Antimicrobial Activity and Polyphenol Profiles of Hydroalcoholic Extracts of Thymus rasitatus Klokov and Thymus eremita Klokov

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Abstract

BACKGROUND: A possible reduction in stocks of medicinal plant raw materials of Thymus serpyllum L. and Thymus vulgaris L. leads to the need to expand the raw material base of the official medicinal plants with using of endemic species of the flora of Kazakhstan, in particular, Thymus rasitatus Klokov, and Thymus eremita Klokov.

AIM: The aim of the study was to study the possibility of using 70% ethanol extracts of T. rasitatus and T. eremita as antimicrobial agents.

MATERIALS AND METHODS: The aerial parts of T. rasitatus and T. eremita were extracted with 70% ethanol using ultrasound assisted extraction. The qualitative and quantitative analyses of the 70% ethanol extracts were determined using the liquid chromatography-detection-ESI-mass spectrometry-(MS)/MS technique. The study of the antimicrobial activity of these extracts was performed for eight strains of Gram-positive bacteria, six strains of Gram-negative bacteria, and four cultures of fungi.

RESULTS: Chromatographic analysis of hydroalcoholic extracts of both investigated Thymus species showed very similar phenolic compounds composition. In both cases, the major components are luteolin-7-O-glucoside and rosmarinic acid. About 70% ethanol extracts of T. rasitatus and T. eremita have a broad spectrum of antimicrobial activity, exhibit the bactericidal or bacteriostatic activity against all tested bacteria and fungi at concentration range of 0.0195–20 mg/ml, but differ in their potency against tested strains of microorganisms.

CONCLUSION: About 70% ethanol extracts of T. rasitatus and T. eremita, endemic plants in the flora of Kazakhstan, can be considered as potential drugs with a wide spectrum of antimicrobial activity. The results of chromatographic analysis could be used for drug standardization.

Introduction

Plants of the Thymus L. genus have been used since ancient times and are currently used in traditional medicine in many countries and peoples. The State Pharmacopoeia of the Republic of Kazakhstan includes the herbs of Thymus serpyllum L. and Thymus vulgaris L. as medicinal plants [1]. In official medicine, these herbs are used as a medicinal plant material with the antibacterial, astringent, anti-inflammatory, sedative, anticonvulsant, expectorant, antispasmodic, choleric, analgesic, diuretic, wound healing, and anthelminthic effect, used in the form of decoctions and infusions [1].

Increased demand for herbal medicines may lead to depletion of medicinal plant materials. This justifies 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 studying the distribution of plants of the genus Thymus L. showed that 15 species grow in the territory of Central Kazakhstan, of which five species are endemic, including Thymus rasitatus Klokov and Thymus eremita Klokov. It should be noted that T. vulgaris L. does not grow in the Karaganda region. In Central Kazakhstan, the most common are T. serpyllum L. and T. rasitatus Klokov. T. eremita Klokov forms large thickets in certain geographical areas, for example, in the Bektauata Mountains [2]. Based on the results of a survey of raw materials in the territory of the Karaganda region, it was found that T. rasitatus and T. eremita have sufficient general operational reserves and possible volumes of annual procurements for use in pharmacy and medicine.
However, composition and the biological properties of T. rasitatus and T. eremita remain practically unexplored. In papers of Atazhanova [3], Sadyrbekov et al. [4], Atazhanova et al. [5] describe the component composition of the essential oil of T. rasitatus. Essential oil of T. rasitatus has a pronounced antimicrobial activity against Staphylococcus aureus and Escherichia coli [6], [7]. It was also found that the essential oil of T. rasitatus suppress the infectious activity of the virus A/FPV/Rostock /34 (H7N1) and has a pronounced analgesic effect [8].

Referring to our earlier research results, it was found that the herbs of T. rasitatus and T. eremita, in addition to essential oils, contain a significant amount of different classes of biologically active substances, namely, flavonoids, phenolic acids, tannins, triterpene compounds, water-soluble polysaccharides, pectin substances, amino acids, and organic acids. Their presence in combination with the quantitative content of many important mineral elements determine the prospects for their use in pharmacy and medicine [9].

