**In vivo and in vitro anti-inflammatory activity of Harrisonia perforata root extract**

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**Introduction:** *Harrisonia perforata* root is one of the five medicinal plants in Bencha-Loga-Wichien remedy used for the treatment of fever in Thai traditional medicine. This study was aimed to investigate the anti-inflammatory effect and the action mechanism of the ethanolic extract from the root of *H. perforata* in vivo and in vitro.

**Methods:** Male Wistar rats were divided into 6 groups and treated with either the ethanolic extract of *H. perforata* at dose 5–400 mg/kg or indomethacin 5 mg/kg or vehicle. Rat paw volume was measured at 1, 2, 3, 4, 5 and 6 h after carrageenan injection. The in vitro mechanism of the anti-inflammatory response was investigated through the measurement of mRNA expression of proinflammatory cytokines, TNF-α, IL-6 and IL-1β in the macrophage cell.

Lipopolysaccharide-stimulated macrophage, J774A.1 cell, were exposed to different concentration of *Harrisonia perforata* root extract (12.5–50 µg/ml). mRNA expression, TNF-α, IL-1β and IL-6, were determined by real-time RT-PCR.

**Results:** *H. perforata* root extract 50–400 mg/kg significantly reduced the effect of acute inflammation in rat paw edema by 28.49%–65.05% at 2 hour after carrageenan injection. Cell viability were not significantly altered by the presence of *H. perforata* extract 3.125–50 µg/ml. It was found that the maximum inhibitory effect for TNF-α, IL-1β and IL-6 were 49.83, 47.27 and 32.16% respectively. The concentration of the extract produced maximum effect were 50 µg/ml for both TNF-α and IL-1β but for IL-6, it was 12.5 µg/ml. **Conclusion:** The ethanolic extract from *H. perforata* root possesses anti-inflammatory effect. This may occur via suppression of proinflammatory cytokines.

**Keyword:** *Harrisonia perforata*, antiinflammation, proinflammatory cytokines.

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**INTRODUCTION**

Inflammation is a process of immune system in response to foreign invader or cells and tissue damage. Inflammation involves the local vascular system, the immune system, inflammatory cells, mediators and cytokines within the injured tissue. It is well established that macrophages play important roles in production of various cytokines, reactive oxygen and nitrogen species, growth factors and chemokines in response to inflammatory trigger substances such as bacteria lipopolysaccharide, chemical mediators.[1] Although modern anti-inflammatory drugs such as steroids and non-steroidal antiinflammatory drugs are effective in the treatment of inflammatory condition, their use are limited because of the side effect associated with the drug treatment. All non-steroidal anti-inflammatory drugs (NSAIDs) carry a risk of upper gastrointestinal complications.[2] There is a need in searching the new potential bioactive compounds which are as effective as the existing product with the lowest side effect for the treatment of the inflammatory diseases.

*Harrisonia perforata* (Blanco) Merr. is belonged to Family Simaroubaceae and known in Thailand as Khon Tha. Apart from Thailand, it is found in Myanmar, Philippines, Malaysia, Laos and Indonesia.[3] This plant is used in Vietnam as a folk medicine for the treatment of itching. In Indonesia and Philippines, the root bark is a remedy for diarrhoea, dysentery and cholera.[4] *H. perforata* root is one of the five medicinal plants in Bencha-Loga-Wichien remedy used for the treatment of fever in Thai traditional medicine. Pharmacology investigation revealed that *H. perforata* (roots and stem) exhibited a bactericidal effect against *Mycobacterium smegmatis*.[5] Moreover, the extracts of the leaves and the branches demonstrated *in vitro* antimalarial activity against *Plasmodium falciparum*.[6]
The powder root also showed antipyretic effect.[7] From our previous study, it was found that the ethanolic root extract of \textit{H. perforata} possessed direct inhibitory effect on macrophage activation.[8] Several chromones, peucenin-7-methyl ether, O-methylalloptaeroxylin (perforatin \textit{A}), perforatin B, perforatic acid, perforatic acid methyl ester and perforatin C-G and perforatinolone, have been isolated from the roots[9,10] leaves[11,12] branches[13] and wood[14]. In acute toxicity study, rats fed with the water extract of \textit{H. perforata} at the dose of 5,000 mg/kg did not show any sign of toxicity over 14-day period of observation. Sub-chronic toxicity study found that the extract at doses up to 1,200 mg/kg body weight caused no significant toxicity to male and female rats over 90 days after the extract feeding including the body and organ weights, hematological and blood clinical chemistry.[15] Although a number of pharmacological activities of this plant have been documented, the supporting evidence for its anti-inflammatory activity is still limited. Therefore this present study is aimed to elucidate the \textit{in vivo} and \textit{in vitro} anti-inflammatory activity of the ethanolic root extract from \textit{H. perforata}.

