

Validation of an HPLC method for quantification of anti-inflammatory markers in an ethanolic extract of Sahastara and its anti-inflammatory activity *in vitro*

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Abstract

Background and purpose: Sahastara (SHT) is a traditional Thai medicine for the treatment of musculoskeletal and joint pain. It consists of 21 plant components. A previous study demonstrated the anti-inflammatory activity of SHT on inhibition of nitric oxide production and prostaglandin E2 (PGE₂) production, however, inhibitory effects on tumor necrosis factor-alpha (TNF- α) has not been reported. In this study, we evaluated the anti-inflammatory activity of SHT on inhibitory effects on TNF- α and PGE₂ production and presented an analytical method for validation of SHT.

Experimental approach: Anti-inflammatory activity was evaluated by inhibitory activity on TNF- α and PGE₂ production in RAW264.7 cells. The validated procedure was conducted according to ICH guidelines. The validated parameters were specificity/selectivity, linearity, range, the limit of detection (LOD), and limit of quantitation (LOQ).

Findings/Results: Ethanolic extract of SHT exerted inhibitory activity on PGE₂ production in RAW264.7 cells with IC₅₀ 16.97 \pm 1.16 μ g/mL. *Myristica fragrans* seed extract showed the highest inhibitory activity on PGE₂ production. *Piper retrofractum* extract showed the highest inhibitory activity on TNF- α production. For the HPLC method, all validated parameters complied with standard requirements. Each analyzed peak showed good selectivity with a baseline resolution greater than 1.51. The linearity of all compounds was > 0.999. The % recovery of all compounds was within 98.0-102.0%. The precision of all compounds was less than 2.0% CV.

Conclusion and implications: Ethanolic extracts of SHT possess anti-inflammatory activity by inhibition of TNF- α and PGE₂ production *in vitro*. This study provides support for the traditional use of SHT. The validated results showed good specificity/selectivity, linearity, precision, and accuracy with appropriate LOD and LOQ. This study is the first report on the validation of the HPLC method of SHT for use as quality control of the SHT extract.

Keywords: Anti-inflammatory; HPLC; Method validation; PGE₂; Sahastara; TNF- α .

INTRODUCTION

Inflammation is a complex biological response protecting the body from harmful stimuli. Several cells and mediators are involved in the inflammatory process such as neutrophils, mast cells, and macrophages.

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Proinflammatory cytokines and inflammatory mediators such as nitric oxide (NO), tumor necrosis factor- α (TNF- α), and prostaglandin E₂ (PGE₂) are produced from these inflammatory cells during the inflammatory process. The over functioning of these mediators cause cell and tissue damage leading to muscle pain, and arthritis (1). Non-steroidal anti-inflammatory drugs (NSAIDs) and steroidal drugs are utilized for the treatment of inflammation and pain. Patients with chronic inflammatory diseases such as rheumatoid arthritis and osteoarthritis need to be treated chronically. Long-term administration of steroids and NSAIDs can cause negative adverse events such as bleeding, indigestion, heart problems, and kidney toxicity (2). Some patients select herbal medicines as alternative treatments. In traditional Thai medicine (TTM), several polyherbal remedies have been used for the management of inflammatory diseases such as Sahastara (SHT) and Lom-Am-Ma-Preuk remedies. These TTM are prescribed according to different types of inflammatory diseases diagnosed by traditional Thai physicians (3).

SHT is a TTM categorised in the group of drugs for the treatment of musculoskeletal symptoms in the National List of Essential Medicines of Thailand. The remedy consists of 21 plant components. *Piper nigrum* (fruit), *Plumbago indica* (root), *Terminalia chebula* (fruit), *Baliospermum montanum* (root), *Acorus calamus* (root), *Piper retrofractum* (fruit), and *Kleinhovia hospita* (Bark) are the major ingredients. Each plant ingredient is combined in a different proportion according to Table 1 (4). Previous pharmacological study reported that plant components in SHT remedy exerted anti-inflammatory activity by inhibiting the production of NO and cyclooxygenase 2 (COX-2) activity (IC₅₀ 2.81 and 16.97 μ g/mL) (5). Some plant components in SHT including *Piper nigrum*, *Piper longum*, *Plumbago indica*, *Acorus calamus*, and *Terminalia chebula* may have anti-inflammatory activity (5). The anti-inflammatory mechanism of an ethanolic extract of SHT suggests anti-inflammatory activity *via* TNF- α and PGE₂ production (6).

Table 1. Plant compositions of Sahastara remedy in 1000 g.

