Anti-inflammatory effect of Phattapitta Recipe in RAW 264.7 macrophages stimulated with lipopolysaccharide

U. Nanna[1*], S. Sireertwong[2], K. Jaijoy[3]

[1]Division of Pharmacology, Department of Preclinical Science, Faculty of Medicine, Thammasat University, Rungsit Campus, Klong Loung, Pathum Thani 12120, Thailand. [2]Department of Pharmacology, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand. [3]McCormick Faculty of Nursing, Payap University, Chiang Mai 50000, Thailand.

ABSTRACT

Pattapitta Recipe has been used for the treatment of cirrhosis in the Thai Traditional Medicine Clinic of Prapokklao Hospital. The liver functions of cirrhotic patients treated with Pattapitta Recipe were improved and recovered to normal function within 5 months. Furthermore, Pattapitta Recipe has been used for the treatment of alcoholic liver cirrhosis, hepatitis-B, hepatitis-C, and cirrhosis due to unidentified causes. However, little is known about the mechanisms underlying its anti-inflammatory activities. This study is mainly focused on the cell viability test of Phattapitta Recipe on macrophages cell line RAW 264.7 and investigation of an anti-inflammatory action of Phattapitta Recipe on lipopolysaccharide (LPS)-induced macrophages cell line, RAW 264.7. The results showed that Phattapitta Recipe had no cytotoxicity in RAW 264.7 cells. Phattapitta Recipe 250 ug/mL possessed anti-inflammatory effects by significantly decreased mRNA levels of interleukin-1β(IL-1β), tumor necrosis factor – α (TNF-α), interleukin-6 (IL-6), inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in macrophages cell line RAW 264.7. Moreover, Phattapitta Recipe also significantly inhibited the production of TNF-α and IL-6 at the protein level. Although Phattapitta Recipe treated cells showed a decreased trend of nitric oxide levels, it was not statistically significant reduction. The results from this study will be beneficial for the development of Phattapitta Recipe as a potential treatment of anti-inflammatory diseases.

Keywords: Anti-inflammatory effect, TNF-α, IL-1β, iNOS, COX-2

*Correspondence to Author:
U. Nanna
Division of Pharmacology, Department of Preclinical Science, Faculty of Medicine, Thammasat University, Rungsit Campus, Klong Loung, Pathum Thani 12120, Thailand.

Introduction

Pattapitta Recipe has been used for the treatment of cirrhosis in the Thai Traditional Medicine Clinic of Prapokklao Hospital. The liver functions of cirrhotic patients treated with Pattapitta Recipe were improved and recovered to normal function within 5 months. Furthermore, Pattapitta Recipe has been used for the treatment of alcoholic liver cirrhosis, hepatitis-B, hepatitis-C, and cirrhosis due to unidentified causes. However, little is known about the mechanisms underlying its anti-inflammatory activities. This study is mainly focused on the cell viability test of Phattapitta Recipe on macrophages cell line RAW 264.7 and investigation of an anti-inflammatory action of Phattapitta Recipe on lipopolysaccharide (LPS)-induced macrophages cell line, RAW 264.7.

Material and methods

Cell cultures (Wang et al., 2009)

RAW 264.7 cells, a murine macrophage cell line, were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin. They were maintained at 37 °C in a humidified atmosphere of 5% CO_2.

Cell viability assay (Vichai et al., 2006)

RAW264.7 cells 2×10^4 cells/well in 96 well plates were incubated for 24 h. Add 10 ml test sample in 10% DMSO to each compound well of a 96-well tissue-culture plate in triplicate. Incubate the remaining assay plates at 37 °C in a humidified incubator with 5% CO_2 for 48 hour. Without removing the cell culture supernatant, gently add 100 ml cold 10% TCA to each well for cell fixation. Add 100 ml of 0.057% SRB solution to each well. Leave at room temperature for 30 min and then quickly rinse the plates four times with 1% acetic acid to remove unbound dye. Add 200 ml of 10 mM Tris base solution (pH 10.5) to each well and shake the plate on a gyratory shaker for 5 min to solubilize the protein-bound dye. Measure the OD at 510 nm in a microplate reader.

Real-time PCR analysis (Wang et al., 2009; Nolan et al., 2006)

RAW 264.7 cells (2×10^5 cells/mL) in 35 mm petri dish were incubated for 24 h. Pretreated with test substance for 1 h before stimulation with 1 µg/mL LPS for 6 h. Total RNA was isolated using Trizol reagent. cell suspension was transfered to microcentrifuge tube and added 100 µL chloroform for 5 min at room temperature. Afterthat, centrifuge at 12,000 g in a refrigerated microfuge at 2-8 °C for 15 minutes. Transfer the aqueous (upper) phase to a new tube and add 500 µL isopropyl alcohol to precipitate RNA, leaving at room temperature for 10 minutes. Centrifuge at 12,000 g in a refrigerated microfuge at 2-8 °C for 10 minutes. Remove the supernatant, wash the tubes two times with 500 µL 75% ethanol, centrifuge at 7,500 g in refrigerated microfuge at 2-8 °C for 10 minutes and remove the ethanol.

