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ANTIBACTERIAL PROPERTIES OF ETHANOLIC EXTRACTS OBTAINED FROM LEAVES OF SOME *THYMUS* L. (LAMIACEAE) REPRESENTATIVES AGAINST *ACINETOBACTER BAUMANNII*

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The *Thymus* L. is one of the most widely used genera in folk medicine, where it is popular for its stimulatory action on all organism functions. The purpose of this study was to evaluate the antimicrobial effects of five ethanolic extracts obtained from leaves of some *Thymus* L. representatives (*Thymus serpyllum* L., *Th. pannonicus* All., *Th.* × *porcii* Borbás, *Th. pulegioides* L., *Th. alpestris* Tausch ex A. Kern.) against *Acinetobacter baumannii* complex isolate, resistant to gentamicin and ciprofloxacin (specimen 3680, UK NEQAS). Freshly leaves were washed, weighed, crushed, and homogenized in 96% ethanol (in proportion 1 : 19) at room temperature. The extracts were then filtered and investigated for their antimicrobial activity. Antimicrobial activity was determined using the agar disk diffusion assay. The present study has shown that ethanolic extracts obtained from leaves of *Thymus* species inhibited mild activity against *A. baumannii*. The mean diameter of inhibition zone for *Th. serpyllum* was (10.45 ±0.81) mm, for *Th. pannonicus* (10.82 ±0.63) mm, for *Thymus* × *porcii* (9.57 ±0.75) mm, for *Th. pulegioides* (10.54 ±0.52) mm, and for *Th. alpestris* (10.62 ±0.54) mm. It should be noted that the most antimicrobially effective plant against *Acinetobacter baumannii* was *Thymus pannonicus*. The present study lays the basis for future research, to validate the possible use of *Thymus* species as a candidate in the treatment of bacterial infections. The knowledge about the chemical profile of the extract will help in explaining the observed activity and designing experiments for activity fractionation for isolation of the active principle. The identification of precise molecular mechanisms addressing how these extracts inhibit bacterial growth needs to be explored.

Keywords: *Thymus* species, leaf extracts, agar disk diffusion assay, antibacterial activity, inhibition zone diameter

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Introduction

Acinetobacter baumannii is an opportunistic pathogen and one of the six most important multidrug-resistant microorganisms in hospitals worldwide responsible for hospital-acquired nosocomial infections (Antunes et al., 2014; Lee et al., 2017). This human pathogen is responsible for a vast array of infections, i.e. ventilator-associated as well as bloodstream infections in critically ill patients, and mortality rates can reach 35% (Antunes et al., 2014). The spread of multidrug-resistant *Acinetobacter* strains is cause for concern (Harding et al., 2018). Due to the prevalence of infections and outbreaks caused by multi-drug resistant *A. baumannii*, few antibiotics are effective for treating infections caused by this pathogen (Lee et al., 2017).