Scientific name	Thai name	Voucher specimen	Part used	Weight (g)	Source
<i>Piper nigrum</i> Linn.	Prik-Thai	SKP146161401	Fruit	240	Thailand
<i>Plumbago indica</i> Linn.	Jet-Ta-Mul-Plerng-Dang	SKP148160901	Root	224	Laos
<i>Terminalia chebula</i> Retz.	Sa-Mhor-Thai	SKP049200301	Fruit	104	Thailand
<i>Piper retrofractum</i> Vahl.	Dee-Plee	SKP146160301	Fruit	96	Thailand
<i>Acorus calamus</i> Linn.	Wan-Nam	SKP015010301	Rhizome	88	Thailand
<i>Baliospermum montanum</i> Muell. A.	Tong-Tank	SKP121021301	Root	80	Thailand
<i>Kleinhovia hospita</i> Linn.	Has-Sa-Khun-Tade	SKP183110801	Root	48	Thailand
<i>Cinnamomum camphora</i> Linn.	Ka-Ra-Boon	SKP096030301	Camphor	14	China
<i>Myristica fragrans</i> Houtt.	Dok-Chan	SKP121130601	Aril of seed	13	China
<i>Myristica fragrans</i> Houtt.	Luk-Chan	SKP121130601	Seed	12	China
<i>Lepidium sativum</i> Linn.	Tien-Dang	SKP057121901	Seed	11	India
<i>Anethum graveolens</i> Linn.	Tien-Ta-Tuk-Ka-Tan	SKP199010701	Fruit	10	India
<i>Ferula assafoetida</i> Linn.	Ma-Ha-Hing	SKP199060101	Resin	10	India
<i>Pimpinella anisum</i> Linn.	Tien-Sut-Ta-But	SKP199160101	Fruit	9	China
<i>Cuminum cyminum</i> Linn.	Tien-Khao	SKP199030301	Fruit	8	India
<i>Merremia vitifolia</i> (Burm. f.) Hallier f.	Jing-Jor	SKP054132201	Root	8	Thailand
<i>Nigella sativa</i> Linn.	Tien-Dum	SKP160141901	Seed	7	China
<i>Anacyclus pyrethrum</i> (L.) DC.	Kote-Kag-Kra	SKP051011601	Root	6	China
<i>Adactylis lancea</i> (Thunb) DC.	Kote-Ka-Mao	SKP051011201	Rhizome	5	China
<i>Picrorhiza kurroa</i> Benth.	Kote-Kan-Prao	SKP177161101	Root	4	India
<i>Terminalia chebula</i> Retz. (gall)	Kote-Pung-Pla	SKP019200301	Gall	3	India

With regard to the phytochemical analysis of SHT, a quantitative analysis of chemical markers of SHT extract by HPLC has been reported by Kanokkangsadal and co-workers in a stability study of SHT extract. They determined only piperine which is a major component of *Piper nigrum* and in the SHT extract (7). A previous study by Asasutjarit and co-workers also showed an HPLC method for the determination of piperine in a film-forming solution as a transdermal delivery system (6). A previous study by Nuengchamrong and Ingkaninan reported an LC-MS/DPPH approach for the determination of antioxidative compounds in SHT, in which they analyzed hydrolyzable tannins (8). All the previously published methods have requisite analytical validation data.

Thus, the objectives of this study were to investigate the anti-inflammatory activity by lipopolysaccharide (LPS)-stimulated TNF- α and PGE₂ productions in macrophage RAW264.7 cells and to develop and validate an HPLC method for the determination of anti-inflammatory markers of SHT ethanolic extract. This study supported the traditional medical use of SHT and the validated method can be used as a quality control method for SHT extract and products.

MATERIALS AND METHODS

Plant materials and preparation of SHT extract

All plant materials were purchased from various sources, as shown in Table 1. The voucher specimens were deposited at the Herbarium of Southern Center of Thai Medicinal Plants at the Faculty of Pharmaceutical Science, Prince of Songkhla University, Songkhla province, Thailand and the voucher specimen IDs are included in Table 1. Plant materials were washed, dried, and cut into small pieces. Each plant component was weighed according to proportions shown in Table 1 and mixed homogeneously. The SHT remedy (1000 g) was macerated with 95% ethanol (3 L) for three days. The extract was filtered and evaporated at 45 °C under vacuum to dryness. The residue of SHT was then re-

extracted two more times. All three extracts were combined and evaporated in a vacuum oven at 45 °C to dryness. The percentage yield of SHT was 8.44% w/w of the dried crude extract. The ethanolic extract of SHT was kept at -20 °C until used.

Chemicals and reagents

Gallic acid, ellagic acid, piperine, β -asarone (Merck, Darmstadt, Germany); plumbagin (Sigma-Aldrich, Seelze, Germany); acetonitrile and phosphoric acid (Labscan, Bangkok, Thailand); purified water was prepared by Milli Q[®] system from Millipore (Bedford, MA, USA); murine macrophage leukemia cell line (RAW 264.7: ATCC[®] TIB-71[™]; American Type Culture Collection, VA, USA); Roswell Park Memorial Institute medium 1640 (RPMI-1640), fetal bovine serum (FBS), penicillin-streptomycin (P/S), and 0.5% trypsin-EDTA (Gibco BRL Life Technologies, NY, USA); phosphate buffer saline (PBS; Amresco, Ohio, USA); dimethyl sulfoxide (DMSO; Fluka, Munich, Germany); LPS and 3-(4,5-dimethyl-2-thiazolyl)-2,5 diphenyl-2H-tetrazolium bromide (MTT) (Sigma-Aldrich Inc., MO, USA); PGE₂ EIA kit (monoclonal; Cayman Chemical Company, MI, USA); mouse TNF- α Quantikine ELISA Kit (R&D Systems Inc., MN, USA).

