How to Cite: Ivasenko, S.A., Shakarimova, K.K., Bokayeva, A.B., Marchenko, A.B., Lavrinenko, A.V., & Kolesnichenko, S.I. (2021) Study of the phenolic compounds of the dry extract of *Thymus crebrifolius* using a combined HPLC–UV and HPLC-ESI-MS/MS method. *Bulletin of the University of Karaganda – Chemistry, 102(2), 18-23.* https://doi.org/10.31489/2021Ch2/18-23

UDC 547.99:543.544 https://doi.org/10.31489/2021Ch2/18-23

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Study of the phenolic compounds of the dry extract of *Thymus crebrifolius* using a combined HPLC–UV and HPLC-ESI-MS/MS method

In world practice interest in herbal medicines is noticeably increasing every year. From this point of view, plants of the *Thymus* L. genus of the *Lamiaceae* family are of undoubted interest. Previously we have obtained a dry extract from the aerial part of an endemic plant of the flora of Kazakhstan *Thymus crebrifolius Klokov* for the first time by double extraction of raw plant materials with 70% ethanol using ultrasound. Dry extract of *Thymus crebrifolius* has a wide spectrum of antimicrobial activity, including against *Helicobacter pylori*, while it is not toxic, and can be used as an antimicrobial agent. The article presents the results of a study of the composition of phenolic compounds of dry extract of *Thymus crebrifolius* using a combined HPLC-UV and HPLC-MS/MS method. 12 phenolic compounds have been identified and quantified in the dry extract of *Thymus crebrifolius*. Four of them are phenolic acids, and eight are flavonoids. The dominant phenolic compounds are luteolin-7-O-glucoside (109.00 mg g^{-1}), rosmarinic acid (30.98 mg g^{-1}), naringenin (24.84 mg g^{-1}), epicatechin (9.98 mg g^{-1}), myricetin (6.15 mg g^{-1}) and gallic acid (3.41 mg g^{-1}). The results of chromatographic analysis will be used to standardize drugs based on dry extract of *Thymus crebrifolius*.

Keywords: Thymus crebrifolius Klokov, dry extract, HPLC-UV, HPLC-ESI-MS/MS, phenolic acids, flavonoids, luteolin-7-O-glucoside, rosmarinic acid.

Introduction

In world practice, interest in herbal medicines is noticeably increasing every year. At the same time, special importance is attached to the study of wild-growing plants and their use both in the form of medicinal raw plant materials and for the production of new drugs.

From this point of view, plants of the *Thymus* L. genus of the *Lamiaceae* family are of undoubted interest. The attention of researchers has long been attracted by plants of the genus *Thymus* L., whose representatives are a source of medicinal plant materials with a wide spectrum of action.

The State Pharmacopoeia of the Republic of Kazakhstan includes the herb *Thymus serpyllum* L. and the herb *Thymus vulgaris* L. as medicinal plants [1]. In official medicine these herbs are used as a medicinal plant material with antibacterial, astringent, anti-inflammatory, sedative, anticonvulsant, expectorant, antispasmodic, choleretic, analgesic, diuretic, wound healing and anthelmintic effect. It is used in the form of decoctions and infusions [1].

At the same time, a possible reduction in stocks of medicinal raw plant materials leads to the need to expand the raw material base of official medicinal plants at the expense of additional plant sources and their complex use. The results of study of the distribution of the genus *Thymus* L. showed that 15 species grow on the territory of Central Kazakhstan, 5 of them are endemic, including *Thymus crebrifolius* Klokov. It should be noted that *Thymus vulgaris* L. does not grow in the Karaganda region. In Central Kazakhstan, the most common *Thymus serpyllum* L. *Thymus crebrifolius* form large thickets in certain geographical areas, for example, in the Ulytau mountains [2]. According to the results of a survey of raw materials on the territory of the Karaganda region, *Thymus crebrifolius* has sufficient general operational reserves and possible volumes of annual procurements for use in pharmacy and medicine. However, the composition and biological properties of *Thymus crebrifolius* remain largely unexplored.

Previously we have obtained a dry extract from the aerial part of *Thymus crebrifolius* Klokov for the first time by double extraction of plant raw materials with 70% ethanol using ultrasound. Dry extract of *Thymus crebrifolius* has a wide spectrum of antimicrobial action, including against *Helicobacter pylori*. It is non-toxic, and can be used as an antimicrobial agent both individually and in complex therapy [3].

