P-ISSN 2586-9000
E-ISSN 2586-9027
Homepage : https://tci-thaijo.org/index.php/SciTechAsia
Science & Technology Asia
Vol.25 No.3 July - September 2020
Page: [60-67]
Original research article

Validated High Performance Liquid Chromatographic (HPLC) Method for Anti-inflammation Activity of Lom-Am-Ma-Preuk Remedy

Krit Piwngam[1], Puritat Kanokkangsadal[2,4], Weerachai Pipatrattanaseree[3], Rodsarin Yamprasert[2], Chadchom Chookong[2], Buncha Ooraikul[4,5], Arunporn Itharat[2,4]*

[1]*Department of Applied Thai Traditional Medicine Faculty of Medicine, Thammasat University, Pathum Thani 12120, Thailand*
[2]*Department of Applied Thai Traditional Medicine, Faculty of Medicine, Thammasat University, Pathum Thani 12120, Thailand*
[3]*Regional Medical Science Center 12 Songkhla, Department of Medical Sciences, Songkhla 90100, Thailand*
[4]*Center of Excellence on Applied Thai Traditional Medicine Research, Faculty of Medicine, Thammasat University, Pathum Thani 12120, Thailand*
[5] *Department of Agricultural food and Nutritional Science, Faculty of Agricultural Life and Environmental Sciences, University of Alberta, Edmonton T6G2R3, Canada*

Received 25 October 2019; Received in revised form 22 March 2020
Accepted 22 May 2020; Available online 21 September 2020

ABSTRACT

Lom-Am-Ma-Preuk (LAP) remedy is a Thai traditional medicine procedure for muscle pain relief. Active ingredients from LAP remedy are eugenol, myristicin and piperine. These standard markers also have been proven to have anti-inflammatory activity. The aim of this study was to develop a validated HPLC method for determination of markers in the LAP remedy to assess the validation parameters. Results show that the validation parameters were specificity, limit of detection, limit of quantification, linearity, accuracy and precision. The developed method was specific to these markers with the retention times of 45.7, 56.6 and 53.9 min for eugenol, myristicin and piperine, respectively. The coefficient of determination (R^2) was more than 0.999. The accuracy and precision were in an acceptable range with good recovery verified by coefficient of variation of less than 2%. The limits of detection (LOD) of eugenol, myristicin and piperine were 0.5, 0.2 and 1.3 µg/mL and limits of quantitation (LOQ) were 1.9, 0.6 and 5 µg/mL, respectively. Total analysis time was approximately 70 min. The contents of eugenol,

***Corresponding author:** iarunporn@yahoo.com **doi:** 10.14456/scitechasia.2020.35

myristicin and piperine in LAP could be obtained in one analysis for the first time. The present method is useful for routine quality control of the LAP remedy.

Keywords: Method validation; HPLC; Eugenol; Myristicin; Piperine; Lom-Am-Ma-Preuk

1. Introduction

Lom-Am-Ma-Preuk is a type of pathology in the vata system (wind element) which involves muscular, skeletal and nervous systems. According to Thai traditional medicine (TTM) Lom-Am-Ma-Preuk is situated in the middle of the body and circulates in loops from head to toe and toe to head. When there is an imbalance of Lom-Am-Ma-Preuk circulation, the following symptoms occur: hemiplegia, hand and foot drop, and loss of functions of muscular and nervous systems. The remedy for these pathological states in Thai traditional medicine is called the Lom-Am-Ma-Preuk (LAP) remedy [1]. The remedy is composed of ten plants: *Zingiber montanum* (J.Konig) Link ex A. Dietr (ZM), *Curcuma zedoaria* (CZ), *Alpinia galanga* (L.) Wild. (AG), *Allium sativum* L. (AS), *Plumbago indica* L. (PI), *Piper nigrum* Linn. (white Peper) (PN), *Myristica fragrans* Houtt. (MF) both aril and seed, *Cleome viscosa* Linn. (CV), *Erythrina variegate* (EV), and two natural chemicals including sodium chloride (NaCl) and camphor. The LAP remedy has been listed in the Thailand National List of Essential Medicine since 2013 for the treatment of muscle pain and numbness.