In this study, the dry extracts from the aerial portions of T. rasitatus and T. eremita were first obtained by double extraction of plant material with 70% ethanol using ultrasound assisted extraction. The study of the antimicrobial activity and polyphenol profiles of the obtained extracts were performed for the 1st time. Thus, the purpose of this research is to determine the possibility of using the dry hydroalcoholic extracts of T. rasitatus and T. eremita as antimicrobial agents against microbial test strains.

Materials and Methods

Plant material

The aerial part of endemic plants was collected in the populations of the Karaganda region of the Republic of Kazakhstan: T. rasitatus Klokov in the Karkaralinsk mountains (N 49°5740′; E 74°3076′), and T. eremita Klokov in the vicinity of Balkhash city in the Bektauat mountains (N 47°2554′; E 74°4738′), in June – July 2016, in the full bloom phase. The 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).

The obtaining of 70% ethanol extracts

Dry extracts from two samples of T. serpyllum L. were 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 is 1:20, in an Ultrasonic Cleaner ultrasonic bath (China) with frequency of the ultrasonic radiation (40 kHz) at room temperature (20–22°C) for 30 min. After an ultrasonic treatment, the liquid extracts were filtered and the extractant was evaporated on Rotavapor® R-100 (Buchi, Switzerland) to dryness at temperature of 50°C [10].

Liquid chromatography-detection-ESI-mass spectrometry (LC-DAD-ESI-MS)/MS analysis

High-performance LC (HPLC) combined with an ultraviolet (UV) DAD and MS (ESI-MS/MS) were used to analyze the polyphenolic compounds present in the obtained 70% ethanol extracts. The following reagents were used in this research: acetonitrile (ACN) for HPLC (≥99.9%, Sigma-Aldrich, France), formic acid (99–100%, AnaLAR NORMAPUR®, VWR Chemicals, France), and highly purified water was prepared with a Milli-Q (Millipore, France) water purification system. The 17 selected phenolic compounds, standards (caffeic acid, gallic acid, chlorogenic acid, ferulic acid, rosmarinic acid, catechin, epicatechin, naringin, rutin, luteolin-7-O-glucoside, dihydroquercetin, myricetin, quercetin, naringenin, apigenin, luteolin, and kaempferol) were purchased from Sigma-Aldrich (USA).

The analysis was performed on an “Agilent 1260 Infinity HPLC system” liquid chromatograph (Agilent Technologies, USA), equipped with G1311C 1260 Pump VL, autosampler G1329B 1260 ALS, thermostated column compartment G1316A 1260 TCC; mass-spectrometer G6130A Quadrupole LC-MS/MS. Operated by Windows NT based ChemStation software was used.

Chromatographic separations were performed on a column with “Zorbax Eclipse Plus C18” reversed-phase sorbent (150 mm × 4.6 mm, 3.5 μm, Agilent Technologies, USA). For 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, 7.00 min 20% B eluent, 7.10 min 30% B eluent, 27.00 min 40% B eluent, 35.00 min 50% B eluent, 35.10 min 20% B eluent, and 40.00 min 3% B eluent. The flow rate was 0.4 mL/min; the column temperature was 30°C. The 70% ethanol extracts and standards were dissolved in a mixture of solvents acetonitrile:water = 1:1 ([v/v]). The injection volume was 20 μL for extracts and 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 MS detection was performed in negative ion mode with the following optimized parameters: Capillary temperature 350°C; drying gas N2, 8 L/min; nebulizer pressure 45 psi. Data gaining was performed using multiple reactions monitoring method that only monitors specific mass transitions during preset retention times.

The identification of each compound was performed by comparing their retention times to
authentic standards and also confirmed by UV and MS spectra. The quantitative content of phenolic compounds in 70% ethanol extracts was calculated by the external standard method. [10].

