

# Determination of Rosmarinic Acid and Caffeic Acid in *Thymus daenensis* and *Thymus lancifolius* Extracts by High-Performance Thin Layer Chromatography

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**Abstract:** *Thymus* species belong to the *Lamiaceae* family, of which 18 species in the flora of Iran, 6 are endemic to Iran. In the current research, high-performance thin-layer chromatography (HPTLC) technique as an easy, fast, reproducible, and low-cost method was used for the determination of rosmarinic acid and caffeic acid in *Thymus lancifolius* (*T. lancifolius*) and two species of *Thymus daenensis* (*T. daenensis*) from Iran. Toluene-ethyl acetate-formic acid with a ratio of 67.72-22.90 and 9.38% was selected as the mobile phase of rosmarinic acid, and ethyl acetate-methanol-formic acid-water with a ratio of 85-8-2 and 5% was designated as the mobile phase of caffeic acid. The highest and lowest amount of rosmarinic acid was observed for *T. daenensis* 1 (10.54±0.12 mg/g) and *T. lancifolius* (0.46±0.01 mg/g), respectively. The amount of rosmarinic acid for *T. daenensis* 2 was obtained as 7.85±0.02 mg/g for each of the dried plants. In the following, HPTLC analysis of caffeic acid for *T. daenensis* 1, *T. daenensis* 2, and *T. lancifolius* was acquired amounts of 0.78±0.007, 0.13±0.007, and 0.26±0.007 mg/g for each of dried plants, respectively. Therefore, regarding the special effects of phenolic acids and properties of the *Thymus* genus, the acquired results are suitable for application in pathogenic research, infections, immunology diseases, and evaluation of the antioxidant activity.

**Keywords:** caffeic acid; high-performance thin-layer chromatography; rosmarinic acid; *Thymus daenensis*; *Thymus lancifolius*.

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## 1. Introduction

The genus *Thymus*, recognized as "Avishan" in Persian, belongs to the *Lamiaceae* family, of which 18 species in the flora of Iran, 6 are endemic to Iran [1, 2]. Antiseptic, antitussive, antibacterial, antifungal, antiviral, expectorant, and antispasmodic properties of *Thymus* species have been reported [3-5]. The aerial parts of *Thymus* species have been suggested in traditional medicine as carminative, digestive, antispasmodic, antitussive, and expectorant [6]. The most medicinal properties of *Thymus* species are related to their essential oils, particularly thymol [1, 7], phenolic acids, flavonoids, and tannins [8]. As a species of the

*Thymus* genus, *Thymus daenensis* (*T. daenensis*) is used for its aromatic properties and medicinal effects [9]. *T. daenensis*, known as "Avishane-denaie" in Persian, grows wildly in the central and northwestern parts of Iran [10]. Various species of the *Lamiaceae* family are very rich in flavonoids, phenolic acids, and phenolic diterpenes, which show great antioxidant effects, impediment or restrain the activity of free radicals [11-15]. Rosmarinic acid (RA) as an ester of caffeic acid (CA) and 3,4-dihydroxy phenyl lactic acid acts like a defense compound against cancer [16]. RA's attendance in medicinal plants, herbs, and spices has valuable and health-stimulating effects [16]. Adstringent, antioxidative, anti-inflammatory, antimutagen, antibacterial, and antiviral activities have been described for RA as a phenolic compound [17]. Caffeic acid (CA) as another phenolic acid significantly influences plants' protein solubility [18]. CA is generally recognized for hepatoprotective and hypolipidaemic activities via inhibition of lipid peroxidation and antioxidant properties.

For the analysis of complex mixtures, high-performance thin-layer chromatography (HPTLC) has several benefits over other analytical techniques: simultaneous determination of various samples, small amounts of the mobile phase, automation, easy sample preparation, rapid analysis, and inexpensive [19].

Despite many studies on the medicinal or functional properties of the *Thymus* genus, only a few reports have been described to measure the phenolic constituents of these plants. The current research aims to determine RA and CA in the *Thymus* genus using HPTLC as a fast, reproducible, and cheap technique.

