Journal of Basic and Applied Pharmacology
Vol. 4 No.2 July - December 2024

Page: [O98-107]

Original research article

Comparison of *in vitro* anti-inflammatory activity of extracts from original Ya-Ha-Rak and adapted formula

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Received 11 September 2024; Received in revised form 26 November 2024
Accepted 26 November 2024; Available online 19 December 2024

ABSTRACT

This study aimed to compare the *in vitro* anti-inflammatory activity of the original Ya-Ha-Rak, and the adapted formula. The original Ya-Ha-*Rak* formula is composed of the roots of *Clerodendrum indicum* (L.) Kuntze, *Capparis micracantha* DC., *Ficus racemosa* L., *Tiliacora triandra* Diels, and *Harrisonia perforata* Merr., whereas the adapted formula is similar to the original formula, except the root of *H. perforata* was replaced with the stem part. The original Ya-Ha-Rak formula, and the adapted formula as well as each plant ingredient, were extracted with 95% ethanol and freeze-dried. The roots of *H. perforata* gave the highest extract yield of 4.26% w/w while that of *H. perforata* stem only gave 1.61% w/w. Dexamethasone was used as the positive control in the measuring of pro-inflammatory cytokines in LPS-stimulated RAW 264.7 cells. Our results demonstrated that the original Ya-Ha-Rak and the adapted formula significantly reduced nitric oxide production with IC_{50} values of 97.38±6.80 and 125.58±9.45 µg/mL respectively, in RAW 264.7 cells after treatment. At a 200 µg/mL dose of both formulas, the release of TNF-α and IL-1β was significantly reduced. The same results were obtained with dexamethasone (40 µM). Interestingly, the 5R extract demonstrated comparable results between PGE2 and dexamethasone. This study implied that the stem of *H. perforata* has the potential to be used in Ya-Ha-Rak provided that the appropriate ratio of the stem ingredient and its biological activities are further evaluated.

Keywords: adapted Ya-Ha-Rak, anti-inflammatory activity, NO, PGE2, TNF-α

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1. Introduction

An antipyretic herbal drug listed in the Thai National List of Essential Medicines is known in Thai as Benjalokawichian (BLW) or Ya-Ha-Rak (5R).[1] It consists of the roots from five plants: *Clerodendrum indicum* (L.) *Kuntze* (CI), *Capparis micracantha* DC. (CM), *Ficus racemosa* L. (FR), *Tiliacora triandra* Diels (TT), and *Harrisonia perforata Merr*. (HP). BLW has been commonly used to reduce fever, which is related to the inflammatory mechanism and has also been utilized on inflamed allergic skin in Thai Traditional Medicine since ancient times.[2] Although BLW (5R) is widely used as an antipyretic by many traditional practitioners in Thailand, limited scientific studies support its efficacy. The antipyretic activity of the BLW formula has been demonstrated using a Baker's yeast-induced fever model in rats[3] and through lipopolysaccharide (LPS)-induced fever in rats compared to that of acetylsalicylic acid (ASA).[4]

Inflammation, triggered by the body's innate immune response to cellular or tissue damage caused by physical, chemical, or biological agents, serves as a protective mechanism.[5,6] It plays a critical role in maintaining normal bodily functions and protecting against external pathogens and internal injuries. However, failure to regulate inflammation can lead to harm to the host organism,[7] contributing to the development of various diseases, such as cancer, rheumatoid arthritis, diabetes, septic shock, and cardiovascular diseases.[8,9] Chronic inflammation is often driven by activated macrophages, which increase the production of inflammatory molecules like nitric oxide (NO), reactive oxygen species (ROS), tumor necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6).[10] In experimental inflammation models, lipopolysaccharide (LPS) is commonly used as an endotoxin. LPS activates signaling pathways within macrophage cells, leading to the activation of nuclear transcription factors such as mitogen-activated protein kinases (MAPKs) and nuclear factor kappa B (NF-κB).[11] Additionally, LPS prompts macrophage cells to release proinflammatory mediators, including TNF-α, IL-6, cyclooxygenase-2 (COX-2), and nitric oxide (NO).[12] Therefore, inhibiting LPS-activated macrophages from releasing cytokines and mediators is a common strategy used to develop and evaluate new anti-inflammatory agents.

