Anti-inflammatory effect of alcoholic extract of *Entada pursaetha* DC in LPS-induced inflammation in mice and RAW264.7 cells

Rashmi Rekha Kumari¹, Madhu Cholenahalli Lingaraju¹, Gaurav Gupta¹, Amar Sunil More¹, Venkanna Balaganur¹, Dhirendra Kumar¹, Pankaj Kumar², Dinesh Kumar¹, Anil Kuma Sharma³, Santosh Kumar Mishra¹ & Surendra Kumar Tandan¹*

¹Division of Pharmacology and Toxicology; ²Division of Medicine; ³Division of Pathology, Indian Veterinary Research Institute, Izatnagar, Bareilly- 243 122, Uttar Pradesh, India

Received 16 June 2016; revised 24 August 2016

*Entada pursaetha* (Fabaceae) is an active, oriental herbal medicine used to treat various inflammatory diseases. Limited information on the use of stem in ethno medicine in painful conditions of joints prompted us to verify its usage for *in vitro* and *in vivo* anti-inflammatory action. The present study was designed to evaluate the anti-inflammatory effects of ethanolic extract of *E. pursaetha* (EPE) on the production of inflammatory mediators in macrophages and LPS (lipopolysaccharide)-induced systemic inflammation. EPE was studied for its anti-inflammatory activity in on the production of inflammatory mediators in systemic inflammation triggered by LPS and LPS-stimulated RAW 264.7 cells. Plasma tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β) proteins and total nitrate and nitrite levels were significantly increased in vehicle-treated control group of mice challenged with LPS (4 mg/kg b.wt.). Pre treatment of EPE at 300 mg/kg b.wt. Continuously for 3-days prior to LPS challenge significantly reduced these cytokine levels and nitrate plus nitrite levels in plasma. EPE treatment also significantly inhibited the LPS-induced production of NO (nitric oxide), IL-1β and TNF-α in RAW 264.7 cell. This study demonstrates the anti-inflammatory effects of EPE in both *in vivo* and *in vitro* model of inflammation potentially justifying the usage of *Entada pursaetha* plant as traditional herbal medicine for the treatment of inflammatory diseases.

**Keywords:** Anti-inflammatory activity, Cytokines, *Entada pursaetha*, Lipopolysaccharide, RAW 264.7 cells

Inflammation, a central feature of many pathophysiological conditions, occurs in response to tissue injury and is part of the host defense against invading microbes. Macrophages are major immune cells that act as the first line of defense against invading agents, and they respond to pathogen attack by release of ROS (reactive oxygen species) and NO, known as cellular signaling molecules and antimicrobial agents¹. However, an exaggerated production of inflammatory mediators by macrophage defensive systems can cause damage to the host². Macrophages are, for example, important contributors to the manifestation of allergic inflammation³ and also involved in TNF-α⁴ and IL-1β production in LPS-stimulated macrophages⁵. During infection LPS acts on cellular toll like receptor 4 (TLR4), activates different pathways in various cells (macrophages, Kupffer cells and hepatocytes)⁶ and induces nitric oxide synthase (iNOS) as well as the production of pro-inflammatory agents, including cytokines (IL-1β, TNF-α, and IL-6), prostaglandins and NO⁷. On the other hand, activated macrophages also produce anti-inflammatory cytokines such as IL-10 and IL-13, which are used to slow down and terminate the inflammatory response⁸. Many pathways have been described to explain the effect of anti-inflammatory compounds on macrophages. A number of studies reported that plant-derived extracts or plant derivatives such as phenolic compounds and flavonoids show anti-inflammatory activity by controlling the levels of various inflammatory cytokines or inflammatory mediators including IL-1, IL-6, IL-10, TNF-α, NF-κB, NO, iNOS and COX-2. Moreover, many crude extracts and chemical constituents of plants have pharmacologic effects and clinical benefits. However, the claims of benefits of many plants or plant based medicines marketed to the general population are only supported by empirical or preliminary scientific data⁹.
Entada pursaetha, (Synonyms: E. rheedi, E. phaseoloides, E. scandens) a very large gigantic woody climbing shrub (liana), is widely distributed in tropical Africa, India, China, Philippines, Guam and Northern Australia\textsuperscript{10}. In India, it is found in the damp forest of Bengal, Bihar, and Orissa, in the forest region of eastern and Western Ghats and hilly forest tract of the northern district of Bengal and Deccan\textsuperscript{11}. This species can be used as narcotic or as a tonic, etc. or used in curing liver troubles, allaying body pains, in warding off cold, curing eye diseases, arthritis, and paralysis\textsuperscript{12}. Mainly seeds, leaves, and stem or stem bark of E. pursaetha are used for different medicinal purposes. Seed- hepatic complaints, paste applied over affected or inflamed swelling to reduce pain; leaf-anthelmintic, antiemetic, jaundice, antiseptic, infantile cold; stem-antiemetic, in diarrhea and skin diseases by various tribal communities of India in ethno botanical surveys\textsuperscript{13-15}. Recently, the anti-inflammatory effects of Entada pursaetha and its synonym E. phasioloides seed extract in vitro\textsuperscript{16,17} and in vivo have been reported\textsuperscript{18}.

