

Research Article

Simultaneous determination of caffeic acid and vitexin contents in *Thunbergia laurifolia* leaf extracts collected from different provinces in Thailand by HPLC

Piyanuch Rojsanga¹,
Pongtip Sithisarn²,
Tanaporn Sanguansataya¹

¹Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand

²Department of Pharmacognosy, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand

*Corresponding author:

Piyanuch Rojsanga
piyanuch.roj@mahidol.ac.th

KEYWORDS:

Thunbergia laurifolia; Rang chuet; Caffeic acid; Vitexin; HPLC

ABSTRACT

Thunbergia laurifolia Lindl. (rang chuet) is a fast growing, perennial climbing vine in Acanthaceae family. Twelve *T. laurifolia* leaf samples were collected from different provinces in Thailand. All samples were identified by analysis of macroscopic and microscopic characteristics and chromatographic fingerprints compared with authentic sample. The simultaneous quantitative analysis of caffeic acid and vitexin was developed and validated using High Performance Liquid Chromatography (HPLC). The analytical method for these two compounds was developed with acceptable validation parameters. Good linearity ranges of caffeic acid and vitexin were 2 – 32 µg/mL. The repeatability and intermediate precision were shown to be precised with relative standard deviations of less than 5%. The average percentage recovery of caffeic acid and vitexin were 95.87% and 101.52% suggesting the acceptable accuracy. The content of caffeic acid and vitexin in *T. laurifolia* leaves collected from different provinces in Thailand analyzed by the validated HPLC method was in the ranges of 0.01 ± 0.00 to 0.28 ± 0.30 and 0.01 ± 0.00 to 0.07 ± 0.02 g/100 g of crude extract, respectively. The leaves collected from Chaiyaphum and Phitsanulok provinces significantly contained the highest caffeic acid and vitexin contents, respectively. The validated HPLC method is rapid, precise, reliable and sensitive for routine analysis of caffeic acid and vitexin contents in *T. laurifolia* leaf raw materials and its commercial products in the future.

1. INTRODUCTION

Thunbergia laurifolia is a fast growing, perennial climbing vine, known in Thai as Rang Chuet. It belongs to family of Acanthaceae. The origin places of *T. laurifolia* are the tropical regions of Africa, Madagascar and southern Asia¹. The stems have round green and basal diameter of 3 cm. Simple leaves are opposite venation, glabrous surface, long-ovate shape, crenate, leaf blades are dark green, 8-10 cm long, 4-5 cm wide, and petioles are 2.5 cm long. Flowers are cymes 3-4 florets per cymes pedicels. The calyx creams have light green, light yellow, light purple or indigo blue color. Fully-blossom flowers are 3 inches in diameter, with a white tube inside. The bract has a green-red spot, light yellowish with violet, 4 stamens, and fruits are sharp-end pods. The aqueous

preparations of leaves and root of *T. laurifolia* have been used in Thai traditional medicine as an anti-inflammatory and antipyretic agent, an antidote for insecticide, ethyl alcohol, arsenic and strychnine poisoning. In Malaysia, juice from the leaves is used in the treatment of menorrhagia and applied for poulticing cuts and boils²⁻⁹. The plant was reported to have antimutagenic, antioxidant, anti-inflammatory, hepatoprotective, neuro-protective activities, acanthamoebicidal and anti-diabetic activities. The aqueous extract of *T. laurifolia* leaves showed antimutagenic activity to inhibit the induction of micronuclei as induced by *Pueraria mirifica* in rat⁴. Orasa et al. reported that fresh and dried Rang Chuet solutions reduced the inflammatory cells in both *Opisthorchis viverrini* infected and N-nitrosodimethylamine administered groups and was correlated with the total antioxidant capacity⁶. The aqueous extract of *T. laurifolia* possesses the hepatoprotective activity against ethanol induced liver injury in both primary cultures of rat hepatocyte and rats⁷. The leaf extract of *T. laurifolia* reduced neuronal cell death and memory loss caused by lead uptake in mice⁹. The chronic toxicity of *T. laurifolia* aqueous extract in Wistar rat has been studied at doses ranging from 20 to 2,000 mg/kg/day. The results revealed that *T. laurifolia* aqueous extract did not affect the body weight, food consumption, behavior or general health of the animals, and did not produce cumulative toxic signs and fatal effects. However, hematological and clinical chemistry values should be monitored in case of prolonged consumption of *T. laurifolia*¹⁰.