**MATERIALS AND METHODS**

**Plant Material and Preparation of Plant Extract**

The ethanolic root extract of \textit{H. perforata} was prepared by Ruangrungsi N, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, the plant was collected from Nongkhai province of Thailand and authenticated. The voucher and number of specimens were deposited at the College of Public Health Sciences, Chulalongkorn University, Thailand. The roots of \textit{H. perforata} were dried under shade and grinded to coarse powders. The powder of roots was macerated in absolute ethanol in closed conical flask for 24 hours. Then the extracts were evaporated to dryness under vacuum and identified by TLC and stored in a solid stage at –4°C until used.

**Preparation of the Extract Solution for In Vitro Study**

The ethanolic root extract of \textit{H. perforata} was dissolved in 100% DMSO and stored at –20°C until used. The stock solution was diluted with sterile double distilled to the constant final concentration of DMSO at 0.2%.

**Preparation of the Extract Solution for In Vivo Study**

The ethanolic roots extract of \textit{H. perforata} was dissolved in 5% Tween 80 solution.

**Cells**

\textit{J774A.1} cells are murine macrophages obtained from ATCC. The cells were maintained in the completed Dulbecco’s Modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C, 97% humidity, 5% \textit{CO}_2. They were subcultured 3 timed weekly during used.

**Experimental Animals**

Male Wistar rats weighing 100–150 g were obtained from National Laboratory Animal Center, Mahidol University, Salaya, Nakhorn Pathom, Thailand. The animals were housed in animal care facility at the Faculty of Medicine, Chulalongkorn University, for at least 7 days before the experiment was performed. They were taken care with standard diet and water in a room under controlled environment; room temperature 25±2°C with 12-h light/dark cycle. All animal handling and experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and with the approval of the Institutional Ethic Committee, Faculty of Medicine, Chulalongkorn University.

**Chemicals and Reagents**

The chemicals and reagents used in this study were in the followings; Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco, USA), penicillin/streptomycin (Hyclone, USA), fetal bovine serum (Gibco, USA), sodium bicarbonate (Baker, USA), dimethyl sulfoxide (DMSO) (Sigma, USA), trypsin blue dye (Sigma, USA), lipopolysaccharide (LPS) (Sigma, USA), nitric oxide assay kit (Promega, USA), reza-surin (Sigma, USA), TRizol reagent (Invitrogen, UK), chloroform (Sigma, USA), isopropanol (Bio Basic, Canada), DEPC (Molekula, UK), ImProm-II® Reverse Transcription system (Promega, USA), agarose gel (Vivantis, Malaysia), primers (Bio Basic, Canada), taq polymerase (Invitrogen, UK), tween 80 (Labchem, New Zealand), indomethacin (Parma, Italy), carrageenan (Sigma, USA), dexamethasone (T.P. drug, Thai).

