Pharmacogn J. 2020; 12(3): 611-618
A Multifaceted Journal in the field of Natural Products and Pharmacognosy
www.phcogj.com

Research Article

Development and Validation of Stability Indicating HPLC Method for Determination of Caffeic Acid, Vitexin and Rosmarinic Acid in *Thunbergia laurifolia* Leaf Extract

Nanthakarn Woottisin[1], Sumet Kongkiatpaiboon[2], Sophida Sukprasert[1,3,*], Korbtham Sathirakul[4]

ABSTRACT

Thunbergia laurifolia has been a popular herb used in Thai traditional medicine for detoxification and as antipyretic. It contains rosmarinic acid (RA), caffeic acid (CA) and vitexin as major compounds. In order to control the herbal quality, the stability indicating high-performance liquid chromatography (HPLC) was developed and validated. The stability study of compounds in *T. laurifolia* leaf extract was investigated. The chromatographic separation was performed using a reversed-phase C18 column and mobile phase consisted of 0.5% acetic acid and methanol using a gradient elution with 1.0 mL/min flow rate. The detection wavelength was set at 330 nm. The method was validated for its linearity, precision, accuracy, limit of detection and limit of quantitation. Forced degradation of three compounds in extract showed that they were stable in oxidative condition, but highly labile under alkaline hydrolytic conditions. All three compounds in *T. laurifolia* leaf extract were stable at room temperature at least 3 months while a remarkable decrease of RA, vitexin and CA in the extract were found in accelerated condition. This finding could be applied for predicting the storage recommendation and expiry of *T. laurifolia* extract and its related pharmaceutical products.

Key Words: Force degradation, Phenolic compound, Phytochemical screening, Stability-indicating method.

Nanthakarn Woottisin[1], Sumet Kongkiatpaiboon[2], Sophida Sukprasert[1,3,*], Korbtham Sathirakul[4]

[1]Division of Integrative Medicine, Chulabhorn International College of Medicine, Thammasat University (Rangsit Campus), Pathum Thani 12120, THAILAND.
[2]Drug Discovery and Development Center, Office of Advanced Science and Technology, Thammasat University (Rangsit Campus), Pathum Thani 12120, THAILAND.
[3]Protein and Proteomics Research Center for Commercial and Industrial Purposes (ProCCI), Khon Kaen University, Khon Kaen 40002, THAILAND.
[4]Department of Pharmacy, Faculty of Pharmacy, Mahidol University, Bangkok 10400, THAILAND.

Correspondence

Sophida Sukprasert

Division of Integrative Medicine, Chulabhorn International College of Medicine, Thammasat University (Rangsit Campus), Pathum Thani 12120, THAILAND; Protein and Proteomics Research Center for Commercial and Industrial Purposes (ProCCI), Khon Kaen University, Khon Kaen 40002, THAILAND.

Phone no: +66-2-564-4440 ext. 4454;

Fax: +66-2-986-9213-9 ext. 7589

E-mail: sophida@staff.tu.ac.th

History

- Submission Date: 03-02-2020;
- Review completed: 22-02-2020;
- Accepted Date: 16-03-2020.

DOI : 10.5530/pj.2020.12.91

Article Available online

http://www.phcogj.com/v12/i3

INTRODUCTION

Thunbergia laurifolia Lindl. (TL) (Family: Acanthaceae), vernacularly named "Rang Chuet" or laurel clock vine, is a fast-growing and popular herb in the tropics.[1] It has been widely used as detoxification and antipyretic drug in Thai traditional medicine for centuries[2] and also included in *"Thailand National List of Essential Medicines"*.[3] Modern pharmacological experiments revealed that the TL possessed antioxidant,[4] anti-inflammatory,[5] hepatoprotective,[6,7] antidote,[8,9] anti-tumor[10] and anti-hyperglycemic activities.[11]

Previous studies of TL reported that rosmarinic acid (RA) is the major anti-oxidative constituent[12] together with its derivatives caffeic acid (CA).[13] Vitexin, an apigenin flavone glucoside was also reported in TL leaf extract.[14] Moreover, HPLC comparison demonstrated that TL extract consisted of pheophorbide a, lutein, chlorophyll b, chlorophyll a, pheophytin b, pheophytin b', pheophytin a, and pheophytin a'.[15]

The decoction of TL is frequently used in traditional medicine. Herbal teas, powders and capsule preparations of TL are commonly available in the herbal and nutraceutical markets.[12] However, the quality control and chemical stability are of serious concern as they affect the safety and efficacy in drug product. It is a mandatory to perform stability studies as an important part of the drug development process.[16] Although a HPLC analytical method has been reported for the quantification of some phytochemical compounds in TL leaves,[13] a fast and simple analytical method for stability study is needed. Therefore, an analytical method was developed for determination of RA, CA and vitexin in TL leaf extract using high performance liquid chromatography coupled with diode array detector (HPLC-DAD) in this study. Stress testing was carried out to demonstrate the specificity of the method. Factors relating the chemical stability were described. Our study could be benefit for predicting the shelf-life of TL extract product.

