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Thymus praecox subsp. *polytrichus:* ANTIMICROBIAL AND ANTIDIABETIC ACTIVITY OF ETHANOL EXTRACTS

ABSTRACT: *Thymus praecox* is one of the most widespread species in the Euro-Siberian region. This study was conducted to determine the *in vitro* antimicrobial and antidiabetic activity of its plant extracts prepared with 70% and 96% ethanol, with and without pretreatment with *n*-hexane, using the Soxhlet apparatus. The MICs and MBCs ranged from 0.035 to 0.150 mg/mL and from 0.075 to 0.300 mg/mL, respectively. Both 96% ethanol extracts showed the most promising antibacterial activity, especially against *Bacillus cereus*, *Enterobacter cloacae* and *Salmonella* Typhimurium. The pretreated 96% ethanol extract also showed remarkable antimicrobial activity against *Staphylococcus aureus*. Bacteria *B. cereus*, *S. aureus* and *S. typhimurium* were also sensitive to the pretreated 70% ethanol extract, while 70% ethanol was most effective against *S. aureus* and *Escherichia coli*. The MICs and MFCs varied from 0.017 to 0.30 mg/mL and from 0.035 to 0.70 mg/mL, respectively, with *Aspergillus versicolor* and *Trichoderma viride* being the most sensitive. The IC_{50} values ranged from 0.94 to 1.40 mg/mL for *α*-amylase and from 76.10 to 610.2 µg/mL for *α*-glucosidase. The 70% ethanol extract, especially with the hexane pretreatment, showed the highest enzyme inhibitory activity in the *α*-glucosidase assay (76.10 µg/mL), even higher than the control, acarbose/glucobay (20.46 µg/mL). The highest *α*-amylase inhibitory effect was obtained for the 96% ethanol extract. The results show that the extracts of *T. praecox* subsp. *polytrichus* have significant antimicrobial and antidiabetic potential and can be used for food preservation and phytopharmacy.

KEYWORDS: *Thymus praecox* subsp. *polytrichus,* extracts, antimicrobial activity, antidiabetic activity

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INTRODUCTION

Today's lifestyle, characterised by constant stress, limited mobility and inadequate nutrition, causes oxidative stress and leads to the development of numerous chronic diseases: cardiovascular, neurodegenerative, inflammation, cancer and diabetes (Kangralkar et al., 2010; Chikezie et al., 2015).

Diabetes mellitus (DM) is a metabolic disease characterised by various symptoms: an elevated level of glucose in the blood (hyperglycaemia) and insufficient production or action of insulin produced by the pancreas (Maritim et al., 2003; Moussa, 2008). Diabetics are considered immune compromised patients and are therefore more affected by microbial infectious diseases and have a higher risk of developing serious complications as a result (Uzunović, 2016). On the other hand, many microorganisms become resistant to existing antibiotics over time due to their excessive and inappropriate use. Therefore, it is necessary to search for new effective medicines and herbs that have antimicrobial effects or could be a good source of them.

The genus *Thymus* L., which belongs to the Lamiaceae family, is widespread in the Old World and has been used in traditional medicine for numerous centuries due to its antioxidant, anti-inflammatory, enzyme-inhibitory and antimicrobial properties (Stahl-Biskup, 2002; Jarić et al., 2007; Kindl et al., 2015; Marković et al., 2020; Şener et al., 2021). For instance, *T. praecox* ssp. *polytrichus* (A. Kern. Ex Borbas) Jalas distributed in south-eastern and western Serbia and Kosovo, in meadows, pastures, on rocks, on limestone in the subalpine and alpine zones (Diklić, 1974), has various biological activities and is therefore used in folk medicine (Jarić et al., 2007, Kindl et al., 2015; Marković et al., 2020).

Considering the traditional use of species of the genus *Thymus*, our idea was to obtain different extracts from *T. praecox* ssp. *polytrichu*s using the Soxhlet apparatus and to investigate their antimicrobial and antidiabetic potential *in vitro*, with the ultimate goal of their possible commercialisation in various applications, in medicine, as food additives or for food preservations.