Inhibitory effect on LPS-induced TNF- α production from RAW 264.7 cells

The inhibitory effect of SHT and its plant components on the release of TNF- α from RAW 264.7 cells was evaluated by using mouse TNF- α quantikine ELISA kit according to the method from Makchuchit *et al.* (9) and manufacturer's instructions. In brief, RAW 264.7 cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 10,000 U/mL penicillin, and 10,000 μ g/mL streptomycin. The cells were maintained in an incubator at 37 °C in a humidified atmosphere containing 5% CO₂. The cells were seeded in 96-well plates at a density of 1×10^5 cells/well and incubated in a humidified atmosphere containing 5% CO₂ at 37 °C for 24 h. After that, the medium was replaced with fresh RPMI 1640 medium containing 5 ng/mL of

LPS (100 µL/well) and treated with various concentrations of test samples (100 µL/well) and then the plate was incubated at 37 °C in 5% CO₂ for 24 h. The stock solution of the test sample was prepared by dissolving the crude extract with DMSO to produce a stock solution at a concentration of 10 mg/mL. Then the stock solution was serially diluted by medium to produce a working solution of the test sample (0.2, 2, 20, 60, 100 µg/mL). The final concentration of DMSO in the treated cell was not more than 0.2% v/v. After 24 h incubation, the supernatant (cultured medium) was collected and the concentration of TNF-α was determined according to the instructions of the mouse TNF-α quantikine ELISA kit. The ELISA reaction was measured by a microplate reader (Bio Tek[®] Vermont, USA) at 405 nm. The inhibition (%) of TNF-α production was calculated by the following equation, and concentration causing 50% inhibition effect (IC₅₀) values were calculated from the Prism program, (GraphPad prism 5,0, California, USA).

$$\text{Inhibition (\%)} = \frac{\text{OD (control)} - \text{OD (sample)}}{\text{OD (control)}} \times 100$$

where, OD (sample) stands for optical density of sample; OD (control), optical density of diluent.

The viability of RAW 264.7 cells was determined by the MTT assay. After collecting the supernatant for determining TNF-α, 10 µL of MTT solution (5 mg/mL) was added to each well and incubated at 37 °C, 5% CO₂, for 2 h. After that, the medium was removed and 100 µL of 0.04 M of hydrochloric acid (HCl) in isopropanol was added to dissolve the MTT formazan. The OD of the solution was measured at 570 nm. Cell survival (%) was calculated and demonstrated > 70% viability.

Inhibitory effect on LPS-induced PGE₂ production from RAW 264.7 cell

The inhibition effect of SHT and its plant components on the release of PGE₂ from RAW 264.7 cells was evaluated using a prostaglandin E₂ EIA kit (monoclonal) according to the manufacturer's instructions. In brief, cell culture and sample treatment protocols were conducted similar to the study protocol of

anti-TNF-α described above except for the concentration of LPS. In this assay, the production of PGE₂ from RAW264.7 was induced by LPS at a concentration of 5 µg/mL. The culture medium after sample treatment (0.1, 1, 10, 30 µg/mL) was collected and the amount of PGE₂ production was determined by the PGE₂ EIA kit, Indomethacin was used as a positive control. The inhibition (%) of PGE₂ production was calculated by the following equation, and IC₅₀ values (Table 2) were calculated from the Prism program.

$$\text{Inhibition (\%)} = \frac{\text{OD (sample)} - \text{OD (active)}}{\text{OD (inactive)} - \text{OD (active)}} \times 100$$

where, OD (sample) stands for optical density of sample (treated in LPS-induced cells); OD (active), optical density of diluent (treated in LPS-induced cells); OD(inactive), optical density of diluent (treated in non-LPS-induced cells).

HPLC analysis

The analytical method for the determination of anti-inflammatory markers in SHT ethanolic extract was carried out by using an HPLC system (Shimadzu, Japan) consisting of a quaternary pump (LC20AD), an autosampler (SIL 20ACHT), a column oven (CT20A), and a diode array detector (SPD M20A). All markers were separated along a C18 HPLC column (5 µm, 4.6 mm × 250 mm; Phenomenex, USA) protecting by a C18 guard cartridge (5 µm, 4.6 mm × 3 mm; Phenomenex, USA). The mobile phase consisted of acetonitrile (A) and 0.1% v/v phosphoric acid in water (B). The gradient elution of mobile phase was programmed as the following: 0-20 min, 5% A:95% B; 21-49 min, 5% A:95% B - 50% A:50% B; 50-55 min, 50% A:50% B - 95% A:5% B; 55-60 min 5% A:95% B. The flow rate was set at 1.0 mL/min. The operating temperature was maintained at room temperature. Ten µL of sample and standard solutions were injected into the chromatographic system and the anti-inflammatory markers were detected at a wavelength of 256 nm.

Preparation of standard solutions

The anti-inflammatory markers of SHT extract analyzed in this study were gallic acid,

ellagic acid, plumbagin, piperine and β -asarone. The stock solution of each standard was prepared in methanol at a concentration of 1 mg/mL. The working solutions of the mixed standard were prepared by diluting the stock solution of each standard in the same container and adjusting to the volume with methanol. The range of each compound is shown in Table 3.

Preparation of sample solutions.

The sample solution of ethanolic extract of SHT was prepared by dissolving an accurate amount of crude extract SHT in methanol to produce a sample solution at a concentration of 10 mg/mL of crude extract. The obtained

sample solution was filtered through a 0.45 μ m membrane filter before injection into the HPLC system.

Validation of the HPLC method

Validation of the analytical method for simultaneous quantification of anti-inflammatory markers: gallic acid, ellagic acid, plumbagin, piperine, and β -asarone in ethanolic extract of SHT was conducted according to the International Conference on Harmonization (ICH) guidelines, ICH Q2 (R1) (10). The validated parameters in this study were specificity/selectivity, LOD, LOQ, linearity, range accuracy, and precision.