2. MATERIALS AND METHODS

2.1. Preparation of *Thunbergia laurifolia* leaf samples

Twelve samples of *T. laurifolia* dried leaves were collected from different provinces in Thailand including Chachoengsao, Chaiyaphum, Chiang Mai, Chumphon, Kanchanaburi, Narathiwat, Phitsanulok, Ratchaburi, Roi Et, Sra Kaeo, Tak and Uthai Thani in 2012. The plant samples were identified by comparing the microscopic characteristics and chromatographic fingerprints compared with an authentic sample (the leaves of *T. laurifolia* collected from Chiang Mai province which was previously identified with a sample number of VK064382). Plant samples were

cleaned and dried in a hot air oven at 60 °C for 6 hrs. The dried samples were then ground and passed through a sieve with mesh number 20.

2.1.1. Macroscopic examination of *T. laurifolia* leaf samples

The macroscopic characteristics of the dried leaf samples were examined and recorded.

2.1.2. Microscopic examination of *T. laurifolia* leaf samples

Each leaf sample was ground and passed through a sieved number 60. Plant powder was cleared with chloral hydrate solution and then stained with aniline sulfate reagent. The characteristics of each plant sample were observed and recorded under a microscope.

2.1.3. Thin-layer chromatographic (TLC) fingerprint of *T. laurifolia* leaf samples

Five milliliters of methanol was added to 5 mg of each *T. laurifolia* leaf sample. The sample was extracted using sonication for 10 minutes. The solution was filtered through a membrane filter (0.45 µm), then 5 µL of each plant extract was spotted on a TLC plate. The plate was developed in 2 solvent systems. Solvent I was toluene: ethyl acetate: formic acid which ratio to 10:9:2 v/v/v and solvent II was ethyl acetate: formic acid: glacial acetic acid: water which ratio to 25:3:2:5 v/v/v/v. The developing distance was 80 mm. After removed from the developing chamber, TLC plate was air dried in a fume hood for 30 minutes and was examined using natural product/ polyethyleneglycol (NP/PEG) reagent and under ultraviolet detector (254 and 366 nm)

2.2. Preparation of *Thunbergia laurifolia* leaf extracts

One hundred grams of each powdered sample was boiled with distilled water at 100°C for 2 hours and repeated three times. The mixture was centrifuged at 15,000 rpm and filtered through Whatman filter paper no. 1. The combined extract was evaporated to dryness on a boiling water bath. The dried residue was cooled in desiccators for 30 minutes, and then accurately weighed. The extraction was performed in triplicate and the yield was reported as mean ± SD.

2.3. Determination of caffeic acid and vitexin in *T. laurifolia* leaf extract by high performance liquid chromatography (HPLC)

2.3.1. Instruments and Chromatographic Conditions

The HPLC (Agilent Technologies 1200 series) instrument was equipped with a model series G13114 quaternary pump, G1316A TCC, G1322A degasser, G1315D diode array detector. Separation and quantification were made on X-terra C18 column, 5 μ m, 150 x 3.9 mm. Data acquisition was performed on Agilent ChemStation software. The analysis was performed at a flow rate of 1 mL/min, detecting wavelength 230 nm at room temperature. The mobile phase consisting of 0.5% glacial acetic acid (solvent A) and acetonitrile (solvent B) at the ratio of 85: 15 was used. The injection volume was 5 μ L.

2.3.2. Preparation of standard and sample solutions

For a standard solution, caffeic acid and vitexin were accurately weighted (5 mg) and transferred to a 25- mL volumetric flask. Methanol was added and the mixture was adjusted to a final concentration 0.2 mg/mL. From this solution, the concentration of caffeic acid and vitexin (2-32 μ g/mL) was prepared and used for the preparation of the calibration curve.

For the sample solution, the sample solution was prepared by accurately weighed the crude extract of each sample (500 mg). Water was added (20 mL) and the mixture was adjusted to pH 3. This mixture was extracted with 3-portion of 20-mL ethyl acetate. The combined ethyl acetate layer was filtered through Whatman filter paper no. 1 and evaporated to dryness by rotary evaporator. The dried residue was kept in desiccators for 30 minutes, and then accurately weighed. The extraction was performed in triplicate and the yield was reported as mean \pm SD. The working solution was prepared at the concentration of 2 mg/mL in methanol.

2.3.3. Method Validation

Selectivity

The UV spectra at 200 - 500 nm of the peak at the retention time corresponding to caffeic acid and vitexin in the sample were compared with those of the standards.

Linearity

The linearity of the method was evaluated by analyzing a series of standard caffeic acid and vitexin ranging from 2.0-32.0 μ g/mL in methanol. Five microliters of each six standard solutions were injected into the HPLC instrument. The elution was carried out as described above and the standard curve was achieved by plotting the concentration of standard caffeic acid and vitexin versus peak area. The slope and intercept values were calculated using the least-square linear regression method. The correlation coefficient was also calculated.