MATERIALS AND METHODS

Plant material

The aerial parts of *T. praecox* subsp. *polytrichus* were collected in July 2022 on Pasjački Vis (mountain Pasjača), Serbia, in full inflorescence and air-dried in the shade. A voucher specimen, no. 16881, was deposited in the Herbarium of the Institute of Botany and Botanical Garden "Jevremovac", Faculty of Biology, University of Belgrade, Serbia (BEOU).

Preparation of plant extracts

Aerial plant parts of *T. praecox* subsp. *polytrichus* were pulverized and 15 g of plant material was extracted using Soxhlet apparatus with 250 mL of

n-hexane for 1 h. The solvent was removed by rotatory vacuum evaporator (Büchi rotavapor R-114). Then, the plant material was dried and extracted again in Soxhlet with 70% and 96% ethanol for 4 h, filtered with filter paper Whatman no. 1 and the solvent was removed by rotatory vacuum evaporator. The other two samples (15 g of aerial plant parts of each) were extracted without previous treatment with *n*-hexane. Prepared extracts were kept in the fridge at +4 °C.

Evaluation of antimicrobial activity using micro-well dilution assay

The antibacterial and antifungal activity of *T. praecox* subsp. *polytrichus* extracts was analyzed using the microdilution method according to Soković et al. (2009) and Kostić et al. (2017). For this purpose, 4 gram(+) (*Bacillus cereus* (clinical isolate), *Micrococcus luteus* ATTC10240, *Staphylococcus aureus* ATCC6538, *Listeria monocytogenes* NCTC 7973), 4gram(-) bacterial strains (*Escherichia coli* ATCC35210, *Pseudomonas aeruginosa* IBRS P001, *Enterobacter cloacae* ATCC 35030, *Salmonella* Typhymurium ATCC 13311) and 8 mycromycetes (*Aspergillus fumigatus* (clinical isolate), *Aspergillus versicolor* ATTC 11730, *Aspergillus ochraceus* ATTC 12066, *Aspergillus niger* ATCC 6275, *Trichoderma viride* IAM 5061, *Penicillium funiculosum* ATCC 36839, *P. ochrochloron* ATCC 9112, *P. verrucosum* var. *cyclopium* (food isolate)) were used. The working solutions of the studied extracts of *T. praecox* subsp. *polytrichus* were prepared in 30% ethanol. Serial dilutions for testing antibacterial/antifungal activity in tryptic soy broth/malt broth were prepared in 96-microtiter plates. Microbial inoculum was added to each well, except the negative control, at a concentration of 1×10^5 CFU/mL to obtain the desired concentrations of the mixture. The microtiter plates containing bacterial inoculum were incubated at 37 °C for 24 hours, while those containing mycotic inoculums were incubated at 28 °C for 72 hours to measure the minimum inhibitory concentrations (MICs). Serial re-inoculations with 10 µL / 2µL of bacterial/fungal inoculum in 100 µL of appropriate sterile broth were performed to estimate the minimum bactericidal/fungicidal concentrations (MBCs/MFCs). The antibiotic ampicillin and the antifungal ketoconazole served as positive controls, and 30% ethanol served as a negative control.

Estimation of enzyme-inhibitory activity in vitro

The inhibitory activity of α-amylase was estimated according to Apostolidis et al. (2011). The amount of 50 µL of the plant extracts concentrations (25–1,000 µg/mL) were mixed with 50 µL of 0.5 mg/mL α-amylase/20 mM phosphate buffer (pH 6.9). The mixture was incubated at 37 °C for 10 min and then 50 µL of 1% starch/20 Mm phosphate buffer was added. After 10 min of incubation at 37 °C, 100 µL of coloring reagent (1% DNS in 0.4 M NaOH/ddH_2O of 12% $KNaC_4H_4O_6 \times 4H_2O$/dd$H_2O$) was added and incubated at 100 °C for 15 min. Finally, the total volume was made up to 1.25 mL with ddH_2O, and

the absorbance was measured at 405 nm. The α-glucosidase inhibitory activity was estimated as described by Wan et al. (2013). Different concentrations of the sample in a final volume of 120 µL were pre-incubated with 20 µL of a 0.6 U/mL α-glucosidase solution in 0.1 M phosphate buffer (pH 6.8), in 96-well microtiter plates at 37 ºC for 15 min. Twenty µL of a 3.5 mM *p*-nitrophenyl-α-D-glucopyranoside (PNPG) solution in 0.1 M phosphate buffer (pH 6.8) was added to initiate the reaction. After 20 min of incubation at 37 ºC, the reaction was stopped by the adding 0.2 M sodium carbonate (Na_2CO_3) and the absorbance was measured at 405 nm. The results obtained in both tests were expressed as IC_{50} values (mg/mL). Glucobay, an official medicine, was used as a positive control.