Air-dry the RNA pellet at room temperature for 5-10 minutes. Resuspend the RNA in 50 ml of TE-buffer. Fill 1 µL RNA into 49 µL DEPC-treated water (1:50 dilution).Measure the absorbance at 260 nm for the RNA concentration and at 280 nm for the protein concentration by using GeneQuant pro (Amersham Biosciences, Germany). Calculate the ratio of OD 260 / OD 280. The value should be more than 1.6. Then calculate the RNA concentration from

Concentration of RNA (µg/mL) = OD260 x 40 x dilution factor

Synthesize cDNA by reverse transcription (Nolan et al., 2006)

RevertAid TM First Strand cDNA synthesis kit (Fermentas, Germany) was used. Briefly, RNA (1 µg) was reverse-transcribed with oligo-(dT)18 primer and AMV reverse transcriptase (M1 reaction buffer). M1 buffer was incubated at 70ºC for 5 minutes and let it cool for 1 minute and then add M2 buffer. Reaction mixture was incubated at 70ºC for 5 minutes, at 4ºC for 1

minutes, and at 42ºC for 60 minutes. cDNA was diluted 5 fold and used as template for RT-PCR reaction.

Expression of TNF-α, IL-1β, IL-6, iNOS และ COX-2 mRNA by Quantitative reverse transcription polymerase chain reaction (RT-qPCR) technique (Nolan et al., 2006)

The primers were choosed from on-line primer design program. SYBR green-based quantitative PCR amplification made using PCR thermal cycler (PCRMax Eco 48 real time PCR system). Each reaction mixture makes PCRMax Eco 48 real time PCR system, which has cycling for PCR amplification.

Stage	Step	Temperature	Duration	Cycles
Polymerase Activation	Step 1	95	00:03:00	1
PCR Cycling	Step 1	95	00:00:15	40
PCR Cycling	Step 2	58	00:00:15	40
PCR Cycling	Step 3	72	00:00:30	40
Melt Curve	Step 1	95	00:00:15	1
Melt Curve	Step 2	55	00:00:15	1
Melt Curve	Step 3	95	00:00:15	1
Total Program Length	1 Hour 5 Minutes 51 Seconds			
Total Cycle Count	40			

Determining the amount of inflammatory substances IL-6 and TNF-α using ELISA (Oh et al., 2012)

The ELISA plate was coated with 100 μL of 1x capture antibody in coating buffer-A and incubated overnight at 2-8 ºC. The plate was washed with wash buffer (0.05% Tween-20 in PBS) four times and then blocked with 200 μL blocking buffer (assay diluent-A) for 1 h at RT. After blocking, the plate was washed with wash buffer four times. Human IL-17A, IL-22, and IL-23 standards were diluted to concentrations ranging from 0-1000 pg/mL in the assay diluent-A and 100 μL standards and 100 μL samples were added to each well in triplicate for 2 h at RT. The ELISA plate was washed four times and 100 μL diluted detection antibody was added and incubated for 1 h at RT and followed by four time washing. Avidin-HRP conjugated (1:1000) (100 μL) was added and incubated for 30 min and then the plate was washed five times. Finally, the freshly mixed TMB substrate solution (100 μL) was added into each well and incubated in the dark for 30 min. The stop solution (2N H_2SO_4) (100 μL) was added to each well and the absorbance at 450 nm and 570 nm was measure within 15 min by using the Synergy™ H4 Hybrid Multi-Mode microplate reader (BioTek Instruments, Inc., Vermont, USA).

Nitric oxide (NO) (Choi et al., 2007)

The synthesis of Nitric oxide (NO) can be measured by measuring nitrite levels in the supernatant of the macrophage culture cells. RAW 264.7 cells ($2×10^5$ cells/mL) in 35 mm petri dish were incubated for 24 h. Pretreated with test substance for 1 h before stimulation with 1 μg/mL LPS for 24 h. The supernatant

was mixed with Griess reagent at the same volume and incubated at room temperature for 5 minutes. Nitrite concentration was measure with a microplate reader at 540 nm wavelength. The amount of Nitrite levels was calculate from the sodium nitrite ($NaNO_2$) standard curve.

Statistical analysis

All experiments were performed in three individual trials using a minimum of three replicates for each trial. All values are expressed mean ± standard error of mean (S.E.M). Differences between treatment groups were analyzed by one-way analysis of variance (one-way ANOVA), followed by *post-hoc* Fisher's least significant difference (LSD) test using the SPSS for Windows (version 20.0). Differences were considered significant at $P < 0.05$.