There have been many studies on the anti-inflammatory activity of phytochemicals that exhibited positive results, including eugenol, myristicin and piperine. Eugenol, a component of *Alpinia galanga* (L.), decreased inflammatory cytokines (COX-2, TNF-α, and IL-6) in male Swiss albino mice [2]. Myristicin, an active compound in *Myristica fragrans* Houtt. (Nutmeg) inhibited the production of nitric oxide (NO), interleukin (IL)-6, IL-10, and interferon inducible protein-10 in dsRNA induced RAW 264.7 cells [3]. Piperine, a major component of *Piper nigrum* Linn., inhibited the expression of IL-6 and MMP-13 and PGE_2 in fibroblast-like synoviocytes from patients with rheumatoid arthritis [4].

A previous report found the LAP remedy to show good potential for anti-inflammatory activity against NO, PGE_2 and TNF-α [5]. Moreover, there have been many reports on the HPLC validation method. For example, eugenol was reported by Farhin Inam, Sujata Deo and Neha Narkhede [6], myristicin by Nagore and team [7], and piperine by Vipul Upadhyay and team [8]. However, there have been no reports on herbal remedies which contain the three compounds together. Therefore, the aim of this study was to develop an HPLC validation method for the detection of chemical markers present in the LAP remedy. The validation of the assay was studied in terms of specificity, linearity, accuracy, precision, limit of detection, and limit of quantitation. We proposed that these markers be used for anti-inflammation analysis and quality control of the crude extract of the remedy.

2. Materials and Methods

2.1 Plant materials

All plant samples were bought from herbal shops in Bangkok Thailand in 2017. The plant materials were washed and dried at 60˚C and powdered.

2.2 Chemicals and reagents

Standard eugenol and myristicin were purchased from Sigma-aldrich (Seelze, Germany). Piperine was purchased from Merck (Darmstadt, Germany). Acetronitrile and purified water

(HPLC grade) were purchased from Labscan (Bangkok, Thailand).

2.3 Apparatus and chromatographic conditions

The HPLC chromatograph (Agilent, Constametric® 4100 Bio) used in this study was equipped with a PDA ultraviolet-visible detector (Spectromonitor® 4100) and automatic injector (Spectra System AS3500). The reversed-phase column used was the Zorbax eclipse XDB-C18 analytical column (4.60 × 250 mm 5 micron; Agilent Inc., USA). The mobile phase was water-acetonitrile using gradient elution as follows: 0 minute, 95:5; 5 minutes, 95:5; 50 minutes, 50:50; 60 minutes 5:95; 65 minutes, 0:100; 65.10 minutes, 95:5; and 70 minutes, 95:5.The mobile phase was filtered by vacuum through membrane filter size 0.45 µm before use. The flow rate was 1 mL/minute with UV absorbance detection at 210 nm for eugenol and myristicin, and 256 nm for piperine.

2.4 Preparation of Lom-Am-Ma-Preuk extracts.

All ingredients were mixed in equal amounts and macerated with 95% ethanol (3 L) for 3 days and filtered. The mace was re-macerated twice and filtered. The combined filtrates were evaporated to dryness producing 5.14% yield. A 10 mg sample was dissolved in 1 mL of methanol and filtered through 0.45 µm before use.

2.5 Preparation of standard solutions

Stock solutions of eugenol, myristicin and piperine were prepared by dissolving in methanol (concentration 10 mg/mL) and storing at -20˚C.

2.6 Validation of HPLC method

The validation of the analytical method for eugenol, myristicin and piperine from the Lom-Am-Ma-Preuk extract was performed in terms of specificity, linearity, accuracy, and precision, as well as the limit of detection (LOD) and limit of quantitation (LOQ).