In fact, our recently conducted study demonstrated beneficial effects of Entada pursaetha stem extract in experimental knee model of OA\textsuperscript{19} and inflammatory bowel disease\textsuperscript{20}. However, the effects of the ethanolic extract of E. pursaetha (EPE) of stem on inflammatory disease are yet to be established. In this study we investigated the effects of EPE on the production of inflammatory mediators by LPS-stimulated RAW 264.7 cells. In this study, to verify our hypothesis that EPE could prevent systemic inflammation triggered by LPS in mice, we demonstrated the effect of Entada pursaetha stem on the release of inflammatory mediators both in vitro and in vivo.

Materials and Methods

Animals

Male albino mice (Livestock Resource Section, Indian Veterinary Research Institute) weighing 20-25 g were used. They were housed at a maximum of four per cage on a 12 h day/night cycle at a temperature of $22 \pm 1^\circ C$. Water and food were provided ad libitum. All animal experimental procedures were duly approved (IAEC/2009/011) by Animals Ethics Committee of Indian Veterinary Research Institute.

Reagents and materials

Preparation of alcoholic extract of E. pursaetha

The mature stem of E. pursaetha was brought from the jungles of Bhawanipatna, District- Kalahandi, Orissa, India and authenticated by Dr. BN Pandey, Department of Botany, Bareilly College, Bareilly, India. A voucher specimen (023/09) was deposited in the Indigenous Drug Laboratory of Division of Pharmacology & Toxicology. The powdered stem of was refluxed twice with 85% ethanol at 95°C for 8 h. Ethanol was removed under vacuum, and the solid extract of E. pursaetha stem was hereafter referred as “EPE”. The yield of the extract was 8.4% with reference to the dry starting material. EPE was used by suspending in a maximum of 0.2% Polysorbate 80. Doses of EPE 30, 100 and 300 mg/kg body weight were used, based on the earlier work carried out in the laboratory (Unpublished data).

In our earlier study, various phytoconstituents were determined in EPE, and the concentration of saponin (glucoside saponin) was highest followed closely by total phenols; total concentration of tannins and flavonoids was almost similar\textsuperscript{19}

In vitro anti-inflammatory activity of EPE in LPS-induced macrophage cell line

Propagation and maintenance of cell line - Subculture

The murine macrophage cell line (RAW 264.7) was procured from NCCS, Pune. On the receipt of monolayer containing 25 cm$^2$ flask, cells were subcultured as soon as they reached confluency. The cells were subcultured using growth medium Dulbecco’s modified Eagle’s medium (DMEM) with 4mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose and supplemented with 10% (v/v) fetal calf serum and antibiotic (100 U/mL penicillin, 100 μg/mL of streptomycin). The growth condition was 37°C temperature and a humidified atmosphere containing 5% CO$_2$ condition. The subculture was done in 10% fetal calf serum containing DMEM medium with the help of cell scraper.