Results

An anti-inflammatory action of Phattapitta Recipe was studied on lipopolysaccharide (LPS)-induced macrophages cell line, RAW 264.7. To examine the cell viability by using MTT and SRB assay of Phattapitta Recipe (PPR), RAW 264.7 cells were treated with different concentrations of PPR. As shown in Fig. **1**, PPR had no cytotoxicity in RAW 264.7 cells. In gene expression of macrophage cell line, PPR at the doses of 125 and 250 μg/mL decreased mRNA levels of interleukin-1β (IL-1β) and tumor necrosis factor –α (TNF-α) as shown in Figure 2 and 3, respectively. Moreover, PPR at the doses of 62.5, 125 and 250 μg/mL decreased mRNA levels of interleukin-6 (IL-6) as shown in Figure 4. PPR at the dose of 250 μg/mL decreased mRNA levels of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in macrophages cell line RAW 264.7 as shown in Figure 5 and 6, respectively. At the protein level, PPR at the dose of 250 μg/mL significantly inhibited the production of TNF-α as shown in Figure 7. PPR at the dose of 125 and 250 μg/mL significantly inhibited the production of IL-6 as shown in Figure 8. Moreover, PPR treated cells showed a decreased trend of nitric oxide levels, it was not statistically significant reduction as shown in Figure 9.
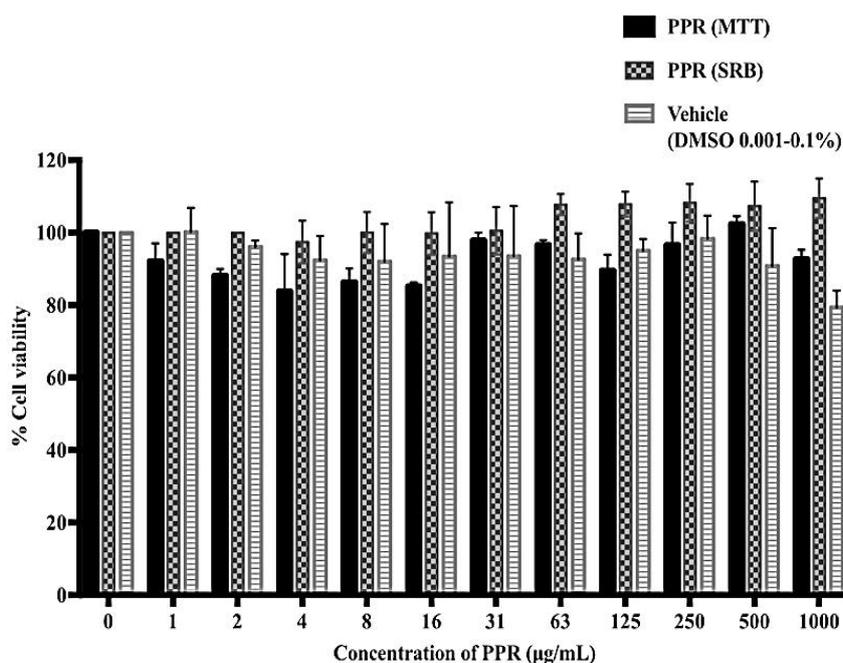

