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Quality Assessment and *In Vitro* Anti-diabetic Activity of *Thunbergia laurifolia* Stems and Leaves

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Abstract

The study aimed to evaluate pharmacognostic parameters for standardization of raw materials, *Thunbergia laurifolia* Lindl. (family: Thunbergiaceae) stems and leaves, as well as their active phytochemical (rosmarinic acid) contents. The antidiabetic potential was evaluated by yeast α-glucosidase inhibitory activity using p-nitrophenyl-α-D-glucopyranoside as substrate. Dried stems and leaves of *Thunbergia laurifolia* were collected from 15 different locations in Thailand. The microscopic anatomical and histological characteristics of stem and leaf were illustrated. The physico-chemical contents, including loss on drying, acid-insoluble ash, total ash, ethanol-soluble extractives, water-soluble extractives, and moisture of dried stems and leaves, were established. TLC-densitometry of rosmarinic acid in dried *T. laurifolia* stems and leaves were developed and revealed contents of 0.120 ± 0.079 and 0.291 ± 0.150 g per 100 g, respectively. Similarly, TLC-image analysis by ImageJ showed contents of 0.127 ± 0.094 and 0.303 ± 0.162 g per 100 g respectively ($p > 0.05$). Both quantitative TLC demonstrated their validity due to specificity, accuracy, repeatability, intermediate precision, limit of detection, limit of quantitation, and robustness. The antidiabetic potential of rosmarinic acid, *T. laurifolia* leaf and stem ethanolic extracts, and acarbose (positive control) exhibited IC_{50} of 0.31, 0.80, 5.89, and 1.48 mg/ml, respectively.

Keywords: *Thunbergia laurifolia*, Rosmarinic acid, α-glucosidase inhibition, TLC-densitometric method, TLC-image analysis

Introduction

Thunbergia laurifolia L., belonging to the Thunbergiaceae family, is a climbing herb which is commonly found in tropical regions [1]. In Thailand, the plant is locally known as "Rang Chuet" and is famous for its therapeutic effects. Various parts of the plant can be used as herbal medicine in fresh or dried crude drug forms. The leaf and stem have been used as traditional medicine to treat fever; inflammation; and poisoning from foods, animals, and chemicals [2]. Some folk medicine healers in Thailand also recommend the use of *T. laurifolia* to treat diabetes. Thus, medicinal plants may be a promising source containing antidiabetic compound which is more effective and less harmful than some modern medicines for treating diabetes mellitus [3].

A study on *T. laurifolia* leaf extract on its hypoglycemic effect was established using alloxan-induced rat model (60 mg/ml/day) for 15 days. The results exhibited the ability of the extract to decrease blood glucose level and recover some of the pancreatic β-cell in diabetic rats [4]. A similar study of *T. laurifolia* leaf extract was conducted in hyperglycemic cats. The cats were treated with the extract (500 mg/kg/day) for 28 days. The results indicated significant lowering of levels of blood glucose in

hyperglycemic cats [5]. In contrast, the stem of *T. laurifolia* seems to be unexplored in terms of its antidiabetic ability or chemical constituents due to the lack of scientific reports. However, a phytochemical study of the aerial part of the plant indicated various constituents, and some of them are currently revealed as potent antioxidant and antidiabetic compounds [6,7].

Rosmarinic acid is an ester composed of caffeic acid and 3,4-dihydroxyphenylacetic acid, which can be found in various parts of medicinal plants, as well as in the leaves of *T. laurifolia* [8]. The medicinal plants containing this natural compound, rosmarinic acid, have shown various remarkable biological activities; for instance, antidiabetic, antioxidant, antinociceptive, anti-inflammatory, antimicrobial, and antimutagenic effects, and so forth [9-14].

Presently, many raw material products of *T. laurifolia* stem and leaf are prepared in various forms, including herbal teas, capsules, and powders, which can be commonly found in herbal markets. Therefore, quality assurance and active phytochemical determination of *T. laurifolia* stem in raw materials are needed for the consumer [15-17].

In this study, pharmacognostic specifications of *T. laurifolia* stem and leaf in Thailand were carried out and quantitative analysis of rosmarinic acid in *T. laurifolia* stem and leaf by TLC-densitometry and TLC-image analysis was developed. Antidiabetic evaluation of *T. laurifolia* stem, leaf ethanolic extracts, and rosmarinic acid was investigated for yeast α-glucosidase inhibitory activity.

