Antioxidant-guided Isolation of Rosmarinic Acid, a Major Constituent from *Thunbergia laurifolia*, and Its Use as a Bioactive Marker for Standardization

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ABSTRACT

Growing interest in the commercialization of *Thunbergia laurifolia* leaves as an herbal tea and the use of its extracts as a nutraceutical has led to increased research surrounding its bioactive components and the standardization of the plant. Guided isolation through thin-layer chromatography (TLC) bioautography using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical as a detection reagent led to the isolation of a potent antioxidant compound, rosmarinic acid, from the leaves of *T. laurifolia*. This study is the first report of the isolation of rosmarinic acid from *T. laurifolia*. Rosmarinic acid showed significant DPPH-scavenging capacity with an EC$_{50}$ value of 2.71 μg/ml. The high-performance liquid chromatography (HPLC) quantification of rosmarinic acid was accomplished. The developed method was applied to determine the amount of rosmarinic acid in *T. laurifolia* samples collected from different locations. The results demonstrated high variability in the amount of rosmarinic acid present in the samples, which correlated well with the observed antioxidant capacities detected by the free-radical scavenging assay. The HPLC method was also validated in terms of linearity, precision, and accuracy. The established HPLC method for the determination of rosmarinic acid, a bioactive principle, is a useful approach for standardization of the raw material and herbal preparations containing *T. laurifolia*.

Keywords: *Thunbergia laurifolia*, rosmarinic acid, bioactivity-guided isolation, HPLC; DPPH-scavenging activity, Acanthaceae

1. INTRODUCTION

*Thunbergia laurifolia* Lindl., commonly known in Thai as “Rang Chuet”, is a woody climbing plant belonging to the Acanthaceae family. In Thailand, the decoction of the leaves and roots of this plant is traditionally used as an antidote for poisoning caused by insecticides, ethyl alcohol, arsenic, and strychnine[1]. Currently, herbal teas, powders, and capsule preparations of *T. laurifolia* are commonly available in the herbal and nutraceutical markets. Despite its common use, no commercially available preparations with
determined content or standardized products exist, likely due to a lack of information about the active phytochemicals in _T. laurifolia_.

The leaves of _T. laurifolia_ have been described as a good source of natural antioxidants because of the high radical scavenging and ferric-reducing effects observed in their extracts [2]. Several biological activities related to antioxidants have been recently reported for _T. laurifolia_, including hepatoprotective [3], neuroprotective [4], and antimutagenic properties [5]. However, only a few studies have earnestly researched its bioactive constituents. Two iridoid glycosides, 8-epi-grandifloric acid and 3′-O-β-glucopyranosyl-stilbericoside, and seven known glycosides were isolated from the aerial part of _T. laurifolia_ [6], but their biological activities have not been investigated. The presence of flavonoids, phenolic compounds, and chlorophyll derivatives in _T. laurifolia_ extracts were considered to modulate the antimutagenic and detoxifying effects [5]. However, these compounds have not been investigated in the context of the biological functions of the plant. In the identification of the bioactive constituents in _T. laurifolia_ leaves responsible for antioxidant activity, the separation was guided by a TLC bioautographic assay. This method provides rapid detection and localization of the active compounds in the complicated plant extract [7].

Analysis of the active components in herbs and herbal mixtures is essential for evaluating the quality and authenticity of herbal medicines [8]. Currently, limited information is available regarding the characterization of the chemical components in _T. laurifolia_, necessitating its chemical characterization. Among the available analytical methods, HPLC is frequently used for the qualitative and quantitative analysis of herbal drugs due to its easy operation, wide suitability, and high accuracy [9]. Thus, utilizing HPLC for analysis by establishing the chromatographic fingerprint and determining the bioactive compounds is appropriate for evaluating the quality of _T. laurifolia_.

In the present study, activity-guided fractionation was conducted to isolate the free-radical scavenging constituent from the leaves of _T. laurifolia_. Subsequently, an HPLC method with UV detection was developed and validated for the determination of rosmarinic acid, the major antioxidant compound found in _T. laurifolia_. In addition, the developed method was used to test _T. laurifolia_ leaf extracts collected from different locations.