Fig. 1. Cell viability was determined by MTT and SRB assay, RAW 264.7 cells were treated with PPR (0-1000 μg/mL). Data are presented as mean ± SEM (n = 4). *$p < 0.05$ compared with untreated cells.
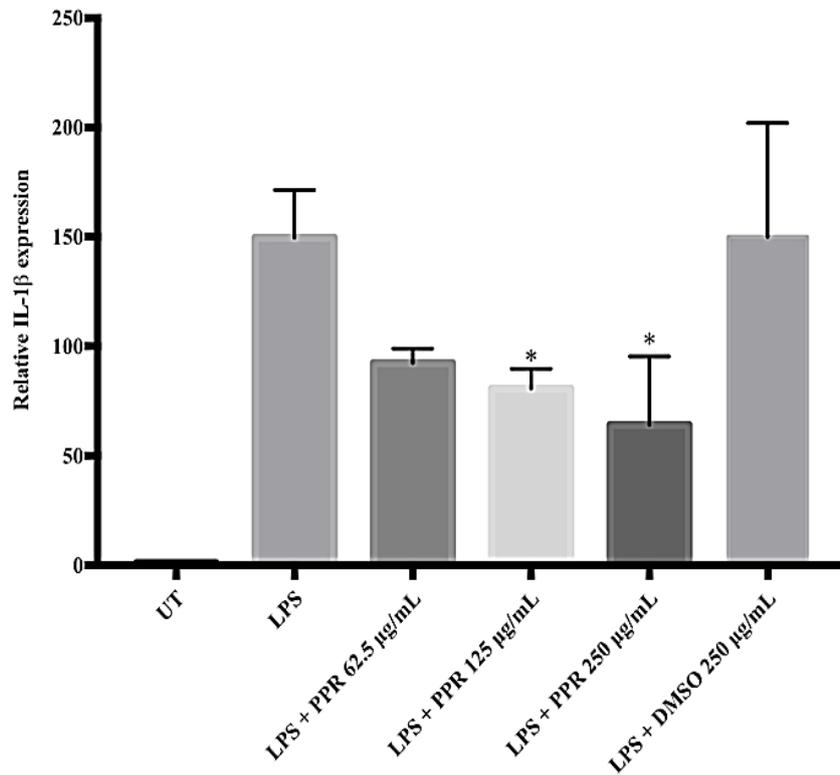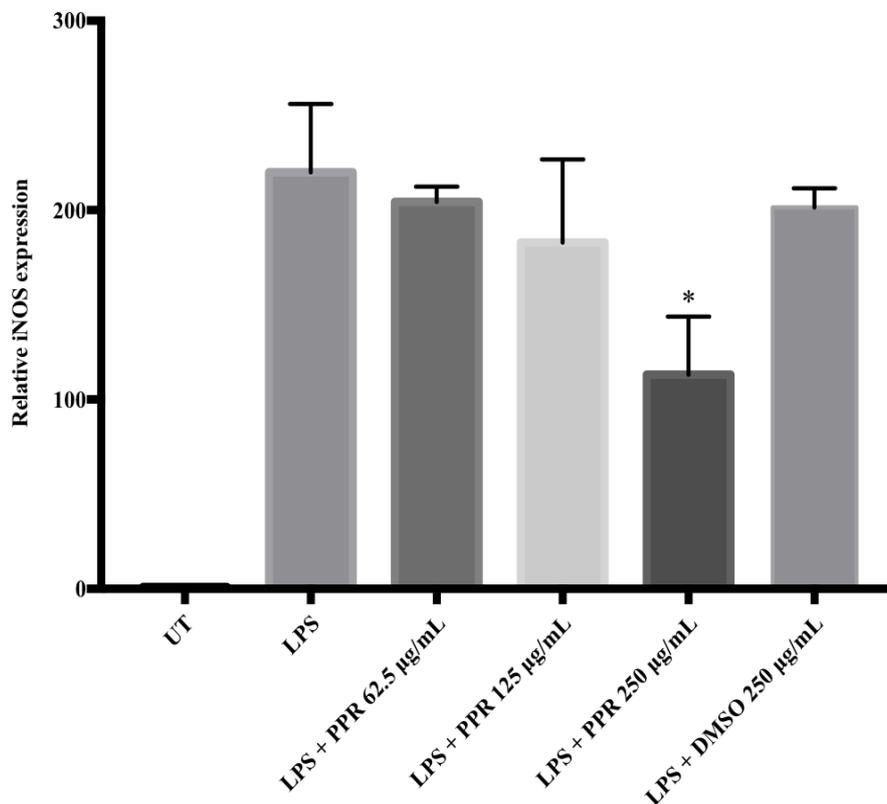
Fig. 2. Effects of PPR on relative expression levels of IL-1β in lipopolysaccharide (LPS)-induced macrophages cell line, RAW 264.7. Data are expressed as mean ± SEM of three independent experiments. *$p < 0.05$.
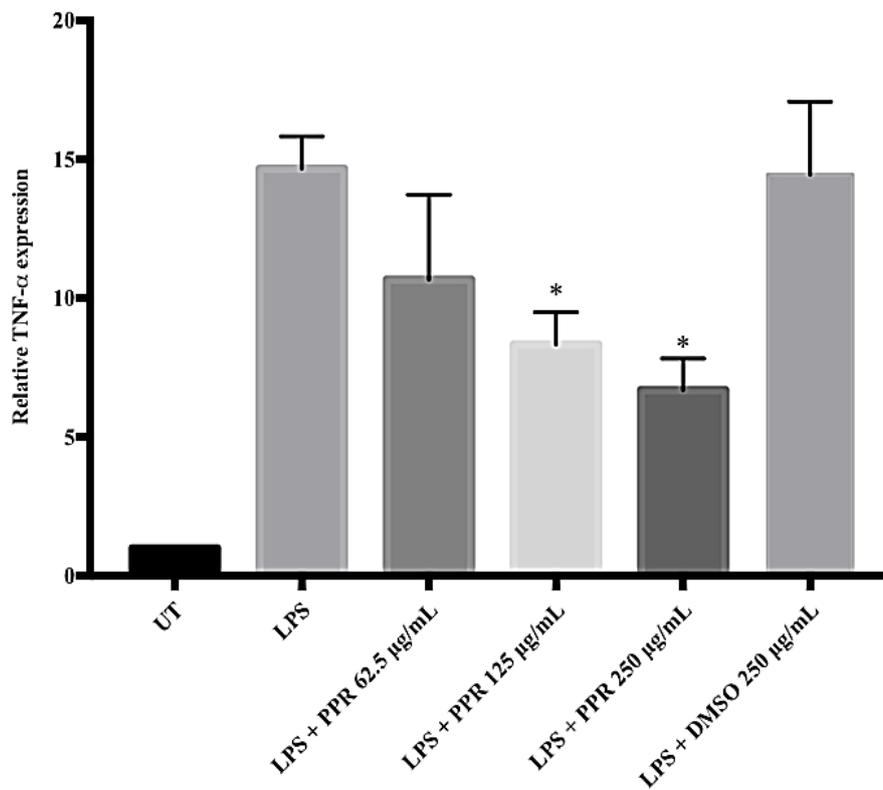