**Carrageenan-induced Paw Edema Test in Rats**

The carrageenan-induced paw edema model was used to evaluate anti-inflammatory activity of the extract as the method described by Winter et al.[16] Rats were weighted and marked with black ink at a ground of lateral malleolus for measuring of the paw volume using plethysmometer. The animals were randomly divided into six groups with 6 animals per group. They were intraperitoneally injected with the extract (50, 100, 200, 400 mg/kg), indomethacin
5 mg/kg (positive control group) or 2% tween 80 (negative control group) one hour before carrageenan injection into sub-plantar side of the right hind paw. Paw volume were measured before and at 1, 2, 3, 4, 5 and 6 h after carrageenan injection following the previous studies.[17,18]

The inhibitory activity of *H. perforata* Ethanolic root extract was determined as the percentage of paw edema inhibition in the following equation.

\[
\% \text{ inhibition} = \left( \frac{(V_t - V_o)_{\text{control}} - (V_t - V_o)_{\text{treated}}}{(V_t - V_o)_{\text{control}}} \right) \times 100
\]

Where \( V_o \): basal volume of paw before carrageenan injection.
\( V_t \): volume of edema paw after carrageenan injection at each time point.

**Cell Viability**

The viability of the cells was assessed in order to evaluate whether the extract possessed cytotoxic effect to J774A.1 murine macrophages cells using the resazurin assay method described by Anoopkumar-Dukie S with some modification.[19] This assay is based on the principle that the enzyme in viable cells can reduce blue color agent resazurin to red color resorufin. The cells were treated with the ethanolic root extract of *H. perforata* at the concentrations 3.125–50 µg/ml. DMSO and 10 µM dexamethasone were used as the negative and positive controls respectively. The cells were stimulated with 100 ng/ml LPS for 4 h in order to determine cytokines expression. Total RNA from the cells was isolated by adding 1 ml Trizol reagent to lyse the cells. Then they were homogenized and incubated at room temperature for 15 minutes. Chloroform 0.2 ml was added and centrifuged at 12,000 × g for 15 minutes at 4°C. The aqueous phase was collected and then isopropanol 0.5 ml was added to precipitate total RNA. The mixture was standed for 10 minutes at room temperature and centrifuged at 12,000 × g for 10 minutes at 4°C to obtain total RNA precipitate. RNA pellet was washed with 75% ethanol, centrifuged and the supernatant was then removed. Dissolved total RNA pellet in DEPC-treated water and determined the concentration and contamination of total RNA at 260 and 280 nm by nanodrop. Stored total RNA at −70°C until use.

**cDNA Synthesis by Reverse Transcription**

cDNA synthesis was performed by adding 1 µl of total RNA of each sample and 1 µl oligo dT15 primer into 0.2 ml PCR tube and mixed. The tubes were heated at 70°C for 5 min and then immediately cooled the tubes on ice for 5 min. Reverse transcription mixture solution was prepared containing 25 mM MgCl₂, mixed dNTP, ribonuclease inhibitor, and reverse transcriptase. Added 15 µl of the mixture solution into each tube. Put the tubes in a thermocycler machine and set up the following condition to generate cDNA, setting the temperature at 25°C for 5 min, then 42°C for 1 hour and 30 min, and finally 70°C for 15 min. Stored cDNA samples at −20°C until use.

**Inflammatory Mediator Genes Amplification by Polymerase Chain Reaction (PCR)**

Each inflammatory mediator gene was prepared by adding 1 µl cDNA of each sample and 24 µl PCR mixture solution containing the corresponding primer for TNF-α, IL-6 and IL-1β gene. Adding dNTP, Taq polymerase and buffer in 0.2 PCR tube and mixed. PCR product on the gel was stained with 0.5 µg/ml ethidium bromide solution. Determined density of the PCR product by gel documentation.[20,21]

**Statistical Analysis**

All data were presented as means ± S.E.M. Data analysis was performed on SPSS 17.0 One-way ANOVA with Tukey’s Honestly Significant Difference (HSD) post hoc test was used to determine the statistical significance analysis. P-value <0.05 was considered as statistically significant.
RESULTS