MATERIALS AND METHODS

Chemicals and reagents

HPLC grade methanol (Fisher Scientific, UK), deionized water (DI) purified by Milli-Q water purification system (Adrona SIA, Latvia), sodium dihydrogen orthophosphate (Loba Chemie, India), phosphoric acid, hydrochloric acid and hydrogen peroxide (Fisher Scientific, UK) were used. CA, RA and vitexin (purify ≥ 98%) (Sigma, St. Louis, MO, USA) were used as HPLC standards. All reagents were of analytical grade.

Plant materials

The mature leaves of TL were collected from a cultivated area in Yasothon Province, Thailand (from June to July 2018). The plant was identified and voucher specimen deposited at Bangkok Herbarium Office, Department of Agriculture, Ministry of Agriculture and Cooperatives, Bangkok, Thailand

Cite this article: Woottisin N, Kongkiatpaiboon S, Sukprasert S, Sathirakul K. Development and Validation of Stability Indicating HPLC Method for Determination of Caffeic Acid, Vitexin and Rosmarinic Acid in *Thunbergia laurifolia* Leaf Extract. Pharmacogn J. 2020;12(3):611-8.

(BK. No. 069396). Leaves were cleaned and dried in green house solar dryer at 40-50 °C for 5 days, then they were ground into coarse powder, kept in sealed containers and protected from light until used.

Sample extraction

The preparation of TL aqueous extract was modified according to previous report.[17] The dried leaf powder was boiled with distilled water (1:20, w/v) for 15 min. The extraction was carried out three times. The solution was combined, filtered, and dried with a spray dryer. The extract was stored in a tight container protected from light at -20 °C until used.

Sample preparation

Reference standard solutions

Stock solutions of caffeic acid, vitexin and rosmarinic acid were prepared by accurately weighing and dissolving with 50% methanol to obtain the final concentration of 1000 µg/mL. Working solution of standard compounds were obtained by diluting the stock standard solutions to achieve the desired concentrations with 50% methanol.

Sample solution

Sample solutions of TL leaf extract was prepared by accurately weighing and dissolving with 50% methanol at the concentration of 2 mg/mL. Each sample was prepared in triplicate. Prior to injection, each sample was filtered through a 0.22 µm nylon membrane.

HPLC apparatus and chromatographic conditions

The experiment was performed on an Agilent 1260 HPLC system (Agilent Technologies, USA) equipped with a 1260 Quat pump VL quaternary pump, 1260 ALS autosampler, 1260 TCC column thermostat, and 1260 DAD VL. The chromatographic separation was achieved on a BDS Hypersil™ C18 column (4.6×100 mm, 3 µm) (Thermo Scientific™, Massachusetts, USA). The mobile phases consisted of (A) 0.5% acetic acid in water and (B) methanol with a gradient elution as follow: Initial solvent proportion of 75:25 A:B with a linear gradient to 35:65 A:B in 15 min was used, followed by 100% B for 20 min. A constant flow rate of 1.0 mL/min was employed throughout the analysis with the controlled temperature at 25ºC. The DAD detection wavelength was set at 330 nm and injection volume was 10 µL.

Degradation studies (Stress Testing)

Five stress conditions, included acid and base hydrolytic, oxidative, photolytic and thermal conditions, were performed on TL extract according to the procedure described by Kongkiatpaiboon *et. al*[18] for the degradation studies.

Acid, base and oxidative studies were done by adding 50 µL of different reagents to 1 mL of TL sample. Concentrated hydrochloric acid (37% w/w), 5 N sodium hydroxide, and hydrogen peroxide (30% w/w) were used as reagent for acid and base hydrolysis, and oxidative stress, respectively. Deionized water was used as control solvent. All spiked solutions were incubated at 60 °C for 60 min.

For photolytic and thermal studies were done by spiking the deionized water. Then, photolytic stress was exposed to light (4500 Lux) for 72 h, whereas the thermal stress was exposed to heat chamber at 80 °C for 72 h.

Each sample was then analyzed with the proposed HPLC method. The peak purity of stressed samples was monitored by DAD in the wavelength range of 200–400 nm. All stress studies were performed in triplicate and % degradation of active compound was calculated.

Method validation

Linearity, precision, accuracy, limit of detection (LOD), and limit of quantitation (LOQ) were validated according to the International Conference on Harmonization (ICH) guidelines.[19]

Linearity

Linearity was determined by using working standard solutions of CA, vitexin and RA at series of concentrations (1-250 µg/mL). Each concentration was analyzed in triplicate. The calibration curves were obtained by plotting the peak area versus the concentration of each standard. Data were evaluated for correlation coefficients (r^2) using linear regression method.