## 2. Materials and Methods

### 2.1. Chemicals.

HPTLC plate silica gel 60 F<sub>254</sub> (20 × 10 cm, Merck, Darmstadt, Germany); MeOH; toluene; ethyl acetate (Samchun, Korea); formic acid purity of 99.9% (Biochem, France); rosmarinic acid and caffeic acid (Sigma-Aldrich, Germany) were purchased. All materials were of analytical grade.

### 2.2. Sample preparation.

The aerial parts of *T. lancifolius*, *T. daenensis* 1, and *T. daenensis* 2 were collected from Moorgol Peak (Kohgiluyeh and Boyer-Ahmad Province, Iran), Sheshpeer (Fars Province, Iran), and Kakan (Kohgiluyeh and Boyer-Ahmad Province, Iran) in July 2013, respectively. Voucher herbarium specimens of the *Thymus* genus were described in Table 1. The plants were dried at a temperature of 28°C and then powdered. The extraction procedure was performed using two methods of reflux and ultrasonic. For the reflux procedure, 2 g of each powdered plant was dissolved in 50 mL of MeOH and heated under reflux for 30 min, four times. The methanolic extracts were combined, concentrated, and diluted using distilled water with a ratio of 1:1. In the following, each solution was extracted four times with 100 mL petroleum ether. The aqueous layers were concentrated to 1 mL and filled up to 10 mL with MeOH to provide a dilution factor ( $\alpha$ ) of 0.1. Finally, 2 mL of the obtained solution was diluted using MeOH to acquire a solution with  $\alpha=0.02$ .

For the ultrasonic procedure, 2 g of each powdered plant was dissolved in 50 mL of MeOH and heated under an ultrasonic water bath for 15 min at a temperature of 80°C. After filtration, the method was followed like the first method.

Stock solutions of 0.05 and 0.005 mg mL<sup>-1</sup> RA and CA were prepared using dissolving appropriate amounts of RA and CA in MeOH.

**Table 1.** Ecological distribution of *Thymus* species.

Plants Name	Herbarium No.	Collection Data
<i>T. lancifolius</i>	HYU 30231	Faculty of Agriculture, Yasouj University, Iran.
<i>T. daenensis</i> 2	HYU 30245	Faculty of Agriculture, Yasouj University, Iran.
<i>T. daenensis</i> 1	568	Department of Pharmacognosy, Shiraz University of Medical Sciences, Shiraz, Iran.

### 2.3. Instrumental parameters

Chromatographic separation at room temperature and 20% humidity was performed by Camag HPTLC, made in Switzerland, equipped with ATS4, ADC2, scanner 3, and visualizer. The samples, in the form of a band, were spotted by ATS4 under N<sub>2</sub> gas (5 bar pressure) on an HPTLC plate silica gel 60 F<sub>254</sub> (20 × 10 cm) with a band length of 6 mm and distance of between tracks 11.3 mm. 15 µl of extracts solution of *T. lancifolius*, *T. daenensis* 1, and *T. daenensis* 2 was applied. Developing of the plates was done by ADC2 to the following parameters: toluene-ethyl acetate-formic acid with a ratio of 67.72-22.90 and 9.38%, respectively as the mobile phase of RA and ethyl acetate-methanol-formic acid-water with a ratio of 85-8-2 and 5%, respectively as the mobile phase of CA; plate preconditioning time 1.0 min; filling volume 10 mL; migration distance 75 mm; and drying time 1.0 min. Scanning of plates at the wavelength of 366 nm and absorption mode was carried out by scanner 3 to the following settings; slit dimension, 6.00 mm × 0.40 mm, macro; scanning speed, 20 mm/s; data resolution, 100 µm/step; and lamp D2. Finally, the image of plates was obtained by visualizer at 254, 366, and visible wavelengths. Recording of data was done by WinCATS software.