First old medium was removed. Secondly, fresh medium was added, thirdly, cells were dislodged using cell scraper and dispensed into a new flask. Flasks were then kept in an incubator with the slightly loose cap. The volume of medium used for subculture was according to the usual ratio of medium volume to surface area, i.e. 0.2-0.5 mL/cm$^2$. The cells were subcultured as soon as they reached confluency and maintained in 2% fetal calf serum containing medium according to our experimental need. Whenever needed, the number of flasks were increased by subculture and maintained in 2% DMEM, changed
3 times per week. Most cell stop growing as the pH falls from 7.0-6.5 and starts to lose viability between 6.5 and 6.0. So if medium goes from red through orange to yellow, the medium should be changed. If there is a rapid change in pH, the medium must be changed frequently, then subculture must be done and seeding density must be reduced.

**Preparation of stock solution of ‘EPE’**

EPE was suspended in DMSO at concentration of 100 mg/mL (0.1% DMSO final concentration). Different concentrations of EPE were prepared from this stock solution in 2% DMEM. Then filter sterilized. For NO estimation all concentration were prepared in phenol red free DMEM.

**Preparation of stock concentration of LPS**

A stock solution of LPS (*Escherichia coli* 055: B5, Sigma-Aldrich) @ 200 μg/mL in PBS, pH 7.4 was prepared and then filter sterilized. [The composition of PBS was Na₂HPO₄ (1.14 g) or Na₂HPO₄·2H₂O (1.44 g), KCl (0.2 g), KH₂PO₄ (0.2 g), NaCl (8 g) per liter of sterilized distilled water].

**MTT Assay for cell viability**

MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay for cell viability was done. MTT, a yellow color dye converted by living cell with active mitochondria into blue color formazan crystals. MTT assay was performed to determine non-cytotoxic concentrations of EPE to be employed for the further study. The different concentrations of EPE were prepared from a stock solution in 2% DMEM. These concentrations were 20, 50, 100, 200 and 400 μg/mL and vehicle control group was treated with 0.1% DMSO containing 2% DMEM. Cells (RAW264.7) were seeded in 10% DMEM at required concentration (2-5 × 10⁶/mL, 100 μL volume) in 96-well plates and incubated for 12 h. After preconditioning, media was decanted and 100 μL of 2% fresh media containing different concentrations of EPE was added to wells. Vehicle control cells were incubated with 0.1% DMSO. After 48 h of incubation, 20 μL of MTT dye (5 mg MTT/mL of PBS) was added in each well and incubated for 4 h in the dark at 37°C and 5% CO₂. Further media was removed by tilting the plate from a side, DMSO (100 μL) was added to each well, formazan crystals formed by viable cells. A violet blue colour develops in wells. Absorbance was taken at 550 nm in ELISA reader (Spectra Max190, Molecular devices). The mean OD value of the content of four well was used as cell viability expression as % of control. The assay was performed in triplicate.

Based on above exploratory work, three increasing non-cytotoxic concentration levels of EPE 20, 30 and 50 μg/mL were selected for assessing the effect of these concentrations of EPE on NO, IL-1β, TNF-α level in medium LPS-stimulated macrophage cell. For each assay, there were six groups I-VI where I was Naïve control, II was vehicle control and treated with LPS at 2 μg/mL (final concentration in well) only, whereas III, IV, V were treated with LPS (2 μg/mL) and 20, 30 and 50 μg/mL concentration of EPE respectively. Group VI treated with LPS and dexamethasone (0.5 μg/mL) and acted as standard/reference treatment group.

**Nitric oxide assay**

For nitric oxide assay, phenol red free DMEM medium was used for seeding step onward. Cells were seeded in 96 well plates at a concentration of 5 × 10⁴ in 10% phenol red free DMEM medium and incubated for 12 h. After removing medium, a cell of group II, III, IV were treated with phenol red free 2% DMEM containing 20, 30 and 50 μg/mL EPE while group VI was treated with dexamethasone at 0.5 μg/mL whereas group I was control. Now Cells of all groups except control were treated with 10 μL of a stock solution of LPS. After incubation for 24 h, supernatant (medium) was collected and stored at −80°C for estimation of nitric oxide. Nitric oxide concentration was determined by the method described by Sastry et al. The details of this methodology are furnished in the estimation of nitrite and nitrate in plasma. Results are expressed as mean ± SE of three replicates of one representative experiment.

