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## CAFFEIC AND ROSMARINIC ACIDS IN THYME SPECIES

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*Key words: thyme species; herb; caffeic acid; rosmarinic acid; hydroxycinnamic acids*

*Hydroxycinnamic acids undoubtedly contribute to the wide range of pharmacological effects of the species of Thyme genus. Rosmarinic acid has antioxidant, hepatoprotective, cardioprotective, nephroprotective, anti-inflammatory, immunomodulatory, anti-allergic and anti-tumor properties. Caffeic acid has the antimicrobial, fungistatic activity, produces a choleric effect. Accumulation of these acids is specific for Lamiaceae family. The aim of our research was to determine the type of hydroxycinnamic acids, the concentration of rosmarinic and caffeic acids and the total content of hydroxycinnamic acids, also to determine parameters of perfect extraction of this type of biological active compounds in the herb of four species of Thyme genus of the European flora. Identification of hydroxycinnamic acids has been carried out using paper and thin layer chromatography in different systems of solvents in comparison with standard compounds. Using TLC and HPLC caffeic and rosmarinic acids have been identified in the herb samples of *Thymus Marshallianus*, *Thymus pulegioides*, *Thymus crenulatus*, *Thymus dimorphus*. The results of HPLC analysis of caffeic and rosmarinic acids in the herb of *Thymus* species show that the concentration of rosmarinic acid ranges from 2343.40 mg/kg to 14351.74 mg/kg; the content of caffeic acid ranges from 74.41 mg/kg to 93.86 mg/kg. The highest concentration of rosmarinic acid, as well as caffeic acid was in *Thymus pulegioides*. The total content of hydroxycinnamic acids has been determined by spectrophotometry, and it ranges from 3.27 to 19.28%. *Thymus crenulatus* is the richest species by accumulation of the total content hydroxycinnamic acids. The optimal parameters for herb extraction such as the size of the crushed herb particles, extractant and extraction time have been found.*

Hydroxycinnamic acids are one of the most wide spread classes of natural compounds containing in herbal drugs. Among them rosmarinic and caffeic acid are the most representative compounds in species of Thyme genus [2, 5, 10, 11, 15]. Hydroxycinnamic acids exhibit a high antioxidant activity, have also the anti-inflammatory, antiviral, immunostimulatory effects [2, 6, 11, 14].

For the first time rosmarinic acid was isolated from plants of *Lamiaceae* family, namely from rosemary by the Italian chemists M.Skorpati and G.Oriente, who offered its name [5, 11, 14]. Rosmarinic acid has the antioxidant, hepatoprotective, cardioprotective, nephroprotective, anti-inflammatory, immunomodulatory, anti-allergic and antitumor properties. It inhibits the activity of acetylcholinesterase, glutathionereductase, aldose-reductase, angiotensin-converting enzyme and reduces genotoxic and cytotoxic effects of ionizing radiation [2, 11].

Caffeic acid has the antimicrobial, fungistatic activity, produces a choleric effect [7, 8, 9].

Rosmarinic and caffeic acids have been found in many types of thyme [2, 5, 6, 9-13, 15]. They undoubtedly contribute to the pharmacological effects of Thyme genus plants. Therefore, research aimed at studying of rosmarinic and caffeic acids in Thyme genus plants, as well as methods for their analysis are highly actual.

### Materials and Methods

The objects of study were herb of species of Thyme genus of Central Russia flora: *Thymus Marshallianus*, *Thymus dimorphus*, *Thymus crenulatus*, *Thymus pulegioides*. Herb samples were harvested at full flowering

time in 2011-2012 in different regions: Belgorod (*Th. Marshallianus*), Bryansk (*Th. pulegioides*), Voronezh (*Th. crenulatus*) and Kursk region (*Th. dimorphus*). After gathering herb samples were dried in proper condition and standardized.

The qualitative composition of hydroxycinnamic acids was analysed using paper chromatography and thin layer chromatography. For this purpose Filtrak No.1, No.5 chromatographic paper, as well as "Silufol" and "Sorbfil" chromatographic plates were applied. The analysis was carried out in the solvent system: chloroform – methanol – water (24:14:3); butanol – acetic acid – water (4:1:2); 2% and 15% acetic acid. After developing the chromatograms were analysed in the UV-light before and after spraying with specific reagents (ammonia vapours; solution of sodium hydroxide) [5, 6, 12, 13].