A study on the *in vitro* anti-inflammatory activity of BLW extract and its components through NO production inhibition showed that BLW exhibited a higheer anti-inflammatory activity (IC_{50}= 40.36 µg/mL) when compared to its component plants, although it was lower than that of indomethacin (IC_{50}= 20.32 µg/mL).[2] The BLW formula comprises the roots of the plants as mentioned earlier. However, commercial crude drugs are often adulterated with the stems.[13] A study performed on the root extract of HP showed that it could significantly reduce acute inflammation in rat paw edema at 2 hours after carrageenan injection. Moreover, *in vitro* testing in the same study for the mechanism of anti-inflammatory response through the measurement of mRNA expression of pro-inflammatory mediators, TNF-α, IL-β, and IL-6 in the LPS-stimulated macrophage cells, revealed a maximum inhibitory effect of 49.83, 47.27, and 32.16% at concentrations of 50 µg/mL, 50 µg/mL and 12.5 µg/mL, respectively. It was concluded that the root extract of HP exerted anti-inflammatory activity via the suppression of pro-inflammatory cytokines.[14] Therefore, the objectives of this study are to investigate the *in vitro* anti-inflammatory activity of 5R and a preparation that contains HP stems in place of HP root or adapted formula (4R), its plant components (CM, CP, FR, TT, and HP), and the stems of HP. This will be achieved through the measurement of NO production inhibition, pro-inflammatory cytokines (TNF-α, IL-β), and PGE2 production in LPS-stimulated RAW264.7 cells using dexamethasone as a positive control. The results of this study will provide evidence on the potential activity of the HP stem in 5R.

2. Materials and Methods

2.1 Plant Materials

Roots of *Clerodendrum indicum* (L.) Kuntze (Lamiaceae) were collected in Udon Thani Province, Thailand, voucher number PBM 006085. Roots of *Capparis micracantha* DC. (Capparaceae) were collected in Prachuap Kiri Khan Province, Thailand, voucher number PBM 006083. Roots of *Ficus racemosa* L. (Moraceae) were collected in Suphan Buri Province, Thailand, voucher number PBM 00617. Roots of *Tiliacora triandra* (Colebr.) Diels (Menispermaceae) were collected in Phitsanulok Province, Thailand, voucher number PBM 006084. The whole plant of *Harrisonia perforata* (Blanco)*Merr. (Rutaceae)* were collected in Ayutthaya Province, Thailand, voucher number PBM 006172. All plants were identified by Dr. Sunisa Sangvirojanapat and deposited at the Mahidol University Herbarium.

2.2 Preparation of the extracts

The roots of each plant and the stem of *Harrisonia perforata* were dried and pulverized. A 100 g root powder aliquot each of *Clerodendrum indicum* (L.) Kuntze (CI), *Capparis micracantha* DC. (CM), Ficus racemosa L. (FR), *Tiliacora triandra* Diels. (TT), *H. perforata* Merr. (HP) and HP stem (S-HP) was macerated at room temperature with 95% ethanol with an L/S ratio of 10:1. After 7 days of maceration the mixture was filtered and evaporated under vacuum, and the liquid residue was subjected to freeze drying. The Ha-Rak remedy (5R) was prepared with an equal portion of each root ingredient. The 4R remedy was prepared with an equal portion of CI, CM, TT, FR, and S-HP. The powder mixture of 4R was treated as mentioned above.