Because of the limited number of available antibiotics, we used medicinal plants with an antibacterial effect. In our previous study, we have revealed that the ethanolic extracts obtained from leaves of *Thymus* L. representatives exhibited different activity against clinical isolates of methicillin-resistant *Staphylococcus aureus*. Results obtained for antibacterial activity exhibited various degrees of the zone of inhibition and significant activity was observed for *Thymus pulegioide*s L. (21–26 mm) followed by *Th. pannonicus* All. (14–18 mm) and *Th. serpyllum* L. (13–18 mm), respectively while some extracts (*Th. × porcii* Borbás and *Th. alpestris* Tausch ex A. Kern.) showed mild activity at the same concentration. The above results revealed that the selected plants can be further utilized for isolation of the active ingredients as the crude extracts were found good for inhibition of MRSA. It is therefore important to investigate the potential of these plants as novel antimicrobial agents, targeting the multidrug-resistant bacteria and clinical importance (Honcharenko et al., 2018a). Additionally, the ethanolic extract obtained from the leaves of *Th. pulegioides* was the most effective plant extracts against *Salmonella enteritidis* studied and presented in the work by Honcharenko et al. (2018b). The antibacterial activity of extracts was greatest for *Th. pulegioides* followed by *Th. pannonicus* (13.1 ±0.85 mm) followed by *Th. alpestris* (12.6 ±0.25 mm), *Th. × porcii* (12.2 ±0.55 mm), and then by *Th. serpyllum* (10.5 ±0.23 mm) (Honcharenko et al., 2018b). Moreover, the ethanolic extracts obtained from leaves of *Thymus* representatives exhibited intermediate activity against β-lactamase producing *Pseudomonas aeruginosa* (Honcharenko et al., 2018c). The mean diameter of inhibition zone for *Th. serpyllum* was (11.3 ±0.3) mm, for *Th. pannonicus* (11.5 ±0.5) mm, for *Th. × porcii* (9.8 ±0.5) mm, for *Th. pulegioides* (11.2 ±0.7) mm, and for *Th. alpestris* (12.8 ±0.8) mm. The effects varied significantly according to the *Thymus* taxa. It should be noted that the most antimicrobial effective plant against β-lactamase producing *P. aeruginosa* was *Th. alpestris*, being highly active with the ethanolic extract (mean diameter of inhibition zone was 12.8 ±0.8 mm). The antibacterial activity of extracts was greatest for *Th. alpestris* followed by *Th. pannonicus* followed by *Th. serpyllum* and then by *Th. pulegioides* (Honcharenko et al., 2018c).

Our previous studies lay the basis for future research, to validate the possible use of *Thymus* species as a candidate in the treatment of bacterial infections and a potential target for the activity-guided isolation of active constituents in order to explore the mechanism of action and relevant uses in the medicine and veterinary. Recent studies have shown that *Thymus* species have strong antimicrobial and antioxidant activities (Jia et al., 2010). The thyme essential oil has also remarkable antibacterial effects, which are associated with the presence of their phenolic components, carvacrol, and thymol. In a study by Yamazaki et al. (2004), carvacrol had the most powerful effect against *Listeria monocytogenes*, followed by thymol, eugenol, cinnamaldeyde, and isoeugenol. In comparative studies including the oregano and thyme essential oils, the two oils demonstrated remarkable antibacterial activity.

Thymol is a naturally occurring phenol monoterpene derivative of cymene and isomer of carvacrol and is one of the major constituents (10–64%) of the essential oils of thyme (*Thymus vulgaris* L.). Nowadays, thymol and thyme present a wide range of functional possibilities in the pharmacy, food, and cosmetic industry due to several studies that have evaluated the potential therapeutic uses of this compound for the treatment of disorders affecting the respiratory, nervous, and cardiovascular systems. Moreover, this compound also exhibits antimicrobial, antioxidant, anti-carcinogenesis, anti-inflammatory, and antispasmodic activities, as well as a potential as a growth enhancer and immunomodulator (Salehi et al., 2018).

Schelz et al. (2006) observed that the essential oil of *Thymus vulgaris* inhibited the growth of *Staphylococcus epidermidis* and *Escherichia coli* more efficiently than the essential oils of orange, eucalyptus, fennel, geranium, cedar, ginger, turpentine, rosemary, and tea tree, whereas of 15 essential oils used against foodborne (*Listeria monocytogenes*, *Salmonella typhimurium*, *E. coli* 0157:H7) and food spoilage bacteria (*Brochothrix thermosphacta* and *Pseudomonas fluorescens*), the oregano, thyme, and cinnamon oils demonstrated the most powerful antibacterial effect (Mith et al., 2014). Additionally, the oregano, thyme, and basil oils exhibited sufficient to moderate effects against multi-resistant clinical isolates of *Acinetobacter baumannii*, *E. coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* (Sakkas et al., 2016).

These data have prompted us to verify the antibacterial effects of four species and one interspecific hybrid of *Thymus* genus sampled in the western part of Ukraine against *Acinetobacter baumanii*. Therefore, the aim of this study was to evaluate the antimicrobial effects of five ethanolic extracts obtained from leaves of some *Thymus* representatives (*Thymus serpyllum*, *Th. pannonicus*., *Th.* × *porcii*, *Th. pulegioides*, *Th. alpestris*) against *Acinetobacter baumanii* strain.

