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THE ANTIBACTERIAL ACTIVITIES OF SOME *THYMUS* (LAMIACEAE) REPRESENTATIVES AGAINST *SALMONELLA ENTERIDITIS* STRAIN LOCALLY ISOLATED

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Some of the plants of the *Thymus* genus were previously reported for their antimicrobial activities. 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* L. emend. Mill., *Th. pannonicus* All., *Th.* × *porcii* Borbás, *Th. pulegioides* L., *Th. alpestris* Tausch ex A. Kern.) against *Salmonella enteritidis* strain. Freshly leaves were washed, weighted, 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 ethanolic extract obtained from the leaves of *Th. pulegioides* was the most effective plant extracts against *Salmonella enteritidis* studied in this work. 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). These plant extracts could be a potential source of new antibacterial agents. Further and more specific studies, *in vivo*, are recommended to determine the efficacy of these plants in the treatment of *Salmonella*-induced bacterial infections.

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

Introduction

Salmonella infection represents a considerable burden in both developing and developed countries and to cause 93 million enteric infections and 155,000 diarrheal deaths each year (Majowicz et al., 2010; Wierup et al., 2017). The majority of clinical disease in animals and humans is caused by serovars within the *Salmonella enterica* subspecies (Pham and McSorley, 2015). Over 50% of the isolates proved resistant to ampicillin, chloramphenicol, and trimethoprim/sulfamethoxazole, the primary treatments of choice for salmonellosis

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(Chu and Chiu, 2006). Use of antibiotics might result in elevation the risk of spawning more resistant strains (O'Brien, 2002). Utilizing the various herbal substitutes in order to replace antibiotics might offer an alternative for the prevention and/or control of this disease (Snow Setzer et al., 2016).

Medicinal herbs have many potential clinical and therapeutic applications in a modern medical setting because they have been reported to contain bioactive components (Kwon et al., 2008; Bielikova et al., 2017). *Thymus* genus is attracting considerable attention from many researchers, and many such herbs have a long history of medicinal use (Viuda-Martos et al., 2011). The analysis and identification of the constituents of the active principals in these herbs has increased our understanding of their individual pharmacological actions as tonics, carminatives, antitussives, aromatic, expectorant, stomachic, antispasmodic, bronchospasmolytic, diuretic, sedative, diaphoretic, and antiseptics, as well as anti-inflammatory, antioxidant, anthelmintic, hepatoprotective and antitumor agents (Khan and Abourashed, 2010; Nabavi et al., 2015). Antimicrobial agents can also be derived from *Thymus* species (Rota et al., 2008; Xu et al., 2008; Palaniappan and Holley, 2010; Mathela et al., 2010; Rivas et al., 2010; Pemmaraju et al., 2013; Kavoosi et al., 2013; de Morais et al., 2014; Marchese et al., 2016). These versatile pharmacological effects can be attributed to the secondary plant metabolites, especially to essential oil and polyphenols. Plants from the genus *Thymus* are rich in different active substances such as thymol, carvacrol, *p*-cymene and terpinene (Nabavi et al., 2015).

However, many species from *Thymus* genus are yet to be explored scientifically and moreover, the need to find a lasting solution to the problem of infectious diseases caused by *Salmonella* strains necessitated further exploration of plants exhibiting antimicrobial activity. This study screened and evaluated selected representatives of the *Thymus* genus for their antibacterial activities against *Salmonella enteritidis* strain locally isolated. This study evaluated the antimicrobial potentials of the four species and one interspecific hybrid of *Thymus* genus sampled in the western part of Ukraine against *Salmonella enteritidis* strain locally isolated.

Material and methodology

Collection of plant materials

Samples were harvested in June-August, 2016. Leaves of *Thymus serpyllum* L. emend. Mill. were collected among 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* All. 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* L. 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* Borbás (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* Tausch ex A. Kern. 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“, 1259 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 attached material and used to prepare ethanolic extracts.

