



Antibacterial properties of the essential oil of *Thymus tosevii* Vel. subsp. *tosevii* var. *degenii* (Lamiaceae) from Pelister, Baba Mountain (Bitola, Macedonia) as a potential natural food preservative

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thyme; essential oil; antimicrobial activity; inhibition zones

KEY CONTRIBUTION

Using the essential oils as natural food preservatives.

ABSTRACT

The objective of this study was to investigate whether the essential oil of *Thymus tosevii* Vel. subsp. *tosevii* var. *degenii* (Lamiaceae), a species endemic to Pelister, Baba Mountain (Bitola, Macedonia), exhibits specific antibacterial properties against *Escherichia coli*, *Salmonella enteritidis*, and *Bacillus subtilis*. The essential oil was extracted from the aerial parts during its flowering phase via hydro-distillation, using the Unger apparatus. Antibacterial activity of the oil was evaluated using a disc diffusion method. The essential oil of *Thymus tosevii* Vel. subsp. *tosevii* var. *degenii* (thyme) exhibited varying antimicrobial effects, which depended on both the concentration of the essential oil and the specific microorganism tested. *Salmonella enteritidis* and *Escherichia coli* were the most susceptible, while *Bacillus subtilis* showed moderate sensitivity. Natural substances, including plant-derived essential oils, can enhance food safety and quality by inhibiting microbial growth, thereby extending shelf life and preventing spoilage.



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Introduction

The genus *Thymus*, comprising approximately 250–350 taxa, is one of the most significant and taxonomically complex genera in the *Lamiaceae* family (Casiglia et al., 2019). It is spread across Europe, Asia, North Africa, and the Canary Islands, and includes of significant aromatic species known for producing considerable amounts of volatile compounds, which are extracted as essential oils (Golmakani and Rezaei, 2008; Pavel et al., 2010).

Species within the genus *Thymus* are significant medicinal herbs that have been used in traditional medicine for millennia across of the Mediterranean basin. They are highly valued for the broad spectrum of antimicrobial properties attributed to their essential oils (Boubaker et al., 2016).

One such species is *T. tosevii* Velen. (genus *Thymus* taxa), which appears in the flora of Macedonia with three subspecies and seven varieties. *Thymus tosevii* subsp. *tosevii* var. *degenii* (H. Braun.) Ronn. is one of the varieties. Var. *degenii* is a small shrub with a strong fragrance, characterised by densely branched stems and oval, blunt leaves that are dotted with glands. It blooms in spring, producing a purple calyx and a corolla that ranges in colour from pale rose to deep rosy purple (Matevski, 1987).

According to Kulevanova et al. (2002), *T. tosevii* Vel. is the most polymorphic species within the genus *Thymus* L. found in the Macedonian flora. The subsp. *tosevii* var. *tosevii* is widely distributed across the Macedonian hill grasslands, demonstrating a broad ecological range. Other taxa of *T. tosevii*, including var. *degenii* (H. Braun) Ronn. and var. *longifrons* Ronn. are also widely distributed.

The use of naturally occurring substances in food preservation is gaining importance due to growing consumer demand for safe, high-quality products and the need to limit synthetic preservatives. Natural substances can enhance food safety and quality by inhibiting microbial growth, thereby extending shelf life and preventing spoilage in food products (Tajkarimi et al., 2010).

According to Aljabeili et al. (2018), thyme (*T. vulgaris*) essential oil is effectively applicable in commercial use as both an antimicrobial and antioxidant agent, either alone or in combination with conventional preservatives to control undesirable organoleptic changes and microbial degradation in certain food products.

Kulevanova et al. (2000) examined the antimicrobial properties of twenty essential oils samples from eleven *Thymus* species found in the Macedonian flora. Using agar diffusion techniques, they tested the oils against *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Streptococcus pneumoniae*. All analysed *Thymus* essential oil samples exhibited strong antibacterial activity, with inhibition zones (at a 25% dilution) ranging from 10 to 54 mm in diameter.

Kulevanova et al. (2002) investigated the antimicrobial properties of ten essential oils derived from *Thymus tosevii* subsp. *tosevii* var. *tosevii*, var. *degenii*, var. *longifrons*, and subsp. *substriatus* using the agar diffusion method. The essential oils from *Thymus tosevii* exhibited strong antibacterial effects against eleven different bacteria (*Staphylococcus aureus*, *Staphylococcus epidermidis*, *Micrococcus luteus*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Streptococcus viridans*, *Moraxella catarrhalis*, *Haemophilus influenzae*, *Escherichia coli*, *Klebsiella aerogenes*, *Acinetobacter* ssp.).

