crosstalk existing between NO and PGE_2 release as far as modulation of inflammation is concerned. Catalytic activity of COX-2 is greatly affected by NO production.[34] However, the mechanism is not fully understood. One probable way is the consequent increase in half-life of COX-2 by NO through the generation of free-radicals (e.g. peroxynitrite), which inhibits auto-inactivation of the COX-2 enzyme. Another possible way is that peroxynitrite formed from a reaction of NO with superoxide anion, initiates lipid peroxidation and liberates from cell membrane arachidonic acid.[35]

The study indicates that, *HEML* could inhibit production of PGE_2 without necessarily inhibiting COX-2 protein expression. This could be attributed to possible inhibition of NO, such that, arachidonic acid levels contributed by NO is curtailed to limit substrate availability to COXs. Moreover, *HEML* could have interfered with catalytic activity of COX-2 by binding irreversibly to the active site, thereby making COX-2 inactive to bind arachidonic acid.

As part of the immuno-regulatory process, inflammatory response proceeds with increasing levels of circulating cytokines, where pro-inflammatory cytokines predominate initially, but their levels are later regulated by increasing anti-inflammatory cytokines.[36] TNF-α and IL-1β are some potent pro-inflammatory cytokines among others.[37] TNF-α causes release of IL-1β and cytokine-activated neutrophil chemo-attractant 1. The process may cause stimulation of COX-2 leading to PGs synthesis. TNF-α activates iNOS, hence, induces synthesis of NO and increases inflammation by increasing neutrophil response to a stimulus.[5] On the contrary, IL-10 is a potent anti-inflammatory cytokine that checks expression of TNF-α, IL-6 and IL-1 by activated macrophages. IL-10 can also boost expression of endogenous anti-cytokines to down-regulate pro-inflammatory cytokine receptors.[36] Thus, it can repress generation and function of pro-inflammatory cytokines at multiple levels. Our study showed that, *HEML* markedly reduced levels of IL-1β and TNF-α whilst boosting IL-10 levels in LPS-activated RAW 264.7 macrophages.

Regulation of inflammatory mediators such as NO, PGs and pro-inflammatory cytokines is mainly through NF-κB pathway.[37] This indicates that *HEML* may be reducing inflammation by regulating NF-κB pathway such that, iNOS expression is strongly affected and this reduces NO generation. Reduced NO levels will mean lower probability of co-activation of COX enzymes. However, further assays are needed to establish this.

An important limitation of the study is its design to assess local paw inflammation and edema over a very short time period of 4 h. Therefore, there was no *in vivo* assessment of the effect of *HEML* and positive control stimulants on systemic levels of any of the inflammatory mediators in rats. Nevertheless, specific effects of *HEML* on these mediators, which include PG, NO and cytokine levels as well as iNOS and COX-2 expression levels, were assessed using murine macrophage cells, RAW 264.7, as complementary data to observations in the rat experiments.

5. Conclusion

Our study has shown that *HEML* has anti-inflammatory effect in carrageenan-induced paw edema of the rats, which is quite comparable to that of the standard drug, diclofenac. The anti-inflammatory activity observed may be due to the presence of flavonoids. The mechanism of action may partly be via minimizing levels of pro-inflammatory mediators such as serotonin and histamine, and reduction of cytokines (TNF-α and IL-1β), PGE_2 and free radical, NO. It is therefore recommended that the active principle in *HEML* be isolated for further studies.

Authors' contributions

LKO, RAO and AAA conceived the study and participated in its design and coordination. FA carried out the experimental studies and data analysis with the assistance of EOA, SO, KAK, MO, GD, NLL and MAB. FA drafted of the manuscript. All authors reviewed the manuscript.

Acknowledgements

The authors are grateful to staff of CPMR and Departments of Clinical Pathology and Parasitology, Noguchi Memorial Institute for Medical Research for their technical support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtcme.2020.07.001.

Funding

This research was supported by West African Centre for Cell Biology of Infectious Pathogens (WACCBIP) and Noguchi Memorial

Institute for Medical Research, College of Health Sciences, University of Ghana; Science and Technology Research Partnership for sustainable Development (SATREPS).

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