**Cytokines: TNF-α and IL-1β assay**

Cells were seeded in 96-well plate at a concentration of 5 × 10⁴ in 10% DMEM medium and incubated for 12 h. After removing medium, cells of group II, III, IV were treated with 2% DMEM containing 20, 30 and 50 μg/mL EPE while cells of group VI were treated with dexamethasone at 0.5 μg/mL whereas group I was control. Macrophage Cells of all groups except control were treated with 10 μL of stock solution of LPS. After incubation for 24 h, cell supernatant will be harvested and stored at −80°C until tested for cytokines. ELISA estimated the quantities of these cytokines following manufacturer’s
instruction. Results were expressed as mean ± SE of three replicates of one representative experiment.

**Determination of anti-inflammatory activity of EPE using in vivo model of inflammation in mice**

*Lipopolysaccharide (LPS)-induced lethality study*

To test if EPE extract can increase survival time in a mouse model of sepsis, mice were injected with a single dose of vehicle or extract before LPS challenge (15 mg/kg IP, *E. coli* 055: B5). The dose of LPS was close to LD95 and dose of extract (30, 100 and 300 mg/kg) were based on earlier studies in the laboratory. Animals were observed for three days for mortality. The groups were as follows:

**Effect of EPE on pro-inflammatory cytokines TNF-α, IL-1β, and nitric oxide levels in LPS-induced systemic inflammation**

Systemic inflammation was induced by a single dose of LPS (4 mg/kg) by intra-peritoneal injection of saline. Mice were divided into five groups of six animals each. Mice in group I served as naive control and received normal saline. Mice in group II were administered vehicle orally while mice in group III, IV and V were administered EPE orally at 30, 100 and 300 mg/kg suspended in the vehicle for three days (Table 1). On the 3rd day after 3h of vehicle or EPE administration, animals from group II, III, IV and V were challenged with LPS (4 mg/kg, i.p.). Blood from mice of all groups was collected in heparinised tube by cardiac puncture, 90 min after LPS administration. Separation of plasma was done in a refrigerated centrifuge tube at 7000 rpm for 10 min and stored at −80°C until analyzed for parameters: TNF-α and IL-1β (EIA kit, E-Bioscience) and Nitrate-nitrite (NOx).

**Estimation of nitrate-nitrite (NOx)**

Nitrate and nitrite in the plasma were estimated following the procedure described Sastry et al.21. The principle of the assay is the conversion of nitrate to nitrite by copper-cadmium alloy and then color development by Griess reagent (Sulfanilamide and N-(1-Naphthyl) ethylenediamine) in acidic medium.

In this procedure, 100 μL of plasma or standard (potassium nitrate) was added to test tubes (12 × 75 mm) to which 400 μL of carbonate buffer was added, followed by small amount (~150 mg) of cadmium filings (washed in the same buffer and dried on a filter paper). The tubes were then incubated at room temperature for 1h with thorough shaking. The reaction was stopped by 400 μL of 120 mM zinc sulfate solution under vortex, and the solution was allowed to stand for 10 min. The tubes were then centrifuged at 400 g for 10 min.

Aliquots (150 μL) of the clear supernatant were transferred to the wells of a microplate (96 well), in triplicate. Then, components of the Griess reagent were added with gentle mixing each time, 75 μL of 1.0 % sulfanilamide and 75 μL of 1.0% N-(1-Naphthyl) ethylenediamine. After 10 min, the absorbance was measured at 545 nm against a blank containing no biological sample in a microplate reader (Spectra Max190, Molecular devices) using the path check option. By using standard curve the amount of nitrate and nitrite present in the sample was calculated.

**Histopathology**

After collecting blood as above, animals were sacrificed by cervical dislocation, liver and lung were removed and fixed in 10% buffered formalin for histopathological studies (H&E Staining).