Preparation of plant extracts

Freshly leaves were washed, weighted, 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 and Agar diffusion susceptibility testing

Antimicrobial activity was determined using the agar disk diffusion technique (Bauer et al., 1966). The clinical isolates of *S. enteritidis* were 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. A loopful of inoculum was transferred by streaking onto a Xylose Lysine Desoxycholate Agar (XLD agar) (Oxoid™ Ltd.). Plates were incubated for 24 hours at 37 °C. Bacterial morphology was confirmed by optical microscopy. Several colonies were collected with a sterile inoculating loop, transferred into a sterile saline solution, and adjusted to the desired concentration using the McFarland nephelometer standards

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 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 millimetres. 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

In order to identify *Thymus* species with antibiotic properties against salmonellosis, the four species and one interspecific hybrid of *Thymus* genus were tested against *Salmonella enteritidis* using the disk-agar method (Figure 1). Ethanol (96%) as the negative control showed inhibition zones of the test strain (8.5 ±0.49 mm). Of the herbal extracts tested, all leaf extracts obtained from *Thymus* species were found to have antibacterial activity against *S. enteritidis* strain tested; inhibition zones ranged from 8 to 16 mm. Moreover, both *Th. alpestris* and *Th. pulegioides* extracts exhibited intermediate antibacterial activity against *S. enteritidis* with statistically significant diameters of inhibition zones (12.6 ±0.25 mm and 14.3 ±0.59 mm, respectively). The inhibition zones produced by leaf extracts obtained from *Th. pannonicus* and *Th.* × *porcii* indicated that both showed effective antimicrobial activities, although these extracts showed slightly higher activity, based on inhibition zone sizes (13.1 ±0.85 mm and 12.2 ±0.55, respectively), these results were non-significantly ($p > 0.05$) (Figure 1).

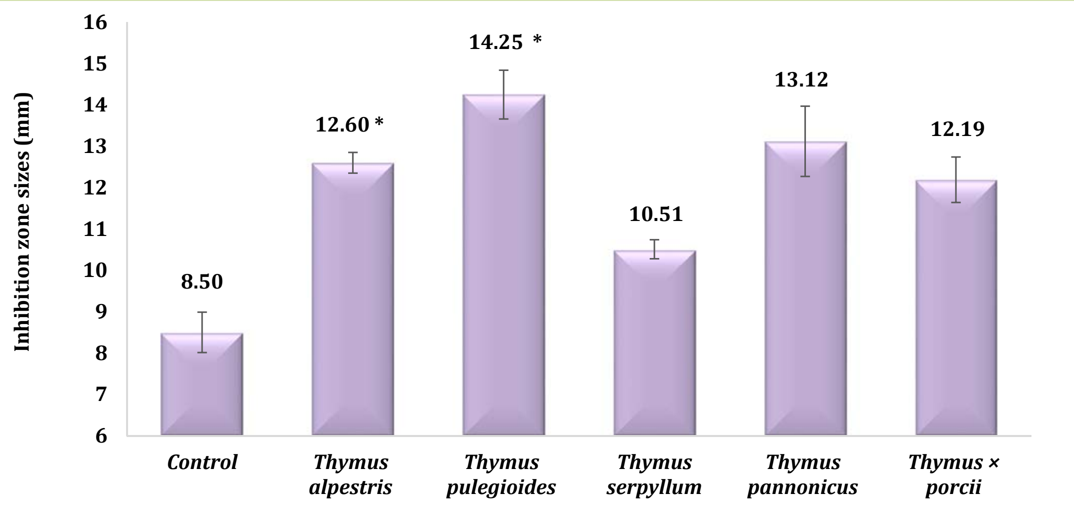

Figure 1 The mean of inhibition zone diameters of ethanolic extracts obtained from leaves of various *Thymus* plants against *Salmonella enteritidis* strain locally isolated (*M* ±m, $n = 8$)
* the changes are statistically significant ($p < 0.05$) compared to the control group (96% ethanol)