To confirm the presence of hydroxycinnamic acids in the herbal raw material the HPLC method was used. The herbal raw accurately weighed (approx. 1.0 g) was ground to a particle size of 2 mm, placed in a 100 ml flask; then 50 ml of 40% ethyl alcohol was added. The alcohol-water mixture with the herbal raw material was attached to a reflux condenser and heated on a boiling water bath for 1 hour after its boiling. After cooling the extract was filtered through a filter paper into a 100 ml volumetric flask and diluted to the volume with 40% ethyl alcohol.

In parallel 0.02% solutions of reference standards of rosmarinic and caffeic acid were prepared in 40% ethyl alcohol. Then the resulting extract (1 ml) was passed

Table 1

## Chromatographic characteristics of hydroxycinnamic acids

Acid	Colour		Rf value	
	UV light	+ ammonia vapour	2% acetic acid	Butanol – acetic acid – water (4:1:2)
Rosmarinic	blue	blue-green	0.42	0.89
Caffeic	blue	blue	0.30	0.92

through the sorbent (1 g octadecyl silica gel with the particle size of 10 micron) and eluted with 40% ethyl alcohol to obtain 10 ml of the eluate. The content and composition of acids in the sample were determined by HPLC on a Shimadzu LC 20 Prominence chromatograph. For analysis a column of Macherey-Nagel Company with the size of 150 X 3 mm filled with the reversed-phase sorbent Nucleosil C 18 AB with 3 micron grain and porosity of 100 Å was used. The sample volume – 2 µl, detection at λ=280 nm, 330 nm, 360 nm, with the scanning frequency of 3 Hz. Elution was performed in a gradient mode of increasing the proportion of solution B (acetonitrile : methyl alcohol : water, 40:40:20, pH 2.5) mixed with solution A (an aqueous solution of HClO<sub>4</sub> pH 1.8) from 0% to 100% within 80 minutes at the temperature of 30°C. Identification of the peaks was performed by comparison of UV spectra with the spectra from databases and by retention times with the reference standards. The mass concentration was determined from calibration curves using reference standards and LC Solutions programme (Shimadzu) [4].

The method of direct spectrophotometry is in the basis of the assay of the total amount of hydroxycinnamic acids [3].

#### Method for the assay of the total amount of hydroxycinnamic acids

Grind the analytical sample of the herbal raw material to a particle size passing through a 2 mm sieve. Place approximately 0.5 g of the crushed raw material (accurately weighed) in a 250 ml flask, add 90 ml of 50% ethyl alcohol and weigh with an accuracy of ±0.01 g. Attach the flask to reflux and extract in a boiling water bath for 75 minutes. After cooling weigh the flask with its contents, if necessary, dilute with 50% ethyl alcohol to the initial mass. Filter the extract through a folded filter paper discarding the first 10 ml of the filtrate.

Transfer 1.5 ml of the extract obtained into a 25 ml volumetric flask and dilute to the volume with 50% ethyl alcohol. Measure the absorbance of the test solution at the wavelength of 328 nm. Use 50% ethyl alcohol as a compensation solution.

Calculate the percentage of the total content of hydroxycinnamic acids, equivalent to rosmarinic acid according to the following formula:

$$X = \frac{A \cdot V_1 \cdot V_2}{E_{1\text{cm}}^{1\%} \cdot m \cdot V_3}$$

where: A – is the absorbance of the test solution at 328 nm; V<sub>1</sub> – is the volume of the volumetric flask used for the extract collection, ml; V<sub>2</sub> – is the volume of the volumetric flask used for dilution and analysis; V<sub>3</sub> – is the

Table 2

#### The content of caffeic and rosmarinic acids in the herb of Thymus species

Thymus species	Rosmarinic acid, mg/kg	Caffeic acid, mg/kg
<i>Thymus Marshallianus</i>	5740.66	58.39
<i>Thymus dimorphus</i>	2343.40	93.86
<i>Thymus crenulatus</i>	10202.46	74.41
<i>Thymus pulegioides</i>	14351.74	80.18

volume of the test solution, ml; E<sub>1cm</sub><sup>1%</sup> – is the specific absorbance of rosmarinic acid equal to 500 at λ=328 nm; m – is the weighed quantity of the herbal raw material, g.

#### Results and Discussion

The presence of caffeic and rosmarinic acids in thyme species under study was determined. Chromatographic characteristics of caffeic and rosmarinic acid are presented in Tab. 1.

