The Effect of Thai Herbal Ha-Rak Formula (HRF) on LPS-Induced Systemic Inflammation in Wistar Rats

Suksalin Booranasubkajorn, Ph.D., Haruethaikan Kanlaya, M.Sc.*, Sukit Huabprasert, M.Sc.*, Natchagorn Lumlerdkij, MRes.**, Pravit Akarasereenont, M.D.*, **, Pinpat Tripatara, M.D., Ph.D.***

**Department of Pharmacology, **Department of Applied Thai Traditional Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand.*

ABSTRACT

Objective: To study effects of Thai herbal Ha-rak formula (HRF) on lipopolysaccharide (LPS)-induced systemic inflammation in rats.

Methods: Male Wistar rats (190-250 g) were orally treated with HRF for 14 days before they were induced with LPS (6 mg/kg, i.v.). The markers of organ injury/dysfunction and pro-inflammatory cytokines were measured at 6 hours after LPS administration.

Results: LPS administration can significantly increase all markers. Intragastric administration of 5 mg/kg indomethacin, the positive control, significantly reduced plasma urea, creatinine, aspartate transaminase (AST), alanine transaminase (ALT), creatinine kinase (CK), tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, and IL-6. HRF trends to attenuate the plasma AST, ALT, CK, TNF-a, and IL-1β, although these effects were not statistically significant. Moreover, all doses of HRF did not increase plasma urea, creatinine, AST, ALT, CK, and lipase, when compared to sham.

Conclusion: HRF trends to protect against endotoxemia-induced organ injuries and pro-inflammatory cytokines. Furthermore, rats that received HRF did not show organ injuries.

Keywords: Thai herbal formula; Ha-rak; lipopolysaccharide; systemic inflammation; anti-inflammatory effect (Siriraj Med J 2017;69: 356-362)

Chemical compounds studied in this article: Lipopolysaccharide (PubChem CID: 11970143); Indomethacin (PubChem CID: 3715)

Abbreviations: ALT: Alanine transaminase; AST: Aspartate aminotransferase; BUN: Blood urea nitrogen; °C: Degrees Celsius; CK: creatine kinase; G: Gram; GMP: Good manufacturing practice; h: Hour; HRF: Thai herbal Ha-rak formula; i.g.: intragastric; indo: indomethacin; IL-1β: Interleukin-1 beta; IL-6: Interleukin-6; i.v.: intravenous; Kg: Kilogram; L: Liter; LPS: lipopolysaccharide; μ: Micro; mM: Millimolar; Mg: Milligram; min: Minute; mL: Milliliter; mm: Millimeter; mmHg: Millimeter of mercury; N: Number of observations; %: Percentage; SEM: Standard error of mean; TNF- α: tumor necrosis factor alpha

INTRODUCTION

The herbal medicines have been widely used in the past few years and continue to grow in admiration each year among health practitioners and the general public. There are many reports about anti-inflammatory plants. In addition, many plants with a medicinal reputation for treating inflammatory diseases have shown anti-inflammatory action in animal or biochemical models used for anti-inflammatory activity screening. These plants can be the alternatives for the non-steroidal anti-

Correspondence to: Pinpat Tripatara
E-mail: pinpat.tri@mahidol.ac.th
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inflammatory drugs (NSAIDs), which currently reported with many side effects. However, scientific evidences concerning either efficacy or safety of herbal products are necessary for supporting the herbal product usage in clinical practice and consumer protection.

Thai herbal Ha-Rak formula (HRF) or Ben-Cha-Lo-Ka-Wi-Chian remedy is a well-known traditional Thai herbal formula consisting of five medicinal plants, *Capparis micracantha DC.*, *Clerodendrum petasites* (Lour.) S. Moore., *Ficus racemosa* L., *Harrisonia perforata* (Blanco) Merr., and *Tiliacora triandra* (Colebr.) Diels, or Chingchi, Thao yaai mom, Konta, Madur-Chumporn, and Yanang in Thai, respectively in equal weight. The formula has been included in Thailand National List of Herbal Medicinal Products since AD 2006 as an antipyretic for both children and adults.[1] These five components have been reported to provide pharmacological properties including antipyretic[2-4], anti-inflammatory[3, 5, 6], anti-oxidant[7-9] and antimicrobial activities.[10-12] The previous studies showed that HRF possessed antipyretic and antinociceptive effects.[2,13] In addition, the phytochemical substances presented in the five medicinal plants in Ha-Rak formulation involved in their biological effects from previous study are as follows: flavonoid hispidulin from *Clerodendrum petasites;*[14] tannins[15,16], flavonoids,[15] bergenin, isocoumarin, ferulic acid, kaempferol and coumarin[17] from *Ficus racemosa*; chromones, perforamone A, B, C, and D, peucenin-7-methyl ether, *O*-methylalloptaeroxylin and limonoids[18] from *Harrisonia perforate;* alkaloids tiliandrine and tiliacorinine from *Tiliacora triandra*.[19] Although the reported efficacies of HRF seem to be similar to the known effects of NSAIDs, there is no report on the anti-inflammatory effects of this formula. Thus, the aim of this study was to investigate the effects on pro-inflammatory cytokines and the safety of this herbal formula in lipopolysaccharide (LPS)-induced systemic inflammation in rats. Therefore, this study serves as an additional scientific support for the use of HRF in Thai traditional medicine in clinical practice.