Materials and methods

Plant material collection

T. laurifolia stems and leaves were collected from 15 locations throughout Thailand. The locations for sample collection were Bangkok, Chachoengsao, Chiang Mai, Chiangrai, Kanchanaburi, Khon Kaen, Lampang, Nakhon Pathom, Nakhon Ratchasima, Nong Khai, Phitsanoulok, Prachuap Khiri Khan, Songkhla, Suphan Buri, and Surin. The collected samples were given numerical codes from 1 to 15 as per the sequence of the aforementioned locations. All samples were authenticated by Associate Professor Dr. Nijsiri Ruangrungsi. The voucher specimens were deposited at the College of Public Health Sciences, Chulalongkorn University, Thailand. Foreign matter was removed, and then the samples were dried in a hot air oven at 40 °C. The dried samples were ground into powder and kept in an airtight container.

Quality evaluation

Macroscopic, microscopic, and physico-chemical evaluations were performed [18]. The physico-chemical evaluations were done in triplicate and calculated as percent by dried weight. The stem and leaf crude drugs were investigated for color, size, and shape. A freehand transverse section and fine powders were investigated for anatomical and histological characteristics under microscope and illustrated by hand drawing.

Three grams of ground sample were placed in a pre-weighed crucible and dried at 105 °C for loss on drying content. It was further incinerated at 500 °C until total ash was obtained, then boiled gently with 25 ml of hydrochloric acid (70 g/L) for 5 min and filtered via ashless filter paper. The insoluble matter was incinerated again at 500 °C to obtain acid-insoluble ash.

For solvent extractable matter, 5 grams of ground sample were macerated in 70 ml of solvent (water/ethanol) under shaking for 6 h and standing for 18 h in room temperature. The extract was filtered, the marc was rinsed with the specified solvent, and the final volume was adjusted to 100 ml. Twenty-five milliliters of the filtrate were transferred to a pre-weighed beaker, evaporated in a water-bath, and then dried in a hot air oven at 105 °C.

Water content of 5 g of ground sample in 200 ml of water-saturated toluene was determined using azeotropic distillation technique.

Quantitative TLC of rosmarinic acid in *T. laurifolia* stem and leaf

Standard solutions of rosmarinic acid (Sigma-Aldrich, Missouri, USA) were prepared in 95 % ethanol. Each of the powder samples (5 g) were exhaustively extracted with 95 % ethanol by a Soxhlet

apparatus and evaporated to dryness *in vacuo* at 50 °C. The ethanolic extract was re-dissolved in 1 ml of 95 % ethanol prior to investigation.

TLC-densitometry

Four microliters of standard and sample solutions were applied by CAMAG Linomat 5 (Muttenz, Switzerland) as bands of 4 mm in length onto a silica gel 60 GF_{254} TLC plate (20 cm × 20 cm, E. Merck, Darmstadt, Germany). Firstly, the plate was developed using toluene-chloroform-acetone-formic acid (5:4:1:0.2, v/v) and removed when the mobile phase migrated to the solvent front. The plate was air-dried and developed again in the second mobile phase using toluene-ethyl acetate-formic acid (5:4:1, v/v). Rosmarinic acid was quantified using CAMAG TLC Scanner 4 at λ_{330} (λmax) and the winCATS version 1.4.9 software. Each sample was done in triplicate.

TLC-image analysis

TLC-image was captured with a Canon PowerShot A560 IS digital camera of 21.1 million pixels resolution, 24x combined zoom with Optical Image Stabilizer System (Canon, USA) in a Spectrolines model CC-80 UV-Fluorescence analysis cabinet with a 365/254 nm UV lamp (New York, USA). The digital image of TLC plate was saved in TIFF format and transformed to chromatographic peak using ImageJ, a free Java-based image processing program for scientific research (National Institutes of Health, USA).