Table 2. Inhibitory effects (IC₅₀) on tumor necrosis factor-alpha and prostaglandin E2 production from RAW264.7 of the ethanolic extracts of Sahastara and its plant components (n = 3).

Plant name	IC ₅₀ (mean \pm SEM; μ g/mL)	
	Tumor necrosis factor-alpha	Prostaglandin E2
<i>Acorus calamus</i>	> 70	41.82 \pm 2.07
<i>Anethum graveolens</i>	> 100	> 50
<i>Adactylis lancea</i>	19.63 \pm 1.13	> 50
<i>Anacyclus pyrethrum</i>	> 100	> 50
<i>Baliospermum montanum</i>	> 70	> 50
<i>Cuminum cyminum</i>	> 70	44.84 \pm 0.31
<i>Cinnamomum camphora</i>	> 100	> 50
<i>Ferula assafoetida</i>	> 100	> 50
<i>Clausena excavata</i>	> 80	> 50
<i>Lepidium sativum</i>	> 100	> 50
<i>Myristica fragrans</i> (a)	> 50	41.46 \pm 1.06
<i>Myristica fragrans</i> (s)	> 100	16.99 \pm 1.11
<i>Merremia vitifolia</i>	> 100	33.31 \pm 0.82
<i>Nigella sativa</i>	> 100	> 50
<i>Piper retrofractum</i>	15.74 \pm 2.50	23.08 \pm 1.79
<i>Pimpinella anisum</i>	> 100	> 50
<i>Plumbago indica</i>	> 100	31.20 \pm 0.33
<i>Picrorhiza kurroa</i>	> 100	> 50
<i>Piper nigrum</i>	20.74 \pm 0.26	17.70 \pm 1.43
<i>Terminalia chebula</i> (g)	> 80	> 50
<i>Terminalia chebula</i> (s)	> 70	45.82 \pm 0.25
Sahastara remedy	> 50	16.97 \pm 1.16
Indomethacin	143.69 \pm 10.01 μ M. (51.42 μ g/mL)	2.80 \pm 1.20 μ M (1.002 μ g/mL)

Table 3. Linearity, the limit of detection and limit of quantitation of gallic acid, ellagic acid, plumbagin, piperine, and β -asarone.

Compounds	Validation parameters			
	Range (μ g/mL)	Linearity	Limit of detection (μ g/mL)	Limit of quantitation (μ g/mL)
Gallic acid	50-400	Y = 17177X-12428, r ² = 0.9999	3.12	6.25
Ellagic acid	2-20	Y = 116003X-149460, r ² = 0.9997	0.625	1.25
Plumbagin	5-200	Y = 30430x + 57568, r ² = 0.9999	0.78	1.56
Piperine	15-500	Y = 25167x - 25086, r ² = 0.9998	0.90	2.31
β -asarone	5-150	Y = 27527x - 17294, r ² = 0.9991	2.50	5.00

Specificity/selectivity

The specificity of the method was evaluated by comparing the chromatograms obtained from the analysis of sample solution, standard solution, and blank (methanol). The UV spectrum of the peaks in the chromatogram of the sample solution corresponded to the respective peaks of markers in the standard solution. The purity of each peak was determined by comparing the similarity pattern of UV spectrum at peak-start, peak-apex, and peak-end.

Linearity and range

The linearity of the method was performed by the coefficient of determination (r^2) of the linear calibration curves. Each calibration curve of markers was constructed by six concentrations of the working standard solution containing gallic acid, ellagic acid, plumbagin, piperine, and β -asarone. The r^2 of all curves were calculated.

Accuracy

The accuracy of the method performed is defined as the closeness of analytical results to the true value. Working standard solutions containing gallic acid, ellagic acid, plumbagin, piperine, and β -asarone were spiked into the SHT solution at three levels; low, medium and high concentrations. The spiked sample and un-spiked sample were analyzed by the HPLC method. Three replications of the spiked samples were analyzed in an analytical run over three consecutive analytical runs. The accuracy was shown as % recovery.

Precision

The precision of the analytical method performed is defined as the closeness of analytical results obtained from multiple analyses. The precision was conducted by analysis of standard solutions containing gallic acid, ellagic acid, plumbagin, piperine and β -asarone at low, medium, and high concentrations. The intra-run and inter-run precisions were performed as the coefficient of variation (CV) which were calculated from triplicate analyses in a run over three consecutive runs (6 days).

LOD and LOQ of the assay

The LOD and LOQ of the method were determined based on the visualization method. The standard solution was diluted and analyzed using the developed HPLC method. The LOD and LOQ of markers were calculated based on three times and ten times signal-to-noise ratio, respectively.

RESULTS

Anti-inflammatory activity of SHT ethanolic extract on LPS-induced TNF- α and PGE₂ production in RAW264.7 cells.

The ethanolic extracts of SHT and its plant components were evaluated for inhibitory effects on LPS-induced TNF- α and PGE₂ production in RAW264.7 cells compared to a positive control, indomethacin. As shown in Table 2, SHT exerted potent inhibitory activity on PGE₂ production with IC₅₀ of 16.97 ± 1.16 μ g/mL. However, SHT extract showed weak inhibitory activity on TNF- α production with an IC₅₀ of more than 50 μ g/mL.