Therefore, the purpose of our work is to study the phenolic compounds of the dry extract of *Thymus crebrifolius* using the HPLC-UV and ESI-MS/MS combined method.

Experimental

For study the aerial part of *Thymus crebrifolius* Klok. was collected in the population of the Karaganda region of the Republic of Kazakhstan, in the vicinity of the city of Zhezkazgan in the Ulytau mountains (N 48°4213′; E 66°5910′) in June 2020 in the full flowering phase. Raw plant materials were dried, crushed and stored in accordance with the requirements of the State Pharmacopoeia of the Republic of Kazakhstan for medicinal plants [1]. Botanical identification was confirmed at the Institute of Botany and Phytointroduction of the Science Committee of the Ministry of Education and Science of the Republic of Kazakhstan (conclusion on the species belonging of plant samples No. 01-04/261).

Dry extract of *Thymus crebrifolius* was obtained by double extraction of air-dry raw materials (leaves, flower baskets and thin stems) with 70% ethanol, without soaking. The ratio of raw material mass and extractant volume was 1:20. This was carried out in an Ultrasonic Cleaner at an ultrasonic frequency of 40 kHz at room temperature (20–22 °C) for 30 minutes. After ultrasonic treatment, the liquid extracts were filtered and the extractant was evaporated on a rotary evaporator to dryness at a temperature of 50 °C [3]. Dry extract of *Thymus crebrifolius* is a dark green mass with a specific odor, which is easily soluble in 70% ethanol, dimethylsulfoxide, partially soluble in purified water, chloroform, ethyl acetate. The yield of dry extract of *Thymus crebrifolius* was 5.76±0.07 %, in terms of air-dry raw materials. The quality indicators of the obtained dry extract of *Thymus crebrifolius* met all the requirements of the analytical regulatory document.

High-performance liquid chromatography (HPLC) combined with an ultraviolet detector (UV) and real-time tandem mass spectrometry (ESI-MS/MS) were used to analyze the polyphenolic compounds of ultrasonic extract. The following reagents were used in the study: acetonitrile (ACN) for HPLC (≥99.9%, Sigma-Aldrich, France), formic acid (99–100 %, AnalaR NORMAPUR®, VWR Chemicals, France), highly purified water was prepared using a Milli-Q water purification system (Millipore, France). The 17 selected phenolic compounds, standards (gallic acid, caffeic acid, chlorogenic acid, ferulic acid, rosmarinic acid, catechin, epicatechin, naringin, rutin, luteolin-7-O-glucoside, luteolin, quercetin, apigenin, kaempferol, dihydroquercetin, myricetin, naringenin) were purchased from Sigma–Aldrich (USA).

The analysis was performed on an "Agilent 1260 Infinity HPLC system" liquid chromatograph (Agilent Technologies, USA), equipped by G1311C 1260 Pump VL, autosampler G1329B 1260 ALS, thermostatted column compartment G1316A 1260 TCC; variable wavelength detector G1314C 1260 VWD VL+ and *mass-spectrometer* G6130A Quadrupole LC-MS/MS. Operated by Windows NT based ChemStation software was used.

Chromatographic separations were carried out on a column with a "Zorbax Eclipse Plus C18" reversed-phase sorbent (150 mm × 4.6 mm, 3.5 μm, Agilent Technologies, USA). For the separations a gradient of mobile phase A (2.5 % (v/v) formic acid in water) and mobile phase B (2.5 % (v/v) formic acid in acetonitrile) was used. The gradient profile was set as follows: 0.00 min 3 % B eluent, 5.00 min 10 % B eluent, 10.00 min 20 % B eluent, 15.00 min 30 % B eluent, 45.00 min 40 % B eluent, 50.00 min 30 % B eluent, 55.00 min 20 % B eluent and 60.00 min 3 % B eluent. The flow rate was 0.4 mL/min, the column temperature was 30 ºC. The ultrasonic extracts and standards were dissolved in a mixture of solvents acetonitrile : water = 1:1 (v/v). The injection volume was 20 μL for ultrasound extracts and for standards. The column effluent passed through a UV detector before arriving in the MS interface. UV detection wavelengths were 280 nm and 360 nm. The electrospray ionization mass spectrometry detection was performed in negative mode with the following optimized parameters: capillary temperature 350 °C; drying gas N_2 8 L/min; nebulizer pressure 45 psi. Data gaining was performed using multiple reactions monitoring (MRM) method that only monitors specific mass transitions during preset retention times.