Method validation

This part was investigated by following ICH guidelines [19]. LOD and LOQ were estimated based on the residual standard deviation of a regression line (σ) and the slope (S), with the equations of $3.3S\sigma/S$ and $10\sigma/S$, respectively. Accuracy was carried out as the percentage recovery of the sample spiked with the known amounts of standard. Precision was performed using 3 concentrations (3 replicates each) in the same day (repeatability) and other 3 different days (intermediate precision) and were expressed as the percentage of relative standard deviation (RSD). Specificity was evaluated according to the similarity of the absorbance spectra at the peak apex among all samples and standard rosmarinic acid, as well as the similarity of the absorbance spectra at up-slope, apex, and down-slope of the peak. Robustness was performed by varying the ratio of the second solvent system and expressed as % RSD of peak area.

Yeast α-glucosidase inhibitory activity

Thirty microliters of the aqueous solutions of the extracts, rosmarinic acid, and positive control (acarbose) were mixed with 30 µl of 0.5 Unit/ml yeast α-glucosidase in 96-well plate, incubated for 10 min at 37 °C, 30 µl of p-nitrophenyl-α-D-glucopyranoside (*1 mM in 0.1 M sodium phosphate buffer, pH 6.9*) added, incubated for 20 min at 37 °C, 80 µl of sodium carbonate added to stop the reaction, and the absorbance measured at 405 nm. The test was done in triplicate and the inhibition percentage was calculated as follows:

$$\% \text{ inhibition} = \frac{(\text{Absorbance of the negative control} - \text{Absorbance of the tested sample}) \times 100}{\text{Absorbance of the negative control}}$$

Results and discussion

Quality specification

The dried stem crude drug was light yellow with gray and reddish-brown bark, while the dried leaf crude drug was greenish-brown (**Figure 1**). The illustrated characteristics can be used as the simplest and most inexpensive tools for this crude drug identification. The histological characteristics of stem powder included fragment of vessel, cork in sectional view, lignified parenchyma, fragment of bordered pitted vessel, fragment of fiber, and raphide crystals (**Figure 2**). Cystoliths were not found in this study, indicating that *T. laurifolia* was distinguished from its related family, Acanthaceae [20]. The histological characteristics of leaf powder included stomata, fragment of epidermis, scale leaf, parenchyma cells,

spiral vessels, raphide crystals, fiber, and calcium oxalate prisms (**Figure 3**). The transverse section of dried stem revealed anatomical characteristics including cork, parenchyma of cortex, endodermis, vessel, and pith (**Figure 4**). Additionally, anatomical characteristics of *T. laurifolia* leaf in the transverse section included upper epidermis, palisade mesophyll, spongy mesophyll, xylem vessel, collenchyma cell, parenchyma cell, phloem, and lower epidermis (**Figure 5**).

Most values in the physico-chemical parameters of *T. laurifolia* leaf were higher than that of *T. laurifolia* stem (**Table 1**). Chemical constituents in particular solvents of the raw material can be estimated from the extractive values. In both raw materials, water-soluble extractive value was about 4 times higher than ethanol-soluble extractive value, and water content value was higher than loss on drying value. This could be used as an indicator to discourage the growth of fungi, yeast, and bacteria. Volatile oil content was undetected in this study, not only because the test samples were dried crude drug, but the result was in agreement with a previous study showing that lipid-containing structure was not evident in the plant cells [20].

Figure 1 *Thunbergia laurifolia* Lindl. dried leaf and stem crude drugs.

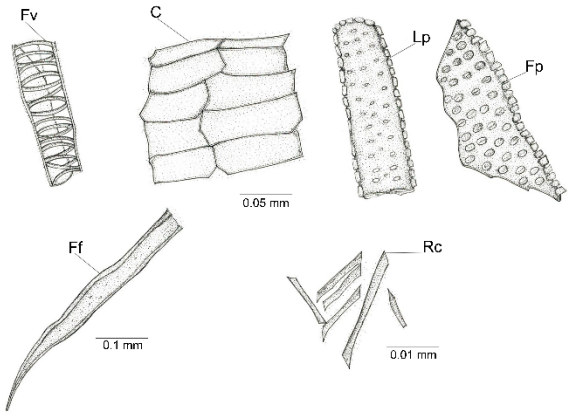


Figure 2 Histological characteristics of *T. laurifolia* stems. (Fv – fragment of vessel, C – cork in sectional view, Lp – lignified parenchyma, Fp – fragment of bordered pitted vessel, Ff – fragment of fiber, Rc – raphide crystals).