**Study of antimicrobial activity**

The hydroalcoholic extracts was screened for antibacterial and antifungal activities by microdilution method using Mueller-Hinton (MH) broth and MH broth with 5% lysed sheep/horse blood for growth of non-fastidious and fastidious bacteria, respectively, or MH broth with 2% glucose for growth of fungi. Minimal inhibitory concentration (MIC) of the tested extract was evaluated for the panel of the reference microorganisms from American Type culture collection (ATCC), including Gram-positive bacteria (S. aureus ATCC25923, Staphylococcus epidermidis ATCC12228, Micrococcus luteus ATCC10240, Bacillus subtilis ATCC6633, Bacillus cereus ATCC10876, Streptococcus pneumoniae ATCC49619, Streptococcus pyogenes ATCC19615, and Streptococcus mutans ATCC25175), Gram-negative bacteria (Salmonella typhimurium ATCC14028, Klebsiella pneumoniae ATCC13883, E. coli ATCC25922, Proteus mirabilis ATCC12453, Pseudomonas aeruginosa ATCC9027, and Helicobacter pylori ATCC43504), and fungi (Candida albicans ATCC102231, Candida parapsilosis ATCC22019, Candida glabrata ATCC 90030, and Candida krusei ATCC 14243).

The 70% ethanol extracts dissolved in dimethylsulfoxide (DMSO) were first diluted to the concentration (20.0 mg/mL) in an appropriate broth medium recommended for bacteria or yeasts. Then, using the same media, serial two-fold dilutions were made to obtain final concentrations of the tested extracts ranged from 20 to 0.156 mg/mL. The sterile 96-well polystyrene microtitrate plates (Nunc, Denmark) were prepared by dispensing 20 μl of appropriate dilution of the tested extracts in broth medium per well. The inocula were prepared with fresh microbial cultures in sterile 0.85% NaCl to match the turbidity of 0.5 McFarland standard and 2 μl were added to wells to obtain final density of 1.5 × 10⁵ CFU/ml for bacteria and 5 × 10⁴ CFU/ml for yeasts; CFU – colony-forming units. After incubation (35°C for 24 h), the MICs were assessed visually as the lowest concentration of the extracts showing complete growth inhibition of the reference microbial strains. Appropriate DMSO control (at a final concentration of 10%), a positive control (containing inoculum without the tested extracts), and negative control (containing the tested extracts without inoculum) were included on each microplate [11].

The MIC of *H. pylori* was determined using two-fold microdilution method in MH broth with 7% of lysed horse blood at extracts concentration ranging from 5.0 to 0.0097 mg/mL with bacterial inocula of 3 McFarland standards. After incubation at 35°C for 72 h under microaerophilic conditions (5% O₂, 15% CO₂, and 80% N₂), the growth of *H. pylori* was visualized by addition of resazurin. The MIC endpoint was recorded as the lowest concentration of extracts that completely inhibits growth [11].

Minimal bactericidal concentration (MBC) or minimal fungicidal concentration (MFC) was obtained by subculturing 5 μl from each well that showed through growth inhibition, from the last positive one and from the growth control onto recommended agar plates. The plates were incubated at 35° for 24 h for all microorganisms but *H. pylori* which was incubated for 72 h in microaerophilic conditions [11, [12]. The MBC/MFC was defined as the lowest concentration of extracts without growth of microorganisms. The MBC/ MFC ratios were calculated to determine the bactericidal or bacteriostatic effect of the assayed extracts [13]. Experiment was repeated in triplicate. Representative data are presented.

**Results**

**Results of extraction**

Under similar conditions for the extraction of each plant materials by ultrasound assisted extraction, the yield of dry 70% ethanol extract from *T. ratisbuta* was 5.4 ± 0.2, which significantly more than the yield of dry 70% ethanol extract from *T. eremita* 3.2 ± 0.1% (p < 0.001).