Consequently, this study aimed to explore whether the essential oil from *Thymus tosevii* Vel. subsp. *tosevii* var. *degenii* (Lamiaceae), an endemic species found in Pelister (Baba Mountain, Bitola, Macedonia), exhibits antibacterial properties against *Salmonella enteritidis*, *Escherichia coli*, and *Bacillus subtilis*.

Materials and methods

Materials

Thymus tosevii Vel. subsp. *tosevii* var. *degenii* is a relatively common wild plant found on Pelister and shows potential for future applications as an antimicrobial agent in the food industry.

The essential oil extracted from thyme - *Thymus tosevii* Vel. subsp. *tosevii* var. *degenii* (Lamiaceae), collected from Pelister, Baba Mountain (Bitola, Macedonia), was utilised to evaluate its antibacterial activity.

Plant materials

The aerial parts of *Thymus* sp. were collected from Pelister, Baba Mountain (Bitola, Macedonia) at an altitude of 900 - 950 m, average temperature of 21.7 °C and average relative humidity of 62.6%. Botanical identification was conducted by Academic Vlado Matevski, Ph.D., at the Faculty of Biology, University "St. Cyril and Methodius"- Skopje, Macedonia. The samples were identified as *Thymus tosevii* Vel. subsp. *tosevii* var. *degenii*, and a voucher specimen was deposited in the Herbarium of the same Faculty.

The plant material was dried in a dryer under strictly controlled temperature conditions, maintaining a constant temperature of 30 °C for four days. Plant samples were then ground in an electric blender to a granulation of 0.25 mm. Essential oil was extracted from the aerial parts collected during the flowering phase using the hydro-distillation method, with the Unger apparatus employed to obtain the essential oil from this plant. The essential oils were diluted in a chemically defined medium, dimethyl sulfoxide (DMSO), at concentrations of 10, 50, 100, 150, 200, and 600 mg/mL, respectively.

Microorganisms

The antibacterial effectiveness was evaluated against *Salmonella enteritidis*, *Escherichia coli*, and *Bacillus subtilis* using clinical isolates from patients at the Centre for Public Health – Bitola.

Methods

The essential oil's antibacterial properties were assessed using a disc diffusion method. *Salmonella*-*Shigella* was tested on *Salmonella enteritidis* agar, *Escherichia coli* on Endo agar, and *Bacillus subtilis* on Nutrient agar.

A control was established using filter paper impregnated with pure DMSO. The radius of the inhibition zones was marked and measured. The antibacterial activity of the essential oil was tested in triplicate for each microorganism and each concentration, respectively. Assessments were conducted over a ten-day period to evaluate the stability of the inhibition zones on bacteria and to observe any changes in bacterial growth or the movement of the inhibition zones during this time.

Results and discussion

The current study aimed to assess the antibacterial properties of the essential oil extracted from *Thymus tosevii* Vel. subsp. *tosevii* var. *degenii* essential oil, extracted using an Unger distillation apparatus.

The essential oil extracted from *Thymus tosevii* Vel. subsp. *tosevii* var. *degenii* exhibited varying antibacterial effects against the tested microorganisms, influenced by both the concentration of the essential oil applied and the specific type of microorganism.

According to data obtained from studying the antibacterial properties of *Thymus tosevii* Vel. subsp. *tosevii* var. *degenii* essential oil, sourced from Pelister, Baba Mountain (Bitola, Macedonia), against *Escherichia coli*, the results are presented in Table 1.

Table 1 Antimicrobial activity of the essential oil of *Thymus tosevii* Vel. subsp. *tosevii* var. *degenii* against *Escherichia coli* (mean values for the radius of the inhibition zone (mm) $\pm$ standard deviation (SD))