RESULTS AND DISCUSSION

Antimicrobial activity of *Thymus praecox* subsp. *polytrichus* extracts

The results of the antibacterial activity of the ethanol extracts of *T. praecox* subsp. *polytrichus* are presented in Table 1. The examined extracts were active against the gram(+) and gram(-) bacteria tested, even more than the commercially used antibiotics, streptomycin/ampicillin. The MICs and MBCs were ranged from 0.035 to 0.150 mg/mL and from 0.075 to 0.300 mg/mL, respectively. The 96% ethanol extracts were most active against *B. cereus*, *E. cloacae* and *S. typhimurium*. Pretreated 96% ethanol extracts exhibited also notable antimicrobial effects against *S. aureus*. Bayoub et al. (2010) previously proved susceptibility of *L. monocytogenes* to *Thymus* sp. ethanol extracts. Moreover, *T. serpyllum* and *T. vulgaris* ethanol extracts affected growth of other pathogenic bacterial strains such as *S. aureus*, *E. cloacae*, *E. coli* and *Acinetobacter baumannii*. Lauk et al. (2015) also reported antimicrobial effects of methanol and hexane extracts of *Thymus spp.* against 17 pathogenic bacteria. The *T. quinquecostatus* ethyl acetate fraction was active against *Kocuria rhizophila* and *S. epidermidis* (Hyun et al., 2014), while the acetone extract of *T. praecox* subsp. *grossheimii* var. *grossheimi* was most potent against *Bacillus cereus* (Ozkan et al., 2016). The methanol extract of *T. praecox* subsp. *skorpilii* var *skorpilii* also showed strong antibacterial effects against the *S. epidermidis* strain, which was explained by high phenolic content (Taşkin et al., 2019). *Thymus praecox* showed antibacterial activity, especially against *S. aureus*, which supports the results presented herein (Şener et al., 2021). Petrović et al. (2016b) found the hexane/ethanol extract less effective against examined bacterial strains than essential oil obtained by supercritical extraction due to lower amounts of thymol.

The examined extracts showed antifungal activity against pathogen fungal strains tested, which was slightly lower than the antibacterial activity, but still higher than the positive control, bifonazole/ketoconazole (Table 2). The MICs and MFCs varied from 0.017 to 0.3 mg/mL and from 0.035 to 0.7 mg/mL, respectively. *A. versicolor* and *T. viride* were the most affected by examined extracts among fungal strains tested. Centeno et al. (2010) previously reported

notable antifungal activity of *T. vulgaris* extracts against *A. flavus* and *A. ochraceus*. The antifungal effects of *T. capitatus* extracts against phytopathogenic fungi of *Citrus sinensis* were previously confirmed by Tabti et al. (2014). The promising antifungal effects of *Thymus* species extracts were usually explained by the presence of major oxygenated monoterpenes, particularly thymol and carvacrol, but also to synergism with other constituents represented in lower amounts (Centeno et al., 2010; Petrović et al., 2016a).

Table 1. Antibacterial activity of examined *T. praecox* subsp. *polytrichus* ethanol extracts

	Gram(+) bacteria				Gram(-) bacteria			
	B. cereus	*M. luteus*	*S. aureus*	*L. monocytogens*	*E. coli*	*P. aeruginosa*	*E. cloacae*	*S.* Typhimurium
	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC
Ethanol 70%*	0.075/0.15	0.1/0.15	0.075/0.15	0.15/0.3	0.1/0.15	0.15/0.3	0.1/0.15	0.075/0.15
Ethanol 96%*	0.035/0.075	0.075/0.15	0.035/0.075	0.075/0.15	0.075/0.15	0.15/0.3	0.035/0.075	0.035/0.075
Ethanol 70%	0.075/0.15	0.1/0.15	0.038/0.15	0.2/0.3	0.038/0.15	0.2/0.3	0.075/0.15	0.1/0.15
Ethanol 96%	0.035/0.075	0.075/0.15	0.075/0.15	0.15/0.3	0.075/0.15	0.15/0.3	0.035/0.075	0.035/0.075
Streptomicin	0.1/0.2	0.2/0.3	0.05/0.1	0.2/0.3	0.2/0.3	0.2/0.3	0.3/0.5	0.2/0.3
Ampicillin	0.3/0.4	0.3/0.4	0.3/0.4	0.4/0.5	0.3/0.5	0.8/1.25	0.4/0.8	0.3/0.5