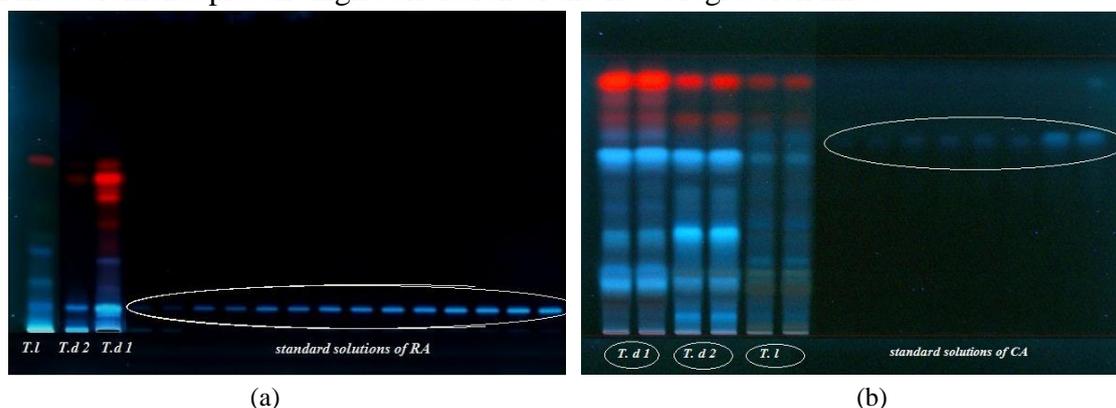
## 3. Results and Discussion

There are different reports about the analysis of essential oil of the *Thymus* genus, its antioxidant and antifungal activities. For example, analysis of essential oil of *T. daenensis* subspecies *daenensis* Celak by Alizadeh *et al.* showed the main components of thymol (66.62-71.49 %), *p*-cymene (5.52-7.12 %),  $\beta$ -caryophyllene (3.91-4.09 %),  $\delta$ -terpinene (3.22-4.3 %) and carvacrol (2.64-2.77 %) [10]. Also, total phenolic content, antioxidant and antifungal activity of *T. daenensis* were investigated by Alizadeh *et al.* [10]. A similar analysis by Amiri showed high antioxidant activity of polar subfraction of *T. daenensis* subspecies *lancifolius* (Celak) Jalas in DPPH assay [2].

The chemical composition of essential oil of investigated plants in the current research was studied by Jaberi *et al.* [20]. Thymol (39.91 %), carvacrol (29.93 %), linalool (5.55 %), caryophyllene (3.5 %) and geraniol (3.09 %) were identified as the major components of the essential oil of *T. daenensis*, but the major components of the essential oil of *T. lancifolius* were as follows: carvacrol (25.55 %), thymol (20.79 %), linalool (16.8 %),  $\alpha$ -terpineol (6.34 %), borneol (4.00 %), caryophyllene (3.98 %), *p*-cymene (3.38 %) and cis-linalool oxide (3.21 %). Furthermore, essential oil composition and antioxidant activity of ethanolic extract of *T. daenensis* Celak was studied by Sabahi *et al.* [21]. Their research demonstrated no antioxidant ability by ferric-reducing antioxidant power (Frap) assay, whereas the extract showed antioxidant activity. Other RA and CA measurements in *Thymus* extracts were performed using LC-MS [22] and HPLC [23-25].

In the current study, CA and RA's determination as phenolic compounds in the *Thymus* genus was investigated. The extraction procedure was done by two methods of reflux and ultrasonic, which caused the same results. So, due to the similarity between data, only the ultrasonic results were reported. Extraction procedures based on ultrasound have shown a great attraction because of their affirmative effects on time and yield of extraction, particle size, and solvent consumption [26, 27]. The best solvent system for chromatographic separation of RA was toluene-ethyl acetate-formic acid with the ratio of 67.72-22.90 and 9.38%, respectively. Also, ethyl acetate-methanol-formic acid-water with a ratio of 85-8-2 and 5%, respectively, was selected as the CA's mobile phase. The spots of RA and CA at the wavelength of 366 nm, seen as blue bonds on the HPTLC plate, were detected at an  $R_f$  value of  $0.11 \pm 0.02$  for RA and  $0.73 \pm 0.02$  for CA (Figure 1). Subsequently, there was no overlap with each other. In the following, TLC scanner 3 was applied for detection and quantitation of RA and CA at the wavelength of 366 nm. Finally, RA and CA with different  $R_f$  values were visualized as blue bonds under 366 nm.