2.6.1 Specificity

Standard solutions of eugenol (50 µg/mL), myristicin (200 µg/mL), piperine (900 µg/mL) and the sample solutions of the ethanolic extract of Lom-Am-Ma-Preuk (10 mg/mL) were prepared by dissolving in methanol.

2.6.2 Linearity

The linearity was validated by preparing six concentrations of the standard eugenol, myristicin and piperine solutions. A volume of 10 µl of each concentration was injected into the HPLC column. Triplicate analyses were performed. The standard curve was analyzed using the linear least-squares regression equation derived from the peak area.

2.6.3 Precision

The precision was determined by preparing standard eugenol, myristicin and piperine solutions at three concentrations. Then 10 µL of each concentration was injected into the HPLC column. Concentrations of all markers were calculated with a linear equation of the standard curve. Both intra-day and inter-day precisions were obtained by triplicate analyses. Coefficient of variation (CV) was calculated as standard deviation (SD) to the mean values from the results of triplicate testing which should not be more than 2%.

2.6.4 Accuracy

The standards of eugenol, myristicin and piperine with known concentrations were spiked to the ethanolic extract of Lom-Am-Ma-Preuk, where the contents of eugenol, myristicin and piperine had been previously determined before the addition. Three injections for each concentration were performed per day over three different days (3 injections × 3 concentrations × 3 days).

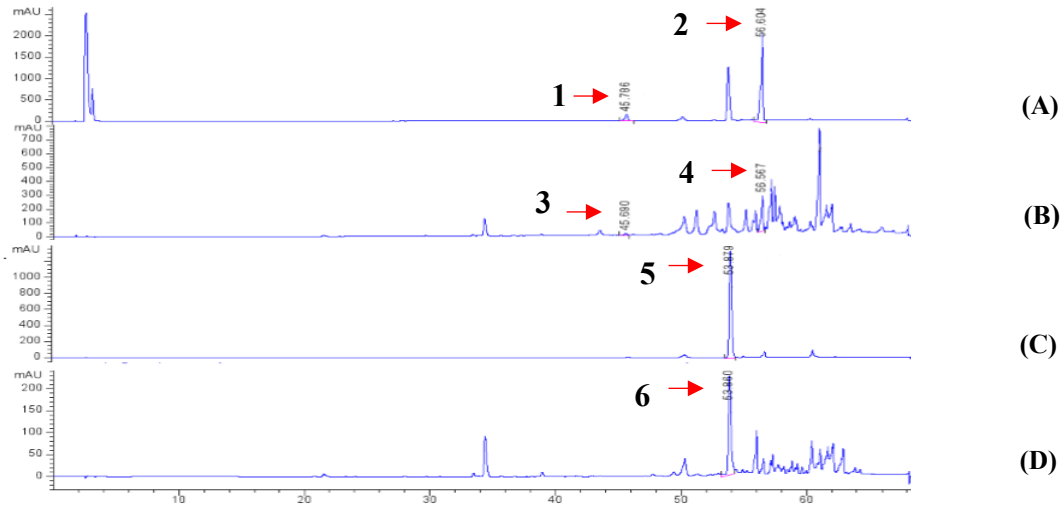

Fig. 1. The HPLC chromatograms of Lom-Am-Ma-Preuk extract and the standard markers. (A) Standard markers at wavelength of 210 nm, 1 eugenol RT= 45.8 min and 2 myristicin RT = 56.6 min. (B) Lom-Am-Ma-Preuk extract at wavelength of 210 nm. 3 eugenol RT= 45.7 min and 4 myristicin RT = 56.6 min. (C) Standard marker at wavelength of 256 nm 5 piperine RT= 53.9 min. (D) Lom-Am-Ma-Preuk extract at wavelength of 256 nm. 6 piperine RT= 53.9 min.