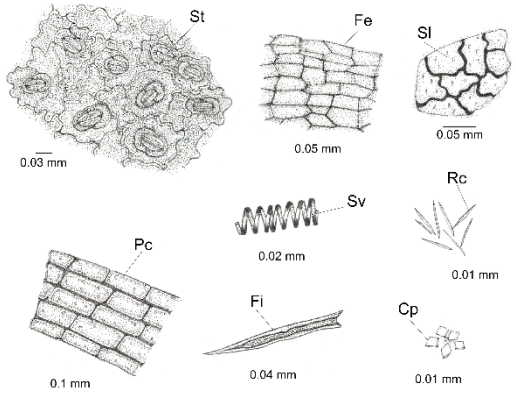


Figure 3 Histological characteristics of *T. laurifolia* leaves. (St – stomata, Fe – fragment of epidermis, Sl – scale leaf, Pc – parenchyma cells, Sv – spiral vessels, Rc – raphide crystals, Fi – fiber, Cp – calcium oxalate prisms).

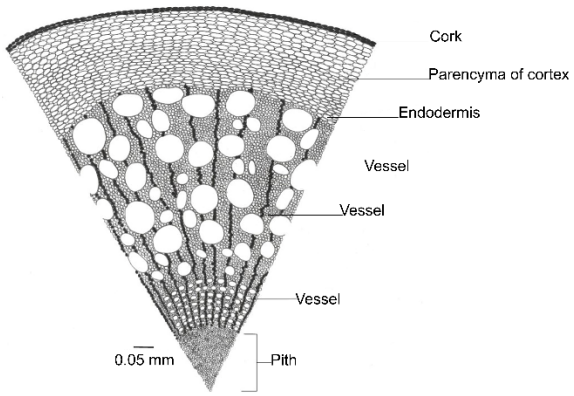


Figure 4 Anatomical characteristics of *T. laurifolia* stem in transverse section.

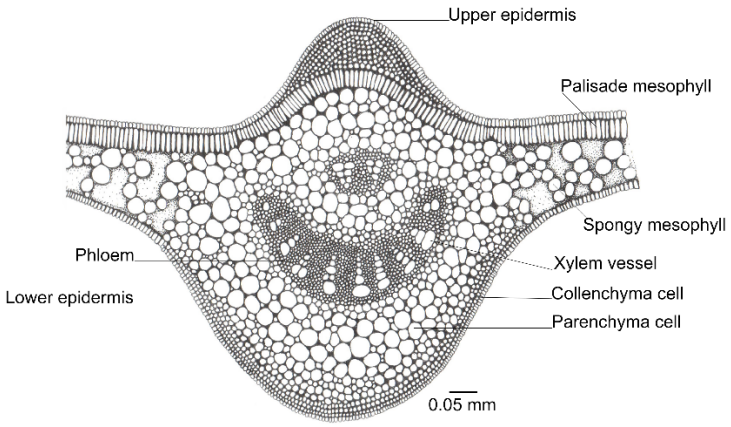


Figure 5 Anatomical characteristics of *T. laurifolia* leaf in transverse section.

Table 1 Physiochemical characteristics of *Thunbergia laurifolia* stems and leaves.

***T. laurifolia* stems content (g/100g)**	**Min-Max**	**Mean ± SD**
Acid-insoluble ash	1.90 – 5.06	3.33 ± 0.39
Total ash	6.03 – 11.02	8.44 ± 0.55
Ethanol-soluble extractive	1.42 – 4.72	3.04 ± 0.19
Water-soluble extractive	4.14 – 16.98	10.95 ± 0.19
Loss on drying	6.04 – 8.03	7.31 ± 0.16
Water content	4.12 – 16.98	10.61 ± 0.43
***T. laurifolia* leaves content (g/100g)**	**Min-Max**	**Mean ± SD**
Acid-insoluble ash	7.11 – 18.88	12.29 ± 0.43
Total ash	13.57 – 27.14	19.71 ± 0.20
Ethanol-soluble extractive	1.25 – 5.41	3.51 ± 0.16
Water-soluble extractive	7.30 – 14.39	10.46 ± 0.08
Loss on drying	5.45 – 11.07	8.42 ± 0.21
Water content	7.30 – 14.39	12.33 ± 0.76