**Results of LC-DAD-ESI-MS/MS analysis**

The results of qualitative and quantitative chromatographic analyses of hydroalcoholic extracts from both *Thymus* species are shown in Table 1.

| Peak number | Retention time (min) | M+H (m/z) | Compound identity | Quantification (mg g⁻¹ dry extract) |
|-------------|----------------------|-----------|-------------------|-------------------------------------|
| 1           | 3.928                | 179       | Methyl caffeic acid | 0.11                               |
| 2           | 4.932                | 169       | Salicylic acid     | 0.34                               |
| 3           | 12.485               | 353       | Chlorogenic acid   | 0.46                               |
| 4           | 13.089               | 289       | Catechin           | 1.20                               |
| 5           | 13.945               | 289       | Epicatechin        | 9.30                               |
| 6           | 14.112               | 609       | Rutin              | 2.96                               |
| 7           | 14.600               | 447       | Luteolin 7-O-glucoside | 78.06                              |
| 8           | 16.299               | 193       | Ferulic acid       | 1.80                               |
| 9           | 16.985               | 359       | Rosmarinic acid    | 30.16                              |
| 10          | 17.448               | 317       | Myricetin          | 42.12                              |
| 11          | 22.303               | 301       | Quercetin          | 0.91                               |
| 12          | 25.267               | 271       | Naringin           | 7.30                               |
| 13          | 27.350               | 269       | Apigenin           | 0.53                               |
| 14          | 28.350               | 285       | Luteolin           | 0.60                               |

In 70% ethanol extract of *T. ratisbuta* 12 phenolic compounds were identified, five of which are phenolic acids and seven are flavonoids. The dominant polyphenolic compounds are luteolin 7-O-glucoside
(79.06 mg g⁻¹), rosmarinic acid (30.16 mg g⁻¹), epicatechin (9.30 mg g⁻¹), naringenin (7.30 mg g⁻¹), and gallic acid (4.97 mg g⁻¹).

In 70% ethanol extract of T. eremita 14 phenolic compounds were identified, five of which are phenolic acids and nine are flavonoids. The main compounds are luteolin 7-O-glucoside (92.00 mg g⁻¹), rosmarinic acid (26.59 mg g⁻¹), epicatechin (9.28 mg g⁻¹), naringenin (9.44 mg g⁻¹), gallic acid (4.96 mg g⁻¹), and myricetin (4.12 mg g⁻¹).

Results of studying the antimicrobial activity

The research results presented in Table 2 demonstrate that the 70% ethanol extracts of T. rastatus and T. eremita have a broad spectrum of an antimicrobial activity at concentration range of 0.0195–20 mg/ml, exhibit the bactericidal (MBC/MIC ≤4) or bacteriostatic (MBC/MIC >4) activity against all test strains.

Table 2: Research results of antimicrobial activity of 70% ethanol extracts of Thymus rastatus Klok., and Thymus eremita Klok

| Microorganism | Thymus rastatus | Thymus eremita |
|---------------|----------------|---------------|
| 70% ethanol extract | MIC (mg/ml) | MBC (mg/ml) | MBC/MIC ratio | MIC (mg/ml) | MBC (mg/ml) | MBC/MIC ratio |
| Staphylococcus aureus | 2.5 | 2.5 | 2.5 | 5 | 2 |
| Staphylococcus epidermidis | 2.5 | 2.5 | 2 | 1.25 | 5 | 4 |
| Micrococcus luteus | 2.5 | 2.5 | 2.5 | 5 | 2 |
| Bacillus subtilis | 10 | 10 | 1 | 5 | 10 | 2 |
| Bacillus cereus | 2.5 | >20 | >8 | 2.5 | >20 | >8 |
| Streptococcus pneumoniae | 2.5 | 2.5 | 1 | 5 | 10 | 2 |
| Streptococcus pyogenes | 2.5 | 2.5 | 4 | 20 | 20 | 1 |
| Streptococcus mutans | 5 | 10 | 2 | 20 | 20 | 1 |
| Gram-positive bacteria | | | | | |
| Salmonella typhimurium | 10 | 10 | 1 | 5 | 10 | 2 |
| Klebsiella pneumoniae | 1.25 | 1.25 | 1 | 1.25 | 1.25 | 1 |
| Proteus mirabilis | 5 | 5 | 1 | 5 | 1 | 1 |
| Escherichia coli | 5 | 10 | 2 | 5 | 10 | 2 |
| Pseudomonas aeruginosa | 5 | 10 | 2 | 5 | 10 | 2 |
| Helicobacter pylori | 0.0195 | 0.250 | 13 | 1.25 | 1.25 | 1 |
| Yeasts | | | | | |
| Candida albicans | 2.5 | 10 | 4 | 5 | 10 | 2 |
| Candida parapsilosis | 5 | 10 | 2 | 5 | 10 | 2 |
| Candida glabrata | 10 | 10 | 1 | 5 | 10 | 2 |
| Candida krusei | 5 | 10 | 2 | 5 | 10 | 2 |