Precision

The intra-day precision was determined by analyzing standard solution containing 100 µg/mL solution of CA, vitexin and RA seven times within one day, while, the inter-day precision was examined for three consecutive days by the proposed method. The precision was expressed as percent relative standard deviation (% RSD).

Accuracy

Recovery was used to evaluate the accuracy of the method. Standard addition was performed with the pre-analyzed standard solution. The three concentration levels of CA, vitexin and RA standard mixture (approximately 50%, 100% and 150% of the determined content of TL leaf extract) were added into working sample solutions. Spiked samples were prepared in triplicate and three determinations were performed in each level. The recovery was calculated as follows:

Recovery (%) = [(observed amount – original amount)/spiked amount] x 100

Limit of detection (LOD) and limit of quantitation (LOQ)

LOD and LOQ under the proposed chromatographic conditions were determined by diluting the working standard solution at the lowest concentration to obtain the signal-to-noise ratios (S/N) of analytes at 3:1 and 10:1, for LOD and LOQ, respectively.

Stability testing

The extract kept in high-density polyethylene (HDPE) solid plastic bottle with screw cap was exposed to two different storage conditions consisting of at room temperature and accelerated condition at 40°C ± 2°C/75% RH ± 5% for 3 months. The samples were obtained at 0, 1st, 2nd and 3rd month. The contents of CA, vitexin and RA in the TL extract were analyzed in triplicate by the proposed HPLC method.

RESULTS AND DISCUSSION

A stability-indicating HPLC method was developed for analyzing CA, vitexin and RA in the TL leaf extract. This method was modified from previous reports.[12,13,20] Optimal condition was achieved with mobile phase mixture of 0.5% acetic acid in water and method using gradient system, which was acetonitrile-free system and has resolution of 6.66, 4.67 and 6.81 for caffeic acid, vitexin and rosmarinic acid, respectively, which could be accepted resolution[21] of all target analytes. The method has been validated and confirmed that it is suitable for intended use. The maximum absorbance of compounds at wavelength at 330 nm was used.

Validation of the method has been performed according to the ICH guideline.[19] The method validation parameters were linearity, precision, accuracy, LOD and LOQ. Specificity of the method was assessed by peak purity using UV spectrum obtained from DAD. The calibration curves were constructed from the peak area versus the concentration

of the standards and showed good linear regressions (all correlation coefficients > 0.999) within the ranges of concentration 1-250 µg/mL. The LOD and the LOQ were less than 0.01 and 0.025 µg/mL for CA and RA, and 0.03 and 0.1 µg/mL for vitexin, respectively (Table 1). Method precision revealed that the %RSD values of the three compounds for intra-day ranged from 0.19 to 0.90%, and for inter-day precision was 0.17% to 0.33% (Table 2), indicating high precision of method. The accuracy of the method represented by the recovery study was shown in Table 3. Adequate recoveries of the three compounds were obtained in the range of 98.77% to 106.74%. Average percentage recovery of CA, vitexin and RA was 103.16, 100.62 and 104.93, respectively, suggesting that the method to be accurate and suitable for intended use. The chemical profile of the 2 mg/mL TL leaf extract and 100 µg/mL reference standards were shown in Figure 1(A) and Figure 1(B), respectively. The chromatogram showed the three compounds from the extract identified as CA, vitexin and RA at retention time (t_R) of 3.9, 7.6 and 10.1 min, respectively.

Stress testing was carried out to demonstrate specificity of the developed method to evaluate the changes in concentration of CA, vitexin and RA in *T. laurifolia* extract. In order to determine the specificity of the method, peak purity analysis was done on line by using diode array detection. Chromatographic profiles of CA, vitexin and RA in *T. laurifolia* extract degradation are shown in Figure 2. Acid (Figure 2A) and base (Figure 2B), oxidative (Figure 2C), photolytic (Figure 2D) and thermal (Figure 2E) conditions were clearly demonstrated. The developed method could separate the potential degradation products from CA, vitexin and RA peaks. The proposed HPLC method was applied for quantitative analysis of the content of CA, vitexin and RA in *T. laurifolia* extract under various stress conditions as shown in Table 4.