2.3 Determination of cell viability

The MTT assay was conducted according to published methods[15] with some modifications. The toxicity to cells of two recipe extracts (4R and 5R) and their respective ingredients (CI, CM, FR, TT, HP, and S-HP) on RAW 264.7 macrophages was assessed. The extracts were diluted in dimethyl sulfoxide (DMSO) and added to the medium at various concentrations. The final concentration of DMSO was 0.1 % in each experiment and control. The cells were initially seeded in 96-well plates at a density of 5×10^4 cells/well and then incubated in a humidified atmosphere of 5% CO_2 at 37°C for 24 hours. Then the cells were exposed to 4R extract, 5R extract (25, 50, 100, 200 µg/mL) or their ingredient components CI (25, 50, 100, 200 µg/mL), CM (12.5, 25, 50, 100 µg/mL), FR (100, 200, 400, 600 µg/mL), TT (25, 50, 100, 200 µg/mL), HP (12.5, 25, 50, 100 µg/mL), and S-HP (12.5, 25, 50, 100 µg/mL) for 24 hours. Following the incubation period, the culture medium was removed, and MTT was added, followed by a 3-hour incubation at 37°C. Subsequently, the medium was aspirated, and 100 µl of DMSO was added to each well. Cell viability was then assessed at 550 nm using a microplate reader. Cells treated with 0.1 % DMSO were used as the control, and their viability was set as 100%.

2.4 Measurement of nitric oxide production

Nitrate and nitrite concentrations were assayed using Griess reagent with a nitric oxide assay kit, as described previously.[16] RAW 264.7 cells (5×10^4 cells/well) were plated in 96-well culture plates overnight. Cells were pretreated with the two recipe extracts (4R and 5R) or their respective ingredients (CI, CM, FR, TT, HP, and S-HP) at various concentrations for 1 hour and subsequently treated with LPS (100 ng/mL) for 24 hours. Dexamethasone (40 µM) was utilized as a positive control. After 24 hours, 100 µL of the supernatants were transferred into 96-well plates, and NO production was determined by measuring the accumulation of nitrite in the culture supernatant using the Griess reagent. The amount of nitrite, which is correlated to the amount of NO, was calculated from nitrite standard curves.

2.5 Measurement of TNF-α, IL-1β, and PGE2

RAW 264.7 cells (5×10^4 cells/well) were pretreated with various concentrations

of two recipe extracts (4R and 5R) or their respective ingredients (CI, CM, FR, TT, HP, and S-HP) for 1 hour and then stimulated with LPS (100 ng/mL) for 24 hours. After the incubation period, the culture supernatants were collected, and the levels of TNF-alpha, IL-1β, and Prostaglandin E2 (PGE2), were determined. PGE2 was quantified using a PGE2 ELISA assay Kit (Abcam, UK), while TNF-α and IL-1β were assayed using the Mouse Cytokine Milliplex MAP assay kit (Millipore Corporation, Billerica, MA, USA).

2.6. Statistical analysis

Data were expressed as the mean ± standard deviation (S.D.). Statistical significance was determined by Dunnett's test after one-way ANOVA for comparisons with the LPS group, with $p < 0.05$ considered significant.

3. Results

3.1 The yield of extracts

The results showed that the ethanolic extract of 5R gave the highest yield (4.58%) followed by root extracts of HP, TT, CI, CM, and FR. The stem of HP (S-HP) gave the lowest yield (1.61%) but the ethanolic extract of 4R was ranked fourth (3.98%) (Table 1)

3.2 Effect of two recipe extracts (4R and 5R) and their respective ingredients on RAW 264.7 cell viability

The optimal concentrations of the two recipe extracts (4R and 5R) and their respective ingredients (CI, CM, FR, TT, HP, and S-HP) that did not induce cytotoxicity in RAW 264.7 cells were determined using the MTT assay before conducting the anti-inflammatory assay. The results revealed that concentrations of 12.5, 25, 50, 100, and 200 µg/mL yielded viability percentages greater than 80% (Fig. 1). Furthermore, the highest concentration of 200 µg/mL resulted in viability percentages of 87.30±4.71% for the 4R and 88±4.66% for the 5R extract (Fig. 1G). Similarly, the ingredients CI, CM, FR, TT, HP, and S-HP also demonstrated non-cytotoxicity at all concentrations tested (Fig. 1A-1F). According to a previous study, treatment can be considered non-toxic to RAW 264.7 cells if the cell viability is greater than 80%.[17]

3.3 Effects of two recipe extracts (4R and 5R) and their respective ingredients on LPS-induced NO release using RAW 264.7 cells