Accuracy

The accuracy was determined by recovery of known amounts of caffeic acid and vitexin standard added to the working sample solution (Chaing Mai province). An aliquot of this standard solution was transferred into a 10-ml volumetric flask containing sample solution (final concentrations of 91.6 μ g/mL) to give final concentrations of five standard solutions at 4-20 μ g/mL. The accuracy of the method was tested by the proposed method. The experiment was conducted in triplicate. The percentage recovery was then calculated.

Precision

Repeatability (intra-day) and intermediate precision (inter-day) were obtained by recovery study of the standard concentration at 16 μ g/mL ($n = 6$) on the same day and on 3 different days, respectively. The relative standard deviation (RSD) was then calculated.

LOD/LOQ

Limit of detection and limit of quantification were defined base on the standard deviation of the slopes of calibration curves. The standard deviation of y-intercepts of regression lines was used as the standard deviation. The LOD and LOQ were then calculated.

2.3.4. Statistical analytical

The experiment was done in triplicate. The results were expressed as mean \pm standard deviation (SD). The average data of the caffeic acid and vitexin content of the ethyl acetate extracts of *T. laurifolia* from difference provinces were statistically analyzed using one way ANOVA with SPSS statistics 17.0 program. The

statistical probability (p -value) less than 0.05 indicated a statistically significant difference between provinces.

3. RESULTS AND DISCUSSION

3.1. Macroscopic examination of *T. laurifolia* leaf samples

The macroscopic characters of all *T. laurifolia* leaf samples were similar compared with the authentic sample collected from Chiang Mai province (Figure 1, TL3). The leaves are simple, opposite venation, glabrous surface, long-ovate shape, entire to create margin and sharpen ends, blades are green to dark green. Leaf is 8-10 cm long, 4-5 cm wide, and petiole length is 2.5 cm. The macroscopic characteristics of *T. laurifolia* leaves collected from different

provinces in Thailand are shown in Figure 1.

3.2. Microscopic examination of *T. laurifolia* leaf samples

The powdered leaf samples of *T. laurifolia* collected from 12 provinces had similar physical characteristics compared with the authentic sample collected from Chiang Mai province. The powders are dark green with a specific odor. All leaf samples were microscopically studied with a total magnification of 400 times. All of the plant samples showed similar microscopic characteristics. The specific microscopic characteristics of *T. laurifolia* leaf powders are laminar in sectional view, upper and lower epidermis showing anisocytic stomata, covering trichome, laminar in surface view and scalariform and spiral vessels (Figure 2).