Degradation of drug substances between 5% and 20% has been considered as reasonable and accepted for validation of chromatographic assay. Some pharmaceutical scientists think 10% degradation is optimal for use in analytical validation for small pharmaceutical molecules, which have 90% acceptable stability limits for label claims.[16] As shown in Table 4, vitexin and CA was stable in acidic hydrolysis, while, RA were degraded up to 22.71%. The rate of hydrolysis in acid was slower as compared to that of alkali. CA, vitexin and RA were found to be highly labile under alkaline hydrolysis which were degraded up to 85.67, 65.29 and 94.07%, respectively. Our result was appeared to be well in line with previous reports. CA was reported to dramatically degrade during alkaline hydrolysis which was probably due to spontaneous oxidation.[22,23] Under oxidative condition, CA, vitexin and RA was stable to 30% hydrogen peroxide with 60 min on heating at 60 °C. Under photolytic condition, vitexin was stable in light 4500 Lx for 72 h, while RA and CA were similar degradation around 22-23%. Moreover, RA and vitexin was stable under thermal condition at 80 °C for 72 h, but

Table 1: Method validation parameters for the quantitation of CA, vitexin and RA.

Parameters	Reference standards		
	CA	Vitexin	RA
Regression equation[a]	$y = 50.032x - 32.811$	$y = 25.839x + 0.1715$	$y = 29.886x - 73.224$
Correlation coefficient (r^2)	0.9999	0.9999	0.9992
Linear range (µg/mL)	1-250	1-250	1-250
LOQ (µg/mL)	0.025	0.100	0.025
LOD (µg/mL)	0.010	0.030	0.010

[a]X is the concentration of reference standard in µg/mL; Y is the peak area at 330 nm.

Table 2: Inter-day and intra-day precision of CA, vitexin and RA.

Reference standards	Intra-day precision[a]			Inter-day precision[a]
	Day 1	Day 2	Day 3	
CA	0.58	0.64	0.34	0.17
Vitexin	0.19	0.72	0.21	0.19
RA	0.90	0.88	0.70	0.33

[a]Results were expressed as %RSD.

Table 3: Recovery study of CA, vitexin and RA in the TL leaf extract.

Serial No.	Theoretical[a] (g/mL)	Found[b] (g/mL)	Recovery[c] (%)
CA			
1	1166.60	1189.20 ± 4.77	101.93 ± 0.41
2	1545.47	1615.60 ± 9.56	104.54 ± 0.62
3	2030.7	2109.90 ± 4.05	103.90 ± 0.20
	Average		**103.46 ± 1.36**
Vitexin			
1	152.70	156.87 ± 1.15	102.73 ± 0.75
2	198.80	198.80 ± 0.72	100.37 ± 0.36
3	294.63	294.63 ± 0.42	98.77 ± 0.14
	Average		**100.62 ± 1.99**
RA			
1	2140.43	2206.60 ± 11.75	103.09 ± 0.55
2	2869.87	3012.03 ± 8.83	104.95 ± 0.29
3	3527.60	3765.30 ± 5.24	106.74 ± 0.15
	Average		**104.93 ± 1.82**

[a]Theoretical values were the amount calculated by original amount plus amount spiked.
[b]Expressed as mean ± SD (n = 3).
[c]Percentage of recovery was expressed as mean ± SD (n = 3).