Pro-inflammatory mediators, such as NO, are critical in the inflammatory response. In this study, NO production was measured in the culture medium to assess the anti-inflammatory activity of the extracts. LPS-activated RAW 264.7 cells were treated with two recipe extracts and their respective ingredients at various concentrations. As shown in Fig. 2, LPS alone markedly induced NO production in the cells compared to the control group. However, treatment with 4R (Fig. 2G) and 5R (Fig. 2H) extracts significantly reduced LPS-stimulated NO production across all concentrations (25-200 µg/mL) in a concentration-dependent manner. The half-maximal inhibitory concentration (IC_{50}) of 4R and 5R were determined to be 125.58 ± 9.45 µg/mL and 97.38 ± 6.80 µg/mL, respectively. Likewise, the ingredients CI (Fig. 2A), CM (Fig. 2B), and FR (Fig. 2C) also led to significantly reduced nitric oxide production compared to the LPS treatment group. HP (Fig. 2E) and S-HP (Fig. 2F) exhibited significant nitric oxide reduction at 25-100 µg/mL compared with the LPS treatment group; however, TT (Fig. 2D) extract did not result in a significant reduction.

3.4 Effect of two recipe extracts (4R and 5R) on TNF-α and IL-1β Production in LPS-stimulated RAW 264.7 Cells

TNF-α and IL-1β are pro-inflammatory cytokines produced by LPS-activated RAW 264.7 cells. The results indicated that 4R at 200 µg/mL and 5R at both 100 and 200 µg/mL significantly reduced the release of TNF-α compared to the LPS group ($p < 0.05$, Fig. 3A, 3B). Moreover, 4R at 100 and 200 µg/mL as well as 5R at all tested concentrations, significantly decreased the release of IL-1β ($p < 0.05$, Fig. 3C, 3D). The results were

comparable to those of the positive control, Dexamethasone (40 µM). Interestingly, 4R at 200 µg/mL as well as 5R at 100 and 200 µg/mL showed comparable results to the positive control.

Table 1 The percentage yields of ethanolic extracts.

No.	Crude drug	Sample weight (g) n=3	Yield dry weight (%) n=3
1	*Clerodendrum indicum* (L.) Kuntze (CI) (CI)	100	3.150 ±0.342
2	*Capparis micracantha* DC. (CM)	100	2.904 ±0.284
3	*Ficus racemosa* L.(FR)	100	2.205 ±0.262
4	*Tiliacora triandra* (Colebr.) Diels (TT)	100	4.002 ±0.290
5	*Harrisonia perforata* Merr. Roots (HP)	100	4.264 ±0.203
6	*H. perforata* Merr. Stem (SHP)	100	1.612 ±0.221
7	4R (1+2+3+4+6 1:1:1:1:1)	100	3.982 ±0.325
8	5R (1+2+3+4+5 1:1:1:1:1)	100	4.581 ±0.412