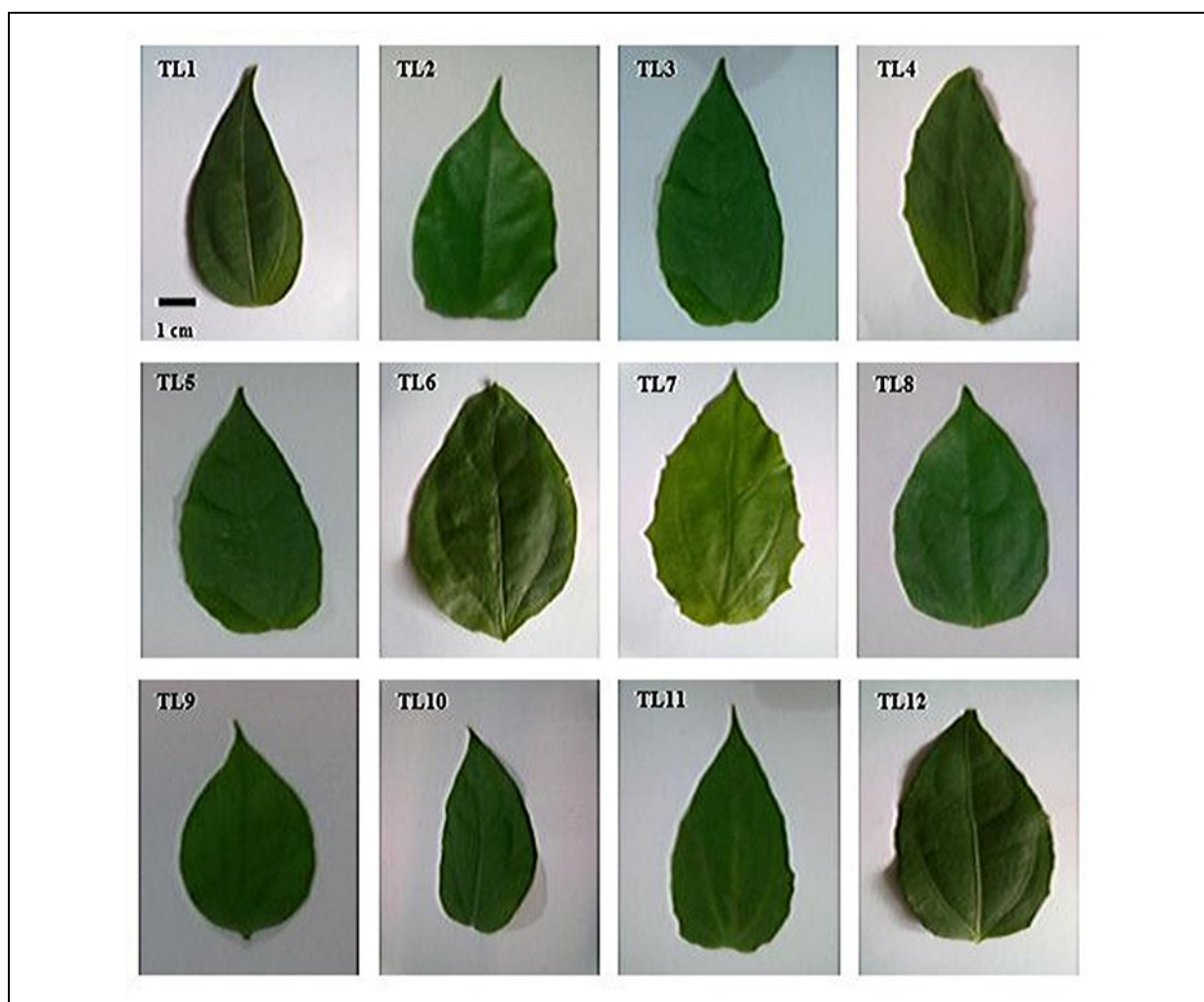


Figure 1. Physical characteristics of *Thunbergia laurifolia* leaf samples collected from 12 provinces in Thailand. *T. laurifolia* collected from; TL1 = Chachoengsao, TL2 = Chaiyaphum, TL3 = Chiang Mai, TL4 = Chumphon, TL5 = Kanchanaburi, TL6 = Narathiwat, TL7 = Phitsanulok, TL8 = Ratchaburi, TL9 = Roi Et, TL10 = Sra Kaeo, TL11 = Tak, TL12 = Uthai Thani.