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

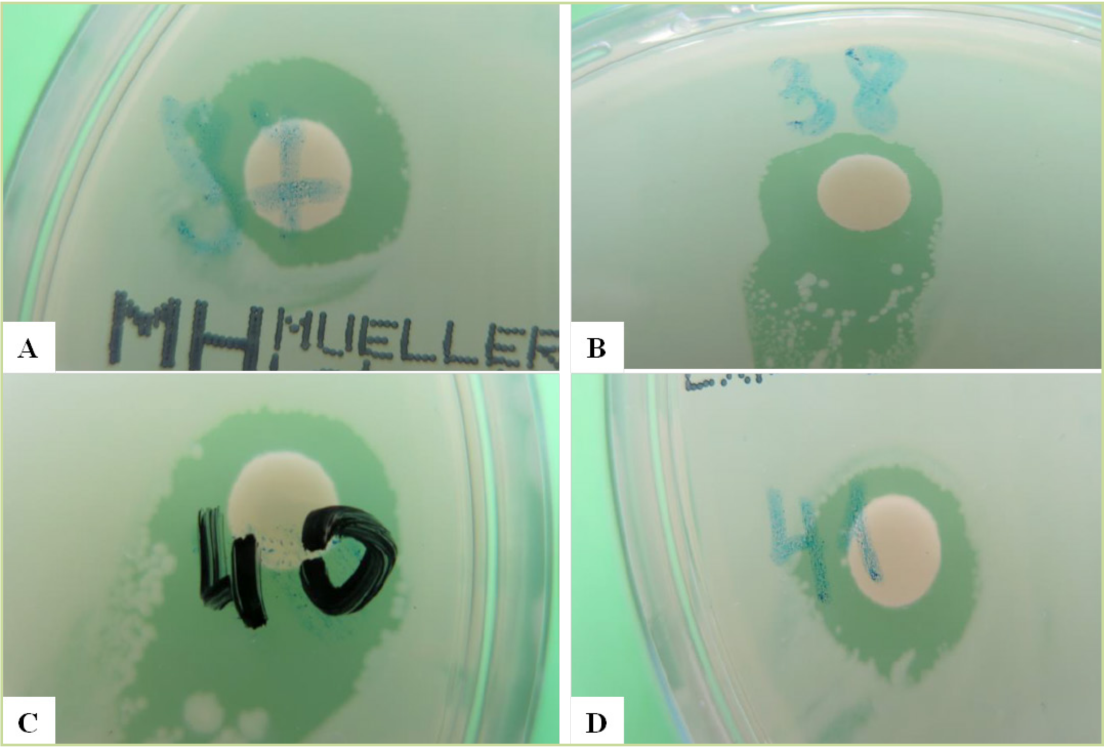

Figure 2 Antimicrobial activity of various ethanolic extracts obtained from leaves of *Th. pannonicus* (A), *Th.* × *porcii* (B), *Th. pulegioides* (C), *Th. alpestris* (D) against *Salmonella enteritidis* strain measured as inhibition zone diameter

It should be noted that the most antimicrobial effective plant against *Salmonella enteritidis* strain was *Th. pulegioides*, being highly active with the mean diameter of inhibition zone 14.3 ±0.59 mm. 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) (Figure 1). Since the antibacterial effectiveness of medicinal plants varies dramatically depending on the phytochemical characteristics of plant families and subfamilies, it is not surprising to note the difference in this efficacy even when using samples taken from the same plant, but from two different regions.

Variation in the chemical profile of extracts could influence their biological activities. Therefore, it was important to know the chemical composition of extracts to correlate with their antimicrobial activities. Most of the antimicrobial activity in essential oils from *Thymus* genus appears to be associated with high amounts of monoterpenoid phenols (thymol and carvacrol) or monoterpenic alcohols (geraniol and linalool) (Petrović et al., 2016). The thymol is responsible for antimicrobial activity (Marchese et al., 2016). Results of this study are in agreement with other research showing that thyme essential oil (especially thymol chemotype) possesses high activity against both Gram-positive and Gram-negative bacteria (Karaman et al., 2001; Rasooli and Mirmostafa, 2002; Rota et al., 2008; Maksimović et al., 2008;

Nejad Ebrahimi et al., 2008; Ruiz-Navajas et al., 2012; Ballester-Costa et al., 2013; Moghimi et al., 2016; Fadli et al., 2012, 2016, 2018; Schött et al., 2017; Semeniuc et al., 2017; Vitali et al., 2017). However, it should be noted that ethanolic extracts have a complex composition and their antimicrobial activities were due to a synergist effect between a large number of components present in small amounts in the extracts.