For 70% ethanol extract of Th. rastatus the maximum bacteriostatic activity was determined with minimum MIC = 0.0195 mg/ml and MBC = 0.250 mg/ml against H. pylori (MIC/MIC = 13). This 70% ethanol extract of T. rastatus exhibits a stronger bactericidal activity against three strains of gram-positive bacteria of S. aureus, S. epidermidis, and S. pneumoniae at a concentration of 2.5 mg/ml (MIC/MIC = 1), while inhibiting the growth of cultures of three strains of M. luteus, B. cereus, and S. pyogenes at concentration of 2.5 mg/ml. Furthermore, at concentration of 1.25 mg/ml, it has a bactericidal effect (MIC/MIC = 1) against the Gram-negative strain of K. pneumoniae. In addition, at concentration of 2.5 mg/ml, it inhibits the growth of the culture of C. albicans fungus [10].

Ethanol extract of T. eremita showed the maximum bacteriostatic effect against H. pylori comparable to the activity of the same extract of the second of the two chemotypes of T. serpyllum (sample 1), growing in the territory of Central Kazakhstan. It also has a similar antimicrobial activity and exhibits stronger bactericidal activity against three strains of gram-positive bacteria S. aureus, S. epidermidis, and S. pneumoniae at concentration of 2.5 mg/ml (MIC/MIC = 1), while inhibiting the growth of cultures of three strains of M. luteus, B. cereus, and S. pyogenes at concentration of 2.5 mg/ml. Furthermore, at concentration of 1.25 mg/ml, it has a bactericidal effect (MIC/MIC = 1) against the Gram-negative strain of K. pneumoniae [10].

The conducted studies showed the possibility of practical use of endemic species of T. rastatus and T. eremita along with the official species – T. serpyllum, which will expand the range of medicinal plant materials.

Differences in the strength of the bactericidal and bacteriostatic effects of the tested 70% ethanol extracts of T. rastatus and T. eremita against the tested bacteria and fungi at concentration of 0.0195 mg/ml to 20 mg/ml are explained by the qualitative composition and quantitative content of the identified phenolic acids and flavonoids. In 70% ethanol extract of Th. rastatus, 12 phenolic compounds were identified, in a 70% ethanol extract of T. eremita – 14 phenolic compounds. The main components of both obtained extracts are luteolin
7-O-glucoside, rosmarinic acid, epicatechin, naringenin and gallic acid, but their quantitative content differs.

Thus, 70% ethanol extracts of T. rasitatus and T. eremita, two endemic plants of the flora of Kazakhstan, were analyzed for the 1st time. Both extracts have a wide spectrum of antimicrobial action, exhibit the bactericidal or bacteriostatic activity against all tested bacteria and fungi at a concentration range of 0.0195–20 mg/ml. 12 Phenolic compounds were identified in 70% ethanol extract of T. rasitatus, while 14 in T. eremita.

Conclusions

Hydroalcoholic extracts of T. rasitatus Klokov and T. eremita Klokov, endemic plants of the flora of Kazakhstan, can be considered as potential drugs of a wide spectrum of antimicrobial action. The results of chromatographic analysis should be used for drug standardization.

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