2.6.5 LOD and LOQ

For limit of detection (LOD) and limit of quantitation (LOQ), serial dilutions of eugenol, myristicin and piperine were made with acetonitrile and then analyzed using the HPLC method. LOD and LOQ were obtained as the analyte concentrations, giving the ratio of signal to noise equal to 3 and 10, respectively.

3. Results and Discussion

3.1 Specificity

Eugenol, myristicin and piperine peaks were well separated with retention times (RT) of 45.8, 56.6 and 53.9, respectively (Fig. 1).

3.2 Quantitation parameter

The standard curves of eugenol, myristicin and piperine obtained from six concentrations showed good linearity and coefficient of variation (0.9997-0.9998) (Table 1, Fig. 2)

The limit of detection (LOD) is the lowest concentration of the marker to be detected by the analytical method and limit of quantitative (LOQ) is the lowest concentration of the marker to be determined by the analytical method. The results of LOD and LOQ for eugenol were found to be 0.5 and 1.9 µg/mL, respectively; those for myristicin were 0.2 and 0.7 µg/mL, respectively. The LOD and LOQ of piperine were 1.3 and 5 µg/mL, respectively. The results indicate that the analytical method for eugenol, myristicin and piperine exhibit good sensitivity for UV- visible detector (Table 1).

3.3 Accuracy and precision

The accuracy of the method is acceptable when the results shows 100% ±5 % recovery. The results showed that mean values for each coefficient of variations did not exceed the recommended values (Table 2).

Table 1. Validation of linearity, range, LOD and LOQ of markers.

Standard markers	Validation parameter			
	Linearity[a]	Range (µg/mL)	LOD[b] (µg/mL)	LOQ[c] (µg/mL)
Eugenol	y = 53.33x-25.99; r^2 = 0.9998	5-50	0.5	1.9
Myristicin	y = 139.02x-790.75; r^2 = 0.9997	40-200	0.2	0.7
Piperine	y = 22.93x-903.99; r^2 = 0.9997	200-900	1.3	5

[a] Y=AX +B, where Y is peak area, X is the concentration of the analyzed sample.
[b] Limit of detection (LOD):signal to noise ratio=3
[c] Limit of quantitation (LOQ):signal tonoise ratio=10

Table 2. Accuracy analysis.

Standard markers	Spiked Level (µg/mL)	Recovery %[a]			Mean	CV %[b]
		1	2	3		
Eugenol	5	100.7±1.6	100.81.1	99.3±0.7	100.2	0.6
	15	102.8±0.5	99.5±0.5	100.6±0.2	100.9	1.2
	30	99.2±0.2	101.9±0.7	100.1±1.1	100.4	0.9
Myristicin	40	99.8±0.7	102.1±0.6	100.9±0.9	100.9	0.8
	80	97.7±0.6	101.9±0.5	102.7±0.5	100.8	1.9
	120	103.4±0.5	102.6±0.9	96.8±0.3	101.7	1.6
Piperine	200	101.4±0.6	103.3±0.3	99.4±0.1	101.4	1.4
	300	101.9±0.8	102.4±0.7	102.4±0.2	102.2	0.2
	500	102.1±1.3	102.5±0.5	100.2±0.1	101.6	0.9

[a] All values are mean ± SD as obtained by triplicate analyses.
[b] Coefficient of variation = SD/mean x 100%