The two main bioactive compounds in thyme essential oil are thymol and carvacrol responsible for most therapeutic aspects of the thyme extracts, i.e. antibacterial, antifungal, anti-inflammatory, and antioxidant activities (Petrović et al., 2016). In a study by Rota et al. (2008), most of the antimicrobial activity in essential oils from *Thymus* genus appears to be associated with phenolic compounds (thymol and carvacrol). In *Th. hyemalis* Lange oil (thymol, thymol/linalool, and carvacrol chemotypes) 49, 51 and 51 components were identified representing about 97, 98.4 and 86.7% of the total detected constituents. Major components quantified for the thymol chemotype were: thymol (43%) followed by *p*-cymene (16.0%) and γ-terpinene (8.4%); for *Th. hyemalis* thymol/linalool chemotype: linalool (16.6%), thymol (16.0%), γ-terpinene (9.8%), 1-8-cineole (5.4%), borneol (4.7%), verbenone (4.8%); and for *Th. hyemalis* carvacrol chemotype were: carvacrol (40.1%), *p*-cymene (19.8%), borneol (5.0%) and thymol (2.9%). Attending to the volatile profile of the essential oils, a richer relative concentration of terpenic hydrocarbons (γ-terpinene), alcohols (linalool, (Z)-verbenol, terpinen-4-ol, α(alpha)-terpineol, geraniol, spathulenol) ketones (camphor, verbenone) and thymol oxygenated derivates (thymol methyl ether) were quantified in *Th. hyemalis* (thymol ch.) when compared to *Th. zygis* L. (thymol ch.) and *Th. vulgaris* L. (thymol ch.). Results of these authors suggest that it could be a synergistic action among phenolic components and these compounds. Carvacrol is another phenolic component that described the chemotype of *Th. hyemalis* essential oil. The assays using this essential oil (40% carvacrol) showed bactericidal and bacteriostatic activities similar to *Th. hyemalis* (43% thymol) since concentrations under 0.2 $\mu l.mL^{-1}$ were enough to achieve the MIC and MBC for 9 of the 10 microorganisms assayed in the study of Rota et al. (2008). The bacteriostatic properties of this oil are suspected to be associated with the carvacrol content (Rota et al., 2008).

The antimicrobial activity of *Thymus* species has been well studied. Since the 1960's, and especially over the past 20 years, a large number of *Thymus* essential oils, extracts, and their isolated compounds have been studied for the antimicrobial activity. These products are of particular interest because no bacterial resistance or adaptation has been described, and low or insignificant side effects have been found both for the essential oils and whole extracts (Nabavi et al., 2015). Semeniuc et al. (2017) have compared the antibacterial effects of several essential oils alone and in combination against different Gram-positive and Gram-negative bacteria associated with food products. Parsley, lovage, basil, and thyme essential oils, as well as their mixtures (1 : 1, v/v), were tested against *Bacillus cereus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Salmonella typhimurium*. Thyme essential oil exhibited the best inhibitory activity against all bacteria evaluated by the Kirby-Bauer disk diffusion test (range 12.16–36.41 mm), followed by basil, lovage, and parsley essential oils. Its zone of inhibition is larger (for *E. coli* and *S. typhi*murium) or similar (for *B. cereus*) to the size of gentamicin zone (the antibiotic used as positive control). Among the tested microorganisms, it produces the largest zone of inhibition against *E. coli* (strong inhibitory effect), followed by *S. typhi*murium (moderate inhibitory effect), *B. cereus* (moderate inhibitory effect), *P. aeruginosa* (mild inhibitory effect), and *S. aureus* (mild inhibitory effect). *P. aeruginosa* is the most susceptible to all essential oils and their combinations. Three essential oils combinations show antagonistic effects against *P. aeruginosa* (parsley/thyme, lovage/thyme, and basil/thyme essential oils), and the other three combinations indifferent effects (parsley/lovage, parsley/basil, and lovage/basil essential oils). Parsley/lovage, parsley/basil, and parsley/thyme essential oil mixtures display significantly higher antibacterial activities than the parsley essential oil. Basil essential oil does not significantly affect the antibacterial activity of lovage/basil essential oil mixture. Thyme essential oil significantly contributes to the antibacterial activity of parsley/thyme and lovage/thyme essential oil mixtures but does not significantly influence the antibacterial activity of basil/thyme essential oil mixture. All pairwise combinations exhibit lower antibacterial activities than the thyme essential oil against all five bacteria. Considering that thyme essential oil has the highest percentage yield and antibacterial potential from all tested formulations, it is therefore recommended to be used alone as the antimicrobial agent (Semeniuc et al., 2017).