**Statistical analysis**

Data were expressed as mean ± SE. Statistical analysis of data was performed using GraphPad prism 4. Data were analyzed by ANOVA and, means were compared with Tukey’s multiple comparison tests. A minimum value of $P <0.05$ was considered statistically significant.

**Results**

*In vitro anti-inflammatory activity of EPE in LPS-induced macrophage cell line*

The objective of study was to measure effect of EPE on pro-inflammatory mediators such as TNF-α, IL-1β proteins and NO production in LPS-stimulated macrophage cell line RAW264.7 cells.

**MTT assay**

In the first place, an MTT assay for cell viability was performed to determine three non-cytotoxic concentrations of EPE. The result of MTT cell viability had been depicted in (Table 2). MTT is a yellow dye which is converted into blue formazan.

<table>
<thead>
<tr>
<th>Group (n=6)</th>
<th>Treatment</th>
<th>EPE (orally 3h before LPS)</th>
<th>LPS (LD95 IP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Control</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>II</td>
<td>Vehicle + LPS</td>
<td>–</td>
<td>15 mg/kg b. wt</td>
</tr>
<tr>
<td>III</td>
<td>EPE + LPS</td>
<td>30 mg/kg b. wt</td>
<td>15 mg/kg b. wt</td>
</tr>
<tr>
<td>IV</td>
<td>EPE + LPS</td>
<td>100 mg/kg b. wt</td>
<td>15 mg/kg b. wt</td>
</tr>
<tr>
<td>V</td>
<td>EPE + LPS</td>
<td>300 mg/kg b. wt</td>
<td>15 mg/kg b. wt</td>
</tr>
</tbody>
</table>
crystals by live cells. Result of cell survival was expressed in percent of control cells with no EPE treatment. The cell viability was 107.4 and 91.95% with 20 and 50 μg/mL concentrations of EPE treatment which were statistically nonsignificant when compared with control cells with no EPE treatment. Cell viability at 100 μg/mL EPE concentrations was 60.94% of control which was significantly less ($P < 0.05$) than control with no EPE. Further with 200 μg/mL and 400 μg/mL EPE concentrations, cell viability was 8.67 and 11.59%, respectively. EPE has cytotoxic effect at 100, 200 and 400 μg/mL and non-cytotoxic at 20 and 50 μg/mL concentrations. Therefore, 20, 30 and 50 μg/mL EPE concentrations were used for further study for cytokine assay and NO levels.

**Effect of EPE on pro-inflammatory cytokines of nitric oxide**

The result of TNF-α, IL-1β proteins and NO levels in culture medium at different concentrations of EPE had been presented in Table 3. TNF-α: TNF-α levels in culture medium was significantly increased ($P < 0.05$) in LPS stimulated cells as compared to unstimulated cells (Table 3). EPE at concentration of 30 and 50 μg/mL significantly reduced ($P < 0.05$) the TNF-α level in culture medium as compared LPS- stimulated cells. EPE at a concentration of 20 μg/mL did not significantly reduce TNF-α level in LPS- stimulated RAW264.7 cells.

IL-1β: As shown in Table 3, after LPS stimulation, with the extract intervention for 24h, secretion of IL-1β was significantly decreased ($P < 0.05$) than cells stimulated with LPS only but not brought to control value of unstimulated cells. NO: The concentration of NO was indirectly measured by measuring total nitrate-nitrite concentration in culture medium. Nitrate-Nitrite level in medium was significantly higher ($P < 0.05$) when cells were stimulated with LPS as compared to unstimulated cells (Table 3). This increase in NO level was reduced in concentration-dependent manner in EPE-treated cells but not brought to unstimulated cells level. Dexamethasone treatment also did not bring the value to unstimulated cells level.

**Determination of anti-inflammatory activity of EPE using in vivo model of inflammation in mice**

**Lipopolysaccharide (LPS) lethality study**

There was no significant reduction in mortality in EPE-treated groups as compared with vehicle-treated groups in LPS-induced sepsis in mice (results not presented).