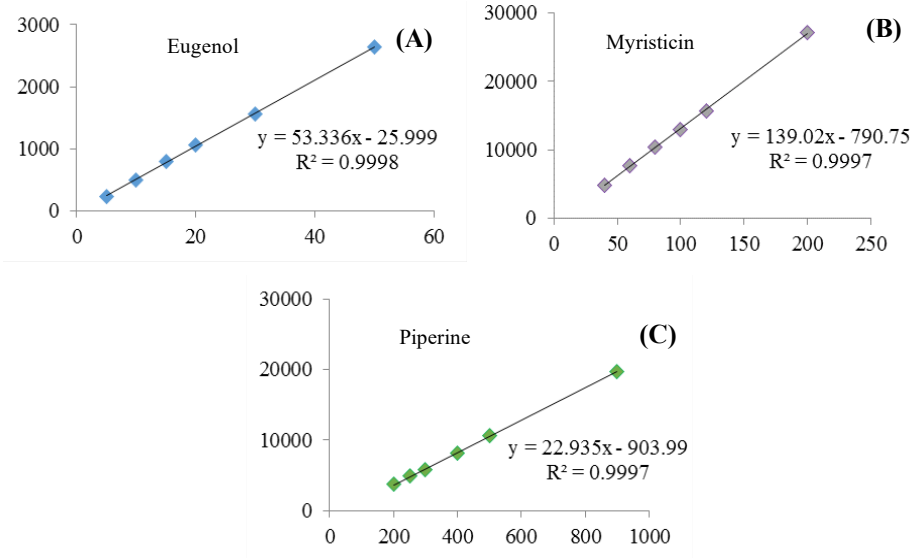

Fig. 2. Linearity of standard markers (A) Eugenol. (B) Myristicin. (C) Piperine.

Table 3. Precision analysis.

Standard markers	Spiked concentration (µg/mL)	Intra-day (n=3)		Inter-day (n=9)	
		Concentration found (µg/mL; Mean±SD)	%CV[a]	Concentration found (µg/mL; Mean±SD)	%CV[a]
Eugenol	5	5.0 ± 0.0	0.6	5.0 ± 0.1	0.8
	15	15.4 ± 0.1	0.8	15.3 ± 0.1	0.9
	30	29.7 ± 0.3	0.9	30.1 ± 0.4	1.
Myristicin	40	39.9 ± 0.4	0.9	40.4 ± 0.4	1.0
	80	78.2 ± 0.5	0.6	81.5 ± 0.6	0.7
	120	124.0 ± 0.1	0.1	123.4 ± 0.7	0.6
Piperine	200	202.8 ± 1.7	0.8	204.3 ± 2.4	1.2
	350	305.1 ± 3.0	1.0	306.7 ± 1.4	0.4
	500	509.8 ± 8.1	1.6	507.9 ± 6.5	1.3

[a] Coefficient of variation=SD/mean x100%.

Table 4. Contents of standard markers of Lom-Am-Ma-Preuk extract.

Extract	Content of markers (mg/g extract) *		
	Eugenol	Myristicin	Piperine
Lom-Am-Ma-Preuk	3.5±0.6	20.7±2.7	175.8±3.3

(*) All values are mean ± SD as obtained by triplicate analysis.

Precision of the method was determined in triplicate analyses. The results of inter-day and intra-day precision for eugenol, myristicin and piperine showed less than 2% coefficient of variations. (Table 3)

3.4 Content determination of marker standards in Lom-Am-Ma-Preuk extract

The ethanolic extract of Lom-Am-Ma-Preuk was analyzed for the contents of eugenol, myristicin and piperine using the validated HPLC method. The contents of eugenol, myristicin and piperine were 3.5±0.6, 20.7±2.7, and 175.8±3.3 (mg/g extract), respectively (Table 4).

4. Conclusion

Drug regulatory agencies like the World Health Organization [9], European Medicines Agency [10], and the International Conference on Harmonization (ICH) [11] have defined the guidelines for maintaining quality, safety, and efficacy of herbal products. The chemical fingerprints and marker compounds are parameters used for the quality control of markers in herbs. Currently, the study on HPLC validation to detect chemical compounds has begun in herbal remedies such as the Benjakul [12] , the Trikatuk [13] and the Benjalokawichien remedy [14]. This is the first report of validated high-performance liquid chromatography (HPLC) method for the Lom-Am-Ma-Preuk remedy. The method can be used to determine three markers (eugenol, myristicin and piperine) for anti-inflammation, standardization and quality control of Lom-Am-Ma-Preuk remedy in one analysis. The method could be used for routine quality control of Lom-Am-Ma-Preuk in terms of anti-inflammatory activity.