Rosmarinic acid was pronounced the most intensely in *Th. pulegioides* and *Th. cretaceus*, and caffeic acid was in *Th. dimorphus* and *Th. pulegioides*.

The analysis by HPLC has confirmed the results of the chromatographic analysis and allowed us to determine the content of rosmarinic and caffeic acids in the herb of Thymus species (Tab. 2).

The results show that the rosmarinic acid content ranges from 2343.40 mg/kg to 14351.74 mg/kg, its highest concentration is in *Th. pulegioides* herb; the caffeic acid content ranges from 74.41 mg/kg to 93.86 mg/kg, the maximum amount of it accumulates in *Th. dimorphus* herb.

When studying the UV-absorption spectrum of the alcohol extract of the thyme species herb it has been found that the absorption maximum of the alcohol extract is at the wavelength of λ=325-330 nm; it suggests that the nature of the absorption curve is determined mainly by hydroxycinnamic acids contained in them, and it gives us the opportunity to use this wavelength for spectrophotometric determination of the total content of acids.

When developing the method for quantitative determination of the total content of hydroxycinnamic acids the following conditions were studied: the herb fineness (the particle size), the type of the solvent for extraction, the ratio of extraction and the time of extraction. The study was carried out using *Th. Marshallianus* herb. The research results are presented in Tab. 3.

From the given data it follows that the optimal parameters are the fineness degree of the raw material – 2 mm,

Table 3

The effect of extraction conditions on isolation of hydroxycinnamic acids from *Thymus Marshallianus* herb

Extraction conditions	Total content of hydroxycinnamic acids, %
Particles size, mm	
1	4.34±0.07
2	5.35±0.10
3	4.64±0.11
Solvent for extraction: alcohol, %	
30	5.32±0.07
50	5.34±0.06
70	5.05±0.07
96	1.97±0.06
Extraction time (50% alcohol, the herb: extract ratio – 1:100)	
30	4.70±0.11
45	4.67±0.06
60	5.01±0.09
75	5.34±0.05
90	5.39±0.11

the extragent – 50% ethyl alcohol, the extraction time – 75 minutes before the extraction equilibrium. To determine the total content of hydroxycinnamic acids we proposed to use rosmarinic acid as a standard sample because it is a dominant compound and accumulates significantly in *Thymus* species herb. The amount of total content of hydroxycinnamic acids in the herb samples was calculated using the specific absorption of rosmarinic acid in 50% ethyl alcohol equal to 500 at the wavelength of 328 nm. The conditions described above allowed us to develop a method for quantitative determination of the total content of hydroxycinnamic acids.

Table 4

The total content of hydroxycinnamic acids in *Thymus species* herb

<i>Thymus species</i> herb	Total content of hydroxycinnamic acids, %
<i>Thymus Marshallianus</i>	4.51±0.17
<i>Thymus pulegioides</i>	10.83±0.34
<i>Thymus crenulatus</i>	19.28±0.50
<i>Thymus dimorphus</i>	3.27±0.14

Several sample of *Thymus* species herb have been analyzed by this method (see results in Tab. 4).

The results show that the total content of hydroxycinnamic acids in *Thymus* species herb ranges from 3.27 to 19.28%. *Thymus crenulatus* herb demonstrates the highest concentration of hydroxycinnamic acids.

#### CONCLUSIONS

1. Using TLC and HPLC the presence of caffeic and rosmarinic acids in the herb samples of *Thymus Marshallianus*, *Thymus pulegioides*, *Thymus crenulatus*, *Thymus dimorphus* has been determined.

2. Using HPLC the content of caffeic and rosmarinic acids in the herb of *Thymus* species has been determined. It has been found that the concentration of rosmarinic acid ranges from 2343.40 mg/kg to 14351.74 mg/kg, the content of caffeic acid ranges from 74.41 mg/kg to 93.86 mg/kg.

3. The method for spectrophotometric determination of the total content of hydroxycinnamic acid in the herb of *Thymus* species has been developed. The results show that the total content of hydroxycinnamic acids in *Thymus* species herb ranges from 3.27 to 19.28%.

4. The optimal parameters for the herb extraction have been found: the size of crushed herb particles – 2 mm, the extragent – 50% alcohol, the extraction time – 75 min.