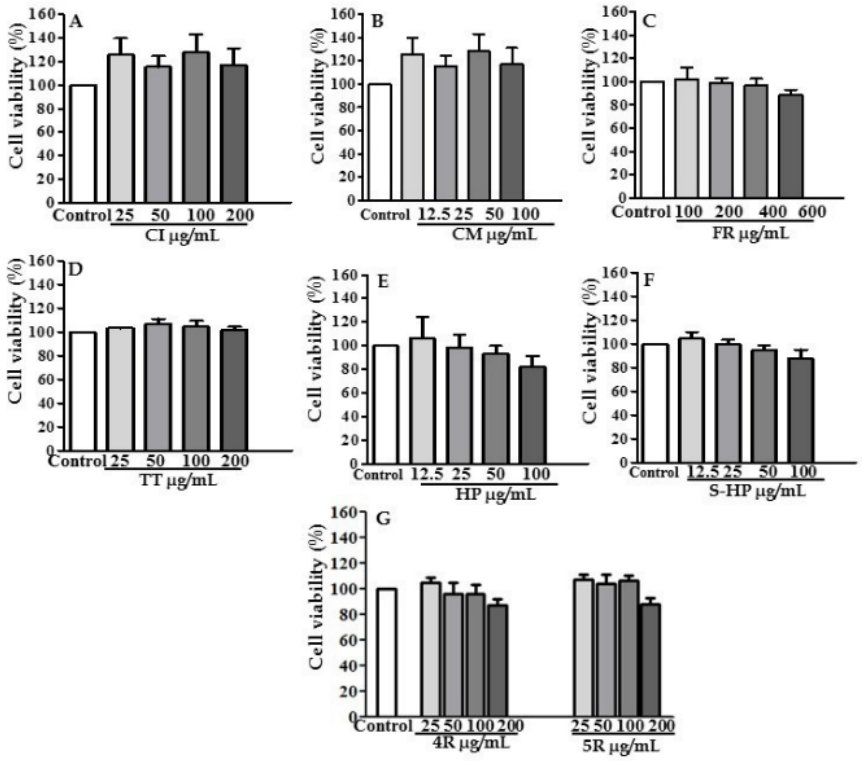

Fig. 1. Cell viability was assessed by the MTT assay in Raw 264.7 cells following exposure to various concentrations of CI extract (A), CM extract (B), FR extract (C), TT extract (D), HP extract (E), S-HP (F) or 4R and 5R extracts (G), for 24 hours. Values represent the mean ± S.D. of three independent experiments, n=3.

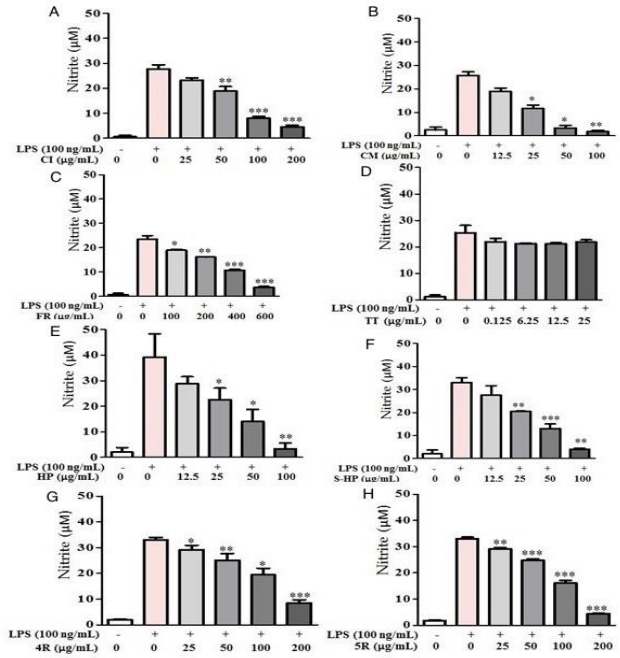

Fig. 2. Effect on NO Production in LPS-stimulated RAW 264.7 cells following exposure to various concentrations of CI extract (A), CM extract (B), FR extract (C), TT extract (D), HP extract (E), S-HP extract (F) or 4R extract (G) and 5R extract (H). Values represent the mean ± S.D. of three independent experiments, n=3. *p < 0.05, **p < 0.01, ***p < 0.001, compared with the LPS group.