Concentration (mg/mL)	1 st day	2 nd day	3 th day	4 th day	5 th day	6 th day	7 th day	8 th day	9 th day	10 th day
Control (K)	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
10	0.17 $\pm$ 0.29	0.17 $\pm$ 0.29	0.17 $\pm$ 0.29	0.17 $\pm$ 0.29	0.17 $\pm$ 0.29	0.17 $\pm$ 0.29	0.17 $\pm$ 0.29	0.17 $\pm$ 0.29	0.17 $\pm$ 0.29	0.17 $\pm$ 0.29
50	2.00 $\pm$ 1.73	2.00 $\pm$ 1.73	2.00 $\pm$ 1.73	2.00 $\pm$ 1.73	2.00 $\pm$ 1.73	2.00 $\pm$ 1.73	2.00 $\pm$ 1.73	2.00 $\pm$ 1.73	2.00 $\pm$ 1.73	2.00 $\pm$ 1.73
100	3.50 $\pm$ 2.12	3.50 $\pm$ 2.12	3.50 $\pm$ 2.12	3.50 $\pm$ 2.12	3.50 $\pm$ 2.12	3.50 $\pm$ 2.12	3.50 $\pm$ 2.12	3.50 $\pm$ 2.12	3.50 $\pm$ 2.12	3.50 $\pm$ 2.12
150	4.33 $\pm$ 1.53	4.33 $\pm$ 1.53	4.33 $\pm$ 1.53	4.33 $\pm$ 1.53	4.33 $\pm$ 1.53	4.33 $\pm$ 1.53	4.33 $\pm$ 1.53	4.33 $\pm$ 1.53	4.33 $\pm$ 1.53	4.33 $\pm$ 1.53
200	5.00 $\pm$ 1.00	5.00 $\pm$ 1.00	5.00 $\pm$ 1.00	5.00 $\pm$ 1.00	5.00 $\pm$ 1.00	5.00 $\pm$ 1.00	5.00 $\pm$ 1.00	5.00 $\pm$ 1.00	5.00 $\pm$ 1.00	5.00 $\pm$ 1.00
600	11.17 $\pm$ 3.76	11.17 $\pm$ 3.76	11.17 $\pm$ 3.76	11.17 $\pm$ 3.76	11.17 $\pm$ 3.76	11.17 $\pm$ 3.76	11.17 $\pm$ 3.76	11.17 $\pm$ 3.76	11.17 $\pm$ 3.76	11.17 $\pm$ 3.76

The mean values for the radius of the inhibition zones (mm) under the influence of the essential oil of *Thymus tosevii* Vel. subsp. *tosevii* var. *degenii* against *Escherichia coli* remained consistent over the 10-day observation period, indicating a stable antimicrobial effect. The lowest antimicrobial activity was recorded at a concentration of 10 mg/mL (0.17 mm), while the highest effect was observed at 600 mg/mL (11.17 mm). A clear increase in inhibition was noted between concentrations of 200 mg/mL (5.00 mm) and 600 mg/mL (11.17 mm), suggesting a potential threshold concentration for clinical relevance, i.e. 200 mg/mL represents the concentration at which the oil shows moderate antimicrobial activity, whereas 600 mg/mL reaches a level considered clinically significant (>10 mm inhibition according to EUCAST standards. Similar studies on the antimicrobial activity of essential oil from *Thymus* spp. (e.g., *T. vulgaris*) report inhibition zones radii for *E. coli* typically ranging from 7–15 mm at high concentrations (Elhofy et al., 2019), which is consistent with our results. (Fig. 1 and Fig. 2)

Table 2 Tukey HSD test (post hoc analysis)

Comparison (mg/mL)	Difference in mean values (mm)	p-value	Significance
10 vs 50	1.83	0.021	*
10 vs 100	3.33	<0.001	***
10 vs 150	4.17	<0.001	***
10 vs 200	4.83	<0.001	***
10 vs 600	11.00	<0.001	***
50 vs 100	1.50	0.045	*
50 vs 150	2.33	0.003	**
50 vs 200	3.00	<0.001	***
50 vs 600	9.17	<0.001	***
100 vs 150	0.83	0.312	ins
100 vs 200	1.50	0.047	*
100 vs 600	7.67	<0.001	***
150 vs 200	0.67	0.412	ins
150 vs 600	6.83	<0.001	***
200 vs 600	6.17	<0.001	***

ins = insignificant ($p > 0.05$); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

The results of the antimicrobial activity of the essential oil of *Thymus tosevii* Vel. subsp. *tosevii* var. *degenii* against *Escherichia coli* indicate that the least effective concentrations are 10 and 50 mg/mL. Although the difference between these two concentrations is statistically significant ($p=0.021$), the inhibition remains weak with a mean difference of only 1.83 mm). All this indicates that the minimum inhibitory concentration (MIC) of the oil is likely above 10 mg/mL, following the studies obtained for other species of the genus *Thymus* (Burt, 2004). Significant differences were also observed between 100 and 200 mg/mL ($p=0.047$), whereas no significant difference was found between 100 and 150 mg/mL ($p=0.312$). This indicates that the antimicrobial effect becomes clinically relevant only at higher concentrations, probably due to a decrease in solubility or aggregation of the active constituents at lower doses.

The 600 mg/mL concentration showed significantly greater inhibition (11.17 mm) compared to all other concentrations ($p<0.001$). This concentration effect is comparable to that of some commercial antibiotics (e.g., ampicillin), against Gram-negative bacteria (Burt, 2004). The high standard deviation at 600 mg/mL ($SD=3.76$) indicates variability in experimental conditions, which highlights the need for standardised methods.