Determination of rosmarinic acid content in *T. laurifolia* stems by quantitative TLC

In this study, TLC-densitometry and TLC-image analysis were used to quantify rosmarinic acid in *T. laurifolia* stem extract. Two mobile phases were developed in this study in order to decrease the spot tailing of rosmarinic acid using toluene-chloroform-acetone-formic acid (5:4:1:0.2, v/v). The methods were shown to be valid (**Figures 6 - 8**). In both methods, the accuracy was found to be around 80 to 100 % of the recovery in the stem and leaf extracts. The repeatability and intermediate precision obtained from both extracts and methods showed %RSD of less than 5.50. LOD and LOQ with suitable precision and accuracy were acceptable. The methods were found to be robust, with no alteration of the peak area in the TLC-densitometric method or the TLC-image analysis method (**Table 2**).

The contents of rosmarinic acid were found to be 0.120 ± 0.079 and 0.127 ± 0.094 % w/w in the stem extract, and 0.291 ± 0.150 and 0.303 ± 0.162 % w/w in the leaf extract, by 2 techniques, respectively (**Table 3**), which were not significant different ($p > 0.05$ by paired t-test). Thus, the 2 methods could be used to determine rosmarinic acid content in *T. laurifolia* stems and leaves. Rosmarinic acid quantification by HPLC in *T. laurifolia* leaves were previously reported as 0.16 to 5.30 % by dry weight [8]. Rosmarinic acid content in *T. laurifolia* stems was found to be less than in *T. laurifolia* leaves. The study of rosmarinic acid in *Rosmarinus officinalis* L. also showed that rosmarinic acid in the leaves was higher than in the stem [21].