Material and methodology

Collection of Plant Materials

Samples were harvested in June–August, 2016. Leaves of *Thymus serpyllum* were collected among the grass on sandy soil in the edge of a pine forest (Baymaky village, Bilohirya district, Khmelnytsky region, Ukraine; N 50° 03´ 58.9´´, E 26° 13´ 37,5´´, 257 m a.s.l.). Leaves of *Th. pannonicus* were harvested among grass in the roadside between the two cultivated fields (Syvky village, Bilohirya district, Khmelnytsky region, Ukraine; N 50° 02´ 09.6´´, E 26° 13´ 19.2´´, 283 m a.s.l.). Leaves of *Th. pulegioides* were collected among grass nearby land parcels (Syvky village, Bilohirya district, Khmelnytsky region, Ukraine; N 50° 02´ 02.8´´, E 26° 14´ 13.9´´, 306 m a.s.l.). Leaves of *Th.* × *porcii* (a hybrid between *Th. pannonicus* and *Th. pulegioides*) were sampled in the grass stand, on the side of the footpath of the race track

(Medovoi Pechery Str., Lviv, Ukraine; N 49° 49‘ 15.1“, E 24° 05‘ 12.5“, 348 m a.s.l.). Leaves of *Th. alpestris* were harvested on the side of the road below the stream, in mountain valley Shumneska (Kvasy village, Rakhiv district, Zakarpattia region, Ukraine; N 48° 09‘ 32.3“, E 24° 21‘ 26.4“, 1,259 m a.s.l.). Identification of these five taxa was made according to Nachychko (2014, 2015) and Nachychko and Honcharenko (2016). The voucher herbarium specimens of plants used in this study were deposited at the Herbarium of M.G. Kholodny Institute of Botany of the National Academy of Sciences of Ukraine (KW). Plant samples were thoroughly washed to remove all the attached material and used to prepare ethanolic extracts.

Preparation of Plant Extracts

Freshly leaves were washed, weighed, crushed, and homogenized in 96% ethanol (in proportion 1 : 19) at room temperature. The extracts were then filtered and investigated for their antimicrobial activity.

Bacterial strain

For the study, specimen 3680 (UK NEQAS, The United Kingdom National External Quality Assessment Service) was used. It contained an *Acinetobacter baumannii* complex isolate, resistant to gentamicin and ciprofloxacin. The organism was borderline susceptible to imipenem and meropenem and only 14.3% of participants reported intermediate or resistant (Seaton et al., 2017).

Agar diffusion susceptibility testing

Antimicrobial activity was determined using the agar disk diffusion technique (Bauer et al., 1966). The *Acinetobacter baumannii* strain was obtained from the Department of Bacteriology, Regional Hospital in Koszalin (West-Pomeranian Voivodeship, Poland). The strain was grown in a test tube containing 45 mL of sterile nutrient broth (Oxoid™ Ltd.) at 37 °C for 24 hours. The purity of the inoculum was confirmed by plating on appropriate selective media and microscopic examination of the Gram-stained smear.

The culture was inoculated onto Mueller-Hinton (MH) agar plates. Sterile filter paper discs impregnated with extracts were applied over each of the culture plates. Isolates of bacteria were then incubated at 37 °C for 24 h. The plates were then observed for the zone of inhibition produced by the antibacterial activity of various ethanolic extracts obtained from leaves of *Thymus* representatives. The presence of inhibition zones around each of paper discs after the period of incubation was regarded as the presence of antimicrobial action while the absence of any measurable zone of inhibition was interpreted as the absence of antimicrobial action. Negative control discs impregnated with sterile ethanol were used in each experiment. The antimicrobial activities of the extracts tested were evaluated at the end of the inoculated period by measuring the inhibition zone diameter around each paper disc in millimeters. The plates were observed and photographs were taken. For each extract, eight replicate trials were conducted. Zone diameters were determined and averaged.