MATERIALS AND METHODS

Materials and reagents

HRF powder was prepared by Herbal Medicines and Products Manufacturing Unit, manufactured under GMP by Ayurved Siriraj, Center of Applied Thai Traditional Medicine (CATTM), Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand (GMP certified since 2009) with quality controlled, using thin layer chromatography (TLC) and ultra performance liquid chromatography (UPLC). The tested specimens were kept in a dry place, at room temperature and protected from light until used. LPS, E. coli serotype 0111:B4 and indomethacin were purchased from Sigma, USA. Thiopental sodium was purchased from Jagsonpal Pharmaceuticals, India. Commercial kits, Amersham tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, IL-6, rat biotrak Enzyme-linked immunosorbent assay (ELISA) system were purchased from GE Healthcare, UK.

Animal care

Male Wistar rats (190-250 g) purchased from the National Laboratory Animal Center, Nakorn Pathom, Thailand, were used in this study. All animals received the standard diet and water ad libitum, and were housed in the animal care facility at the Department of Anatomy, Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand, under standard conditions. The animals were acclimatized at least 1 week before the experiments. All experimental protocols were approved by Siriraj Animal Care and Use Committee (Si-ACUC 013/2553), Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand.

LPS-induced systemic inflammation

The dose range of HRF in humans is 3,000-4,500 mg/day. The animal dose equivalent can be calculated by using the body surface normalization method.[20] HRF (300, 1,000, or 3,000 mg/kg/day) or indomethacin (5 mg/kg/day) or sterile water (HRF and indomethacin vehicle) was intragastrically administered once daily for 14 days. The model of LPS-induced endotoxemia and organ injuries[21] was modified to study the effects of HRF and indomethacin on LPS induced organ injuries. Briefly, on day 15 rats were anesthetized with sodium thiopental (110 mg/kg, i.p.). The right carotid artery was cannulated for the measurement of mean arterial pressure (MAP) and heart rate (HR) using Powerlab 8/30 and Chart v6 software (AD Instruments, Australia), and for blood sample collection. The right jugular vein was cannulated for administration of intra-operative anesthetic agent and treatment solutions. The respiratory rate (RR) was counted and the rectal temperature was kept in range of 37±1°C. Ten minutes after surgical preparation, LPS or vehicle were intravenously administered.

At 6 h after the induction of endotoxemia, blood and organ samples (kidney, liver, lung, heart, and terminal ileum) were collected.

Measurement of biochemical parameters

The samples were centrifuged (6,000 g for 10 min) to separate plasma. All samples were stored at -80°C and analyzed for plasma levels of urea and creatinine for kidney injury; aspartate transaminase (AST) and

alanine transaminase (ALT) for liver injury; creatine kinase (CK) for heart and muscle injury, and lipase for pancreatic injury by using automatic clinical biochemistry A15 analyzer (Biosystems S.A., Barcelona, Spain).

Measurement of plasma cytokines

Carotid blood was taken after 90 min for TNF-α measurement and 6 h after LPS administration for IL-1β and IL-6 measurement and centrifuged at 6,000 g for 10 min. ELISA was performed according to the manufacturer's protocol. Samples were analyzed using a microplate reader set to 450 nm and corrected at a wavelength of 540 nm. All samples were analyzed in duplicate.