**Table 2 — MTT assay for cell viability. Values are expressed as means ± SE.**

<table>
<thead>
<tr>
<th>EPE concentration (μg/mL)</th>
<th>0</th>
<th>20</th>
<th>50</th>
<th>100</th>
<th>200</th>
<th>400</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell survival (% of control)</td>
<td>100.0 ± 0.00\textsuperscript{a}</td>
<td>107.4 ± 2.765\textsuperscript{a}</td>
<td>91.95±3.48\textsuperscript{a}</td>
<td>60.94 ± 16.4\textsuperscript{b}</td>
<td>8.763 ± 1.56\textsuperscript{c}</td>
<td>11.59 ± 1.382\textsuperscript{c}</td>
</tr>
</tbody>
</table>

Result shown is atypical result of three independent experiments with similar results. Cell survival is expressed as % of control. Value with dissimilar superscript differs significantly.

**Table 3 — Effects of EPE on LPS-induced production of pro-inflammatory cytokine and nitric oxide in RAW 264.7 cells. Values are expressed as mean ± SE.**

<table>
<thead>
<tr>
<th>Treatments</th>
<th>TNF-α (pg/mL)</th>
<th>IL-1β (pg/mL)</th>
<th>Total nitrate &amp; nitrite (nM/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.63±1.131\textsuperscript{a}</td>
<td>8.630±1.662\textsuperscript{a}</td>
<td>22.03±2.275\textsuperscript{a}</td>
</tr>
<tr>
<td>LPS (2 µg/mL)</td>
<td>104.8±21.54\textsuperscript{b}</td>
<td>975.7±40.86\textsuperscript{b}</td>
<td>106.3±1.289\textsuperscript{b}</td>
</tr>
<tr>
<td>LPS+EPE (20 µg/mL)</td>
<td>97.83±11.09\textsuperscript{b}</td>
<td>449.3±24.77\textsuperscript{c}</td>
<td>99.10±1.249\textsuperscript{c}</td>
</tr>
<tr>
<td>LPS+EPE (30 µg/mL)</td>
<td>50.61±11.08\textsuperscript{a}</td>
<td>77.84±5.918\textsuperscript{a}</td>
<td>89.11±0.5888\textsuperscript{d}</td>
</tr>
<tr>
<td>LPS+EPE (50 µg/mL)</td>
<td>26.51±2.663\textsuperscript{a}</td>
<td>61.95±4.946\textsuperscript{a}</td>
<td>79.02±0.8329\textsuperscript{e}</td>
</tr>
<tr>
<td>LPS+DEXA (0.5 µg/mL)</td>
<td>9.65±1.410\textsuperscript{a}</td>
<td>31.05±3.800\textsuperscript{a}</td>
<td>49.48±0.3543\textsuperscript{f}</td>
</tr>
</tbody>
</table>

Three independent experiments were performed. Value with dissimilar superscript differs significantly.
Effect of EPE on Pro-inflammatory cytokines IL-1β, TNF-α and nitric oxide levels in systemic inflammation

Results of plasma levels of cytokines IL-1β, TNF-α proteins and nitric oxide are summarized in Table 4 following systemic inflammation induced by a single dose of LPS (4 mg/kg). Plasma TNF-α and IL-1β levels were significantly increased ($P < 0.05$) in vehicle-treated control group of mice challenged with LPS. Pretreatment of EPE at 300 mg/kg body wt. continuously for 3 days prior to LPS challenge significantly reduced ($P < 0.05$) these cytokine levels in plasma.

Results also showed that LPS challenge to mice caused significant increase in nitrate and nitrite plasma levels as compared to naive control group of mice and this increase was significantly ($P < 0.05$) attenuated by pretreatment of EPE at the rate of 300 mg/kg body wt.

Histopathology

In the naive control group, light microscopy showed no infiltration of polymorph nuclear cells (PMNs) in the liver (Fig. 1A) or lungs (Fig. 2A). In contrast, increased infiltration of PMNs in both liver (Fig. 1B) and lungs (Fig. 2B) was noted at 90 min after injection of LPS. In mice pretreated with EPE, infiltration of PMNs in liver (Fig. 1C) and lungs (Fig. 2C) was reduced.