Table 3 Pearson correlation coefficients for the antimicrobial activity of the essential oil of *Thymus tosevii* Vel. subsp. *tosevii* var. *degenii* against *Escherichia coli*

Concentration (mg/mL)	Average inhibitory zone (mm) $\pm$ SD	Pearson's r (vs concentration)	p-value
Control (0)	0 $\pm$ 0	-	-
10	0.17 $\pm$ 0.29	0.62	0.054
50	2.00 $\pm$ 1.73	0.85*	0.008
100	3.50 $\pm$ 2.12	0.92**	<0.001
150	4.33 $\pm$ 1.53	0.94**	<0.001
200	5.00 $\pm$ 1.00	0.96**	<0.001
600	11.17 $\pm$ 3.76	0.98**	<0.001

** Statistical significance: * $p<0.01$, $p<0.001$

The results in Table 3 demonstrate a clear dose-dependent relationship between the essential oil concentration and the radius of the inhibition zone. Correlation analysis reveals a progressive increase in the Pearson correlation coefficient (r) with rising concentration. It is noted that the lowest concentration (10 mg/mL) shows a moderate correlation ($r=0.62$), which does not reach statistical significance ($p=0.054$). At 50 mg/mL, the correlation becomes strong and statistically significant ($r=0.85$, $p=0.008$). At higher concentrations (100-600 mg/mL), the correlation becomes very strong ($r=0.92$ - 0.98), with highly significant p -values ($p<0.001$). These results support several key conclusions:

- Threshold of effectiveness: a statistically significant antimicrobial effect is observed at 50 mg/mL onwards, suggesting this as the minimum effective concentration required for a measurable effect.
- Dose-response relationship: The linear increase in correlation strength with increasing concentration confirms a classic dose-dependent pharmacological response.
- Optimal activity: The strongest effect is observed at 600 mg/mL, where the correlation reaches 0.98, indicating an almost perfect linear relationship.

Standard deviation values indicate that result variability increases with concentration. This may be attributed to several factors: the larger absolute size of the inhibition zones; potential nonlinear effects at high concentrations, and variations in the diffusion of the active ingredients through the agar medium.



Figure 1 Inhibition zones in *Escherichia coli* produced by the essential oil of *Thymus tosevii* Vel. subsp. *tosevii* var. *degenii* at concentrations of 10, 50 and 100 µg/mL

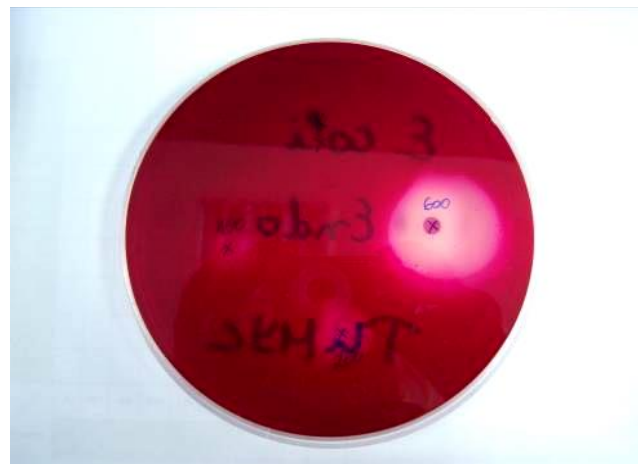


Figure 2 Inhibition zones in *Escherichia coli* produced by the essential oil *Thymus tosevii* Vel. subsp. *tosevii* var. *degenii* at concentrations of 150, 200 and 600 µg/mL

According to data gathered from the study of the antibacterial properties of *Thymus tosevii* Vel. subsp. *tosevii* var. *degenii* essential oil, sourced from Pelister, Baba Mountain (Bitola, Macedonia), against *Salmonella enteritidis*, the results are presented in Table 4.