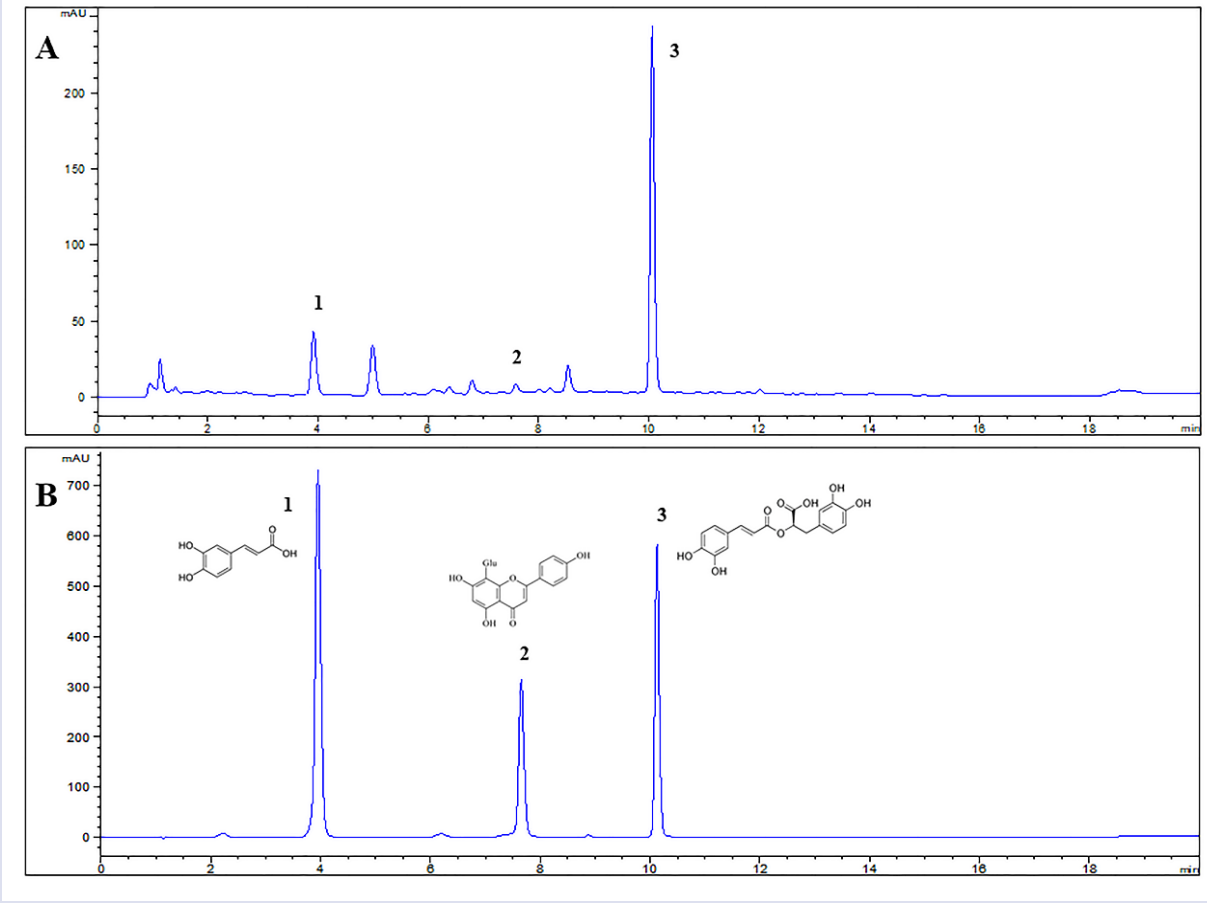

Figure 1: HPLC chromatograms of the TL leaf extract **(A)** and authentic compounds **(B)**; CA, vitexin and RA peaks were numbered as 1, 2 and 3, respectively.

Table 4: Stress testing result of CA, vitexin and RA in the TL leaf extract.

Degradation type	Spiked Reagent[a]	Condition[a]	Assay (µg/mL)	Relative Amount[b] (%)
CA				
Control	Water	-	6.2882 ± 0.1119	100
Acid hydrolysis	37% HCl	60 °C, 60 min	6.2222 ± 0.0381	98.95 ± 0.61
Base hydrolysis	5 N NaOH	60 °C, 60 min	0.9010 ± 0.1129	14.33 ± 1.80
Oxidative	30% w/w H_2O_2	60 °C, 60 min	6.1656 ± 0.3059	98.05 ± 4.86
Photolytic	Water	4500 Lux, 72 h	4.8501 ± 0.2015	77.13 ± 3.20
Thermal	-	80 °C, 72 h	5.2468 ± 0.1239	83.39 ± 1.97
Vitexin				
Control	Water	-	1.4937 ± 0.0388	100
Acid hydrolysis	37% HCl	60 °C, 60 min	1.5711 ± 0.0112	105.18 ± 0.75
Base hydrolysis	5 N NaOH	60 °C, 60 min	0.5184 ± 0.0684	34.71 ± 4.58
Oxidative	30% w/w H_2O_2	60 °C, 60 min	1.4950 ± 0.0039	100.09 ± 0.26
Photolytic	Water	4500 Lux, 72 h	1.5758 ± 0.0153	105.50 ± 1.02
Thermal	-	80 °C, 72 h	1.5008 ± 0.0184	100.48 ± 1.23
RA				
Control	Water	-	47.3396 ± 0.1694	100
Acid hydrolysis	37% HCl	60 _C, 60 min	36.5887 ± 1.3876	77.29 ± 2.93
Base hydrolysis	5 N NaOH	60 _C, 60 min	2.8093 ± 0.1110	5.93 ± 0.23
Oxidative	30% w/w H_2O_2	60 _C, 60 min	46.3123 ± 0.0435	97.83 ± 0.09
Photolytic	Water	4500 Lux, 72 h	36.8134 ± 0.9690	77.76 ± 2.05
Thermal	-	80 °C, 72 h	46.9940 ± 0.3197	99.27 ± 0.68

[a]Stress testing was performed by adding 50 µL of reagent to 1 mL of TL sample and incubated in condition as stated above. DI was used as control solvent.
[b]Percentage of relative amount was expressed as mean ± SD (n = 3).