Discussion

Plant products have been historically important in the prevention and treatment of illness. Plants are a
rich source of active ingredients for health care products with many blockbuster drugs being directly or indirectly derived from plants. The pharmacological activities of many high-value plant-derived natural products remain undiscovered or unexplored. We used ethanolic extract of *E. pursaetha* (EPE) for exploring its anti-inflammatory activity. *E. pursaetha* is an active, oriental herbal medicine used to treat various inflammatory diseases. In spite of its famous legacy, the pharmacological effects have not been fully explored. The present study was undertaken to elucidate the pharmacological and biological effects of EPE on the production of inflammatory mediators in macrophages and LPS-induced systemic inflammation.

We showed that EPE suppressed the production of TNF-α, IL-1β proteins and NO in LPS-stimulated RAW264.7 cells which are primary peritoneal macrophages. Macrophages actively participate in inflammatory responses by releasing pro-inflammatory cytokines (TNF-α and IL-1β) and inflammatory factors (NO and PGE2) that recruit additional immune cells to sites of infection or tissue injury. The production of these inflammatory molecules by macrophages can be induced in response to LPS stimulation. Therefore, inhibitors of these inflammatory molecules have been considered as a candidate for an anti-inflammatory drug to alleviate a variety of disorders caused by activation of macrophages.

RAW 264.7 macrophages provide us with an excellent model for anti-inflammatory drug screening and for subsequently evaluating the inhibitors of the pathways that lead to the induction of pro-inflammatory enzymes and the production of pro-inflammatory cytokines. EPE has been reported to possess anti-inflammatory activity in carrageenan-induced inflammation in mice. However, no report has been available on the mode of action involved. In this study, we show that EPE acts as an anti-inflammatory substance *in vitro*. NO is a free radical that plays a pivotal role in cell survival and death and plays various pro-inflammatory effects on many cell types. High levels of NO generated by iNOS in inflamed tissue have been shown to be cytotoxic in studies of many types of inflammatory diseases including asthma, arthritis, and cardiovascular diseases leading to cell death. LPS stimulation significantly increased the concentrations of NO. Therefore, NO inhibition in inflammation has the potential therapeutic implications. We found that EPE effectively decreased NO production possibly via suppressing the mRNA and protein expressions of iNOS in LPS-stimulated RAW264.7 cells. Moreover, the inhibitory effects on the LPS-induced expressions were not due to the cytotoxicity of EPE as assessed by MTT assay.

TNF-α and IL-1β proteins are major pro-inflammatory cytokines in various immune cells such as macrophages, monocytes and T cells and have various pro-inflammatory effects in chronic inflammatory diseases. In this study, EPE effectively inhibited the production of these two cytokines by possibly suppressing their mRNA expressions in LPS-stimulated RAW 264.7 cells. The results of this study demonstrate that EPE inhibited the generation of pro-inflammatory cytokines in a concentration-dependent manner that is paramount in the generation of an inflammatory response in activated macrophages. In our experiments, LPS was used as the prototypical inflammatory stimulus because of its ability to initiate a range of pro-inflammatory mediators. LPS, as well as TNF-α and interferon gamma (IFN-γ), are effective activator of NF-κB. NF-κB is known to play a critical role in regulating cell survival genes and controlling most inflammatory responses by induction of inducible enzymes (iNOS and COX-2), pro-inflammatory cytokines (IL-1, IL-2, IL-6 and TNF-α), chemokines (IL-8, MIP-1β, MCP-1 and RANTES), adhesion molecules (ICAM, VCAM and E-selectin), growth factors, some of the acute phase proteins, and immune receptors. Therefore, blocking the NF-κB transcriptional activity in the macrophage nucleus can suppress the expression of inflammatory mediators. EPE might have reduced the pro-inflammatory cytokines and NO by blocking NF-κB.