With regard to plant components of SHT, the extract of seed of *M. fragrans* exerted the highest activity of inhibition of PGE₂ production with IC₅₀ of 16.99 ± 1.11 μ g/mL following by *P. nigrum* (17.70 ± 1.43 μ g/mL), and *P. retrofractum* (23.08 ± 1.79 μ g/mL). All extracts exerted inhibitory activity less than indomethacin. For the inhibitory activity on TNF- α production, *P. retrofractum* showed the highest inhibitory activity with IC₅₀ of 15.74 ± 2.50 μ g/mL following with *A. lancea* (19.63 ± 1.13 μ g/mL) and *P. nigrum* (20.74 ± 0.26 μ g/mL).

Validation of HPLC method

Specificity/selectivity

The analytical method for the simultaneous determination of five anti-inflammatory compounds in SHT ethanol extract was developed and validated. As shown in Fig. 1, the chromatogram of the sample was compared to the chromatogram of standard solution and blank (methanol). The retention time of gallic acid, ellagic acid, plumbagin, piperine, and β -asarone in the sample solution were 10.27, 37.51, 50.18, 50.41, and 50.74 min, respectively.

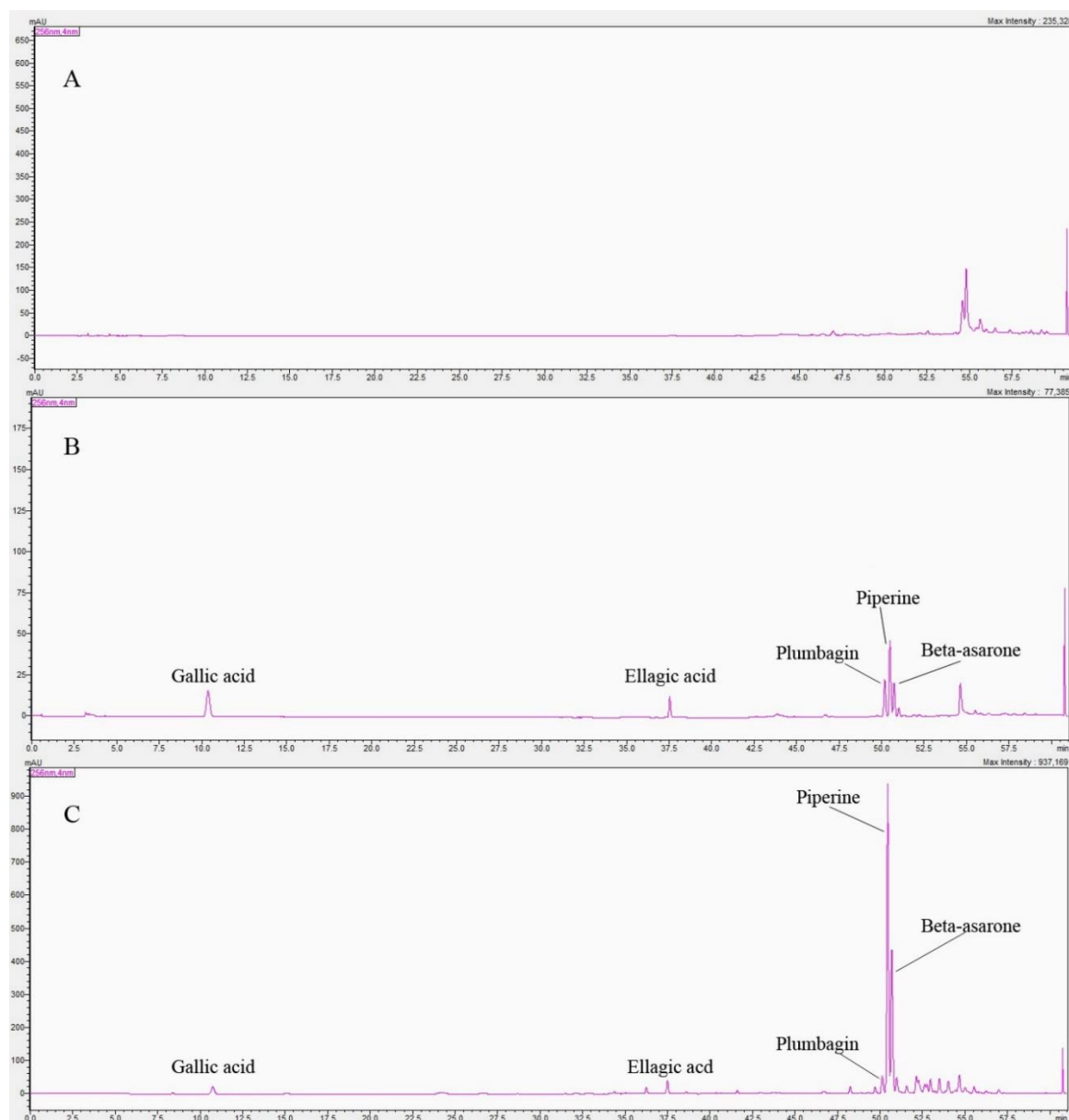


Fig. 1. The HPLC chromatogram of (A) methanol, (B) mixed standard solution, and (C) Sahastara sample solution. X-axis is retention time (min) and Y-axis is intensity (milliabsorbance unit as mAU).