Statistical analysis

All values described in the text and figures are expressed as mean ± standard error of the mean (SEM) for N observations. For repeated measurements (biochemical data of hemodynamics data), a two-way analysis of variance was performed, followed by Bonferroni *post hoc* test. Data without repeated measurements were analyzed by one-way analysis of variance with Dunnett's *post hoc* test for multiple comparisons. All analyses were conducted by using GraphPad Prism version 5.03 for Windows (Graph Pad Software Inc., San Diego, CA); and a P-value of less than 0.05 was considered to be statistically significant.

RESULTS

Effects of HRF on vital signs after endotoxemia in rats

Baseline values of vital signs were similar in all experimental groups (Table 1). Intravenous LPS (6 mg/kg) significantly biphasically decreased MAP at 1-6 h, increased HR at 3 to 6 h, but did not significantly alter endotoxemia-induced RR (Fig 1). Body temperature

TABLE 1. Baseline characteristics of the animals.

	BW (g)	MAP (mmHg)	HR (beats/min)	T (°C)	RR (breaths/min)
sham	214.6 ± 3.8	140 ± 2	445 ± 7	36.9 ± 0.1	74 ± 4
sham-300	210.5 ± 2.1	138 ± 3	437 ± 9	36.9 ± 0.1	92 ± 1
sham-1000	220.9 ± 2.9	135 ± 3	438 ± 6	36.8 ± 0.1	80 ± 4
sham-3000	212.7 ± 4.3	144 ± 2	429 ± 1	36.9 ± 0.2	78 ± 6
LPS	214.9 ± 1.6	139 ± 1	440 ± 5	36.9 ± 0.1	72 ± 4
LPS-300	215.6 ± 2.4	135 ± 3	431 ± 7	36.8 ± 0.1	86 ± 2
LPS-1000	219.3 ± 3.9	137 ± 2	428 ± 5	37.0 ± 0.1	76 ± 2
LPS-3000	214.8 ± 2.4	133 ± 3	431 ± 5	36.8 ± 0.1	84 ± 7
LPS-indo5	216.3 ± 6.0	140 ± 2	438 ± 6	37.0 ± 0.2	80 ± 6
***p*-value**	0.558	0.122	0.616	0.953	0.640

Data are expressed as the mean ± S.E.M. P<0.05 was considered statistically significant using one-way ANOVA. N = 12-18 animals per group.

Abbreviations: BW: Body weight; MAP: Mean arterial pressure; HR: Heart rate; T: Body temperature; RR: Respiratory rate.

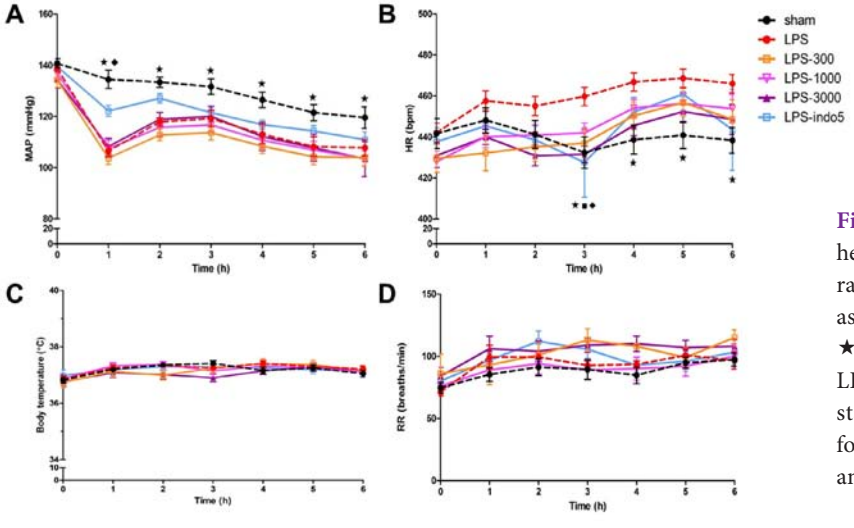

Fig 1. Effects of HRF on (A) blood pressure, (B) heart rate, (C) body temperature, (D) respiratory rate after LPS injection in rats. Data are expressed as mean ± S.E.M. for N number of observation. ★*P*<0.05 sham vs. LPS; ◆*P*<0.05 LPS-indo5 vs. LPS; ■*P*<0.05 LPS-3000 vs. LPS was considered statistically significant using two-way ANOVA followed by Bonferroni post hoc test. N = 12-18 animals per group.

was controlled in range 37.2 ± 0.5°C. When compared to the LPS-control group, treatment with any dose of HRF did not affect the fall in MAP caused by endotoxin at any time point. Indomethacin significantly improved MAP only at 1 h after the induction of endotoxemia (Fig 1). Interestingly, treatment of 3,000 mg/kg/day HRF and 5 mg/kg/day indomethacin significantly attenuated tachycardia associated with endotoxemia at 3 h after LPS administration whereas lower doses (300 or 1,000 mg/kg/day) of HRF did not have significant effect on HR (Fig 1). When compared to the sham-control group, animals which received HRF alone (sham-300, sham-1000 and sham-3000) exhibited similar MAP, HR, RR, and body temperature throughout the experiment (Fig 2).