* praetreatment with *n*-hexane

Table 2. Antifungal activity of examined *T. praecox* subsp. *polytrichus* ethanol extracts

	A. fumigatus	*A. versicolor*	*A. ochraceus*	*A. niger*	*T. viride*	*P. funiculosum*	*P. ochrochloron*	*P. verrucosum* var. *cyclopium*
	MIC/MFC	MIC/MFC	MIC/MFC	MIC/MFC	MIC/MFC	MIC/MFC	MIC/MFC	MIC/MFC
Ethanol 70%*	0.15/0.70	0.15/0.30	0.15/0.30	0.15/0.30	0.075/0.15	0.075/0.30	0.3/0.7	0.15/0.3
Ethanol 96%*	0.075/0.15	0.035/0.075	0.075/0.15	0.075/0.15	0.017/0.035	0.035/0.075	0.075/0.15	0.075/0.15
Ethanol 70%	0.15/0.3	0.075/0.15	0.075/0.15	0.15/0.3	0.075/0.15	0.15/0.3	0.15/0.3	0.3/0.7
Ethanol 96%	0.15/0.30	0.035/0.15	0.15/0.30	0.15/0.30	0.075/0.15	0.15/0.30	0.3/0.7	0.3/0.7
Bifonazole	0.15/0.2	0.1/0.2	0.15/0.2	0.15/0.2	0.15/0.2	0.2/0.25	0.2/0.25	0.2/0.3
Ketokonazol	0.2/0.5	0.5/1.0	2.5/3.0	0.2/0.5	2.5/2.5	0.2/0.5	1.5/2.0	1.5/2.0

* praetreatment with *n*-hexane

In vitro antidiabetic activity of *T. praecox* subsp. *polytrichus* ethanol extracts

The *α*-amylase and *α*-glucosidase inhibitory activities of investigated ethanol extracts of *T. praecox* subsp. *polytrichus* were determined using acarbose (Glucobay), a clinical drug, as a positive control. As shown in Table 3, the IC_{50} values for *α*-glucosidase inhibitory activity of examined *Thymus* extracts were in a range from 76.1 to 610.16 μg/mL. The 70% ethanol extract, especially those previously treated with hexane (76.1 μg/mL), showed better activity in inhibiting *α*-glucosidase. On the other hand, the IC_{50} values for *α*-amylase inhibitory activity were in the range of 0.941–1.398μg/mL. The results of the *α*-amylase inhibition test show that pretreatment of the samples with hexane do not contribute to an increase in activity. The 70% ethanol extracts also showed the best enzyme inhibitory activity (0.941 μg/mL), while the 96% ethanol extracts, both pretreated with hexane and untreated with hexane, showed close values (1.352 μg/mL and 1.398 μg/mL, respectively).

Despite the increasing interest in medicinal plants, as potential new sources for the development of drugs with fewer side effects than hypoglycemic agents currently used in the treatment of diabetes mellitus, there are no data in the literature on the antidiabetic effects of extracts from *T. praecox* subsp. *polytrichus*. The potential antidiabetic role of other *Thymus* species through inhibition of *α*-amylase and *α*-glucosidase, key enzymes involved in the hydrolysis of carbohydrates and their absorption, has been confirmed earlier by several authors (Hyun et al., 2014; Lauk, 2015; Cam et al., 2017; Ekin et al., 2019). The crude methanol extract and its ethyl acetate fraction from *T. serpyllum* possessed strong antidiabetic activity, which the authors attributed to the presence of various phenolic compounds such as catechin, rutin, chlorogenic and rosmarinic acid, while the *α*-glucosidase inhibitory activity of the hexane fraction of *T. vulgaris* was positively associated with thymol (Hyun et al., 2014). *Thymus vulgaris* extracts significantly reduced the blood glucose levels in diabetic rabbits, and the authors suggested their use alone or in combination with insulin to manage DM and its associated complications (Mushtaq et al., 2016). Similar to our results, Dessalegn et al. (2019) showed higher *α*-glucosidase than *α*-amylase inhibitory activity of *T. shimpery* and *T. vulgaris*. They also demonstrated the influence of solvent polarity on the revealed antidiabetic properties.