Fig. 3. Effects of PPR on relative expression levels of TNF-α in lipopolysaccharide (LPS)-induced macrophages cell line, RAW 264.7. Data are expressed as mean ± SEM of three independent experiments. *$p < 0.05$.
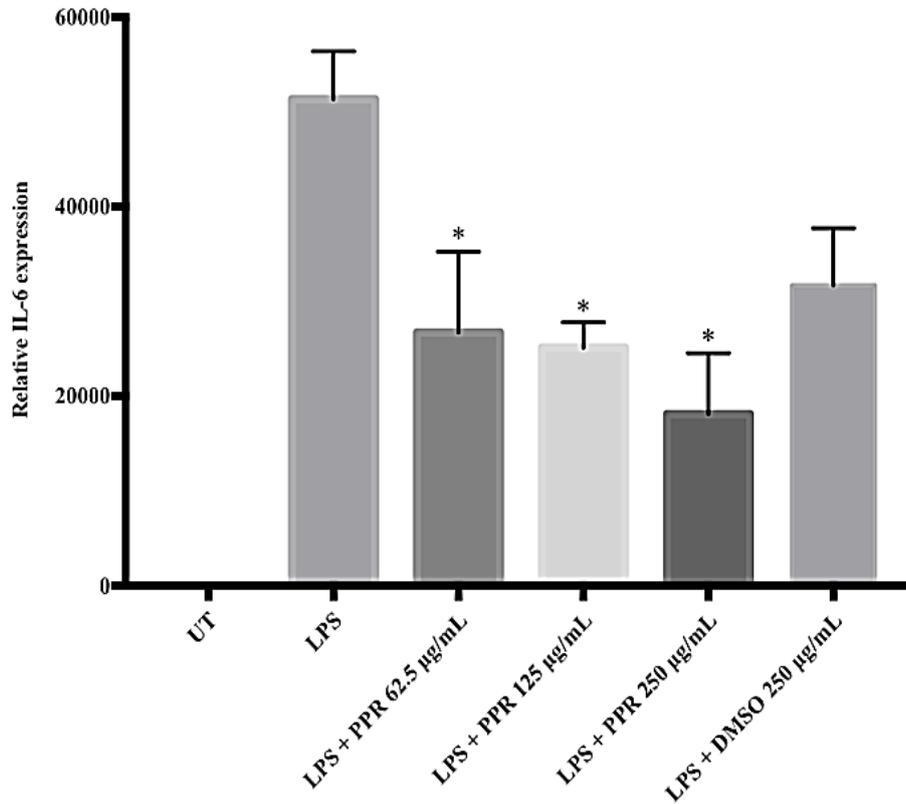
Fig. 4. Effects of PPR on relative expression levels of IL-6 in lipopolysaccharide (LPS)-induced macrophages cell line, RAW 264.7. Data are expressed as mean ± SEM of three independent experiments. *$p < 0.05$.
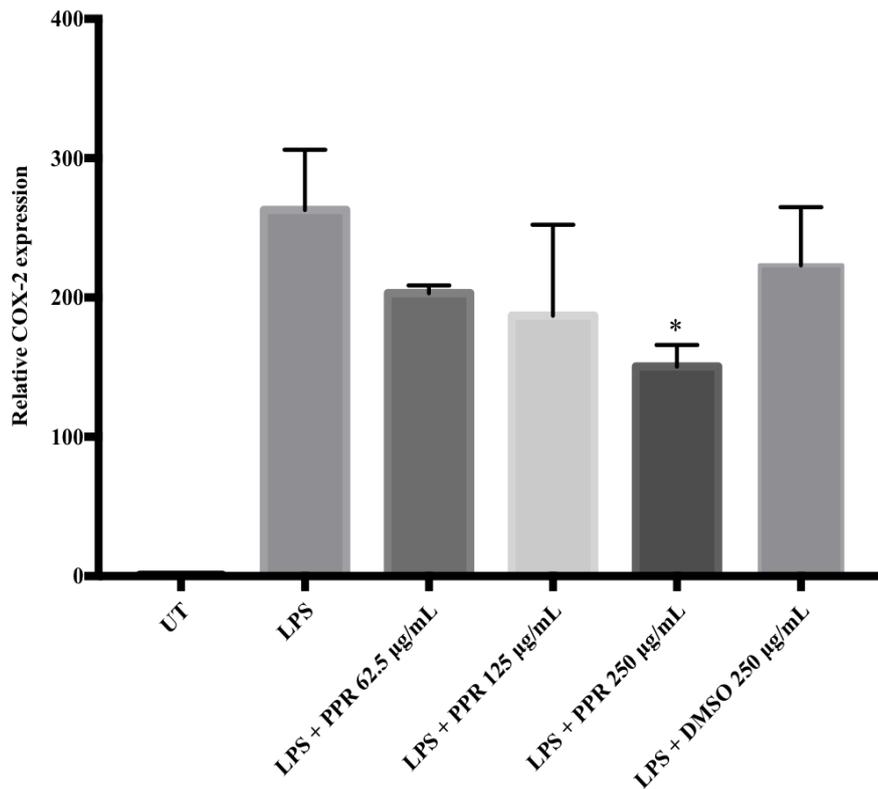