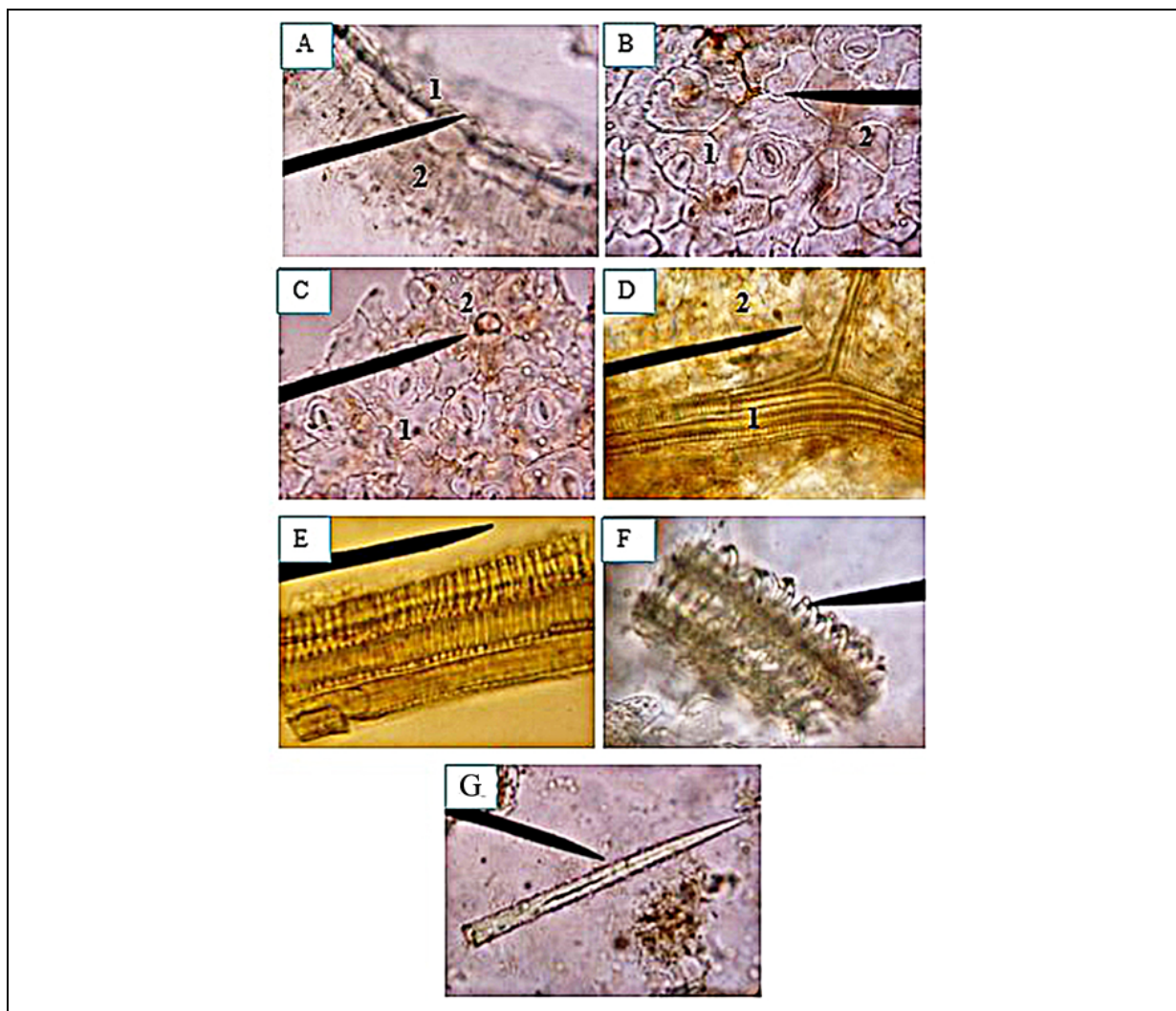


Figure 2. Microscopic characteristics of *T. laurifolia* leaves. Magnification x 400, chloral hydrate solution with aniline sulfate dyeing reagent. A = Laminar in sectional view showing the upper epidermis (1) and palisade layer (2), B = Epidermis in surface view showing anisocytic stomata (1) and a cicatrix (2), C = Lower epidermis in surface view showing anisocytic stomata (1) and calcium oxalate crystal (2), D = A lamina in surface view showing part of a vein (1) and calcium oxalate crystals (2), E = Scalariform vessel from vessel, F = Spiral vessel, G = Covering trichome.).

3.3. Thin- layer chromatographic (TLC) fingerprint of *T. laurifolia* leaf samples

TLC fingerprints of the methanolic leaf extracts of *T. laurifolia* collected from different provinces in Thailand developed in 2 different solvent systems are shown in Figure 3. TLC chromatograms from all leaf samples promoted the same fingerprints with chromatographic bands that appeared as dark quenching bands under UV 254 nm while some of them promoted the blue, red and yellow fluorescence bands under UV 366 nm suggesting the presences of compounds with

chromophore suggesting phenolics and flavonoids (Figure 3; 1A, 1B, 2A and 2B). After spraying with NP/PEG and detect under UV 366 nm, there are some bright blue and yellow fluorescence chromatographic bands confirming the presence of phenolics and flavonoids (Figure 3; 1D and 2D).

3.4. *T. laurifolia* leaf extracts

All *T. laurifolia* leaf aqueous extracts appeared as dark brown semisolid. The yields of extracts from the leaves of *T. laurifolia* collected from 12 provinces in Thailand ranged from 20.93 ± 1.57 to 32.68 ± 0.27 %w/w (Table 1).