Table 3. In vitro α-amylase and *α*-glucosidase inhibitory effects of examined *T. praecox* subsp. *polytrichus* ethanol extracts.

	α-amylase IC_{50} (μg/mL)	*α*-glucosidase IC_{50} (μg/mL)
Ethanol 70%*	1.300 ± 0.26	76.10 ± 18.35
Ethanol 96%*	1.398 ± 0.58	610.16 ± 28.28
Ethanol 70%	0.941 ± 0.35	121.26 ± 5.12
Ethanol 96%	1.352 ± 0.68	449.26 ± 22.15
Acarbose/Glucobay	3.40 ± 0.92	20.46 ± 2.91

* pratreatment with *n*-heksane

CONCLUSION

In this study, extracts of *T. praecox* subsp. *polytrichus* were found to have valuable antimicrobial and antidiabetic potential, and could therefore be exploited as a new source of bioactive ingredients in antidiabetic supplements, in products for the treatment of infectious diseases and as food preservatives.

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Thymus praecox subsp. *polytrichus*: АНТИМИКРОБНА И АНТИДИЈАБЕТИЧНА АКТИВНОСТ ЕТАНОЛНИХ ЕКСТРАКАТА

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РЕЗИМЕ: *T. praecox* subsp. *polytrichus* једна је од најраспрострањенијих биљних врста у евро-сибирском региону. У овој студији је тестирана *in vitro* антимикробна и антидијабетична активност екстраката који су припремљени у Soxhlet апарату коришћењем 70% и 96% етанола, са и без предтретмана хексаном. Минималне инхибиторне концентрације (МИК) и минималне бактерицидне концентрације (МБК) биле су у опсегу од 0,035 до 0,150 mg/mL и од 0,075 до 0,300 mg/mL, редоследом. Оба 96% етанолна екстракта су показала значајну антибактеријску активност, посебно на бактерије *Bacillus cereus*, *Enterobacter cloaceae* и *Salmonela* Typhymurium. Екстракт 96% етанола који је претходно третиран хексаном, такође је испољио значајну антибактеријску активност на *Staphylococcus aureus*, *B. cereus*, *S. aureus* и *S.* Typhymurum су такође биле сензитивне на 70% етанолни екстракт (са предтретманом хексаном), док је 70% етанолни екстракт без предтретмана хексаном био ефективнији против *S. aureus* и *Escherichia coli*. Минималне инхибиторне (МИК) и минималне фунгицидне концентрације (МФК) биле су у опсегу од 0,017 до 0,300 mg/mL и од 0,035 до 0,700 mg/mL, редом, при чему су *Aspergillus versicolor* и *Trichoderma viride* показале највећу осетљивост. Вредности инхибиторних концентрација (IC_{50}) су варирале између 0,94 и 1,4 mg/mL у α-амилаза тесту и између 76,1 и 610,2 μg/mL у α-глукозидазном тесту. Седамдесетопроцентни етанолни екстракти, посебно онај предтретиран хексаном показао је изразито високу ензимску инхибиторну активност у α-глукозидазном тесту (76,10 μg/mL), бољу чак и у односу на контролу (glucobay 20,46 μg/mL). Највећи инхибиторни ефекат у α-амилаза тесту је показао 96% етанолни екстракт. Добијени резултати показују да етанолни екстракти *T. praecox* subsp. *polytrichus* испо-

њавају значајан антимикробни и антидијабетични потенцијал и могу се користити у фитофармацији и презервацији хране.

КЉУЧНЕ РЕЧИ: *T. praecox* subsp. *polytrichus,* екстракти, антимикробна активност, антидијабетична активност