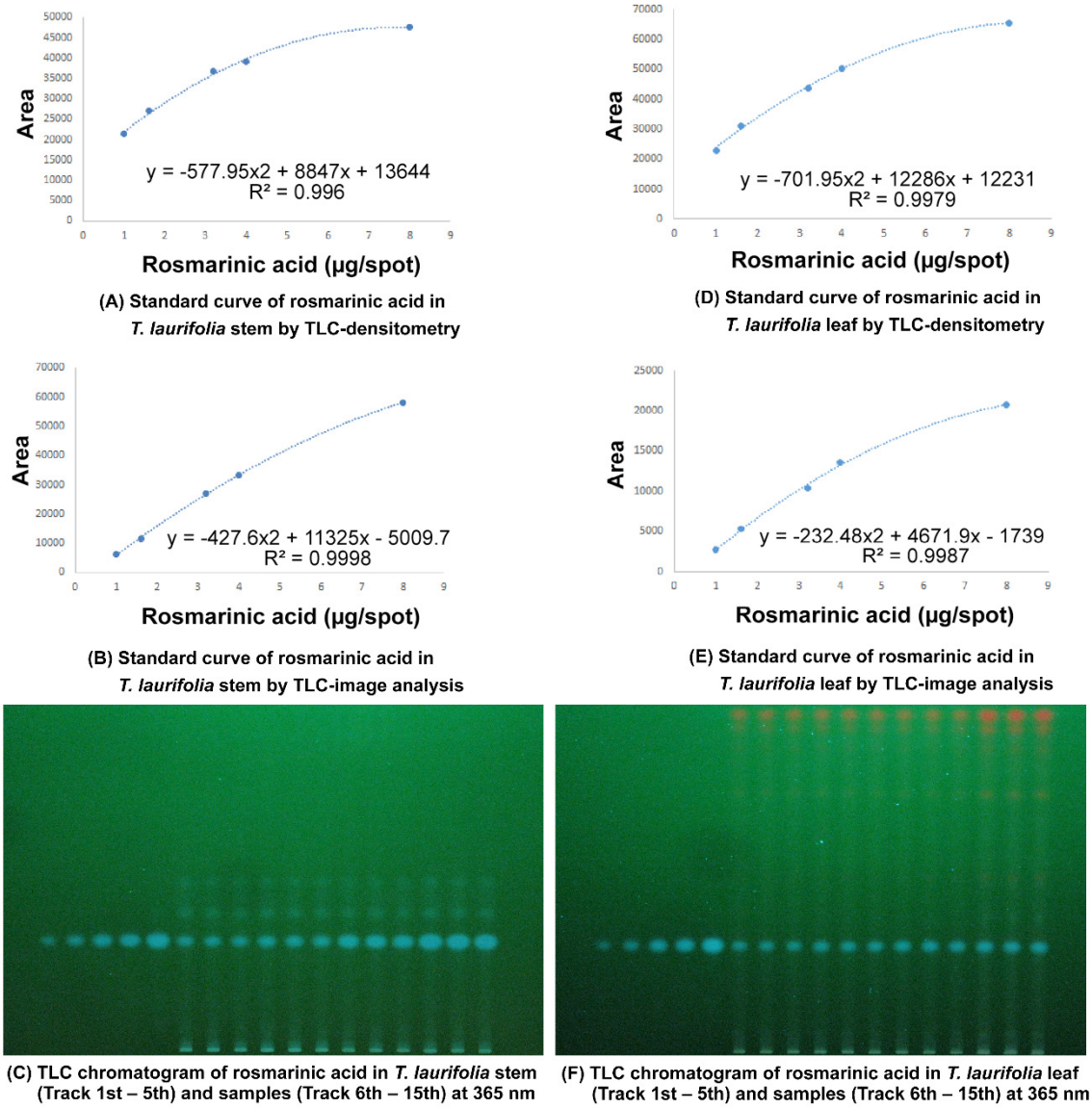


Figure 6 Standard curves and TLC chromatograms of rosmarinic acid in *T. laurifolia* stem and leaf.

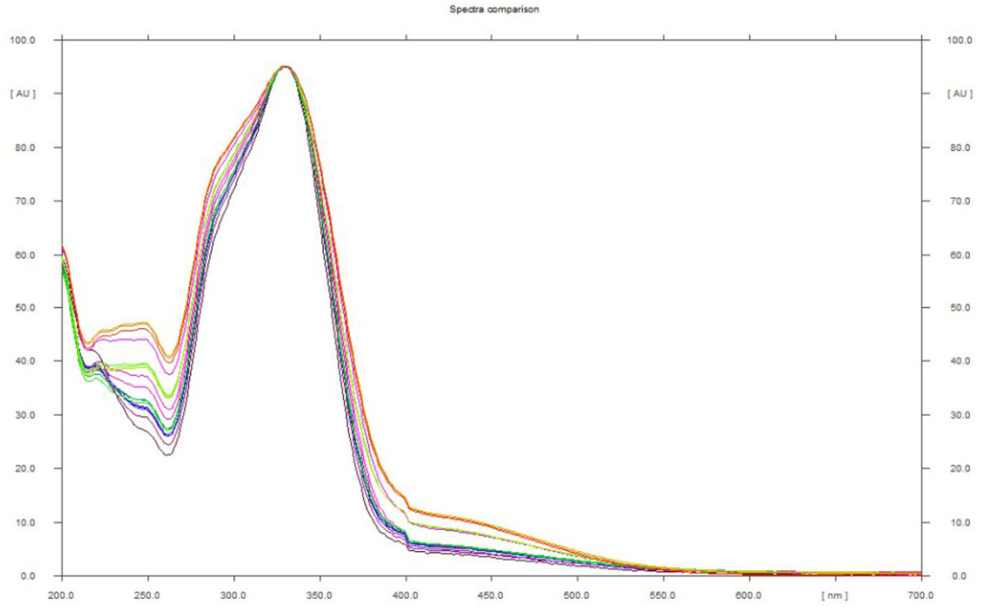


Figure 7 Peak identity determination by comparison of the ultraviolet absorbance spectra of rosmarinic acid in samples with standard rosmarinic acid.