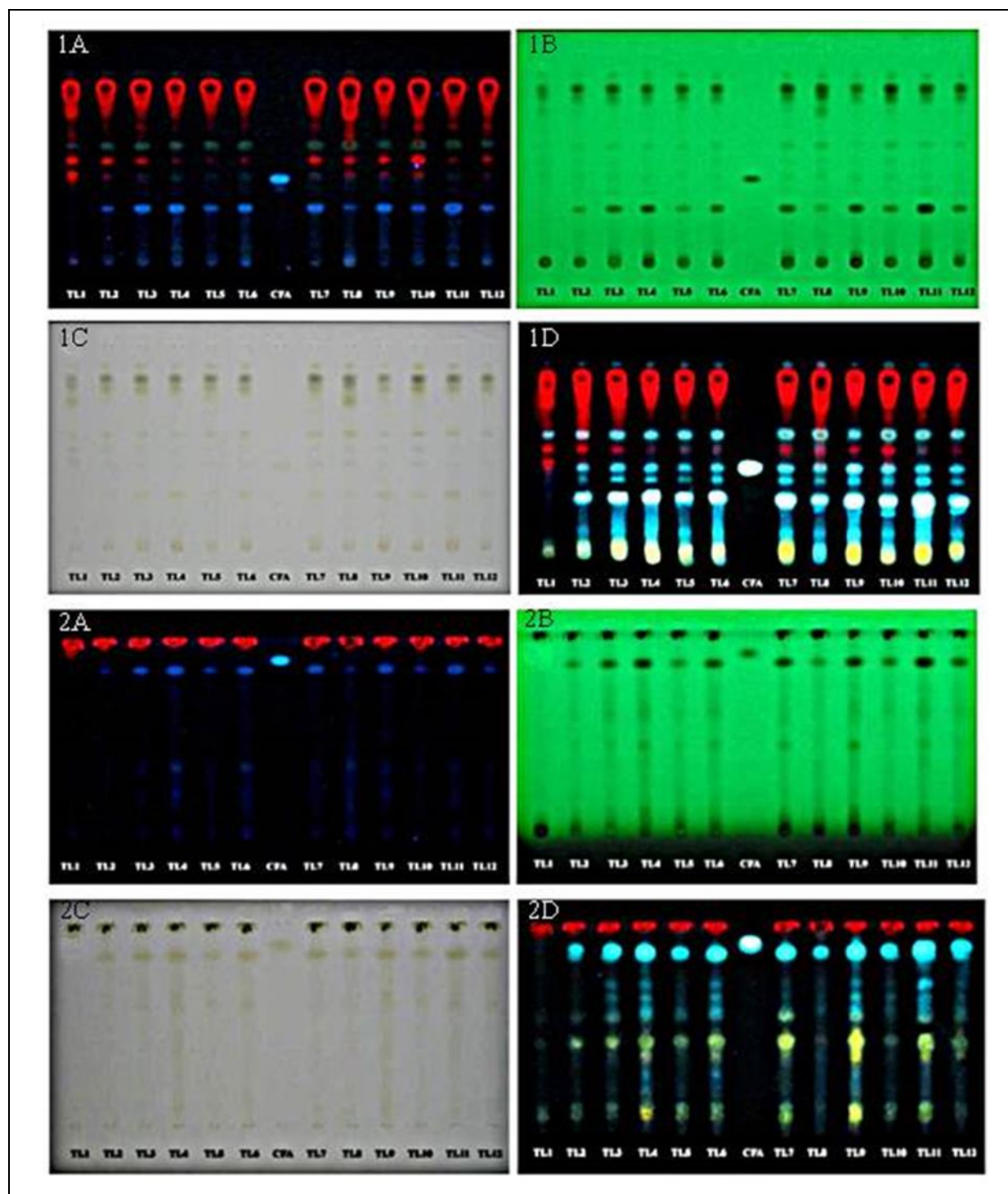


Figure 3. TLC fingerprint of methanolic extract of *T. laurifolia* leaves from 12 locations in Thailand Absorbent: silica gel 60 GF254 Solvent system: 1 = toluene: ethyl acetate: formic acid (10:9:2), 2 = ethyl acetate: formic acid: glacial acetic acid: water (25:3:2:5), 2 = Detector: A = UV 366 nm, B = UV 254 nm), C = white light, D = NP/PEG reagent under UV 366 nm. *T. laurifolia* collected from; TL1 = Chachoengsao, TL2 = Chaiyaphum, TL3 = Chiang Mai, TL4 = Chumphon, TL5 = Kanchanaburi, TL6 = Narathiwat, TL7 = Phitsanulok, TL8 = Ratchaburi, TL9 = Roi Et, TL10 = Sra Kaeo, TL11 = Tak, TL12 = Uthai Thani, CFA = standard caffeic acid.

3.5. Method validation

HPLC method for simultaneous quantitative analysis of caffeic acid and vitexin in the leaf extract of *T. laurifolia* was validated by the

evaluation of selectivity, linearity, precision, accuracy, limit of detection (LOD) and limit of quantitation (LOQ). The proposed HPLC method showed acceptable validation parameters (Table 2).

Table 1. The yields (%w/w) of extracts from the leaves of *T. laurifolia* collected from 12 provinces in Thailand

| Sample | Yield (%w/w) |
|--------|--------------|
| TL1 | 25.91 ± 0.47 |
| TL2 | 27.18 ± 1.11 |
| TL3 | 32.68 ± 0.27 |
| TL4 | 27.09 ± 1.25 |
| TL5 | 30.49 ± 0.30 |
| TL6 | 27.15 ± 0.51 |
| TL7 | 30.35 ± 0.85 |
| TL8 | 26.43 ± 1.14 |
| TL9 | 26.36 ± 1.65 |
| TL10 | 25.32 ± 1.34 |
| TL11 | 26.22 ± 3.40 |
| TL12 | 20.93 ± 1.57 |

TL1 = Chachoengsao, TL2 = Chaiyaphum, TL3 = Chiang Mai, TL4 = Chumphon, TL5 = Kanchanaburi, TL6 = Narathiwat, TL7 = Phitsanulok, TL8 = Ratchaburi, TL9 = Roi Et, TL10 = Sra Kaeo, TL11 = Tak, TL12 = Uthai Thani.