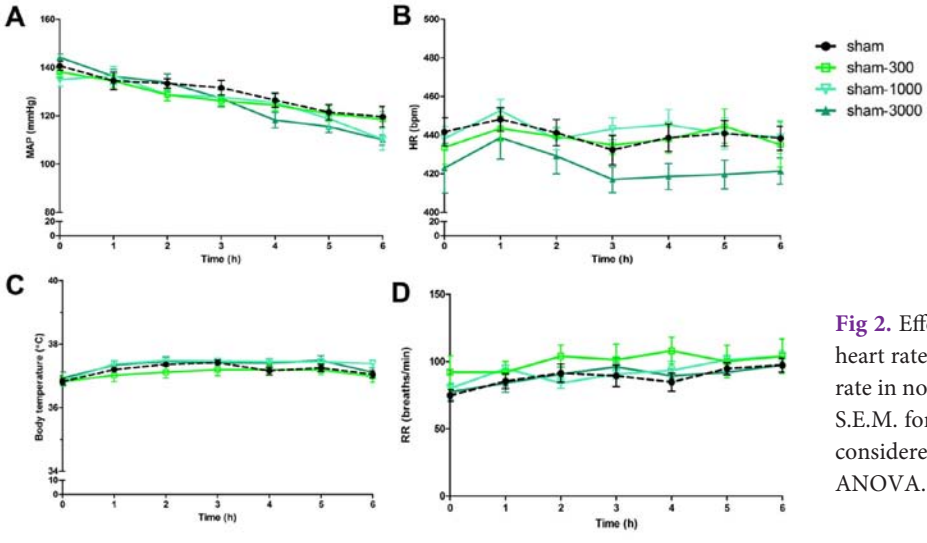

Fig 2. Effects of HRF on (A) blood pressure, (B) heart rate, (C) body temperature, (D) respiratory rate in normal rats. Data are expressed as mean ± S.E.M. for N number of observation. *P*<0.05 was considered statistically significant using two-way ANOVA. N = 12-18 animals per group.

Effects of HRF on the organ injuries caused by endotoxemia and organ parameters

Endotoxemia for 6 h significantly increased plasma levels of urea, creatinine, AST, ALT, CK and lipase. When compared to LPS-control, indomethacin markedly reduced kidney, liver and heart/muscle injuries, whereas treatment with HRF did not significantly attenuate the increase in the plasma levels of any markers of organ injury/dysfunction caused by endotoxemia (Fig 3). When compared with the sham-control group, animals which received HRF alone exhibited similar plasma levels of urea, creatinine, AST, ALT, CK and lipase (Fig 4).