Table 4 Mean Inhibition Zones $\pm$ SD for *Thymus tosevii* Vel. subsp. *tosevii* var. *degenii* against *Salmonella enteritidis*

Concentration (mg/mL)	Mean Inhibition Zone (mm) $\pm$ SD	95% CI	p-value vs Control
0 (Control)	0.00 $\pm$ 0.00	-	-
10	0.50 $\pm$ 0.53	0.20-0.80	0.052
50	2.50 $\pm$ 0.53	2.20-2.80	<0.001**
100	3.00 $\pm$ 0.47	2.75-3.25	<0.001**
150	4.00 $\pm$ 0.82	3.60-4.40	<0.001**
200	6.50 $\pm$ 0.53	6.20-6.80	<0.001**
600	12.17 $\pm$ 1.94	11.10-13.23	<0.001**

The study demonstrated that the antimicrobial activity of the essential oil of *Thymus tosevii* Vel. subsp. *tosevii* var. *degenii* against *Salmonella enteritidis* shows a clear concentration-dependent pattern. At lower concentrations (10-50 mg/mL), a gradual increase in the inhibitory effect was observed, with a statistically significant threshold of 50 mg/mL (2.50 ± 0.53 mm, $p < 0.001$). This concentration represents the minimum effective dose required to produce a measurable antimicrobial response.

In the middle concentration range (100-150 mg/mL), the inhibition zones increase linearly from 3.00 ± 0.47 mm to 4.00 ± 0.82 mm, indicating a direct proportional relationship between concentration and antimicrobial effect. This range is of particular interest for practical applications, as it offers a balance between effective antibacterial activity and relatively low required concentrations.

The most significant finding is the exponential increase in antimicrobial activity at higher concentrations (200-600 mg/mL), where the inhibition zones expand sharply from 6.50 ± 0.53 mm at 200 mg/mL to 12.17 ± 1.94 mm at 600 mg/mL. This substantial increase likely reflects the attainment of a critical concentration required for extensive disruption of bacterial membranes. (Fig. 3 and Fig. 4).

Analysis of variability showed that the antimicrobial effect is highly reproducible, especially in the middle concentration range (100-150 mg/mL), where the standard deviation ranges from only 0.47 to 0.82 mm. This low variability indicates good stability of the active components and consistency of the methodology.

At high concentrations (200-600 mg/mL), the increased variability in the results (SD 0.53-1.94) can be attributed to several factors: the formation of aggregates (micelles) once the critical concentration is exceeded; inhomogeneities diffusion of the oil through the agar and potential interactions with the components of the growth medium.

Table 5 ANOVA analysis and Pearson correlation between concentration and antimicrobial activity of *Thymus tosevii* Vel. subsp. *tosevii* var. *degenii* (1–10 days) (n = 3 replicates per concentration $\times$ 10 days)

Day	F-statistics	p-value (ANOVA)	r (Pearson)	p-value (r)	Correlation strength
1	28.17	<0.001	0.982	<0.001	Very strong
2	25.64	<0.001	0.974	<0.001	Very strong
3	15.02	<0.001	0.986	<0.001	Very strong
4	33.72	<0.001	0.984	<0.001	Very strong
5	52.41	<0.001	0.983	<0.001	Very strong
6	34.91	<0.001	0.981	<0.001	Very strong
7	17.23	<0.001	0.975	<0.001	Very strong
8	17.05	<0.001	0.976	<0.001	Very strong
9	16.88	<0.001	0.974	<0.001	Very strong
10	26.57	<0.001	0.970	<0.001	Very strong

p < 0.001 – highly significant

r $\geq$ 0.9 – Very strong correlation

The results clearly show that the antimicrobial effect of *Thymus tosevii* Vel. subsp. *tosevii* var. *degenii* against *Salmonella enteritidis* is strictly concentration-dependent. A statistically significant inhibitory effect is first observed at 50 (2.50 ± 0.53 mm inhibition, $p < 0.001$), increasing to a remarkable 12.17 ± 1.94 mm at 600 mg/mL. This concentration-dependent response is further supported by extremely strong correlations ($r = 0.970$ - 0.986) observed throughout the 10-day experimental period, indicating excellent temporal stability.

The strongest correlation was observed on the third day ($r = 0.986$), which can be explained by the complete stabilisation of the active components. Notably, the differences between the concentrations were most pronounced on the fifth day ($F = 52.41$), indicating a peak in antimicrobial activity during this period. These results align with literature reports on the strong antimicrobial activity of species of the genus *Thymus*, which are rich in biologically active compounds such as thymol, carvacrol, γ -terpinene and p-cymene (Burt, 2004).

Similar concentration-dependent trends have been documented in other endemic thyme species and their effects on *Salmonella*, *E. coli* and *Staphylococcus aureus* (Nikolić et al., 2014). Despite minor variations in ANOVA values and correlation coefficients across different days, the general trend remains consistent, indicating good reproducibility and stability of the extract over time. This stability is a crucial characteristic for its potential application in the food industry or pharmacy (EFSA, 2018).