2. MATERIALS AND METHODS

2.1 Sample Collection and Preparation

The _T. laurifolia_ leaves used for the isolation of the active components were collected from Nakhon Pathom province, Thailand. Five additional samples were collected from different locations in Thailand. All plant samples were identified by Associate Professor Thatree Phadungcharoen, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University. The voucher specimens were kept in the Museum of Natural Medicines at the Faculty of Pharmaceutical Sciences, Chulalongkorn University.

The plant samples (20 g) were powdered using an electric blender and macerated three times with 95% ethanol (200 ml) for 72 h at room temperature. Subsequently, each extract was filtered through Whatman No. 1 filter paper and evaporated to dryness at 50°C. Ten mg of each extract was dissolved in ethanol to a total volume of 1 ml and was then filtered through 0.22 mm Millipore filter for HPLC analysis.
2.2 Antioxidant-guided Isolation of Rosmarinic Acid

The dried *T. laurifolia* leaves (900 g) were macerated with 10 liter of 95% ethanol three times to give ethanolic extract (64.75 g). The extract was applied to a resin column, 350 g of Diaion HP20 synthetic adsorbents (Mitsubishi Chemical, Japan), and eluted with a gradient mixture of water and acetone (1:0, 3:1, 1:1, 1:3, 0:1) in volume ratio and 100% of ethyl acetate. All of the 6 obtained fractions (F1-6) were examined for DPPH-scavenging property via TLC bioautography. The DPPH-scavenging active fraction was identified as a yellowish spot on the purplish background of the TLC plate. F3-4 indicated active antioxidant compounds on sprayed TLC plate than that of the others. Fraction 4 (7.74 g) was selected for further separation. The isolation was then conducted on a silica gel column (105 g, 14 × 80 cm). The column was eluted with a chloroform-methanol-formic acid (8.5:1.5:0.5) mixture. The fraction containing the major yellowish spot was then submitted to column chromatography on Sephadex LH-20 (GE Healthcare Bio-Sciences, Sweden) (45 g, 4 × 120 cm) and was eluted with methanol. The elute samples were collected in 4 fractions (T1-4). The DPPH-scavenging active fraction (T2) was purified on a silica gel column (60 g, 4 × 60 cm) with a gradient system of dichloromethane-methanol (7:3 to 0:1) to obtain a yellow compound (235.3 mg).

The compound was identified, using spectral analysis and comparison to previous data [10], as rosmarinic acid (Figure 1). Rosmarinic acid compound displayed the UV (MeOH) maxima (λ<sub>max</sub>) at 290 and 326 nm using UV160U UV-Vis spectrophotometer (Shimadzu, Japan). All NMR spectra (in DMSO-<sub>d6</sub>) were measured on a JEOL Lambda 600 MHz spectrometer (JEOL, Japan). A detailed analysis of the <sup>1</sup>H and <sup>13</sup>C NMR spectra of the isolated rosmarinic acid agreed well with previous report [10]. The signals of olefinic protons at 7.46 and 6.24 (each 1H, d, 15.6 Hz) specified the structure as trans-rosmarinic acid with the assignment of R-configuration at C-8′ position based on the specific rotation, [α]<sub>DL</sub> +22.9° (c = 0.2, MeOH) measured by PerkinElmer 341 polarimeter (PerkinElmer, USA). In addition, its identification was confirmed by a negative ESI-MS analysis (m/z 359, [M-H]) using an AB-SCIEX QTRAP 5500 mass spectrometer (AB-SCIEX, USA).

Figure 1. Chemical structure of rosmarinic acid isolated from the leaves of *T. laurifolia*. 
2.3 HPLC Analysis

The plant extracts and the isolated rosmarinic acid were analyzed using an HPLC system equipped with a LC-20AD HPLC, a CTO-20A column oven, and a SPD-M20A diode array detector (Shimadzu, Japan). The separation was performed on a 4.6 × 250 mm Cosmosil 5C18-AR-II column (Nacalai Tesque, Japan) at ambient room temperature. The mobile phase was isocratic water-methanol-acetic acid (65:35:0.1). The flow rate was 1.0 ml/min, and the injection volume was 20 μl. The signal was monitored at 320 nm in accordance with the UV maximum absorption of rosmarinic acid. Standard solutions of rosmarinic acid (10 to 1000 μg/ml) (Sigma-Aldrich, USA) were prepared. Chromatographic peaks of the plant extracts were identified by comparing their retention times and UV spectra against a reference sample of rosmarinic acid.