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**КОФЕЙНА ТА РОЗМАРИНОВА КИСЛОТИ В РОСЛИНАХ РОДУ ЧЕБРЕЦЬ****В.М.Бубенчікова, Н.В.Попова, Ю.А.Старчак****Ключові слова:** види чебрецю; кофейна, розмаринова кислоти; гідроксикоричні кислоти

Гідроксикоричні кислоти обумовлюють широкий спектр фармакологічної активності рослин роду чебрець. Розмаринова кислота продукує антиоксидантну, гепатопротекторну, кардіопротекторну, нефропротекторну, протизапальну, імуномодулюючу, протиалергічну та протипухлинні властивості. Кофейна кислота чинить антимікробну, фунгістатичну, жовчогінну дію. Накопичення цих кислот специфічне для рослин родини Ясноткові. Метою дослідження було визначення похідних гідроксикоричної кислоти, концентрації розмаринової та кофейної кислот, а також суми гідроксикоричних кислот, визначення параметрів досконалої екстракції цього класу біологічно активних сполук у траві чотирьох видів роду чебрець європейської флори. Ідентифікацію похідних гідроксикоричної кислоти визначали за допомогою паперової та тонкошарової хроматографії у ряду систем розчинників у порівнянні з вірогідними речовинами. За допомогою ТШХ і ВЕРХ аналізу ідентифікували кофейну і розмаринову кислоти у траві видів чебрецю *Thymus Marshallianus*, *Thymus pulegioides*, *Thymus crenulatus*, *Thymus dimorphus*. Результати ВЕРХ аналізу показують, що вміст розмаринової кислоти варіює від 2343,40 мг/кг до 14351,74 мг/кг, а кофейної – від 74,41 мг/кг до 93,86 мг/кг. Найвища концентрація розмаринової кислоти, також як і кофейної була відзначена у траві *Thymus pulegioides*. Вміст суми гідроксикоричних кислот встановлювали спектрофотометричним методом і він складає 3,27-19,28%. Найвищий рівень суми гідроксикоричних кислот спостерегається у траві *Thymus crenulatus*. Визначені оптимальні параметри екстракції трави чебрецю: розмір частинок, тип екстрагенту і час екстракції.

**КОФЕЙНАЯ И РОЗМАРИНОВАЯ КИСЛОТЫ В РАСТЕНИЯХ РОДА ТИМЬЯН****В.Н.Бубенчикова, Н.В.Попова, Ю.А.Старчак****Ключевые слова:** виды тимьяна; кофейная, розмариновая кислоты; гидроксикоричные кислоты

Гидроксикоричные кислоты несомненно обуславливают широкий спектр фармакологической активности растений рода тимьян. Розмариновая кислота проявляет антиоксидантные, гепатопротекторные, кардиопротекторные, нефропротекторные, противовоспалительные, иммуномодулирующие, противоаллергические и противоопухолевые свойства. Кофейная кислота обладает противомикробным, фунгистатическим и желчегонным действием. Накопление этих кислот специфично для растений семейства Яснотковые. Целью исследований было определение производных гидроксикоричной кислоты, содержания розмариновой и кофейной кислот, а также суммы гидроксикоричных кислот, анализ параметров оптимальной экстракции этого класса биологически активных соединений травы четырех видов рода тимьян европейской флоры. Идентификацию производных гидроксикоричной кислоты проводили с помощью бумажной и тонкослойной хроматографии в ряде систем растворителей в сравнении с достоверными веществами. С помощью ТСХ и ВЭЖХ анализа идентифицировали кофейную и розмариновую кислоты в траве видов тимьяна *Thymus Marshallianus*, *Thymus pulegioides*, *Thymus crenulatus*, *Thymus dimorphus*. Результаты ВЭЖХ анализа показывают, что содержание розмариновой кислоты варьирует от 2343,40 мг/кг до 14351,74 мг/кг, а кофейной – от 74,41 мг/кг до 93,86 мг/кг. Самое высокое содержание розмариновой кислоты, так же как и кофейной было характерно для травы *Thymus pulegioides*. Содержание суммы гидроксикоричных кислот устанавливали спектрофотометрически и оно составляло 3,27-19,28%. Наивысший уровень суммы гидроксикоричных кислот наблюдался в траве *Thymus crenulatus*. Определены оптимальные параметры экстракции травы тимьяна: размер частиц, тип экстрагента и время экстракции.