Carrageenan-induced Paw Edema Test in Rats

*H. perforata* ethanolic root extract at concentration 50–400 mg/kg and indomethacin 5 mg/kg given by intraperitoneal injection one hour before carrageenan-induced paw edema produced significant inhibitory effect on rat paw edema at every time point of the study time period. The peak inhibitory response to carrageenan occurred at the second hour after *H. perforata* 50, 100, 200, 400 mg/kg administration with the percentage of inhibition of 28.49, 31.18, 47.85 and 65.05 respectively, while indomethacin 5 mg/kg caused 37.10% inhibition of rat paw edema at the corresponding time. The highest inhibition of indomethacin (47.44%) was seen at 5 hour after carrageenan injection. (Table 1, Figure 1)

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Control (2% ween 80)</th>
<th>Indomethacin (5 mg/kg)</th>
<th><em>H. perforata</em> (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% inhibition (paw volume)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>28.70*</td>
<td>25.93*</td>
<td>44.44*</td>
</tr>
<tr>
<td>2</td>
<td>0.26±0.04</td>
<td>(0.27±0.04)</td>
<td>(0.26±0.04)</td>
</tr>
<tr>
<td>3</td>
<td>0.28±0.05</td>
<td>31.18*</td>
<td>47.85*</td>
</tr>
<tr>
<td>4</td>
<td>0.27±0.05</td>
<td>13.44*</td>
<td>24.24*</td>
</tr>
<tr>
<td>5</td>
<td>0.22±0.03</td>
<td>13.13*</td>
<td>20.71*</td>
</tr>
<tr>
<td>6</td>
<td>0.22±0.03</td>
<td>13.25*</td>
<td>19.23*</td>
</tr>
</tbody>
</table>

Cell viability were not significantly altered by the presence of 0.2% DMSO, 10 µM dexamethasone and *H. perforata* extract at the concentration used (3.125–50 µg/ml) (Figure 2). Thus the value of non-toxic concentration obtained were used in proinflammatory cytokines determination.

Effects of *H. perforata* on Pro-inflammatory Cytokine mRNA Expression

Stimulation of J774A.1 with LPS caused the generation of mRNA expression of pro-inflammatory cytokines. Pre-treated the cells with *H. perforata* 12.5–50 µg/ml showed the inhibitory activity on mRNA expression of TNF-α and IL-1β in dose dependent manner. The
highest inhibition was found to be 49.83±2.71% and 47.27±3.77%, for TNF-α and IL-1β respectively at the concentration 50 µg/ml (Figures 3, 4, and Table 2). In contrast, it was found that *H. perforata* 50 µg/ml significantly increased mRNA expression of IL-6 by 43.93±5.65%, however the expression of IL-6 was inhibited at the lowers concentration (12.5 and 25 µg/ml). Dexamethasone 10 µM also inhibited TNF-α, IL-1β and IL-6 mRNA expression by 30.06±4.09, 77.96±2.09, 89.44±0.54 respectively (Figure 5, Table 2).

**DISCUSSION**

As previously described, *H. perforata* root is one component of a Thai traditional medicine known as Bencha-Loga-Wichien used for relieving of fever. The extract obtained from various parts of this plant demonstrated antibacterial, antiplasmodium falciparum, antipyretics effect. In addition, the ethanolic root extract possessed direct inhibitory effect on macrophage activation by inhibition of mRNA expression of cyclooxygenase (COX) and iNOS. Edematous formation due to carrageenan-induced paw edema in rat used in this model is biphasic phase.[22–24] The first phase begins immediately after carrageenan injection and diminishes in two hours, the second phase begins at the end of the first phase and lasts for three to five hours. In this present study, all dose of the extract (50–40 mg/kg) effectively inhibited rat paw edema at every time point measured. The peak time of inhibition was seen at the second hour after carrageenan injection. The dose that produced maximum inhibition was 400 mg/kg (65.05%) while indomethacin 5 mg/ml

![](image)

**Figure 3.** Inhibitory effect of *H. perforata* ethanolic root extract at the concentrations 12.5–50 µg/ml and 10 µM dexamethasone (Dex) on IL-1β mRNA expression in LPS-stimulated J774A.1 macrophage cells. The data are expressed as mean ± S.E.M from 3 independent experiments (n=3). *P < 0.05 compared to untreated cells (control).