Table 2. Validation parameters by the proposed HPTLC method

| Parameter | Caffeic acid | Vitexin |
|---|--------------------|--------------------|
| Range of linearity | 2 – 32 µg/ml | 2 – 32 µg/ml |
| Regression equation (n=6) | Y = 13.43X – 0.252 | Y = 9.247X + 1.050 |
| Correlation coefficient (r ²) | 0.999 ± 0.000 | 0.999 ± 0.000 |
| % RSD Intraday precision (n = 6) | 101.98 ± 2.06 % | 100.79 ± 0.95 % |
| % RSD Interday precision (n = 18) | 103.65 ± 2.80 % | 104.49 ± 3.29 % |
| % Recovery | 95.87 ± 0.03 % | 101.52 ± 0.03 % |
| Limit of detection (LOD) | 0.08 µg/ml | 0.69 µg/ml |
| Limit of quantitation (LOQ) | 0.25 µg/ml | 2.08 µg/ml |

X = concentration of caffeic acid and vitexin (µg/ml), Y = peak area

Table 3. Caffeic acid and vitexin contents in leaf extracts and dried powders of *T. laurifolia* leaves collected from 12 provinces in Thailand determined by validated HPLC method

| Sample | Content (%w/w) | | | |
|--------|----------------------------------|------------------------------|--------------------------------|--------------------------------|
| | Caffeic acid | | Vitexin | |
| | In crude extract | In leaf powder | In crude extract | In leaf powder |
| TL1 | 0.01 ± 0.00 ^a | 0.00 ± 0.00 ^a | 0.01 ± 0.00 ^a | 0.00 ± 0.00 ^a |
| TL2 | 0.28 ± 0.30 ^b | 0.08 ± 0.09 ^b | 0.04 ± 0.03 ^{a,b} | 0.01 ± 0.01 ^b |
| TL3 | 0.10 ± 0.07 ^{a,c} | 0.03 ± 0.02 ^{a,b} | 0.03 ± 0.02 ^{b,c} | 0.01 ± 0.01 ^{a,b} |
| TL4 | 0.21 ± 0.08 ^{b,c,d} | 0.06 ± 0.02 ^{b,d} | 0.05 ± 0.02 ^{b,d,g} | 0.02 ± 0.01 ^{b,c} |
| TL5 | 0.12 ± 0.05 ^{a,b,d} | 0.04 ± 0.02 ^{a,b} | 0.04 ± 0.00 ^{b,e} | 0.01 ± 0.00 ^b |
| TL6 | 0.10 ± 0.06 ^{a,b,d} | 0.03 ± 0.02 ^{a,b} | 0.07 ± 0.03 ^{d,f} | 0.02 ± 0.01 ^{b,d} |
| TL7 | 0.04 ± 0.05 ^{a,d,e} | 0.01 ± 0.02 ^{a,c,d} | 0.07 ± 0.02 ^{f,g} | 0.02 ± 0.01 ^{c,d,f,g} |
| TL8 | 0.06 ± 0.02 ^{a,d,f} | 0.02 ± 0.00 ^{a,c} | 0.02 ± 0.01 ^{a,c,e} | 0.00 ± 0.00 ^{a,b} |
| TL9 | 0.09 ± 0.01 ^{a,d,g} | 0.03 ± 0.00 ^{a,c} | 0.07 ± 0.01 ^{b,f,h} | 0.02 ± 0.00 ^{b,e,f} |
| TL10 | 0.12 ± 0.08 ^{a,b} | 0.03 ± 0.02 ^{a,b} | 0.01 ± 0.00 ^a | 0.00 ± 0.00 ^a |
| TL11 | 0.18 ± 0.09 ^{a,b} | 0.05 ± 0.02 ^{a,b} | 0.06 ± 0.02 ^{b,f} | 0.02 ± 0.01 ^{b,e,g} |
| TL12 | 0.21 ± 0.08 ^{b,c,e,f,g} | 0.05 ± 0.02 ^{a,b} | 0.05 ± 0.01 ^{a,c,e,h} | 0.01 ± 0.00 ^{a,b} |

* values are express as mean ± SD, (n = 3), different letters in the same column indicated significant differences ($P < 0.05$). TL1 = Chachoengsao, TL2 = Chaiyaphum, TL3 = Chiang Mai, TL4 = Chumphon, TL5 = Kanchanaburi, TL6 = Narathiwat, TL7 = Phitsanulok, TL8 = Ratchaburi, TL9 = Roi Et, TL10 = Sra Kaeo, TL11 = Tak, TL12 = Uthai Thani.