The identification of each compound was performed by comparing their retention times with authentic standards and also confirmed by an Agilent G6130A LC-MS/MS spectrometer equipped with an electrospray ionization source. The quantitative content of phenolic compounds in the ultrasonic extract was calculated by the method of an external standard [4].

Results and Discussion

The composition of phenolic compounds of dry extract of *Thymus crebrifolius* was studied for the first time using HPLC-UV and ESI-MS/MS combined method. The composition of the phenolic substances of the dry extract of *Thymus crebrifolius* and the mass spectra for the identified compounds in the negative ionization mode are listed in Table 1. The HPLC-UV and HPLC-ESI-MS/MS chromatograms of the test extract and the identified phenolic compounds are shown in Figure 1.

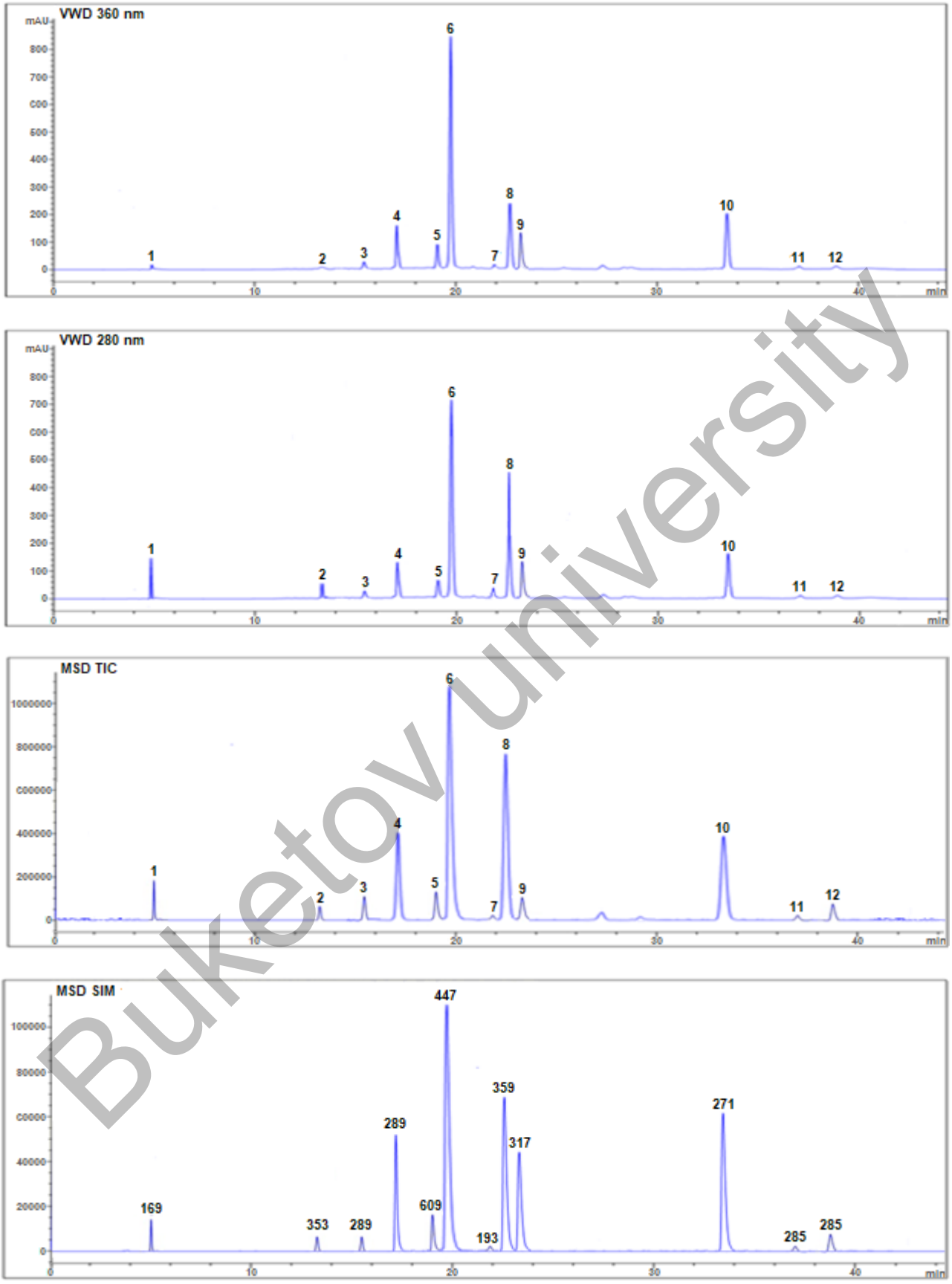

Figure 1. HPLC-UV and HPLC-ESI-MS/MS chromatograms, the total ion chromatogram (TIC) and of the identified phenolic compounds of dry extract *Thymus crebrifolius* (SIM)