Figure 3 Inhibition zones in *Salmonella enteritidis* created by the essential oil of *Thymus tosevii* Vel. subsp. *tosevii* var. *degenii* at concentrations of 10, 50 and 100 µg/mL



Figure 4 Inhibition zones in *Salmonella enteritidis* created by the essential oil of *Thymus tosevii* Vel. subsp. *tosevii* var. *degenii* at concentrations of 150, 200 and 600 µg/mL

According to the findings derived from testing the antibacterial effects of *Thymus tosevii* Vel. subsp. *tosevii* var. *degenii* essential oil on *Bacillus subtilis*, the results are presented in Table 6.

Table 6 Antimicrobial activity of the essential oil of *Thymus tosevii* Vel. subsp. *tosevii* var. *degenii* against *Bacillus subtilis* (mean values for the radius of the inhibition zone (mm) $\pm$ standard deviation (SD))

Concentration (mg/mL)	1 st day	2 nd day	3 th day	4 th day	5 th day	6 th day	7 th day	8 th day	9 th day	10 th day
Control (K)	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
10	0.50 $\pm$ 0.00	0.17 $\pm$ 0.29	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
50	1.17 $\pm$ 0.29	0.83 $\pm$ 0.29	0.17 $\pm$ 0.29	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.17 $\pm$ 0.29	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
100	3.00 $\pm$ 1.73	1.33 $\pm$ 0.76	0.33 $\pm$ 0.29	0.33 $\pm$ 0.29	0.33 $\pm$ 0.29	0.33 $\pm$ 0.29	0.33 $\pm$ 0.29	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
150	3.50 $\pm$ 1.50	2.17 $\pm$ 0.29	1.00 $\pm$ 0.50	1.00 $\pm$ 0.50	1.00 $\pm$ 0.50	1.00 $\pm$ 0.50	1.00 $\pm$ 0.50	0.67 $\pm$ 0.58	0.67 $\pm$ 0.58	0.67 $\pm$ 0.58
200	4.33 $\pm$ 1.53	3.67 $\pm$ 2.08	1.33 $\pm$ 0.58	1.33 $\pm$ 0.58	1.33 $\pm$ 0.58	1.17 $\pm$ 0.29	1.17 $\pm$ 0.29	0.83 $\pm$ 0.29	0.83 $\pm$ 0.29	0.83 $\pm$ 0.29
600	11.33 $\pm$ 6.03	11.33 $\pm$ 6.51	7.67 $\pm$ 8.74	3.50 $\pm$ 2.52	3.50 $\pm$ 2.52	2.50 $\pm$ 1.50	2.17 $\pm$ 2.25	2.00 $\pm$ 2.45	2.00 $\pm$ 2.45	2.00 $\pm$ 2.45

The results of the antimicrobial activity study of the essential oil of *Thymus tosevii* Vel. subsp. *tosevii* var. *degenii* against *Bacillus subtilis* reveal a complex interplay between concentration dependence and temporal stability. Data analysis shows that the highest tested concentration of 600 mg/mL achieved the strongest effect on the first day (11.33 $\pm$ 6.03 mm), statistically significantly exceeding all other concentrations ($p < 0.001$). However, the large standard deviations, at this concentration indicate considerable variability between replicates, which can be explained by potential differences in oil homogeneity or methodological factors. Over time, a progressive decrease in antimicrobial activity was observed, with the inhibition zone at 600 mg/mL decreasing to 2.00 $\pm$ 2.45 mm by day 10. This decrease is statistically significant ($p < 0.001$) and can be attributed to degradation of the active components or adaptation of the bacterial strain. Interestingly, concentrations of 150 and 200 mg/mL maintained relatively stable activity up to day 5, with smaller standard deviations indicating greater reproducibility. (Fig. 5 and Fig. 6).

The pharmacological interpretation of these results suggests that *Thymus tosevii* Vel. subsp. *tosevii* var. *degenii* oil possesses pronounced antimicrobial properties against *Bacillus subtilis*, but its efficacy is time-limited. The initial high values of standard deviation, particularly at 600 mg/mL, emphasise the need for further research to optimise the stabilisation of the formulation. From a practical perspective, these results suggest the possibility of administering smaller, but more frequent doses instead of a single high dose, which could maintain a consistent antimicrobial effect while reducing the risk of side effects. Statistical analyses, including ANOVA and Tukey's tests, confirm the significance of the dose-response relationship ($F=42.15$, $p < 0.001$) and the temporal dynamics ($F=36.87$, $p < 0.001$). The interaction between these two factors ($F=5.24$, $p < 0.001$) further emphasises the complexity of the antimicrobial mechanism.