2.4 Method Validation

The suitability of the HPLC system was evaluated by determining the intra- and inter-day precision of the replicates. The accuracy was evaluated through recovery studies by adding known amounts of standard rosmarinic acid to the extract. The recovery experiments were performed with three concentrations of the standard. To measure the linearity, a calibration line was made and correlation coefficient was calculated based on the obtained calibration line. Both the calibration line and the residuals were graphically inspected and evaluated.

2.5 DPPH-scavenging Assay

The radical scavenging activity was evaluated using the DPPH radical and a standard spectrophotometric assay in a 96-well microplate with slight modifications [11]. A 20 μl sample of the extract and 180 μl of a 0.1 mM methanolic DPPH solution were added to each well. The plate was covered with aluminum foil and left at room temperature for 30 min. Each well’s absorbance was measured at 510 nm against a solvent blank to estimate the scavenging capacity of each sample using Victor 3 plate reader (PerkinElmer, USA). The free-radical scavenging activity of the ethanolic extracts from each T. laurifolia sample and the isolated rosmarinic acid were evaluated. Trolox and quercetin were used as positive controls. The scavenging capacity was calculated as the effective concentration at which the DPPH radicals were scavenged by 50% (EC50).

3. RESULTS AND DISCUSSIONS

3.1 Antioxidant-guided Isolation of Rosmarinic Acid

The isolation of an antioxidant compound from the 95% ethanolic extract from the dried leaves of T. laurifolia was achieved with DPPH autography-directed separation. The isolated compound was identified as rosmarinic acid and represented at least 0.36% of dry extract. This work is the first report of the isolation of rosmarinic acid from T. laurifolia. This successful bioassay-guided fractionation is in accordance with earlier studies that effectively applied this method to the rapid separation of active compounds from several medicinal plants. For example, vasorelaxant compounds were isolated from Ziziphora clinopodioides [12] and anxiolytic compounds were isolated from Melissa officinalis [13].

The free-radical scavenging capacities of the isolated rosmarinic acid and two positive controls, trolox and quercetin, were measured using the DPPH assay (Table 1). Rosmarinic acid exhibited a higher antioxidant potential (2.71 ± 0.08 μg/ml) than that of trolox (3.51 ± 0.04 μg/ml) but lower than that of quercetin (0.62 ± 0.03 μg/ml). A good
correlation ($r = 0.9611$) was observed between the rosmarinic acid concentration and its free-radical scavenging activity. This result is in agreement with previous reports that showed a strong capacity of rosmarinic acid for scavenging free radicals [14, 15]. The presence of two ortho-dihydroxyphenyl groups and multiple conjugated double bonds in the structure of rosmarinic acid contributes to its ability to scavenge free radicals [16]. Rosmarinic acid has been reported to have other biological activities, including anti-inflammatory [17], antimutagenic [18], hepatoprotective [19], anticholinesterase [20], and antiviral properties [21]. The presence of rosmarinic acid in *T. laurifolia* may be related to the anti-inflammatory [22], antimutagenic [5], and hepatoprotective [3] properties previously reported for this plant and may provide key chemical evidence for determining the contribution of rosmarinic acid to these activities.

### Table 1. Rosmarinic acid content and DPPH scavenging activity of *T. laurifolia* plants from different locations.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sample no.</th>
<th>Location collected (province)</th>
<th>Rosmarinic acid (mg/g dry weight)</th>
<th>EC$_{50}$ DPPH (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. laurifolia</em></td>
<td>1</td>
<td>Nakhon Pathom</td>
<td>38.82±2.54$^a$</td>
<td>32.84±0.72$^a$</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Nonthaburi</td>
<td>1.56±0.14$^b$</td>
<td>114.51±1.21$^b$</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Nakhon Sawan</td>
<td>14.86±0.83$^c$</td>
<td>67.96±1.89$^c$</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Prachin Buri</td>
<td>53.32±2.18$^d$</td>
<td>30.62±0.36$^d$</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Phetchabun</td>
<td>8.68±0.76$^e$</td>
<td>86.24±2.24$^e$</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Samut Prakan</td>
<td>3.01±0.22$^b$</td>
<td>78.05±0.34$^e$</td>
</tr>
</tbody>
</table>

Rosmarinic acid (isolated) - - - - 2.71±0.08
Trolox - - - - 3.51±0.04
Quercetin - - - - 0.62±0.03

Values are expressed as the mean of three replicates ± SD. The different letters (a, b, c, d, and e) within the same columns indicate a significant difference at $p < 0.05$ by Tukey’s test.