Cutillas et al. (2018) have supported the potential use of *Thymus* sp. essential oils as natural preservatives of natural food, cosmetic and pharmaceutical ingredients. They have analyzed by gas chromatography coupled with mass spectrometry detection six samples of red thyme (*Th. zygis*) and two samples of winter thyme (*Th. hyemalis*) essential oils obtained from plants cultivated in south-eastern Spain and extracted by steam distillation. Thymol (30–54%), *p*-cymene (14–27%) and γ-terpinene (8–28%) were the most abundant components of *Th. zygis* essential oil, while 1,8-Cineole (3–37%), *p*-cymene (1–29%), linalool (8–13%) and thymol (0–19%) were the most abundant components in the case of *Th. hyemalis* essential oil. Enantioselective gas chromatography identified (-)-linalool, (-)-borneol and (+)-limonene as the main enantiomers. Several methods to evaluate antioxidant capacities were applied to the essential oils, concluding that their activities were mainly due to thymol and linalool. The inhibition of lipoxygenase activity, mainly due to thymol, *p*-cymene, and linalool, suggested their possible use as anti-inflammatories. The high antibacterial and antifungal activities determined for the essential oils means that they can be used as natural preservatives (Cutillas et al., 2018). Moreover, the antimicrobial activity of five plant extracts against *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella typhi* using agar disc diffusion technique was investigated by Mostafa et al. (2018). Ethanolic extracts of *Punica granatum* L., *Syzygium aromaticum* (L.) Merr. & L. M. Perry, *Zingiber officinale* Roscoe, and *Thymus vulgaris* were potentially effective with variable efficiency against the tested bacterial strains at a concentration of 10 $mg.mL^{-1}$. These plant extracts which proved to be potentially effective can be used as natural alternative preventives to control food poisoning diseases and preserve foodstuff avoiding health hazards of chemically antimicrobial agent applications (Mostafa et al., 2018).

In research of Safarpoor et al. (2018), *Thymus daenensis* Čelak. and *Silybum marianum* (L.) Gaertn. extracts with and without the presence of silver nanoparticles (Ag-NPs) were prepared and used against some pathogenic bacteria and fungi to detect new sources of antimicrobial agents. The antimicrobial activities of *Th. daenensis* and *S. marianum* extract with and without the presence of Ag-NPs were investigated at concentrations from 12.5 to 50 mg.mL^{-1} against *Staphylococcus aureus* (Gram-positive organism) and *Escherichia coli* (Gram-negative organism), and fungal strains were *Aspergillus oryzae* and *Candida albicans*. Antimicrobial activity determined using agar disc diffusion method revealed that the activities of Ag-NPs/*Th. daenensis* were superior to Ag-NPs/*S. marianum* and extracts (*Th. daenensis* and *S. marianum*). The medicinal plant extract can be used to synthesize the Ag-NPs as an eco-friendly and inexpensive method in large scale. The results of Safarpoor et al. (2018) showed that the prepared Ag-NPs/extracts as good antibacterial and antifungal agents can be potentially applied against rapidly increasing antibiotic resistance.

To increase the sensibility of *Salmonella typhimurium* strain, a mixture of *Thymus vulgaris*, *Rosmarinus officinalis* L. and *Myrtus communis* L. essential oils were used in combined treatment by experimental design methodology (mixture design) in the study of Fadil et al. (2018). The chemical composition of essential oils was firstly identified by gas chromatography and gas chromatography/mass spectrometry and their antibacterial activity were evaluated. The results of this first step have shown that thymol and borneol were the major compounds in *Th. vulgaris* and *M. communis* L. essential oils, respectively, while 1,8-cineole and α-pinene were found as major compounds in *R. officinalis*. The same results have shown a strong antibacterial activity of *Th. vulgaris* essential oil followed by an important power of *M. communis* essential oil against a moderate activity of *R. officinalis* essential oil. The optimization of mixtures antibacterial activities has highlighted the synergistic effect between *Th. vulgaris* and *M. communis* essential oils. A formulation comprising 55% of *Th. vulgaris* and 45% of *M. communis* essential oils, respectively, can be considered for the increase of *Salmonella typhimurium* sensibility (Fadil et al., 2018).

Conclusions

The ethanolic extract obtained from the leaves of *Th. pulegioides* was the most effective plant extracts against the *Salmonella enteritidis* studied in this work. 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). These plant extracts could be a potential source of new antibacterial agents. Further and more specific studies, *in vivo* as well are recommended to determine the efficacy of these plants in the treatment of *Salmonella*-induced bacterial infections.

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