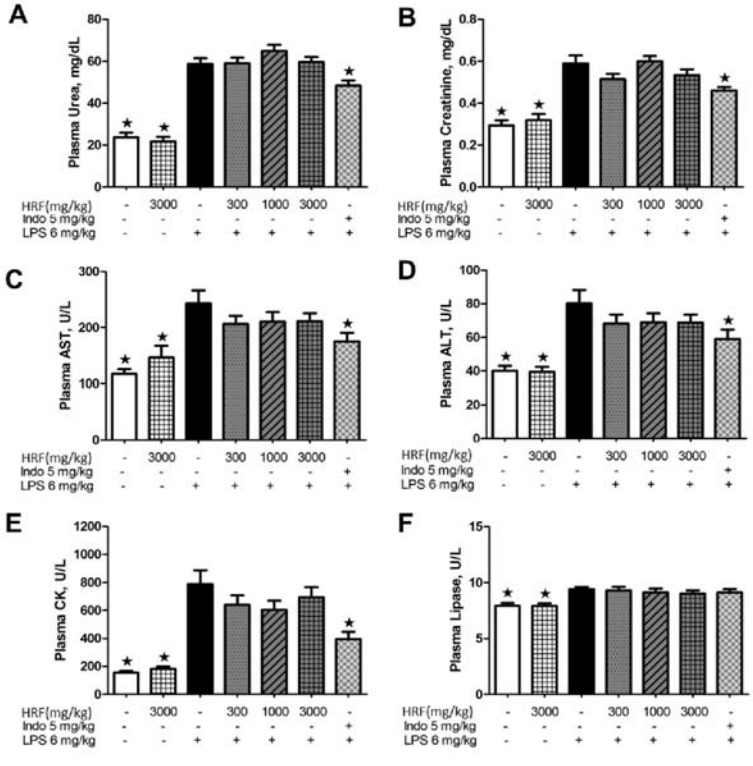

Fig 3. Effects of HRF on (A) plasma urea, (B) plasma creatinine, (C) plasma AST, (D) plasma ALT, (E) plasma CK and (F) plasma lipase at 6 h after LPS injection in rats. Data are expressed as mean ± S.E.M. for N number of observation. ★*P*<0.05 vs. LPS was considered statistically significant using one-way ANOVA followed by Dunnett's post hoc test. N = 12-18 animals per group.

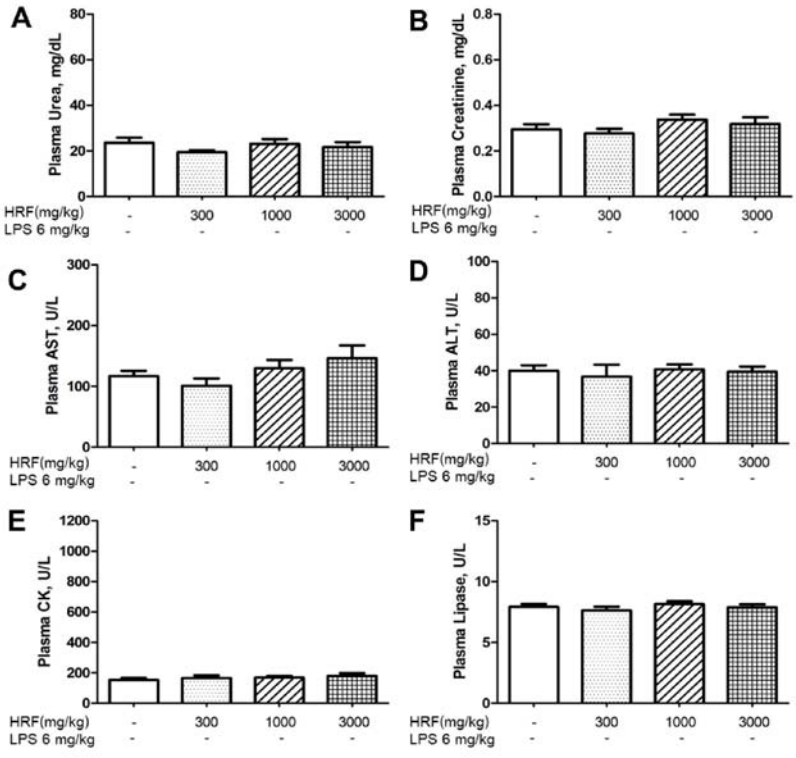

Fig 4. Effects of HRF on (A) plasma urea, (B) plasma creatinine, (C) plasma AST, (D) plasma ALT, (E) plasma CK and (F) plasma lipase in normal rats. Data are expressed as mean ± S.E.M. for N number of observation. $P<0.05$ was considered statistically significant using one-way ANOVA. N = 12-18 animals per group.