Fig. 5. Effects of PPR on relative expression levels of iNOS in lipopolysaccharide (LPS)-induced macrophages cell line, RAW 264.7. Data are expressed as mean ± SEM of three independent experiments. *$p < 0.05$.

Fig. 6. Effects of PPR on relative expression levels of COX-2 in lipopolysaccharide (LPS)-induced macrophages cell line, RAW 264.7. Data are expressed as mean ± SEM of three independent experiments. *p < 0.05.
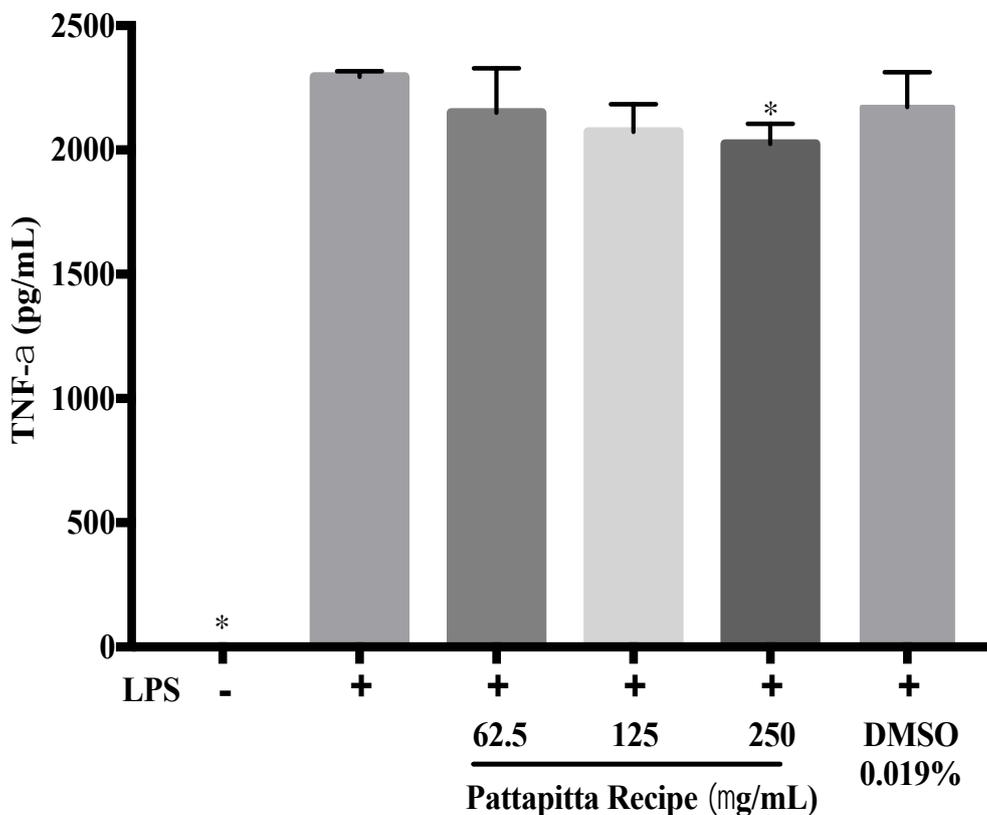