Statistical analysis

Statistical analysis of the data obtained was performed by employing the mean ± standard error of the mean (S.E.M.). All variables were tested for normal distribution using the Kolmogorov-Smirnov test (p >0.05). In order to find significant differences (significance level, p <0.05) between groups, the Kruskal-Wallis test by ranks was applied to the data (Zar, 1999). All statistical analyses were performed using Statistica 8.0 software (StatSoft, Poland). The following zone diameter criteria were used to assign susceptibility or resistance of bacteria to the phytochemicals tested: Susceptible (*S*) ≥15 mm, Intermediate (*I*) = 11–14 mm, and Resistant (*R*) ≤10 mm (Okoth et al., 2013).

Results and discussion

Antimicrobial activity of various ethanolic extracts obtained from leaves of *Thymus* species against *Acinetobacter baumannii* measured as inhibition zone diameter is shown in Figure 1 and 2. The present study has shown that ethanolic extracts obtained from leaves of *Thymus* species inhibited mild activity against *A. baumannii*. The mean diameter of inhibition zone for *Th. serpyllum* was (10.45 ±0.81) mm, for *Th. pannonicus* (10.82 ±0.63) mm, for *Thymus* × *porcii* (9.57 ±0.75) mm, for *Th. pulegioides* (10.54 ±0.52) mm, and for *Th. alpestris* (10.62 ±0.54) mm (Figure 1 and 2).

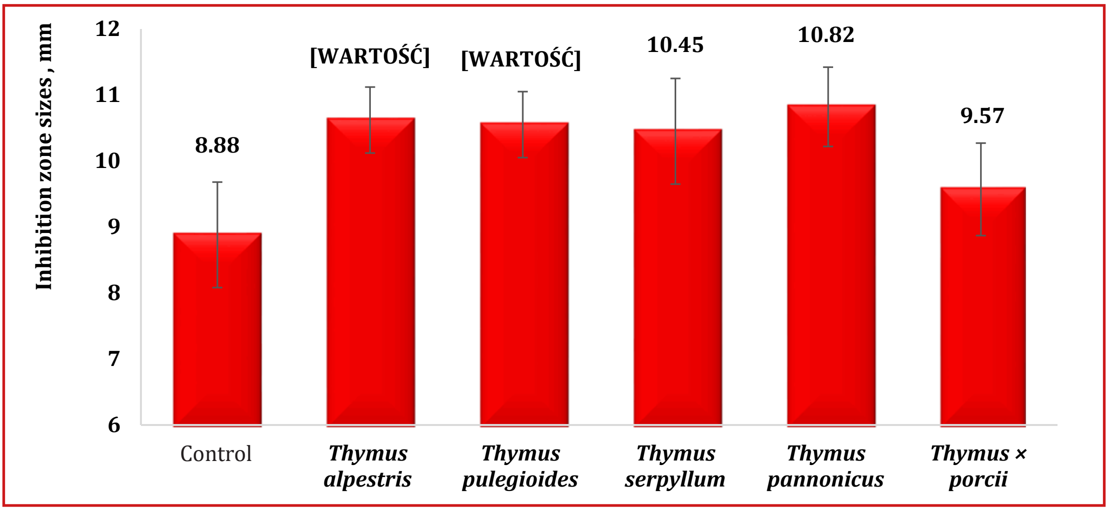

Figure 1 The mean of inhibition zone diameters of various ethanolic extracts obtained from leaves of *Thymus* species against *Acinetobacter baumannii* (M ±m, n = 8)

Detailed data regarding the zones of inhibition by the various plant extracts were recorded and presented in Figure 2.

The main purpose of our study was to assess the antimicrobial effects of five ethanolic extracts obtained from leaves of *Thymus* species against *Acinetobacter baumannii* complex isolate, resistant to gentamicin and ciprofloxacin (specimen 3680, UK NEQAS). Ethanolic extracts of the plant materials decrease the growth of the strain studied. The effects varied significantly according to the *Thymus* species. It was observed in the present study that ethanolic extracts inhibited the growth of bacteria tested moderately. The highest antimicrobial effect was recorded for *Th. alpestris*, *Th. pannonicus*, and *Th. pulegioides* (Figure 1 and 2). It should be noted that the most antimicrobially effective plant against *Acinetobacter baumannii* was *Th. pannonicus* (mean diameter of inhibition zone was 10.82 ±0.63 mm) (Figure 1).