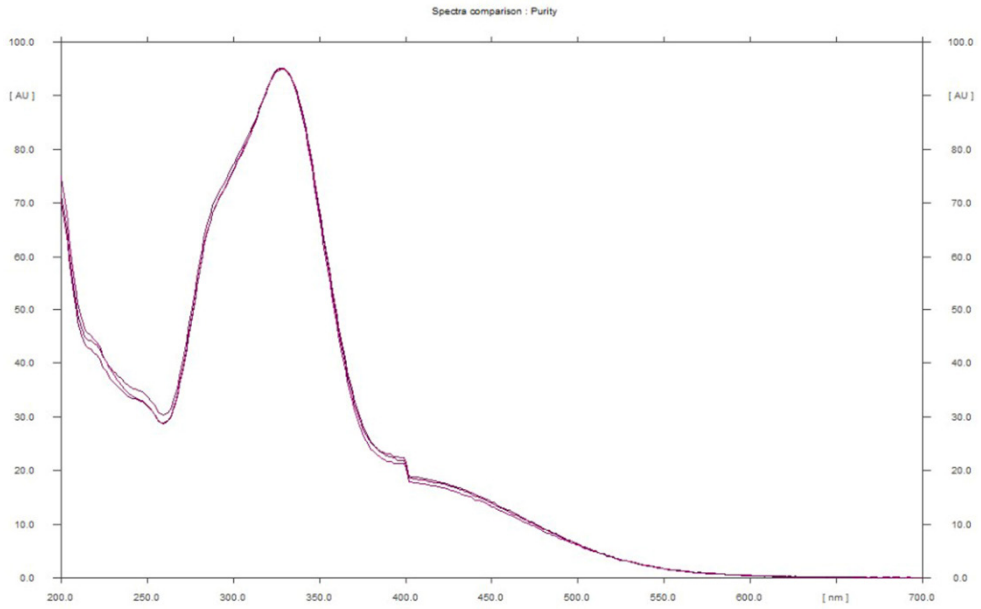


Figure 8 Peak purity determination using up-slope, apex, and down-slope of the peak.

Table 2 Method validity of TLC-densitometry and TLC-image analysis.

***T. laurifolia* stems** **Parameter**	**TLC-densitometry**	**TLC-image analysis**
Accuracy (%recovery)	98.1 – 102.6	97.5 – 99.3
Repeatability (%RSD)	1.25 – 2.20	1.27 – 3.45
Intermediate precision (%RSD)	4.01 – 5.08	1.53 – 4.05
Robustness (%RSD)	0.32 – 2.76	0.46 – 2.17
Limit of detection (LOD)	0.240	0.202
Limit of quantitation (LOQ)	0.726	0.612
***T. laurifolia* leaves** **Parameter**	**TLC-densitometry**	**TLC-image analysis**
Accuracy (%recovery)	89.3 – 96.4	89.5 – 98.1
Repeatability (%RSD)	1.54 – 3.89	1.04 – 1.76
Intermediate precision (%RSD)	1.29 – 4.88	2.14 – 3.00
Robustness (%RSD)	2.32 – 4.55	0.50 – 3.66
Limit of detection (LOD)	0.140	0.200
Limit of quantitation (LOQ)	0.423	0.606

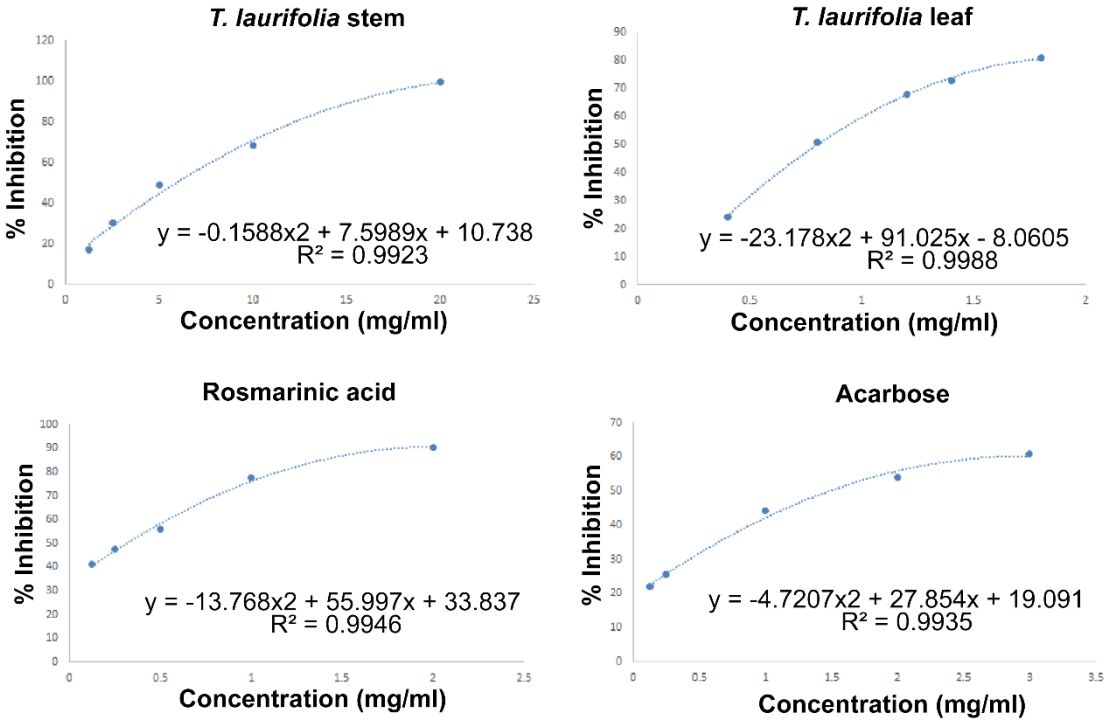
Table 3 Rosmarinic acid content (g/100g) in *T. laurifolia* stem and leaf from 15 sources throughout Thailand (each source was done in triplicate).