Fig. 7. Effects of PPR at the protein level on the production of TNF-α in lipopolysaccharide (LPS)-induced macrophages cell line, RAW 264.7. Data are expressed as mean ± SEM of three independent experiments. *p < 0.05.
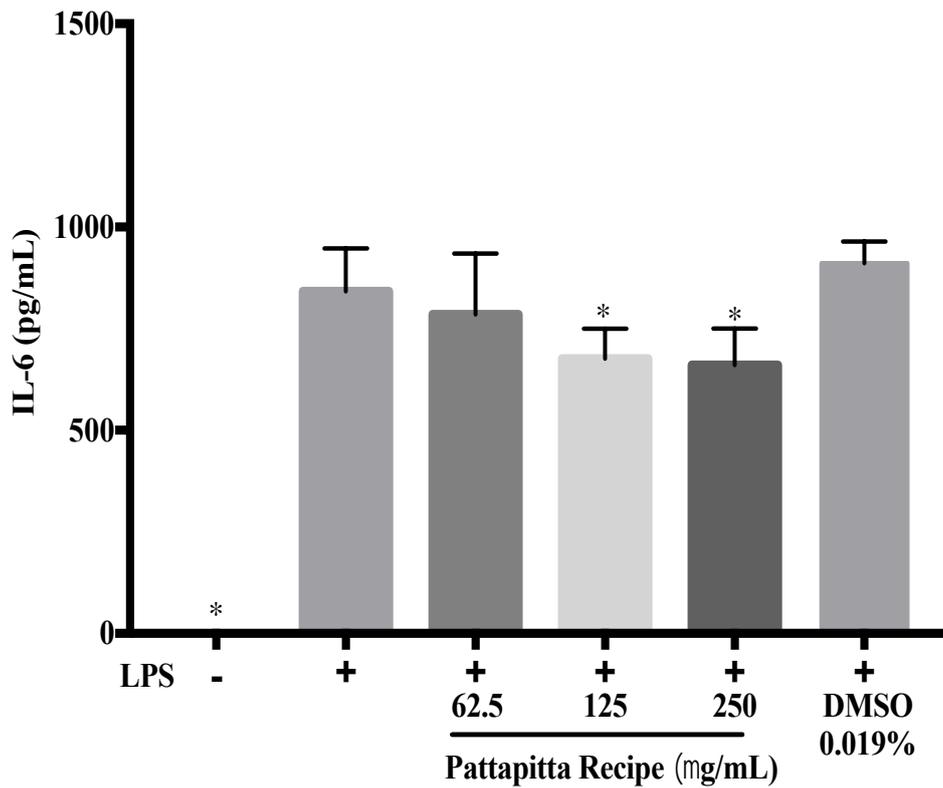
Fig. 8. Effects of PPR at the protein level on the production of IL-6 in lipopolysaccharide (LPS)-induced macrophages cell line, RAW 264.7. Data are expressed as mean ± SEM of three independent experiments. *$p < 0.05$.
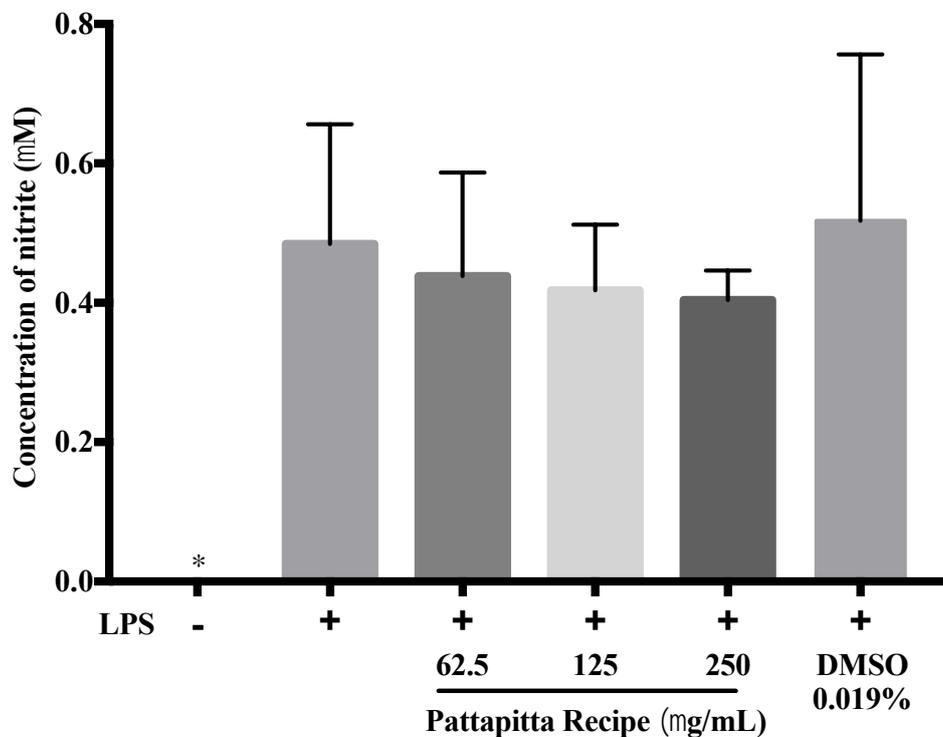

Fig. 9. Effects of PPR on the concentration of nitrite in lipopolysaccharide (LPS)-induced macrophages cell line, RAW 264.7. Data are expressed as mean ± SEM of three independent experiments. *$p < 0.05$.