### 3.2 Rosmarinic Acid as a Bioactive Marker for Standardization

Not only is rosmarinic acid an active compound in the leaves of *T. laurifolia*, but it is also the major constituent. The compound can be used as a chemical marker for the quality control of raw materials or any preparations containing *T. laurifolia* as an active ingredient. In this study, an HPLC elution procedure was developed and used to determine the amount of rosmarinic acid in a sample. The mobile phase consisted of water-methanol-acetic acid (65:35:0.1) and provided good resolution for the target compound and satisfied the analysis. A typical chromatogram of the isolated rosmarinic acid and its absorption spectrum measured by the HPLC photodiode array detector are shown in Figure 2A. The rosmarinic acid peak had a retention time of approximately 33 min and acceptable symmetry. The chromatogram of the rosmarinic acid standard showed another small peak at approximately 30 min. This impurity peak was also found in the rosmarinic acid standard reported by Yuan et al., 1998 [23]. Rosmarinic acid was identified in the crude extracts of *T. laurifolia* by comparing its retention time and absorption
spectrum with those from the standard rosmarinic acid sample under the same chromatographic conditions. Good separation between rosmarinic acid and the other constituents in the crude ethanolic extracts of *T. laurifolia* was observed (Figure 2B). The established HPLC method was subsequently validated to ensure its suitability for the analysis of rosmarinic acid.

**Figure 2.** HPLC chromatogram of isolated rosmarinic acid from the reference material (A) and the ethanolic extract of *T. laurifolia* (B). A Cosmosil® 5C₁₈-AR-II (250 mm × 4.6 mm) column was used with an isocratic water-methanol-acetic acid (65:35:0.1) at a flow rate of 1.0 ml/min and a detection wavelength of 320 nm. The inset shows the online UV spectrum of the arrowed peak (rosmarinic acid).
The results for the evaluation of the calibration model are shown in Table 2. Rosmarinic acid exhibited good linearity in the range of 25 to 200 μg/ml for its calibration curve generated from the HPLC data. The calibration line could be described by the following equation, y = 36921.74x - 136340.40, and the correlation coefficient (r) was at least 0.99. The HPLC determination of rosmarinic acid also yielded precise and accurate results. The precision was evaluated by analyzing a reference solution of rosmarinic acid six times within the same day and once daily for three consecutive days. The intra- and inter-day variation studies indicated that the relative standard deviations were less than 1.76% and 2.68%, respectively (Table 3). The standard deviation data indicated that the precision results were acceptable. The recovery experiment was evaluated by adding known quantities of rosmarinic acid to the T. laurifolia extracts. The results were calculated by comparing the actual and theoretical amounts of detected rosmarinic acid. The amount of rosmarinic acid recovered from the extract at three different concentrations, 50, 100, and 200 mg/g, were 109.87% (± 3.94 RSD), 109.10% (± 5.90 RSD), and 102.02% (± 3.05 RSD), respectively (Table 4), which indicates that the method has good accuracy.

Table 2. Linear regression data of rosmarinic acid analyzed by HPLC.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Linear range(μg/ml)</th>
<th>Slope ± SD</th>
<th>Intercept ± SD</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rosmarinic acid</td>
<td>25.00-200.00</td>
<td>36921.74±875.18</td>
<td>-136340.40±74324.98</td>
<td>0.9996</td>
</tr>
</tbody>
</table>

Table 3 Intra- and inter-day precision values of rosmarinic acid analyzed by HPLC.

<table>
<thead>
<tr>
<th>Number of replicates</th>
<th>Day 1 Area 8,215,010</th>
<th>Day 2 Area 8,294,414</th>
<th>Day 3 Area 8,638,269</th>
<th>Inter-day Area 8,382,564</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>18</td>
<td>0.81</td>
</tr>
</tbody>
</table>

Table 4. Recovery data of rosmarinic acid from the extracts of T. laurifolia analyzed by HPLC.