3.6. Determination of caffeic acid and vitexin in *T. laurifolia* leaf extract by HPLC

The caffeic acid and vitexin contents in the *T. laurifolia* leaf extracts were determined by the validated HPLC method. The contents of caffeic acid in the crude extract and in dried powder ranged from 0.01 ± 0.21 to 0.28 ± 0.30 %w/w and 0.002 ± 0.001 to 0.078 ± 0.087 %w/w,

respectively while vitexin contents in the crude extract and in dried powder ranged from 0.01 ± 0.00 to 0.07 ± 0.02 %w/w and 0.002 ± 0.001 to 0.021 ± 0.007 %w/w, respectively. Leaf sample collected from Chaiyaphum province significantly contained the highest caffeic acid content while the leaves collected from Phitsanulok province contained the highest vitexin content. Caffeic acid and vitexin contents

in leaf extracts and dried powders of *T. laurifolia* leaves collected from 12 provinces in Thailand determined by a validated HPLC method are shown in Table 3.

4. CONCLUSION

Thunbergia laurifolia leaves collected from 12 provinces in Thailand were identified by macroscopic, microscopic and chromatographic characteristics comparing to the characteristics of the authentic sample. From thin layer chromatographic analysis, there were chromatographic bands corresponded to some phenolics and flavonoids. HPLC analytical method was developed and validated for the analysis of caffeic acid and vitexin in the leaf extracts of *T. laurifolia* with acceptable validation parameters. Chaiyaphum and Phitsanulok provinces were found to be the good sources for *T. laurifolia* leaf raw material with the highest amounts of caffeic acid and vitexin contents, respectively. The information from this study could be a guideline for the quality control of *T. laurifolia* leaf extract and raw material in pharmaceutical and medicinal industries in the future.

5. ACKNOWLEDGEMENTS

The authors would like to thank The Agricultural Research Development Agency (Public Organization) for financial support.

Conflict of interest

None to declare

Funding

None to declare

Ethical approval

None to declare

Article info:

Received March 30, 2017

Received in revised form December 22, 2017

Accepted February 09, 2018

REFERENCES

1. Starr F, Starr K, Loope L. *Thunbergia laurifolia*. United States Geological Survey- Biological Resources Division. 2003:1–4.
2. Thongsard W, Marsden CA, Morris P, Prior M, Shah YB. Effect of *Thunbergia laurifolia*, a Thai natural product used to treat drug addiction, on cerebral activity detected by functional magnetic resonance imaging in the rat. *Psychopharmacology (Berl)*. 2005;180(4):752–60.
3. Thongsard W, Marsden CA. A herbal medicine used in the treatment of addiction- mimics the action of amphetamine on in vitro rat striatal dopamine release. *J Neurosci Lett*. 2002; 329(2):129–32.
4. Saenphet K, Kantaoop P, Saenphet S, Aritajat S. Mutagenicity of *Pueraria mirifica* Airy Shaw & Suvatabandhu and antimutagenicity of *Thunbergia laurifolia* Linn. Southeast Asian. *J Trop Med Public Health*. 2005;36(Suppl. 4): 238–41.
5. Oonsivilai R, Cheng C, Bomser J, Ferruzzi MG, Ningsanond S. Phytochemical profiling and phase II enzyme- inducing properties of *Thunbergia laurifolia* Lindl.(RC) extracts. *J Ethnopharmacol*. 2007;114(3):300–6.
6. Wonkchalee O, Boonmars T, Aromdee C, Laummaunwai P, Khunkitti W, Vaeteewoottacharn K, et al. Anti-inflammatory, antioxidant and hepatoprotective effects of *Thunbergia laurifolia* Linn. on experimental opisthorchiasis. *J Parasitol Res*. 2012;111(11):353–9.
7. Pramyothin P, Chirdchupunsare H, Rungsipipat A, Chaichantipyuth C. Hepatoprotective activity of *Thunbergia laurifolia* Linn extract in rats treated with ethanol: in vitro and in vivo studies. *J Ethnopharmacol*. 2005;102(3):408–11.
8. Aritajat S, Wutteerapol S, Saenphet K. Anti-diabetic effect of *Thunbergia laurifolia* Linn. aqueous extract. *Southeast Asian J Trop Med Public Health*. 2004;35(Suppl. 2):53–8.
9. Tangpong J, Satarug S. Alleviation of lead poisoning in the brain with aqueous leaf extract of the *Thunbergia laurifolia* (Linn.). *Toxicol Lett*. 2010;198(1):83–8.
10. Songpol Chivapat, Pranee Chavalittumrong, Aimmanas Attawish, Jaree Bansiddhi, Songpol Padungpat. Chronic Toxicity of *Thunbergia laurifolia* Lindl. extract. *Journal of Thai Traditional and Alternative Medicine*. 2009;(7)1:17–25.