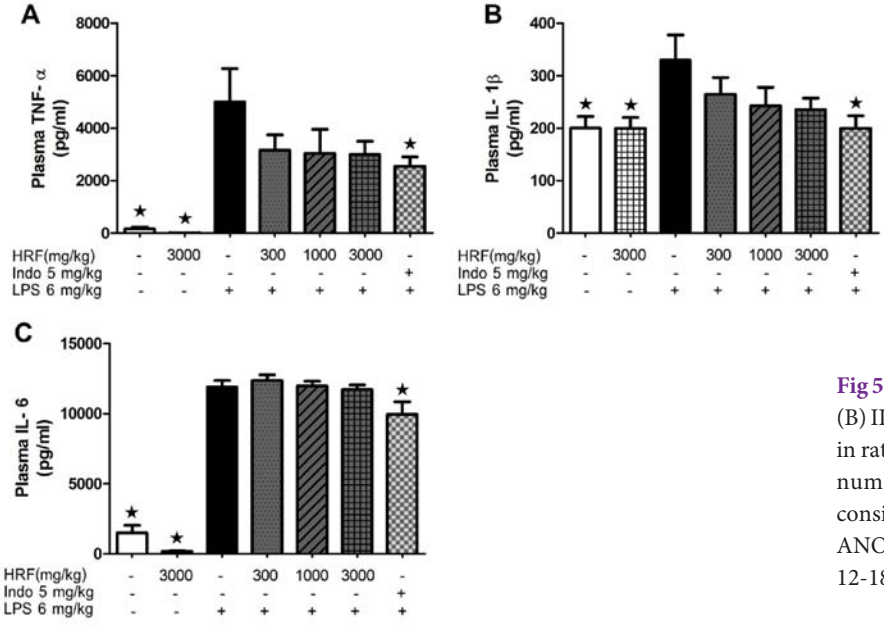

Fig 5. Effects of HRF on plasma level of (A) TNF-α, (B) IL-1β and (C) IL-6 at 90 min after LPS injection in rats. Data are expressed as mean ± S.E.M. for N number of observation. ★ $P<0.05$ vs. LPS was considered statistically significant using one-way ANOVA followed by Dunnett's post hoc test. N = 12-18 animals per group.

Effects of HRF on the increased circulating levels of TNF-α, IL-1β and IL-6 caused by endotoxemia

Administration of LPS caused a significant increase in TNF-α, IL-1β and IL-6 production. When compared to LPS-control, indomethacin significantly decreased all plasma levels, whereas treatment with HRF trended to reduce the increase in plasma levels of TNF-α and IL-1β, although it was not statistically significant. (Fig 5)

DISCUSSION

The bacterial endotoxin, LPS, initiates a cascade of systemic, cellular, and molecular events that ultimately manifest in the typical signs and symptoms of infection.[22,23] This study demonstrated that the systemic administration of LPS resulted in the development of acute renal injury, liver injury, neuromuscular injury and pancreatic injury. Furthermore, development of circulatory failure

(hypotension and tachycardia) was observed as indicator of systemic inflammatory response. The results showed that pretreatment with HRF tends to protect against endotoxemia-induced organ injuries, although these effects were not statistically significant.

LPS significantly reduced the MAP and increased HR that was defined as a progressive failure of the circulation. Although pretreatment with HRF at high dose (3,000 mg/kg/day) significantly reduced tachycardia at 3 h after LPS administration, it did not affect the hypotension caused by LPS. Thus these findings suggested that HRF had no clinically significant hemodynamic effects on blood pressure or heart rate.

In response to endotoxemia, the organism provokes release of pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6 and certain secondary mediators, such as leukotrienes and prostaglandins into surrounding tissues, thereby causing tissue damage and organ failure.[24-26] HRF trends to reduce the rise in the circulating levels of TNF-α and IL-1β inflammatory cytokines associated with endotoxemia.

For the safety assessment, there were no renal injury, liver injury, neuromuscular injury and pancreatic injury or hemodynamic changes shown in the treatment of HRF. These results suggest that oral administration of HRF up to 3,000 mg/kg/day, equivalent to the dose used in humans, for 14 days should be relatively safe.

Indomethacin is a NSAID acting as non-selective COX inhibitor and is commonly used to treat pain and inflammation despite the risk of major gastrointestinal complications. In the present study, indomethacin attenuated and prevented the endotoxin-induced decrease in MAP and increase in HR, respectively, while 3,000 mg/kg/day HRF prevented the LPS effect on HR only. The results showed the protective effects of indomethacin on LPS-induced multiple organ injuries and the circulating levels of TNF-α, IL-1β and IL-6, which were associated with endotoxemia, whereas HRF tends to reduce these organ injuries, TNF-α and IL-1β pro-inflammatory cytokines. This is the first study that investigated the anti-inflammatory effects of HRF on the LPS-induced systemic inflammation model in vivo.

CONCLUSION

In summary, our results demonstrated that pretreatment with HRF tends to attenuate circulatory failure, liver and neuromuscular injuries and pro-inflammatory cytokines associated with exdotoxemia. Moreover, HRF at the animal dose equivalent to that used in humans does not show toxicity in rats. Therefore, this study thus provides a scientific evidence for HRF.

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Conflict of interest: The authors declare no conflict of interest.

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