The analyzed peaks in the chromatogram of the sample solution corresponded to the respective standards in the chromatogram of the standard solution. Comparison of each peak in the chromatogram of the sample at peak-start, peak-apex, and peak-end position showed a similar pattern (Fig. 2). These results indicated the specificity and selectivity of this method.

Linearity and ranges

The linearity of the developed method was determined by r^2 calculated from the constructed calibration curves of each marker. As shown in Table 3, the r^2 values of gallic acid,

ellagic acid, plumbagin, piperine, and β -asarone were 0.9999, 0.9997, 0.9999, 0.9998, 0.9991, respectively. The linear range of each marker was varied relating to the contents of the SHT extract and their absorptivity at a wavelength of 256 nm.

LODs and LOQs

LODs and LOQs of the markers were analyzed according to the visualization shown in Table 3. The LODs of gallic acid, ellagic acid, plumbagin, piperine, and β -asarone were 3.12, 0.625, 0.78, 0.9, 2.5 $\mu\text{g/mL}$, respectively. The LOQs were 6.25, 1.25, 1.56, 2.31, 5 $\mu\text{g/mL}$, respectively.

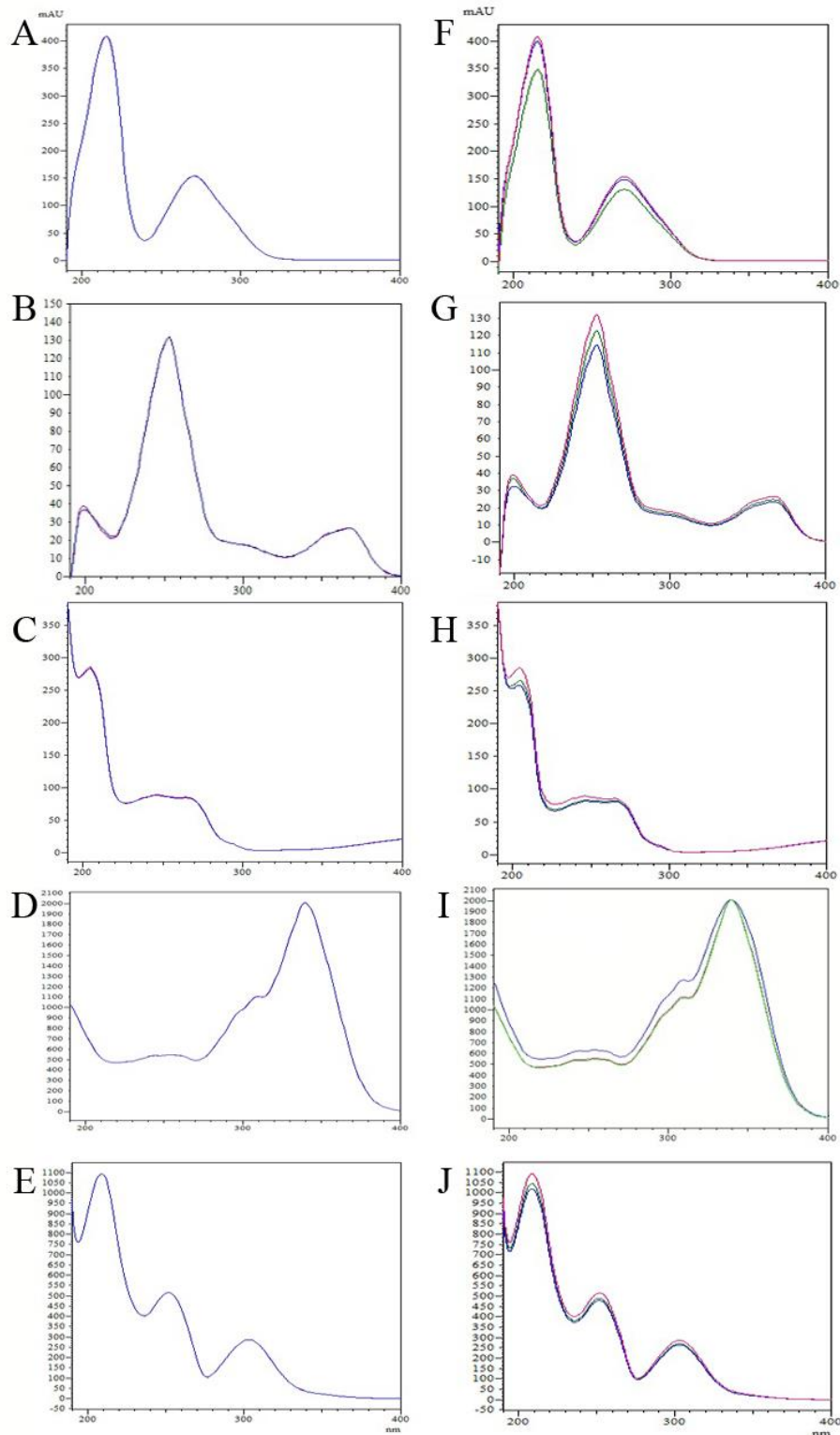


Fig. 2. UV-spectrum of anti-inflammatory markers; (A and F) gallic acid, (B and G) ellagic acid, (C and H) plumbagin, (D and I) piperine, and (E and J) β -asarone. Panels A-E perform a comparative UV-spectrum of each marker's peak in the sample solution to its respective standard. Panels F-J performs a comparative UV-spectrum of each marker in sample solution at peak-start, peak-apex, and peak-end. X-axis is the wavelength (nm) and Y-axis is absorbivity (milliabsorbance unit as mAU).