Source	**TLC-densitometric method**		**TLC-image analysis method**	
	stems	**leaves**	**stems**	**leaves**
1	0.152	0.551	0.136	0.578
2	0.079	0.117	0.081	0.132
3	0.308	0.513	0.384	0.623
4	0.222	0.356	0.206	0.405
5	0.104	0.064	0.109	0.070
6	0.026	0.353	0.030	0.348
7	0.044	0.090	0.048	0.100
8	0.089	0.333	0.086	0.332
9	0.088	0.252	0.082	0.257
10	0.113	0.348	0.127	0.358
11	0.061	0.176	0.068	0.241
12	0.241	0.215	0.277	0.198
13	0.074	0.188	0.081	0.166
14	0.086	0.351	0.091	0.338
15	0.113	0.454	0.105	0.401
Mean ± SD	0.120 ± 0.079	0.291 ± 0.150	0.127 ± 0.094	0.303 ± 0.162

Yeast α-glucosidase inhibitory activity

Inhibition of the key enzyme α-glucosidase, which is located in the brush border of the small intestine, has been of concern for hyperglycemia management. Acarbose is one of the currently-used medicines to treat diabetes mellitus. It retards the rate of glucose absorption by inhibiting the key enzyme to digest carbohydrate into glucose. The inhibitory activity of the stem extract, leaf extract, rosmarinic acid, and acarbose was depicted with IC_{50} of 5.89, 0.80, 0.31, and 1.48 mg/ml, respectively (**Figure 9**).

Zhu *et al.* [22] reported IC_{50} of rosmarinic acid on α-glucosidase from *Bacillus stearothermophilus* as 0.95 mg/ml, and the inhibitory potential of rosmarinic acid rich fraction of *Perilla frutescens* leaves was higher than *Perilla* leaf extract.



Materials	IC50 (mg/ml)
T. laurifolia stem extract	5.89
T. laurifolia leaf extract	0.80
Rosmarinic acid	0.31
Acarbose	1.48

Figure 9 Yeast α-glucosidase inhibitory activities of *T. laurifolia* stem extract, leaf extract, rosmarinic acid, and acarbose.

Conclusions

Pharmacognostic specification with reference to the rosmarinic acid content of the *Thunbergia laurifolia* stem and leaf was established for quality assessment of the crude drug. Yeast α-glucosidase inhibitory activity indicated that the stem and leaf possess antidiabetic potential. Developed TLC-densitometry and TLC-image analysis were valid for quantitative determination of rosmarinic acid in ethanolic extracts of *Thunbergia laurifolia* stem and leaf. TLC-image analysis using a free analytical software, ImageJ, a Java-based image processing program, could be used as an alternative method for

rosmarinic acid quantification in *T. laurifolia* stem and leaf, due to its simplicity, reduced time consumption, and inexpensiveness of the instruments.

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