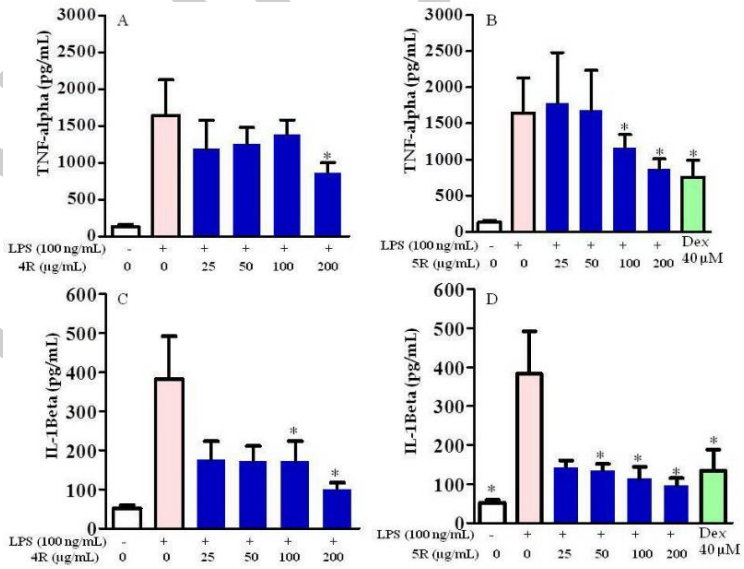

Fig. 3. Effect of 4R and 5R extracts on TNF-α and IL-1β production in LPS stimulated RAW264.7 cells compared with the LPS group. Values represent the mean ± S.D. of three independent experiments, n=3. *p < 0.05 compared with the LPS group.

3.5 Effect of two recipe extracts (4R and 5R) on PGE2 Production in LPS-stimulated RAW264.7 Cells

PGE2 is a pro-inflammatory cytokine produced by LPS-activated RAW 264.7 cells. The results revealed that 4R at 200 µg/mL and 5R at both 100 and 200 µg/mL significantly reduced the release of PGE2 compared to the LPS group ($p < 0.05$). Interestingly, 5R extract showed comparable results to the positive control (Fig. 4B).

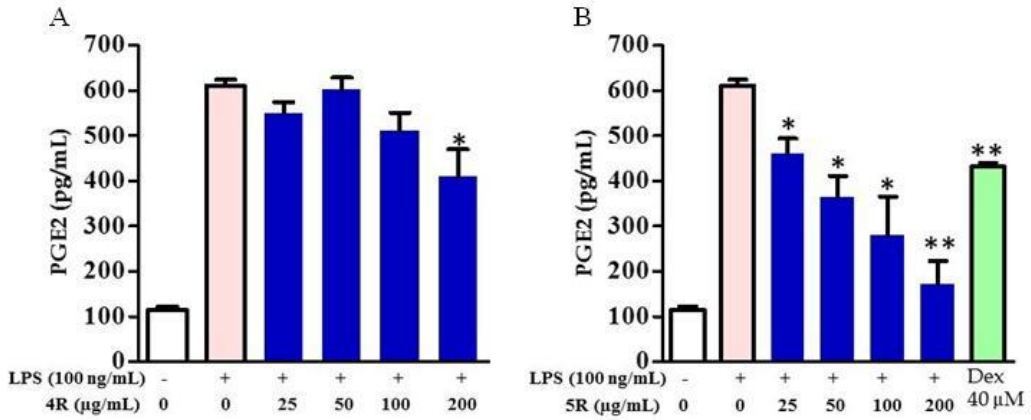

Fig. 4. Effect of 4R (A) and 5R (B) extracts on PGE2 Production in LPS Stimulated RAW 264.7 cells with Dexamethasone (40 µM) as a positive control. Data are expressed as mean ± S.D., n=3. * $p < 0.05$; ** $p < 0.01$ compared with the LPS group.

Table 2 Comparison between IC_{50} (µg/ml) of 4R and 5R (n = 3).

	IC_{50} (µg/ml)			
	4R	5R	HP	S-HP
NO	125.58 ± 9.45	97.38 ± 6.80*	33.53 ± 5.14	38.37 ± 1.96
TNF-α	196.16 ± 23.51	103.96 ± 31.37*		
PGE2	>200	60.09 ± 26.12		

Data are expressed as mean ± S.D. n=3. * $p < 0.05$

4. Discussion

All extracts of Ya-Ha-Rak (5R) ingredients at concentrations of 12.5 – 200 µg/mL were not cytotoxic to cells, particularly the FR extract, which at 400 and 600 µg/mL demonstrated over 80% cell viability. The original 5R remedy extract and the adapted formula (4R), which contained the stem of HP (S-HP) in place of the root extract also gave more than 80% cell viability (Fig. 1). A previous study on stem adulteration in Benchalokawichian (BLW) or Ya-Ha-Rak revealed the presence of stems in all commercial ingredients of BLW.[13] Therefore, in this study, only the authenticated plant materials were used (Table 1). The yield of ethanolic extracts of S-HP was only 1.61% while that of the root (HP) was 4.26%, which is 2.65 times the S-HP yield. This finding suggested that S-HP at the same proportion, could not replace the roots (HP) in Ya-Ha-Rak.