Table 4. Accuracy of the analytical method for determination of gallic acid, ellagic acid, plumbagin, piperine and β -asarone in SHT extract.

Compounds	Spiked Concentration ($\mu\text{g/mL}$)	Intra-run (n = 3)			Inter-run (n = 9)		
		Concentration found ($\mu\text{g/mL}$; mean \pm SD)	Recovery (%)	CV (%)	Concentration found ($\mu\text{g/mL}$; mean \pm SD)	Recovery (%)	CV (%)
Gallic acid	50	50.65 \pm 0.49		0.96	50.25 \pm 0.47		0.74
	150	150.59 \pm 1.38	99.73% - 101.88%	0.69	150.33 \pm 1.09	99.00% - 101.88%	0.60
	200	201.43 \pm 0.62		0.31	201.33 \pm 1.33		0.63
Ellagic acid	4	4.03 \pm 0.02		0.60	4.03 \pm 0.02		0.09
	12	12.16 \pm 0.08	99.26% - 101.87%	0.68	12.12 \pm 0.08	99.11% - 101.87%	0.56
	16	16.02 \pm 0.18		1.11	16.05 \pm 0.11		0.18
Plumbagin	10	10.05 \pm 0.08		0.75	10.02 \pm 0.08		0.39
	100	100.49 \pm 1.26	99.09% - 101.79%	1.26	100.34 \pm 0.79	99.00% - 101.79%	0.45
	150	150.21 \pm 2.03		1.35	150.45 \pm 1.06		0.18
Piperine	30	30.19 \pm 0.10		0.34	30.19 \pm 0.20		0.19
	150	150.26 \pm 0.90	99.76% - 101.02%	0.60	150.86 \pm 1.02	99.00% - 101.86%	0.49
	250	250.90 \pm 0.62		0.25	251.38 \pm 0.96		0.24
β -asarone	15	14.97 \pm 0.10		0.66	15.03 \pm 0.09		0.37
	50	50.40 \pm 0.37	99.20% - 101.47%	0.74	50.34 \pm 0.23	99.11% - 101.47%	0.25
	100	100.45 \pm 1.12		1.12	100.46 \pm 0.76		0.33

Accuracy and precision

The accuracy of the method was presented as % recovery of each compound shown in Table 4. The % recovery of all standards was within 99.09-101.88%. The precision of the analytical method, both intra- and inter-run, was performed as % CV which was less than 1.35.

Content of anti-inflammatory markers from ethanolic extract of SHT remedy

The ethanolic extract of SHT remedy was analyzed for content using the developed and validated HPLC method. The results found the amount of gallic acid, ellagic acid, plumbagin, piperine, β -asarone were 18.26, 4.50, 11.48, 246.90, 104.30 mg/g, respectively.

DISCUSSION

Inflammation is a complex biological response protecting the body from harmful stimuli. Several cells and mediators are involved in the inflammatory process including pro-inflammatory cytokines, chemokines, and inflammatory immune cells (1). PGE₂ is an important inflammatory mediator produced from the COX pathway. It is the most abundant PGE produced in the body. PGE₂ involves the classical signs of inflammation including redness, swelling, and pain (11). TNF- α is an important pro-inflammatory cytokine expressed during the inflammatory process. It plays an important role in the immune system resulting in the pathophysiology of inflammatory diseases including arthritis and osteoarthritis (2).

SHT is a traditional Thai medicine approved by the Thailand National List of Essential Medicines. It has been indicated for the treatment of muscle and joint pain (4). According to the traditional methods used, in this study, we investigated inhibitory effects of SHT and its components on PGE₂ and TNF- α production in LPS-induced RAW264.7 macrophage cells. The ethanolic extract of SHT exerted anti-inflammatory activity *via* inhibition of PGE₂ production from LPS-induced RAW 264.7 cells and weak activity on inhibition of TNF- α production. This study is the first report of SHT ethanol extract on

in vitro inhibitory effects on PGE₂ and TNF- α production. A previous study reported that the ethanolic extract of SHT exerted anti-inflammatory activity by inhibition of NO production in RAW264.7 cells with an IC₅₀ of 2.56 μ g/mL (4). A clinical study by Pinsornsak *et al.* reported that oral 1000 mg SHT powdered (in capsule preparation), given 3 times daily for 28 days, significantly reduced pain and improved the symptoms of osteoarthritis patients (12). There was no significant change in the blood chemistry of renal and liver functions in SHT treated group but the patients who took diclofenac showed significant increases in their aspartate aminotransferase, alanine transaminase, and alkaline phosphatase (12). These results demonstrated the potential of SHT powder capsules for treating osteoarthritis patients. However, the ethanolic extract of SHT should be studied further for safety and efficacy in animal models prior to commencing clinical trials as an anti-inflammatory therapy in human.