Table 1

Identification and content of phenolic compounds of dry extract of Thymus crebrifolius

Peak	Retention time (min)	$M-H^-$ (m/z)	Compound identity	Quantification (mg g^{-1} dry extract)
1	4.932	169	Gallic acid	3.41±0.16
2	13.466	353	Chlorogenic acid	0.82±0.08
3	15.588	289	Catechin	1.27±0.09
4	17.187	289	Epicatechin	9.98±0.12
5	19.166	609	Rutin	3.43±0.18
6	19.880	447	Luteolin-7-O-glucoside	109.00±1.18
7	21.926	193	Ferulic acid	0.04±0.01
8	22.699	359	Rosmarinic acid	30.98±0.24
9	23.327	317	Myricetin	6.15±0.37
10	33.723	271	Naringenin	24.84±32
11	37.057	285	Luteolin	0.10±0.01
12	38.924	285	Kaempferol	0.60±0.05

The use of a "Zorbax Eclipse Plus C18" column with a particle size of 3.5 μm and a gradient elution mode made it possible to achieve a satisfactory separation of $\alpha > 1$. Identification and assignment of each compound was performed by comparing their HPLC-UV retention times and HPLC-ESI-MS/MS mass-spectra with data from authentic phenolic standards and by the addition of witness taps. Phenolic compounds were quantified by the integration peaks on HPLC-UV chromatograms using an external standard method.

The dry extract of *Thymus crebrifolius* contains such phenolic acids, as gallic acid, chlorogenic acid, ferulic acid and rosmarinic acid. The flavonoids identified in the studied extract belong to the groups flavanols (catechin, epicatechin), flavones (luteolin-7-O-glucoside, luteolin), flavonols (rutin, myricetin, kaempferol), flavanone (naringenin). The dominant phenolic compounds are luteolin-7-O-glucoside (109.00 mg g^{-1}), rosmarinic acid (30.98 mg g^{-1}), naringenin (24.84 mg g^{-1}), epicatechin (9.98 mg g^{-1}), myricetin (6.15 mg g^{-1}) and gallic acid (3.41 mg g^{-1}).

The literature reports that the qualitative composition and quantitative content of phenolic compounds in plants of the genus *Thymus* L. vary depending on the species, geographical region, climatic conditions, growing environment and growing season. Rosmarinic acid is the most common phenolic acid in extracts of the genus *Thymus* L. It has a wide spectrum of biological activity: anti-inflammatory, antitumor, antiproliferative, antiviral and others. Rosmarinic acid is one of the most effective natural antioxidants. Luteolin-7-O-glucoside is the most common flavonoid in extracts of the genus *Thymus* L. It has a wide spectrum of biological effects, including suppressing oxidative stress [5–7].

Luteolin-7-O-glucoside and rosmarinic acid are identified as the main components not only in dry extract of *Thymus crebrifolius* Klok., but also in dry extracts of two chemotypes *Thymus serpyllum* L. and two endemic species *Thymus rasitatus* Klok., *Thymus eremita* Klok. Therefore, luteolin-7-O-glucoside and rosmarinic acid can be considered as chemical markers of plants of the genus *Thymus* L. growing on the territory of Central Kazakhstan.

Conclusions

As a result of the study, the composition of the phenolic compounds of the dry extract of the endemic plant of the flora of Kazakhstan *Thymus crebrifolius* Klokov was studied for the first time using the HPLC-UV and ESI-MS/MS combined method. In the dry extract of *Thymus crebrifolius* 12 phenolic compounds have been identified and quantified, four of them are phenolic acids, and eight are flavonoids. The dominant phenolic compounds are luteolin-7-O-glucoside, rosmarinic acid, naringenin, epicatechin, myricetin, and gallic acid. The results of chromatographic analysis will be used to standardize drugs based on dry extract of *Thymus crebrifolius*.