Acknowledgements

The project was supported by the National Research University Project of Thailand Office of Higher Education Commission, Center of Excellence in Applied Thai Traditional Medicine Research (CEATMR) and Faculty of Medicine, Thammasat University, Thailand. Assoc. Prof. Dr. Omboon Vallisuta was instructive in writing this manuscript.

References

[1] Foundation of Thai Traditional Medicine and Ayuvedathamrong school. Tamrakarnphaetthaideim (Phaetthayasat songkhro conservation issue) vol.1. Bangkok : Supawanichkarnpimm. (in Thai); 2007

[2] X.Huang, Y. Liu, Y. Lu, C. Ma. Anti-inflammatory effects of eugenol on lipopolysaccharide-induced inflammatory reaction in acute lung injury via regulation inflammation and redox status. Int Immunopharmacol. 2015;26(1):265-7.

[3] Lee JY, Park W. Anti-inflammatory effect of Myristicin on RAW 264.7 Macrophages Stimulated with Polyinosinic-Polycytidylic Acid. Molecules. 2011;16(8):7132-42

[4] Bang JS, Oh DH, Choi HM, Sur BJ, Lim SJ, Kim JY, et al. Anti-inflammatory and antiarthritic effects of piperine in human interleukin 1beta-stimulated fibroblast-like synoviocytes and in rat arthritis models. Arthritis Res Ther. 2009;11(2):R49.

[5] Thongmee P. Anti-inflammatory and antioxidant activity of Thai traditional remedy for stroke called Lom-Am-Ma-Pruek and its plant ingredients [dissertation]. Faculty of Medicine: Thammasat University; 2015.

[6] Inam F, Deo SU, Narkhede NE. HPLC–UV method development and quantification of eugenol from methanolic extracts of some spices. Int J Chem Phys Sci. 2014 Nov;3:96-102.

[7] Nagore DH, Kuber V, Patil P, Deshmukh T. Simultaneous assessment and validation of reverse phase-high performance liquid chromatography method for quercetin, eugenol, myristicin, and safrole from nutmeg, fruit and mace. Chronicles of Young Scientists. 2013 Jan 1;4(1):9.

[8] Upadhyay V, Sharma N, Joshi HM, Malik A, Mishra M, Singh BP, Tripathi S. Development and validation of rapid RP-HPLC method for estimation of piperine in Piper nigrum L. Int. J. Herb. Med. 2013;1(4):6-9.

[9] Schwertner HA, Rios DC. High-performance liquid chromatographic analysis of 6-gingerol, 8-gingerol, 10-gingerol, and 6-shogaol in ginger-containing dietary supplements, spices, teas, and beverages. J Chromatogr B Analyt Technol Biomed Life Sci. 2007;856(1):41-7.

[10] European Medicines Agency [Internet]. London: The european medicines agency; c1995-2018. [cited 2018 Jan 27] Available from: https://www.ema.europa.eu/documents/scientific-guideline/guideline-quality-herbal-medicinal-products-traditional-herbal-medicinal-products_en.pdf

[11] Sachan AK, Kumar A. Stability testing of herbal products. J Chem Pharm Res. 2015;7(12):511-4.

[12] Itharat A, Sakpakdeejaroen I. Determination of cytotoxic compounds of Thai traditional medicine called Benjakul using HPLC. J Med Assoc Thai. 2010 Dec;93 Suppl 7:S198-203.

[13] Pattanacharoenchai N. Anti-allergic activity of Trikatuk, Triphala and Trisarn remedy [dissertation]. Faculty of Medicine: Thammasat University; 2016.

[14] Sakpakdeejaroen I, Juckmeta T, Itharat A. Development and validation of RP-HPLC method to determine anti-allergic compound in Thai traditional remedy called Benjalokawichien. J Med Assoc Thai. 2014 Aug;97 Suppl 8:S76-80.