With regard to plant components of SHT, *P. nigrum* and *P. retrofractum* exerted anti-inflammatory activity as measured with both PGE₂ and TNF- α production. *A. lancea* showed inhibitory activity on TNF- α production. *P. nigrum* is the major ingredient of SHT with a proportion of 24% while *P. retrofractum* is 9.6%. For other major components, gallic acid and ellagic acid are the two anti-inflammatory compounds in *Terminalia spp.*(13), plumbagin is the major compound found in *P. indica* (14), piperine in *Piper spp.*(15), and β -asarone in *A. calamus* (16). These compounds have been previously reported for their anti-inflammatory activities. Piperine inhibited the production of interleukin (IL6) and PGE₂ in IL1 β -stimulated fibroblast-like synovio-cytes (17,18). Ellagic acid and gallic acid inhibited the stimulation of COX-2 production in the LPS-induced RAW264.7 cells (19,20). Gallic acid also suppressed the release of IL-6, CCL7, and CXCL8, in IL-31- and IL-33-treated eosinophils-dermal fibroblasts co-culture (21). β -asarone suppressed the production of TNF- α and IL-1 β (22,23). Plumbagin inhibits NF- κ B, resulting in a reduction of TNF- α , IL-1 β , and IL-6, COX-2, and iNOS expression (24). A previous study of Pinkaew and co-worker

reported the clinical efficacy and safety on the treatment of osteoarthritis patients of *Phyllanthus amarus* cream. Gallic acid was the marker of this preparation. The results demonstrated that *Phyllanthus amarus* cream decreased symptoms of knee osteoarthritis as evaluated by the visual analog scale and Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) (25). A previous clinical study by Schell *et al.* demonstrated that freeze-dried strawberry powder as a dietary supplement improved pain and inflammation in obese adults as evaluated by Intermittent and Constant Osteoarthritis Pain (ICOAP). Ellagic acid was a marker of the preparation and its plasma level was monitored (26). A previous clinical study of a traditional Thai medicine named Benjakul on safety and efficacy in osteoarthritis patients demonstrated that Benjakul decreased the visual analog scale pain score and 100-meter walking times. Piperine and plumbagin are the chemical markers of Benjakul remedy (27). Therefore, gallic acid, ellagic acid, plumbagin, piperine, and β -asarone were selected as the markers for anti-inflammatory activities in SHT.

The present study aimed at developing and validating of an HPLC method for the determination of the main anti-inflammatory constituents of an SHT extract. The method can be used as a quality control method for SHT extracts for further use as a traditional drug formulation in patients. However, there is no previous analytical method reported for quality control of these anti-inflammatory markers in SHT. This present study is the first study presenting validation results of an HPLC method for the determination of anti-inflammatory markers; gallic acid, ellagic acid, plumbagin, piperine and β -asarone.

A previous study from Kanokkangsadal and co-workers determined only the content of piperine in SHT ethanol extract (7). Asatjarit *et al.* also determined the content of piperine in film-forming containing SHT (6). A previous study by Nuengchamnon and Ingkaninan analyzed the antioxidant compounds using an on-line LC-MS/DPPH approach which determined the hydrolysable tannins (8). All previous HPLC methods did not report ICH validation results.

An HPLC method for simultaneous quantification of anti-inflammatory markers, gallic acid, ellagic acid, plumbagin, piperine, and β -asarone in SHT extract was developed and validated. In the present study, 0.1% v/v phosphoric acid was used as a component in the mobile phase for suppressing the ionization of phenolic compounds, such as gallic acid and ellagic acid, causing the reduction of peak tailing. The gradient elution was adjusted to separate peaks of piperine and β -asarone to obtain a peak resolution value of more than 1.5. The concentration range of the calibration curve of each marker was varied depending on the content of the markers in SHT extract. All markers were detected at a wavelength of 256 nm. The developed HPLC method showed good specificity for the determination of the anti-inflammatory markers in SHT ethanolic extract. The calibration curves showed good linearity with $r^2 > 0.999$ at the analyzed concentration ranges. The LODs for all markers ranged between 0.625-3.12 $\mu\text{g/mL}$ and the LOQs ranged between 1.25-6.25 $\mu\text{g/mL}$. The accuracy of each marker ranged between 99.55-101.35%. The intra-run and inter-run precisions presented as %CV were less than 1.35%. The method validation showed excellent results that can be used for quality control of the SHT extract.

CONCLUSION

SHT is a traditional Thai medicine for muscle and joint pain. In this study, SHT possessed anti-inflammatory activity as measured by inhibition of PGE₂ production *in vitro*. This study provided proof of anti-inflammatory activity supporting the traditional treatment of SHT. For quality control of the SHT ethanol extract, the developed HPLC method for simultaneous quantification of anti-inflammatory markers was validated. The validated results showed good selectivity, linearity, precision, and accuracy with appropriate LOD and LOQ. This study is the first report of validation of an HPLC method of SHT for use in quality control of the SHT extract and products.

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Conflict of interest statement

The authors declared no conflict of interest in this article.

Authors' contribution

N. Kakatum conducted all the research. A. Itharat as the supervisor of N. Kakatum helped in the scope of research, the idea of the work, and participated in extraction, biological testing, chemical analyses, and interpretation of all data. A. Itharat, head of the Center of Excellence in Applied Thai Traditional Medicine Research (CEATMR), provided all funding and facilities. W. Pipatrattanaseree helped N. Kakatum for validation method of HPLC and writing the HPLC part. P. Kanokkangsadal assisted for training N. Kakatum on biological test. A. Itharat, W. Pipatrattanaseree and N. M. Davies wrote the article. N. M. Davies and A. Itharat helped in interpreting, revising, and editing the article.

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