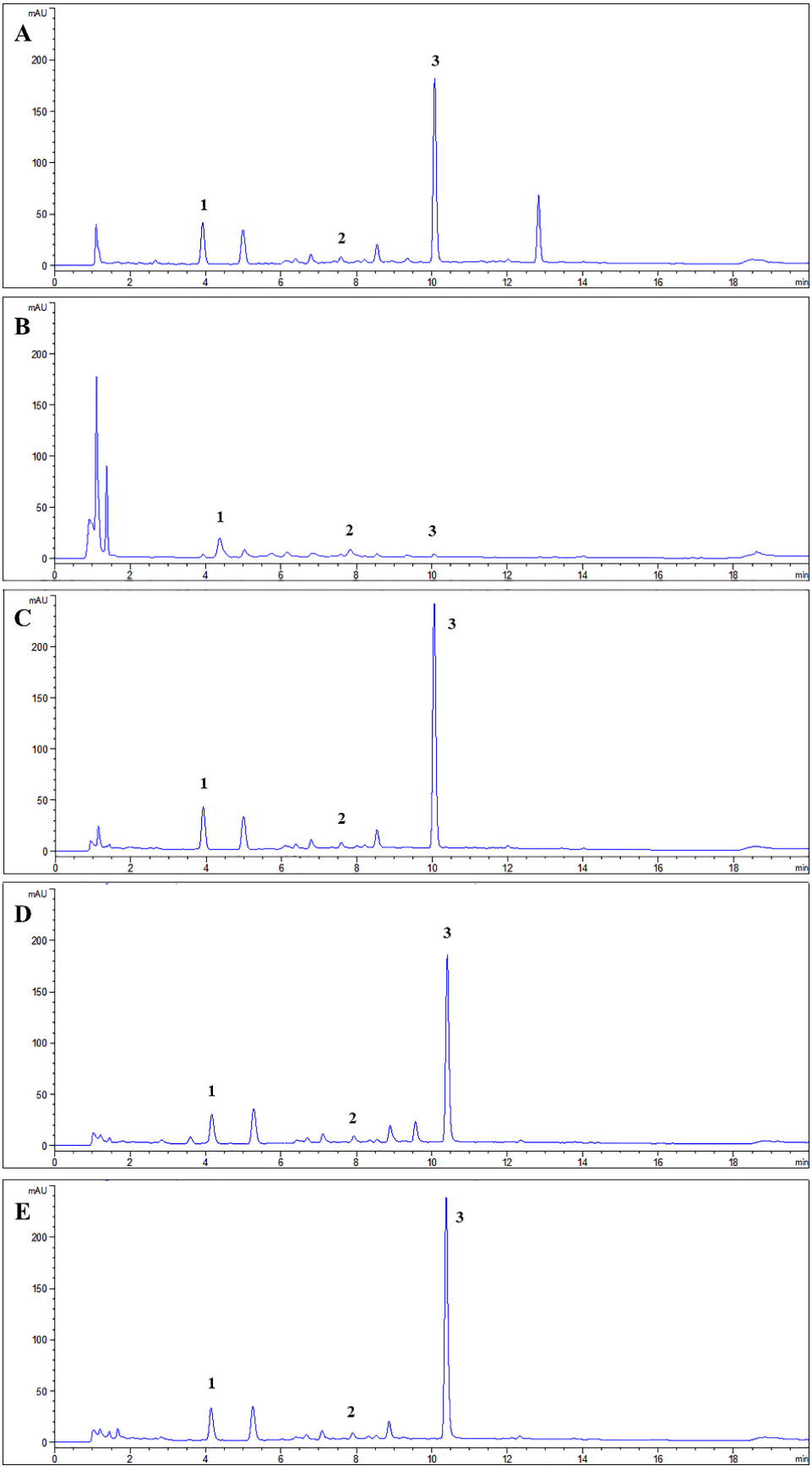

Figure 2: Representative HPLC chromatogram of CA, vitexin and RA in the TL leaf extract under acid **(A)** and base **(B)** hydrolytic; oxidative **(C)**; photolytic **(D)** and thermal **(E)** stress conditions.

Table 5: The content of three phenolic compounds in the TL leaf extract analyzed by the validated HPLC method.

	Content of compounds (mg/g)[a]		
	CA	Vitexin	RA
TL leaf extract	3.1727 ± 0.08	0.7674 ± 0.04	24.6019 ± 0.58

[a]Content of compounds were expressed as mean ± SD (n = 3).

Table 6: Remaining percentage of CA, vitexin and RA in the TL leaf extract under room temperature and accelerated storage conditions for 3 months.

Storage conditions	Time (months)			
	0	1	2	3
		% remaining of CA		
Room temperature	100	100.02 ± 0.88	99.48 ± 1.71	100.79 ± 0.60
40°C /75% RH	100	85.99 ± 0.66	69.73 ± 0.63	48.55 ± 0.80
		% remaining of vitexin		
Room temperature	100	99.15 ± 0.44	100.85 ± 1.03	102.30 ± 3.40
40°C /75% RH	100	88.08 ± 1.03	85.45 ± 1.21	80.93 ± 1.80
		% remaining of RA		
Room temperature	100	99.90 ± 0.50	100.37 ± 0.93	100.50 ± 0.20
40°C /75% RH	100	96.97 ± 1.53	93.66 ± 1.35	91.00 ± 1.54

Results were expressed as mean ± SD (n = 3).