The results of *in vitro* studies on reduced cytokines production (NO, TNF-α, IL-1β, and PGE2) in LPS-stimulated RAW 264.7 cells showed that most extracts (CI,

CM, FR, HP, S-HP) could significantly reduce the release of NO compared with the LPS-treated group, although TT did not show a significant reduction. Interestingly, HP root and stem extracts exhibited similar potency against NO production, the IC_{50} for HP was 33.53 ± 5.14 µg/mL, and for S-HP was 38.37 ± 1.95 µg/mL (Table2). Increasing the proportion of S-HP could possibly yield similar activity. Moreover, both the adapted formula of Ya-Ha-Rak (4R) and the original formula (5R) could significantly reduce NO production with a lower IC_{50} value in 5R (97.38 µg/mL) than in 4R (125.58 µg/mL) (Fig. 2). Similar results for 4R and 5R extracts were observed in the experiments with TNF-α, IL-1β, and PGE2, where the release of cytokines was significantly reduced in a dose-dependent manner. The IC_{50} values of 5R extract against NO and TNF-α production were significantly lower than those of the 4R extract (Table 2). Interestingly, the 5R extract exhibited higher efficacy than 4R in suppression of PGE2 production with an IC_{50} value of 60.09 ± 26.12, but that of the 4R extract could not be determined since increasing the concentration would be toxic to the cells. This could suggest a significant difference in efficacy between 4R and 5R. Prostaglandin E2, a well-known pain mediator abundantly produced in inflamed tissues, contributes to the genesis of inflammatory and neuropathic pain conditions[18,19] thus forming the basis for the extensive clinical application of the inhibitors to treat chronic pain conditions. In addition to this, at a concentration of 200 µg/mL, the 5R extract performed similarly to the positive control, dexamethasone (40 µM). The 5R extract was more active than the 4R extract (Fig. 3 and Fig. 4). This could be explained by the lower extract yield of the stem compared to the root. A previous study revealed TLC fingerprints of the root extract (HP) and the stem extract (S-HP), which demonstrated common components but were present in lower amounts in the stem extract.[13] As stated earlier, the yield of the root extract was 2.65 times that of the stem extract; therefore, when replacing the root with the stem, the proportion should be closer to 2.65 units instead of 1 unit in the adapted formula. Moreover, several studies have been performed on HP stems, demonstrating bioactive phytochemicals. Isolated quassinoids showed anti-Parkinson's disease potential in an *in vitro* study.[20] Two isolated chromones, heteropeucenin-5-methoxy-7-methyl ether and heteropeucenin-7-methyl ether, from the HP stem, showed NO production inhibition in LPS-stimulated RAW 264.7 macrophages.[21] The methanolic extract of the stem also exhibited antimicrobial activity against *Staphylococcus aureus* and *Bacillus subtilis*.[22] This study provides evidence that the stem of *Harrisonia perforata* has the potential to be used in Ya-Ha-Rak provided that the appropriate ratio of the stem ingredient and its biological activities are further evaluated.

5. Conclusion

Both ethanolic extracts of *Harrisonia perforata* stem and root, ingredients in Ya-Ha-Rak, exhibited anti-inflammatory activity as evidenced by the reduction of cytokines released from LPS-stimulated RAW 264.7 macrophages. However, due to the stem extract's lower yield, as compared to that of the root, it is essential to determine the appropriate proportion of the stem extract in Ya-Ha-Rak, as well as its efficacy and safety.

Acknowledgements

This research was partly supported by the Research Fund 2562//2 of the Research and Development Institute, Bansomdej-chaopraya Rajbhat University. The authors also gratefully acknowledged the Center of Excellence for Innovation in Chemistry (PERCH-CIC), Ministry of Higher Education, Science, Research and Innovation is gratefully acknowledged.

Conflicts of Interest

The authors declare no conflict of interest.

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