Discussion

The study of the effect of Phatthapatta Recipe (PPR) on the viability of macrophages cells line. It was found that the PPR does not have toxic effects on macrophages in vitro. The anti-inflammatory effect of PPR was studied by using macrophage cells RAW 264.7, is a prototype cell in the study. Lipopolysaccharide (LPS) is a component of bacterial cell wall. LPS act as a catalyst for the release of inflammatory mediator such as tumor necrosis factor –α (TNF-α), interleukin-6 (IL-6) and nitric oxide (NO). Therefore, inhibition of tumor necrosis factor –α (TNF-α) interleukin-6 (IL-6) and nitric oxide (NO) is used as an index that indicates the ability to reduce inflammation of the PPR (Diaz et al., 2012; Buapool et al., 2013). In this study, it has been found that PPR has anti-inflammatory effect due to the PPR at a concentration of 250 μg/mL, inhibiting tumor necrosis factor –α (TNF-α) and interleukin-6 (IL-6) in macro cells. However, there was no statistical difference of PPR towards the reduction of nitric oxide (NO) levels in macrophages. This result is expected from the inhibiting the expression of tumor necrosis factor –α (TNF-α), interleukin-6 (IL-6).

This result found that "Phatthapitta Recipe" has anti-inflammatory effects due to inhibiting the releases of inflammatory mediator such as tumor necrosis factor –α (TNF-α) and interleukin-6 (IL-6) and inhibiting gene expression of TNF-α and IL-6.

Conclusion

Phatthapitta Recipe can reduce the risk factor, severity, and delay the progression of alcohol-induced cirrhosis, hepatitis B virus infection and hepatitis C virus infection, which can be seen from the Thai Traditional Medicine Clinic of Phrapokklao Hospital, have used Phatthapitta" Recipe to treat cirrhosis. Moreover, blood tests showed better liver function the blood returned to normal levels in the 5th month. In addition, this herbal formula is also used to treat cirrhosis caused by alcohol, hepatitis B virus, hepatitis C virus or unknown cause.

Conflict of interest

The authors declare no conflicts of interests.

Acknowledgements

References

1. Buapool, D., Mongkol, N., Chantimal, J., Roytrakul, S., Srisook, E., Srisook, K., 2013. Molecular mechanism of anti-inflammatory activity of *Pluchea indica* leaves in macrophages RAW 264.7 and its action in animal models of inflammation. J. Ethnopharmacol. 4, 495-504.

2. Choi, H.J., Kang, O.H., Park, P.S., Chae, H.S., Oh, Y.C., Lee, Y.S., Choi, J.G., Lee, G.H., Kweon, O.H., Kwon, D.Y., 2007. Mume Fructus water extract inhibits pro-inflammatory mediators in lipopolysaccharide-stimulated macrophages. J. Med. Food. 10, 460–466.

3. Diaz, P., Jeong, S.C., Lee, S., Khoo, C., Koyyalamudi, S.R., 2012. Anti-oxidant and anti-inflammatory activities of selected medicinal plants and fungi containing phenolic and flavonoid compounds. J. Chin. Med. 7, 26.

4. Nolan, T., Hands, R.E., Bustin, S.A., 2006. Quantification of mRNA using real-time RT-PCR. Nat. Protocols. 1(3), 1559–1582.

5. Oh, Y.C., Cho, W.K., Im, G.Y., Jeong, Y.H., Hwang, Y.H., Liang, C., Ma, J.Y., 2012. Anti-inflammatory effect of Lycium Fruit water extract in lipopolysaccharide-stimulated RAW 264.7 macrophage cells. Int. Immunopharmacol. 13(2), 181–189.

6. Vichai, V., Kirtikara, K., 2006. Sulforhodamine B colorimetric assay for cytotoxicity screening. Nat. Protocols. 1(3), 1112–1116.

7. Wang, J.X., Hou, L.F., Yang, Y., Tang, W., Li, Y., Zuo, J.P., 2009. SM905, an artemisinin derivative, inhibited NO and pro-inflammatory cytokine production by suppressing MAPK and NF-[kappa]B pathways in RAW 264.7 macrophages. Acta. Pharmacol. Sin. 30(10), 1428-1435.