CA was degraded up to 16.61%. However, there was previous report mentioned that RA did not degrade appreciably under different thermal and light exposure conditions for the duration of 13-day study.[24]

Developed HPLC was able to determine CA, vitexin and RA in the TL extract. The content of CA, vitexin and RA in the TL extract was 3.1727, 0.7674 and 24.6019 mg/g extract, respectively (Table 5). As such, the three phytochemical compounds were reported as the main chemical constituents found in TL leaves.[13,14,20,25] RA showed the highest content as the major identified phytochemical compound. This result was appeared to be well in line with previous reported studies that the aqueous extract of TL leaves contained the RA as major constituent following by CA,[13,20] and small amount of vitexin.[14]

RA, CA and vitexin were of interested compounds in leaf decoction of TL with broad biological effects.[13] RA and CA showed antidote activity[26-28] and acted as a chemopreventive agent against cancer[29,30] and diabetes mellitus.[31] These effects are attributed to its excellent anti-inflammatory and anti-oxidant activity.[32-36] Wide range pharmacological effects of vitexin was reported, i.e. anti-oxidant, anti-cancer, anti-inflammatory, anti-hyperalgesia, and neuroprotective effects.[37] Therefore, the three phenolic compounds may be the candidate natural compounds for drug development and require further investigation.

The percentage remaining contents of CA, vitexin and RA in TL extract stored at two different storage conditions for 3 months was presented in Table 6. These results showed that CA, vitexin and RA in the TL extract were stable at room temperature, but they were gradually degraded under accelerated condition at 40°C and 75% RH up to 3 months. The rate of decomposition of CA was dramatically increased within 3 months at elevated temperature and moisture. Vitexin showed more stable than CA in that condition. Interestingly, RA was more stable than the others at 40°C /75% RH condition. Only 10% loss of RA was found, whereas the decrease in concentration of vitexin and CA were found to be 20% and 50% under accelerated condition at 40°C and 75% RH for 3 months, respectively. However, no stability of RA and vitexin in the TL extract has been reported till now. Increasing temperature led to the rise in decomposition and the shorter half-lifes for the CA in the TL extract.[38]

CONCLUSION

This is the first degradation report of CA, vitexin and RA in the *Thunbergia laurifolia* leaf extract. Stability-indicting HPLC method was simultaneously developed and validated for determining the three active compounds in the *T. laurifolia*. This method was simple, specific, linear, precise, and accurate which could be employed in routine analysis. Force degradation revealed that CA, vitexin and RA showed similar trend under tested conditions. All three compounds were stable in oxidative condition, but labile in basic conditions. Although the concentrations of three compounds were gradually decreased under accelerated condition at 40°C and 75% RH, they were stable at room temperature up to 3 months. As these findings suggested that this method could be of benefit and be applied for predicting *T. laurifolia* extract shelf-life and its related pharmaceutical products.

ACKNOWLEDGMENTS

The authors gratefully acknowledged the financial support provided by grant number CRP 6105021210 from the Agricultural Research Development Agency (ARDA) (Public Organization), Thailand. The research team would like to express our gratitude to Chulabhorn International College of Medicine and Drug Discovery and Development Center, Advanced Science and Technology, Thammasat University, Thailand for laboratory facilities. We would like to thank Miss Suwanna Meanpuen for plant collection and Bangkok Herbarium Office, Department of Agriculture, Ministry of Agriculture and Cooperatives, Bangkok, Thailand for plant identification. The authors also thank Dr. Noel Pabalan, Chulabhorn International College of Medicine, Thammasat University for his critical English review of manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHOR'S CONTRIBUTIONS

NW planed and conducted the experiment, analyzed the data and drafted the manuscript and figures. SK advised HPLC condition and reviewed the manuscript. SS coordinated research protocol, conducted the experiment and finalized the manuscript. KS advised for degradation conditions and reviewed the manuscript.

ABBREVIATIONS

TL, *Thunbergia laurifolia*; HPLC-DAD, high-performance liquid chromatography coupled with diode array detector; LOD, limit of

detection; LOQ, limit of quantitation; RA, rosmarinic acid; CA, caffeic acid.