Figure 2 Antimicrobial activity of various ethanolic extracts obtained from leaves of *Thymus*. pannonicus (37), *Thymus* × *porcii* (38), *Th. pulegioides* (39), *Th. serpyllum* (40), *Th. alpestris* (41, 42) against *Acinetobacter baumannii* measured as inhibition zone diameter

Many works support the traditional use of *Thymus* species as valuable plant-based compounds for the treatment of wounds. Ustuner et al. (2019) have observed that *Thymus sipyleus* Boiss. subsp. *rosulans* (Borbas) Jalas (TS) extracts can promote the healing process by enhancing fibroblast migration, proliferation, and collagen synthesis as well as suppressing pro-inflammatory cytokines.

Fresh and dried *Thymus* species, as well as their processed products, have been widely used as flavorings since ancient times; however, during the last few decades, they also have become a subject for a search of natural antioxidants and antimicrobial agents (Jia et al., 2010). For example, Demirci et al. (2018) have evaluated *in vitro* antimicrobial activities of the essential oil derived from *Thymus sipyleus* Boiss. subsp. *sipyleus* var. *sipyleus* by agar diffusion, microdilution, and vapor diffusion methods against selected rhinosinusitis associated strains such as *Staphylococcus aureus*, methicillin-resistant *S. aureus* (MRSA), *S. epidermidis*, *Streptococcus pyogenes*, *S. pneumoniae*, *Pseudomonas aeruginosa*, *Haemophilus influenzae*, and *Moraxella catarrhalis*. Additionally, the *in vitro* anti-inflammatory activity was evaluated by 5-lipoxygenase (5-LOX) inhibitory effect of the essential oil spectrophotometrically. The antibacterial activity against rhinosinusitis pathogens varied between 160 and 1,250 μg/

mL minimum inhibitory concentrations, with the best inhibitory effects observed against the *S. aureus*, *S. pyogenes* and *M. catarrhalis*. The anti-inflammatory activity of the oil was determined as 12.1 ±1.8% in 100 μg/mL. The results Demirci et al. (2018) showed the *in vitro* antimicrobial and anti-inflammatory potential of the oil also in the vapor phase against sinusitis supporting the traditional use.

Antimicrobial activities of the essential oils from *Thymus marschallianus* Will. and *Thymus proximus* Serg. growing in the wild in Xinjiang against *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, yeast, *Rhizopus*, and *Penicillium* were investigated by Jia et al. (2010). The inhibition zones (IZ) and minimum inhibitory concentration (MIC) values were 5.0 to 35.7 mm in diameter and 1.81 to 4.52 μL/mL, respectively. Benameur et al. (2019) have evaluated the susceptibility of blaESBL producing Enterobacteriaceae to Slovakian *Thymus vulgaris* essential oil alone and in combination with cefotaxime. Thymol (34.5%), *p*-cymene (22.27%) and linalool (5.35%) were the major components present in the essential oil. The identified strains were multi-drug resistant (MDR). *Thymus vulgaris* essential oil showed high activity against all MDR strains, including blaESBL producing isolates, with inhibition zones and MIC values in the range of 24–40 and 2.87–11.5 μg/mL, respectively. *Thymus vulgaris* essential oil in combination with cefotaxime showed a synergistic action against blaSHV-12 producing *Escherichia coli* (FICI 0.28) and an additive effect vs ESBL producing *Enterobacter cloacae* (FICI 0.987).