<table>
<thead>
<tr>
<th>Final concentration</th>
<th>% Inhibition</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>TNF-α</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td></td>
</tr>
<tr>
<td>10 µM</td>
<td>30.06±4.09*</td>
</tr>
<tr>
<td><em>H. perforata</em></td>
<td></td>
</tr>
<tr>
<td>12.5 µg/ml</td>
<td>2.54±0.30*</td>
</tr>
<tr>
<td>25 µg/ml</td>
<td>20.49±1.61*</td>
</tr>
<tr>
<td>50 µg/ml</td>
<td>49.83±2.71*</td>
</tr>
</tbody>
</table>

Table 2. Inhibitory effect of *H. perforata* ethanolic root extract 12.5–50 µg/ml and 10 µM dexamethasone on pro-inflammatory cytokine mRNA expression in LPS-stimulated J774A.1 cells. The data are expressed as mean ± S.E.M from 3 independent experiments (n=3). *P < 0.05 compared to untreated cells.
produced 37.10% inhibition at the corresponding time point. This result indicated that *H. perforata* ethanolic root extract was able to attenuate the acute inflammatory process in response to carrageenan and its effect was predominately occurred during the first phase of reaction. On contrary, indomethacin, a non selective cyclooxygenase inhibitor, exhibited the maximum inhibitory effect at the fifth hour after the induction of paw edema (47.44%). This phenomenon is a well known fact that indomethacin acts by inhibiting prostaglandin synthesis at the late phase of inflammation.

The effect of *H. perforata* *in vitro* was further investigated for its anti-inflammatory mechanism in stimulated macrophage. As it is already known that macrophages are key innate immune cells contribution to diverse functions including the phagocytosis of foreign substances, expression of reactive oxygen species, production of proteins or enzymes involved in tissue remodeling, expression of chemokines and proinflammatory cytokines. Thus macrophages are involved in modulating the inflammatory process during the pathogenesis and resolution of tissue injury and inflammation.[23] Lipopolysaccharides (LPS) is known to be one of the best – stimuli in macrophage to induce transcription of genes encoding proinflammatory protein and cytokines such as TNF-α, IL-1β, IL-6, increase production of eicosanoids, oxygen and nitric oxide species. Over production of these substances cause acute and chronic inflammatory condition. In this present investigation *H. perforata* root extract significantly reduced TNF-α and IL-1β mRNA expression in LPS-stimulated macrophage J774A.1 cells in dose-dependent manner. Maximum inhibitory concentration were seen at 50 µg/ml of the extract as shown in Table 2, Figures 3, 4. As a result, the extract showed anti-inflammatory activity. However dexamethasone 10 µM exerted more potent inhibitory activity on IL-1β, IL-6 than the extract. Furthermore, it was found that the extract suppressed IL-6 expression both at low and medium concentration (12.5, 25 µg/ml) but not at the high concentration used in this study (50 µg/ml) since it stimulated IL-6 expression. This could be explain at least in part that the different in TNF-α, IL-1β, IL-6 mRNA reduction in LPS-stimulated macrophage were due to the difference in magnitude and time point in response to lipopolysaccharide activation.[26–28] Our previous report also supported this antiinflammatory phenomenon because the extract was able to inhibit nitric oxide production and the expression of iNOS and COX-2.[8] All of these consistent evidences indicated the anti-inflammatory potential of the extract.

In relation to the phytochemical constituents of the extract, it contained several chromones and limonoid compounds, there has been a number of studies report anti-inflammatory action of these phytochemical constituents.[29–31] Therefore, these phytochemical components could be responsible, in part, for the edematous inhibition and the reduction in inflammatory cytokines expression in the *in vivo* and *in vitro* model performed in this present study.

Taken together, it could be concluded that the ethanolic extract from the root of *H. perforata* possessed anti-inflammatory activity in carrageenan-induced paw edema in rat. The ability of the extract to cause edematous inhibition was partly mediated through inhibition of proinflammatory cytokines production. The antiinflammatory potential of the extract needs further systematic investigation.

**ACKNOWLEDGEMENT**

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