Standard solutions of  $0.05$  and  $0.005$   $\text{mg mL}^{-1}$  RA and CA in amount per fraction mode was used to obtain calibration curves of RA at concentrations of 4, 6, 8, 10, 12, 14, and 20  $\text{ng/spot}$  and 2, 4, 6, 10, 14, 18, and 20  $\text{ng/spot}$  for CA by plotting the amounts of standard compounds versus equivalent area of each bond. Statistical parameters and equations were shown in Table 2. The RA quantitation was led to amounts of  $0.46 \pm 0.01$ ,  $10.54 \pm 0.12$ , and  $7.85 \pm 0.02$   $\text{mg/g}$  for *T. lancifolius*, *T. daenensis* 1, and *T. daenensis* 2, respectively. Furthermore, the CA determination was caused to values of  $0.26 \pm 0.007$ ,  $0.78 \pm 0.007$ , and  $0.13 \pm 0.007$   $\text{mg/g}$  for *T. lancifolius*, *T. daenensis* 1, and *T. daenensis* 2, respectively. It is seen the highest amount of RA, and CA was obtained for *T. daenensis* 1. However, the geographical distribution of *T. daenensis* 2 and *T. lancifolius* are the same. The amount of RA for *T. daenensis* 2 was higher than *T. lancifolius*. and value of CA in *T. daenensis* 2 was lower than *T. lancifolius*. Also, the measurement for RA and CA evaluations was validated. Therefore, the calibration curve was assessed via plotting chromatographic peak areas versus standards concentration based on  $\text{ng/spot}$ . Limit of detection (LOD) and limit of quantification (LOQ) using the equations of  $\text{LOD} = 3 \times \text{SD}/b$  and  $\text{LOQ} = 10 \times \text{SD}/b$  were calculated to determine the quantitation sensitivity, which SD displays the standard deviation of peak areas of the standard and b is the slope of the linear equation. As shown in Table 2, LODs of 30.19 and 3.65  $\text{ng/spot}$  were achieved for RA and CA, respectively. Similarly, LOQs of 91.53 and 11.06  $\text{ng/spot}$  were obtained for RA and CA, respectively. High correlation amounts ( $r^2$ ) for RA and CA measurements present high correlation of the fitted regression lines.



**Figure 1.** HPTLC chromatograms of *Thymus* genera under 366 nm (T.d 1: *T. daenensis* 1, T.d 2: *T. daenensis* 2, T.l: *T. lancifolius*, RA: rosmarinic acid, CA: caffeic acid). (a) determination of rosmarinic acid; (b) determination of caffeic acid.

**Table 2.** The amounts of rosmarinic acid and caffeic acid in methanolic extracts of different *Thymus* genera.

	Rosmarinic acid	Caffeic acid
R <sub>f</sub> values	0.11 ± 0.02	0.73 ± 0.02
Linear equation	y = 7.18 x + 602.97	y = 9.64 x - 297.03
R <sup>2</sup>	0.994	0.994
Linear range (ng)	100-700	30-200
LOD (ng/spot)	30.19	3.65
LOQ (ng/spot)	91.53	11.06

#### 4. Conclusions

In the current study, two reflux and ultrasound methods were applied as the extraction procedures, which resulted in the same data. Concerning the HPTLC technique's benefits, the developed method can be used in food and drug industries as routine analysis. Compared to published reports about essential oil compositions of various *Thymus* species, surveys on phenolic compounds' determination are limited. The comparison of achieved results exposed that there are significant variations even within the two plants from the same locations of Iran (*T. lancifolius* and *T. daenensis* 2). It seems that the acquired differences in quantitation might have related to numerous differences such as type of the genus, climatic, seasonal, geographical, and geological conditions.

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#### Conflicts of Interest

The authors declare no conflict of interest.

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