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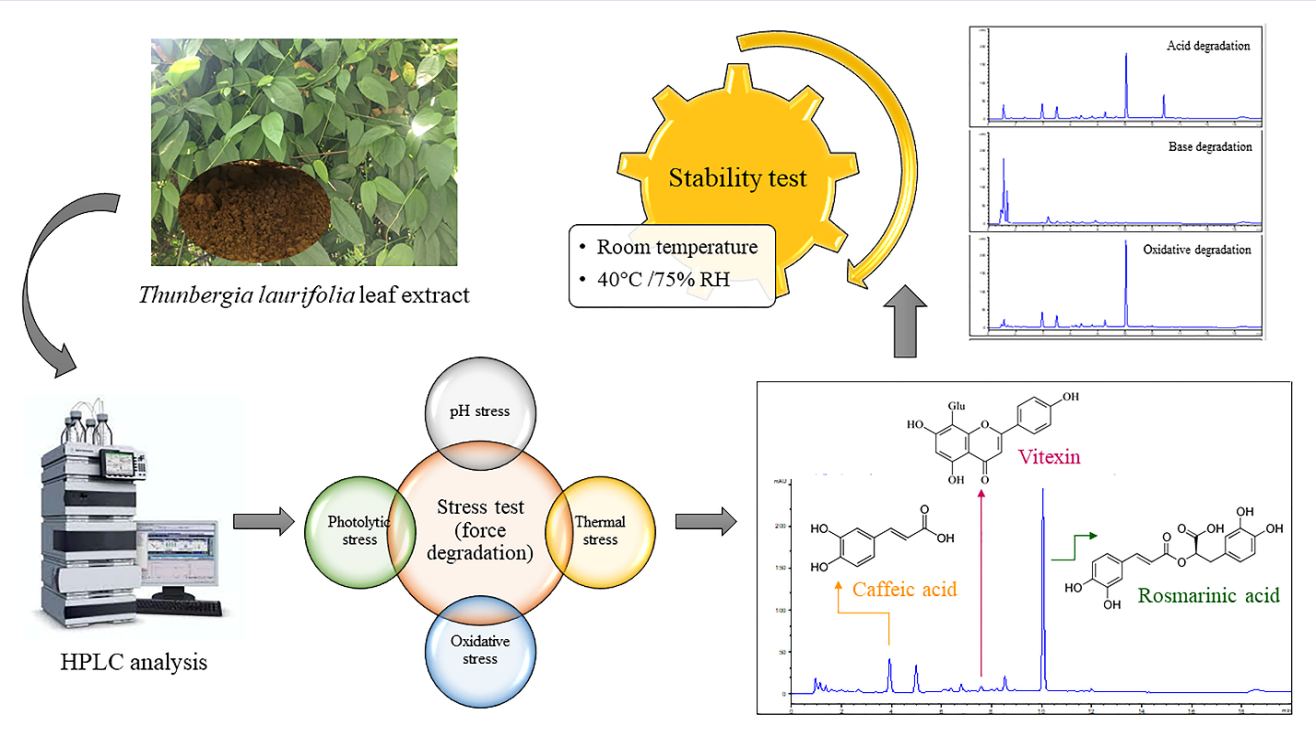
GRAPHICAL ABSTRACT



ABOUT AUTHORS

- **Nanthakarn Woottisin; M.Sc. (Applied Thai Traditional Medicine):** is now a PhD candidate in Integrative Medicine, Chulabhorn International College of Medicine, Thammasat University (Rangsit Campus), THAILAND. She is presently working at School of Integrative Medicine, Mae Fah Luang University, THAILAND. Her research is focused on the development of Thai medicinal plants or recipes used in Thai traditional medicine by conducting preclinical and clinical researches.
- **Sumet Kongkiatpaiboon; Ph.D. (Pharmaceutical Chemistry and Phytochemistry):** Presently work at Drug Discovery and Development Center, Office of Advanced Science and Technology, Thammasat University (Rangsit Campus), THAILAND. His research is focused on phytochemistry, quality control and standardization, pharmacological testing and development of herbal medicinal products.
- **Sophida Sukprasert; Ph.D. (Biochemistry):** Presently work at Chulabhorn International College of Medicine, Thammasat University (Rangsit Campus), THAILAND. Her research is focused on medicinal plants which possess antidote and anti-diabetic properties. Furthermore, immunomodulatory effect of any substance has been also investigated *in vivo* and clinical studies.
- **Assoc. Prof. Korbtham Sathirakul; Ph.D. (Biopharmaceuticals and Pharmacokinetics):** Presently work at Department of Pharmacy, Faculty of Pharmacy, Mahidol University, THAILAND. His research is focused on *in vitro*/*in vivo* extrapolation of biopharmaceutical properties of the herbal compounds. He has also conducted pharmacokinetic studies both preclinic and clinic of those new chemical candidates. He has also responsibility for not only biopharmaceutical and pharmacokinetics data handling using various mathematical modeling techniques but also using those techniques for overall data integration aiming for clinical trial simulation.

Cite this article: Woottisin N, Kongkiatpaiboon S, Sukprasert S, Sathirakul K. Development and Validation of Stability Indicating HPLC Method for Determination of Caffeic Acid, Vitexin and Rosmarinic Acid in *Thunbergia laurifolia* Leaf Extract. Pharmacogn J. 2020;12(3):611-8.