The study was carried out with the financial support of the Science Committee of the Ministry of Education and Science of the Republic of Kazakhstan, within the framework of grant funding project No. AP08956260 "Development of the composition and technology of original herbal medicines for the treatment and prevention of Helicobacter pylori-associated diseases".

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ЖТСХ–УК және ЖТСХ-МС/МС аралас әдісін қолдануымен *Thymus crebrifolius* құрғақ сығындысының фенолдық қосылыстарын зерттеу

Әлемдік тәжірибеде жыл сайын өсімдік тектес дәрілік препараттарға деген қызығушылық айтарлықтай артып келеді. Осы тұрғыдан алғанда, *Lamiaceae* тұқымдасының *Thymus* L. тұқымдас өсімдіктері сөзсіз қызығушылық тудырады. Бұған дейін ультрадыбысты қолдана отырып, өсімдік шикізатын 70 % этанолмен екі рет экстракциялау арқылы *Thymus crebrifolius* Klokov Қазақстанның эндемикалық флорасының жер үсті өсімдігінен құрғақ сығынды алынған болатын. *Thymus crebrifolius* құрғақ сығындысы микробқақарсы әсердің кең спектріне ие, соның ішінде *Helicobacter pylori*-ге қатысты, улы емес және оны микробқақарсы препарат ретінде пайдалануға болады. Мақалада ЖТСХ-УК және ЖТСХ-МС/МС аралас әдісін пайдалана отырып, Қазақстан флорасының эндемикалық өсімдігі *Thymus crebrifolius* Klokov құрғақ сығындысының фенолдық қосылыстарының құрамын зерттеу нәтижелері ұсынылған. *Thymus crebrifolius* құрғақ сығындысында 12 фенолдық қосылыстар сәйкестендірілді және сандық түрде анықталды, олардың төртеуі фенол қышқылдары, сегізі – флавоноидтар. Доминантты фенолдық қосылыстар лютеолин-7-О-глюкозид (109,00 мг/г), розмарин қышқылы (30,98 мг/г), нарингенин (24,84 мг/г), эпикатехин (9,98 мг/г), мирицетин (6,15 мг/г) және галл қышқылы (3,41 мг/г) болып табылады. Хроматографиялық талдау нәтижелері *Thymus crebrifolius* құрғақ сығындысына негізделген дәрі-дәрмектерді стандарттау үшін қолданылады.

Кілт сөздер: Thymus crebrifolius Klokov, құрғақ сығынды, ЖТСХ-УК, ЖТСХ-МС/МС, фенол қышқылдары, флавоноидтар, лютеолин-7-О-глюкозид, розмарин қышқылы.

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Изучение фенольных соединений сухого экстракта *Thymus crebrifolius* с использованием комбинированного метода ВЭЖХ–УФ и ВЭЖХ-МС/МС

В мировой практике с каждым годом заметно возрастает интерес к лекарственным препаратам растительного происхождения. С этой точки зрения особое внимание привлекают растения рода *Thymus* L. семейства *Lamiaceae*. Нами ранее впервые получен сухой экстракт из надземной части эндемичного растения флоры Казахстана *Thymus crebrifolius* Klokov двукратной экстракцией растительного сырья 70 %-ным этанолом с использованием ультразвука. Сухой экстракт *Thymus crebrifolius* обладает широким спектром антимикробного действия, в том числе в отношении *Helicobacter pylori*, при этом он не токсичен и может быть использован в качестве антимикробного средства. Авторами представлены

результаты исследования состава фенольных соединений сухого экстракта *Thymus crebrifolius* с использованием комбинированного метода ВЭЖХ-УФ и ВЭЖХ-МС/МС. В сухом экстракте *Thymus crebrifolius* идентифицировано и количественно определено 12 фенольных соединений, из них 4 — фенольные кислоты, 8 — флавоноиды. Доминирующими фенольными соединениями являются лютеолин-7-О-глюкозид (109,00 мг/г), розмариновая кислота (30,98 мг/г), нарингенин (24,84 мг/г), эпикатехин (9,98 мг/г), мирицетин (6,15 мг/г) и галловая кислота (3,41 мг/г). Результаты хроматографического анализа будут использованы для стандартизации лекарственных средств на основе сухого экстракта *Thymus crebrifolius*.

Ключевые слова: Thymus crebrifolius Klokov, сухой экстракт, ВЭЖХ-УФ, ВЭЖХ-МС/МС, фенольные кислоты, флавоноиды, лютеолин-7-О-глюкозид, розмариновая кислота.

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