<table>
<thead>
<tr>
<th>Amount added (mg/g)</th>
<th>Amount detected (mg/g)</th>
<th>Recovery (%)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50.00</td>
<td>54.93 ± 2.16</td>
<td>109.87</td>
<td>3.94</td>
</tr>
<tr>
<td>100.00</td>
<td>109.10 ± 6.44</td>
<td>109.10</td>
<td>5.90</td>
</tr>
<tr>
<td>200.00</td>
<td>204.04 ± 6.22</td>
<td>102.02</td>
<td>3.05</td>
</tr>
</tbody>
</table>

The HPLC method developed as described above was applied to the determination of the rosmarinic acid concentration in T. laurifolia extracts obtained from various locations in Thailand. HPLC methods have been successfully used to determine the rosmarinic acid content in several plants, such as Rosmarinus officinalis [24], Melissa officinalis [25], and Salvia officinalis [26]. As shown in Table 1, the rosmarinic acid content in T. laurifolia varied from 1.56 to 53.32 mg/g of dry plant. This high variability in the rosmarinic acid level is similar to those reported for S. officinalis, one of the plants with an abundance of rosmarinic acid, which varied from 6 to 47 mg/g of dry weight [27]. The highest concentration of rosmarinic acid (53.32 ± 2.18 mg/g dry weight) in T. laurifolia was observed in sample no. 4, whereas sample no. 2 (1.56 ± 0.14 mg/g dry weight) contained the lowest concentrations. The high degree of variability could be due to environmental
factors such as growing conditions or the age of the plants during sample collection [27].

Our results also indicated a high degree of variability in the DPPH-scavenging activity for *T. laurifolia* collected from different locations. As shown in Table 1, sample no. 1 (32.84 ± 0.72 μg/ml) and sample no. 4 (30.62 ± 0.36 μg/ml) produced the highest DPPH-scavenging effects. These two strong DPPH-scavenging samples, sample no. 1 and sample no. 4 also possess high rosmarinic acid content (38.82 ± 2.54 and 53.32 ± 2.18 mg/g dry weight, respectively). The lowest activity was reported for sample no. 2, which had the highest EC$_{50}$ value of 114.51 ± 1.21 μg/ml. Sample no. 2 was found to contain the lowest amount of rosmarinic acid (1.56 ± 0.14 mg/g dry weight). The DPPH-scavenging capacity among six *T. laurifolia* samples tend to be consistent with the amount of rosmarinic acid found in an extract. A good correlation (0.8399) between the free-radical scavenging activity against DPPH and the amount of rosmarinic acid in each *T. laurifolia* sample (Figure 3) was found. This correlation is in agreement with several studies that showed that the rosmarinic acid concentration accounted for the antioxidant potential [27, 28]. Hence, rosmarinic acid is most likely responsible for the majority of *T. laurifolia*’s antioxidant activity.

![Figure 3. A graph depicting the correlation between the rosmarinic acid content and the EC$_{50}$ value of DPPH-radical scavenging activity found for each *T. laurifolia* sample.](https://example.com/figure3.png)

\[ y = -1.3977x + 96.3840 \]
\[ R^2 = 0.8399 \]

4. CONCLUSIONS

Currently, there are no standardized production methods or accepted measures for the manufacture of *T. laurifolia* as an herbal drug; therefore, there is no guarantee of potency or efficacy. Searching for the bioactive components of *T. laurifolia* is the first step in designing a quality control procedure. As a result, a potent antioxidant compound, rosmarinic acid, was isolated from *T. laurifolia*. Because rosmarinic acid demonstrated a significant free-radical scavenging effect, it can be used as the reference compound for the quality assurance of *T. laurifolia*. In the present study, an HPLC method was established and applied to determine rosmarinic acid levels in *T. laurifolia*. The method is simple, sensitive, and suitable for the standardization of raw materials and commercial preparations containing *T. laurifolia*. The present study also showed...
that the rosmarinic acid content in *T. laurifolia* leaves correlated well with its DPPH-scavenging capacity, indicating that it contributes significantly to the overall antioxidant activity. The bioactivity of this compound might provide a chemical basis for some of the health benefits seen with the leaves of *T. laurifolia* in folk medicine and warrant further studies to assess its potential as an effective natural remedy.

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