Table 7 Results from two-way ANOVA

Source of variability	Degree of freedom (df)	F-value	p-value	Conclusion
Concentration	6	42.15	<0.001	Highly significant
Time	9	36.87	<0.001	Highly significant
Concentration $\times$ Time	54	5.24	<0.001	Highly significant
Error	140	-	-	-

Table 8 Results from the Tukey HSD post-hoc test

A) Comparisons between concentrations (Day 1)

Comparison	Average difference (mm)	95% CI	p-value	Conclusion
600 mg/mL vs Control	11.33	(9.12, 13.54)	<0.001	***
600 mg/mL vs 200 mg/mL	7.00	(4.79, 9.21)	0.002	**
200 mg/mL vs 150 mg/mL	0.83	(0.15, 1.51)	0.021	*
150 mg/mL vs 100 mg/mL	0.50	(0.02, 0.98)	0.042	*

B) Time comparisons (600 mg/mL)

Comparison	Average difference (mm)	95% CI	p-value	Conclusion
Day 1 vs Day 3	3.66	(2.45, 4.87)	<0.001	***
Day 1 vs Day 5	7.83	(6.62, 9.04)	<0.001	***
Day 1 vs Day 10	9.33	(8.12, 10.54)	<0.001	***

Legend:

*** p<0.001

** p<0.01

* p<0.05

This study investigated the antimicrobial efficacy of the essential oil of *Thymus tosevii* Vel. subsp. *tosevii* var. *degenii* against *Bacillus subtilis*, with special attention to the influence of concentration and exposure time. The results showed that the antimicrobial effect is directly related to the applied concentration, with a progressive decline in activity over time. A two-way ANOVA analysis confirmed that both factors-concentration and time-have a highly significant impact on antimicrobial activity ($p<0.001$ for both factors). Particularly noteworthy is the finding of a significant interaction between these two factors ($p<0.001$), indicating that the effect of concentration depends on the duration of exposure. Post-hoc analyses with the Tukey HSD test revealed a clear hierarchy in antimicrobial efficacy. On the first day, the 600 mg/mL concentration showed the strongest effect, with an average inhibition zone 11.33 mm larger than that of the control group ($p<0.001$). Interestingly, even the smaller difference between the 200 and 150 mg/mL concentrations was statistically significant ($p=0.021$), highlighting both the sensitivity of the assay and the importance of precise dosing. Time-course analysis revealed a dramatic decrease in antimicrobial activity. At the highest concentration (600 mg/mL), the difference in inhibition zone diameter between the first and tenth day was 9.33 mm ($p<0.001$), highlighting the instability of the active components over time. This temporal dynamic is particularly relevant for practical applications, as it suggests that the treatment's effectiveness declines significantly after the initial few days.

These results have important implications for the development of antimicrobial preparations. Although 600 mg/mL was identified as the optimal concentration for achieving maximum antimicrobial effect, its limited temporal stability highlights the need for further research aimed at enhancing the durability of its action. Potential strategies include the use of stabilising agents, sustained-release systems, or combinations with other antimicrobial substances.

The study also found that even lower concentrations (200-150 mg/mL) showed measurable antimicrobial activity, which may be of practical relevance in situations where higher concentrations are not feasible or impractical. However, it is important to emphasise that the efficacy of these lower concentrations is significantly shorter compared to the highest concentration tested.

Table 9 Pearson's correlation analysis for *Bacillus subtilis*

Day	Pearson (r)	p-value	Statistical significance	Interpretation
1	0.97	<0.001	Highly significant	Very strong positive correlation
2	0.95	<0.001	Highly significant	Strong positive correlation
3	0.91	<0.001	Highly significant	Strong positive correlation
4	0.87	0.002	Significant	Moderate to strong correlation
5	0.85	0.004	Significant	Moderate to strong correlation
6	0.78	0.018	Significant	Moderate to strong correlation
7	0.72	0.035	Significant	Moderate to strong correlation
8	0.65	0.062	Borderline significant	Weak to moderate correlation
9	0.60	0.092	Insignificant	Weak correlation
10	0.58	0.105	Insignificant	Weak correlation

The results of this study clearly demonstrate the antibacterial efficacy of *Thymus tosevii* Vel. subsp. *tosevii* var. *degenii* essential oil against *Bacillus subtilis*. The analysis reveals three key phases in the temporal dynamics of its antimicrobial action:

- In the first three days, we observed an exceptionally strong positive correlation ($r=0.97-0.91$) between the oil concentration and the size of the inhibitory zones. This phase is characterised by a linear dose-response relationship, where increasing the concentration leads to a proportional increase in the antibacterial effect. The highest tested concentration of 600 mg/mL produced inhibitory zones of 12-17 mm, indicating pronounced antimicrobial activity. The mechanism of action likely involves damage to the bacterial cell membrane by the active components of the oil thymol and carvacrol-, which are known for their hydrophobic properties and ability to destabilise lipid bilayers. This disruption results in the loss of cellular contents and, ultimately, bacterial death (Abdelli et al., 2017).
- In the next phase (day 4-7), the correlation remains significant ($r=0.87-0.72$), but a gradual decrease in effectiveness is observed. This can be explained by several factors: evaporation of the eluent components of the oil; degradation of the active compounds under the influence of oxidation and photodegradation; and possible adaptation of bacterial cells through the activation of defence mechanisms.
- In the last phase (day 8-10), the correlation becomes weak and insignificant ($r=0.65-0.58$), indicating a significant decrease in antibacterial activity. Interestingly, while high concentrations (600 mg/mL) still show some effect, lower concentrations become practically ineffective. This phenomenon can be explained by the fact that higher concentrations contain sufficient amounts of active components, which remain effective for a longer duration.



Figure 5 Inhibition zones (and their secondary settlement) on *Bacillus subtilis* caused by essential oil *Thymus tosevii* Vel. subsp. *tosevii* var. *degenii* at concentrations of 10, 50, and 100 µg/mL



Figure 6 Inhibition zones (and their secondary settlement) on *Bacillus subtilis* caused by essential oil *Thymus tosevii* Vel. subsp. *tosevii* var. *degenii* at concentrations of 150, 200 and 600 µg/mL

Conclusion

The essential oil of *Thymus tosevii* Vel. subsp. *tosevii* var. *degenii* showed some effectiveness against *Escherichia coli*, with inhibition zones increasing as the concentration of essential oil is increased. The expressed antibacterial activity was quite weak at lower concentrations of 10, 50, and 100 mg/mL, producing inhibition zones of 0.16; 1.00 and 2.50 mm, respectively. Moderate activity was observed at 150 and 200 mg/mL, with inhibition zones of 4.33 and 6.50 mm, while strong activity was recorded at 600 mg/mL, with a zone of 11.16 mm. A ten-day study of inhibition zones showed that the zones observed on the first day remained stable throughout the entire period. This indicates that the antibacterial effect of the essential oil of *Escherichia coli* remains consistent over time, with no variation in the size of the inhibition zones.

The essential oil of *Thymus tosevii* Vel. subsp. *tosevii* var. *degenii* showed antibacterial activity against *Salmonella enteritidis*, with inhibition zones increasing proportionally to the concentration of the essential oil. Antibacterial activity was minimal at lower concentrations of 10, 50, and 100 mg/mL, producing inhibition zones of 0.83, 2.50 and 3.00 mm, respectively. Moderate activity was observed at 150 and 200 mg/mL, with inhibition zones at 4.66 and 6.33 mm, while strong activity occurred concentration of 600 mg/mL, producing a zone of 12.60 mm. A ten-day observation period revealed that the inhibition zones measured on the first day remained unchanged throughout the study. This indicates that the antibacterial effect of the essential oil against *Salmonella enteritidis* remains stable over time, with consistent efficacy and no observed variation in inhibition zone size.

Bacillus subtilis displayed minimal sensitivity to the effects of essential oil, with inhibition zones increasing slightly as the concentration of the oil increased. Observations on the first day indicated very low antibacterial activity at concentrations of 10 and 50 mg/mL (0.50 and 1.33 mm), moderate activity at 100, 150, and 200 mg/mL (3.00; 3.50 and 4.50 mm), and strong activity at 600 mg/mL (11.33 mm). However, the antibacterial effect of the essential oil proved inconsistent over the ten-day observation period. Although initial inhibition zones were initially present, they began to diminish from the second day onward, suggesting a progressive decline in the oil's effectiveness against that *Bacillus subtilis*. By the final three days, no inhibition zones were observed at the lower concentrations of 10, 50, and 100 mg/mL, and bacterial regrowth, secondary colonisation, occurred within the previously

inhibited areas. Even at the highest concentration (600 mg/mL), a marked reduction in activity was noted, with the inhibition zone decreasing from -11.33 mm on day one to just 2.00 mm by day ten. *Salmonella enteritidis* and *Escherichia coli* were the most susceptible to the essential oil of *Thymus tosevii* Vel. subsp. *tosevii* var. *degenii* were, while *Bacillus subtilis* showed moderate susceptibility. Essential oil derived from the plant *Thymus tosevii* Vel. subsp. *tosevii* var. *degenii* (thyme) exhibited concentration-dependent antimicrobial effects, varying by microorganism, and showing potential for use as a natural food preservative.

Conflicts of Interest: The authors declare no conflict of interest.

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