Thymus vulgaris essential oil could be an alternative to classical antibiotics against bacterial biofilms, which show increased tolerance to antibiotics and host defense systems and contribute to the persistence of chronic bacterial infections (Perez et al., 2019). Lagha et al. (2019) have studied the antibacterial activity of five medicinal plant essential oils against urinary tract infections caused by *E. coli* using disc diffusion and minimal inhibition concentration (MIC) methods. In addition, the biofilm inhibitory action of oils was realized by crystal violet. Gas chromatography-mass spectrometry analysis showed variability between oils in terms of compound numbers as well as their percentages. Antibacterial activity was observed only in cases of *Origanum majorana*, *Thymus zygis*, and *Rosmarinus officinalis*, while *Juniperus communis* and *Zingiber officinale* did not show any effect towards *E. coli* isolates. *T. zygis* essential oil demonstrated the highest antibacterial activity against *E. coli* isolates, followed by *O. majorana* and *R. officinalis*. Further, oils showed high biofilm inhibitory action with a percentage of inhibition that ranged from 14.94 to 94.75%. *R. officinalis* oil had the highest antibiofilm activity followed by *T. zygis* and *O. majorana* (Lagha et al., 2019).

The synthesized silver nanoparticles (AgNPs) using *Thymus kotschyanus* Boiss. & Hohen. revealed significant antibacterial activity against Gram-positive bacteria such as *Staphylococcus aureus* and *Bacillus subtilis*, while the growth inhibition of AgNPs at 1,000–500 μg/ml occurred against *Klebsiella pneumonia* and at 1000–250 μg/ml of AgNPs was observed against *E. coli* (Gholami et al., 2018).

Manconi et al. (2018) have extracted, characterized, and formulated *Thymus capitatus* (*Tymbra capitata*) essential oil in phospholipid vesicles: liposomes, glycerosomes and Penetration Enhancer-containing Vesicles (PEVs). The antibacterial activity of the oil was demonstrated against cariogenic *Streptococcus mutans*, *Lactobacillus acidophilus*, and commensal *Streptococcus sanguinis*. The combination of antioxidant and antibacterial activities of *Thymus* essential oil formulations may be useful for the treatment of oral cavity diseases.

In vitro assessment of antioxidant and antimicrobial activities of methanol extracts and essential oil of *Thymus hirtus* sp. *algeriensis* was carried out by Fatma et al. (2014). The essential oils of *Thymus hirtus* sp. *algeriensis* were screened for their antibacterial activity against six common pathogenic microorganisms (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enteridis*, *Staphylococcus aureus*, *Bacillus subtilis*, and *Listeria monocytogenes*) by well diffusion method and agar dilution method (MIC). All the essences were found to inhibit the growth of both Gram-positive and Gram-negative bacteria organisms tested. These activities were correlated with the presence of phenolic compounds in the active fractions. HPLC confirmed the presence of phenolic compounds in methanol extracts (Fatma et al., 2014).

The biological activities of *Thymus* plants and essential oils depend on their chemical composition, which is determined by the genotype and influenced by environmental conditions (Jia et al., 2010). The main constituents of *Thymus vulgaris* leave essential oil are 2 phenolic compounds, thymol (2-isopropyl-5-methylphenol) and its conformational isomer, carvacrol (5-isopropyl-2-methylphenol). Further components in the essential oil are thymol methyl ether, cineol, cymene, α-pinene, and borneol (Pina-Vaz et al., 2004). The antimicrobial activities of *Thymus vulgaris* oil are mostly believed to be related to the thymol and carvacrol contents of the oil (Fani and Kohanteb, 2017). The main compounds identified in the infusion of *Th. pulegioides* essential oil were: carvacrol (38.96%), thymol (25.17%), linalool (9.75%) and thymoquinone (7.27%), while in hydrodistillate: carvacrol (63.20%), linalool (16.95%) and thymol (15.55%) (Rădulescu et al., 2009). It is well documented that the presence of these chemicals is responsible for various medicinal properties of plants. There are many reports available to support the role of phytochemicals and their activity against specific diseases.

CONCLUSIONS

The ethanolic extracts obtained from leaves of *Thymus* species showed different antibacterial activities against *Acinetobacter baumannii* complex isolate, resistant to gentamicin and ciprofloxacin (specimen 3680, UK NEQAS). It should be noted that the most antimicrobially effective plant against *Acinetobacter baumannii* was *Thymus pannonicus*. The present study lays the basis for future research, to validate the possible use of *Thymus* species as a candidate in the treatment of bacterial infections.

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