Severe sepsis may not be reversible and result in high mortality. In this study, we established a mild systemic inflammation model in which mice were challenged i.p. by a single dose of LPS (4 mg/kg b. wt.). According to our preliminary data, the LPS-challenged mice were sick from LPS challenge, but none died during one week of observation period. A sub-lethal endotoxin shock murine model has been established. The sub-septic doses of endotoxin LPS can activate distinct immune responses such as systemic inflammation and have been used in previous studies for different purposes. Using our established sub-septic (sublethal) animal model, the effects of EPE pretreatment *in vivo* on acute systemic inflammation...
inflammation were measured. Our results showed that LPS challenge (vehicle control) significantly increased plasma cytokine levels of IL-1β or TNF-α suggesting that systemic inflammation was induced. Pretreatment of EPE (300 mg/kg) 3h before systemic inflammation by LPS significantly decreased plasma IL-1β and TNF-α level in mice (Table 4). IL-1β and TNF-α play a critical role in the manifestation of systemic inflammation and are believed to be a pro-inflammatory and EPE pretreatment before acute systemic inflammation alleviates acute inflammation status via inhibiting plasma IL-1β and TNF-α production. Macrophages are large mononuclear phagocytic cells involving innate and adaptive immunity in vivo. These are migratory cells and are found in most tissues of the body. The releases of IL-1β and TNF-α by macrophages may induce local protective effects but they can have damaging effects, when released systemically.

TNF-α is the target for development of new medication for disease involving inflammation such as rheumatoid arthritis. Inhibition of elevated TNF-α induced by LPS has been a common practice for evaluation of anti-inflammatory effect of drug candidates. Out results showed lower production of TNF-α not only in LPS-stimulated RAW 264.7 cells pretreated by EPE as stated above but also in plasma from LPS-challenged mice pretreated with EPE (300 mg/kg) suggests TNF-α as a target in anti-inflammatory strategy.

Recently, LPS-injected animal models have been introduced to evaluate the effects of medicine, food, or nutrition on systemic inflammation. It has been proposed that NO production in response to LPS regulates important aspects of septic shock. Therefore, to investigate whether EPE has a protective effect on LPS-induced systemic inflammation, the plasma nitrite-nitrate levels in LPS-treated mice was examined in this study. It was found that pretreatment of mice with EPE (300 mg/kg, orally) inhibited plasma nitrite-nitrate production in the LPS challenged mice (Table 4). This finding further supports the hypothesis that EPE has a potent anti-inflammatory activity in vivo.

A large amount of inflammatory mediators produced in the body is thought to contribute to the LPS-induced symptoms of septic shock and mortality. Amongst the inflammatory mediators, NO is known to be closely associated with hypotension and hypo responsiveness to vasoconstrictors in endotoxin-induced sepsis. Production of NO by iNOS is beneficial in fighting bacteria, but its overproduction can be harmful as shown in endotoxic shock. There is increasing evidence that overproduction of TNF-α during infection also leads to severe systemic toxicity and even death. Evidence supporting this hypothesis comes from reports indicating that mediators (e.g. TNF-α and interleukins) produced by endotoxin challenge can induce iNOS expression and produce large amounts of NO. This point was further supported by the results in which EPE reduced plasma nitrate-nitrite concentration possibly by reducing iNOS protein expression in tissues challenged by LPS.

In systemic inflammation, histopathology section of liver and lung (Fig. 1 & 2) also showed inflammatory changes such as blood vessel dilatation and infiltration of neutrophils in liver and lung parenchyma in LPS challenged animals. But the EPE (300 mg/kg) pretreatment at this dose resulted in less infiltration of leucocytes in liver and lung (Figs. 1 & 2). Further, reduced cytokines and NO plasma levels at this dose in mice were associated with decreased leukocyte infiltration in lung and liver.

Phenolic compounds were detected in high amounts in *Entada africana*. Qualitative analysis was done with *Entada africana* crude extract also revealed the presence of polyphenols in this extract. The biological effects of polyphenols have been reported to be related to their ability to influence cell signaling pathways and to modulate pro and anti-inflammatory gene expression. Therefore, we suggest that the phenolic compounds present in *Entada pursaetha* may be responsible for the observed effects on RAW264.7 cells and in vivo inflammatory cytokines and NO in mice. Overall, this study demonstrates the anti-inflammatory effects of EPE in both in vivo and in vitro model of inflammation suggesting that *Entada pursaetha* might have substantial therapeutic potential for treatment of inflammation and inflammatory diseases.

References


