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Investigation of antibacterial activity of combinations of *Thymus vulgaris* essential oil and some conventional antibiotics

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The essential oils of *T. vulgaris* have antiseptic, antiviral, and antimicrobial properties. Many studies have reported that the synergistic combination of essential oils (EOs), and conventional antimicrobial agents is an effective solution for developing preparations with increased antimicrobial properties and low toxicity to the organism. The present study aims to evaluate the antimicrobial activity of thyme essential oil and standard antibiotics combination against *Staphylococcus aureus*, *Escherichia coli*, and *Klebsiella pneumoniae*. The essential oil was analyzed using gas chromatography coupled with mass spectrometry (GC-MS). The analysis of the essential oil of *Thymus vulgaris* revealed the main components to be thymol (45.74%), *p*-cymene (21.05%), *ɣ*-terpinene (12.37%). For *S. aureus*, we combined thyme essential oil (TEO) with penicillin (P), cefoxitin (FOX), erythromycin (ERY), gentamicin (GEN), and tetracycline (TET), and we combined *Escherichia coli* and *Klebsiella pneumoniae*, respectively, with ampicillin, ceftriaxone, meropenem, ciprofloxacin and gentamicin, as suggested by EUCAST, 2024.

TEO showed *in vitro* antibacterial activity against all tested bacterial strains and increased the antimicrobial activity of the tested antibiotics. For all tested combinations of TEO with antibiotics against *S. aureus* (penicillin, cefoxitin, erythromycin, gentamicin and tetracycline), an increase in the zone of inhibition was observed in a large part of the strains - respectively P-TEO in 3/6 strains, FOX-TEO in 5/ 6 strains, ERY-TEO - 3/6 strains, GEN-TEO - 5/6 and TET-TEO – 4/6 strains of *S. aureus.* TEO also increased the antimicrobial action of ampicillin, ceftriaxone, meropenem, ciprofloxacin and gentamycin against most of the *Escherichia coli* and *Klebsiella pneumoniae* strains. The joint application of TEO and classic antibiotics can be one of the ways to overcome the development of bacterial resistance and side effects of the antibiotic preparations application.

Keywords: antimicrobial effects, antibiotics, thymus essential oil, combination

INTRODUCTION

In 2019, the World Health Organization (WHO) defined antimicrobial resistance (AMR) as one of the 10 global health threats facing humanity [1]. Antimicrobial resistance is observed after introducing almost every microbial agent in the clinical practice. For instance, in the mid-1940s, only a few years after the introduction of penicillin, penicillin-resistant *Staphylococcus aureus* spread in the hospital environment and within a decade became a serious public problem [2, 3]. The incidence of antibiotic resistance to ciprofloxacin, commonly used to treat UTIs, was 43.1% for *Escherichia coli* and 36.4% for *Klebsiella pneumoniae* in the countries reporting data to the Global Antimicrobial Resistance Surveillance System in 2019 [4]. In some countries, nearly half of the patients infected with *Klebsiella pneumoniae* carry strains that are resistant to carbapenems, significantly limiting the options for effective treatment [5]. The AMR problem requires using new, safe, and effective substances [6]. In recent years, special attention has been paid to products of natural origin due to their low toxicity, biodegradability, and broad spectrum of action

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compared to synthetic essential oils (EOs) due to their strong and broad-spectrum action against microorganisms, their relative safety for humans, and undetermined (so far) microbial resistance to their components [6, 7], therefore they may help in limiting antibiotic resistance. The action mechanism of EOs has been reported in detail in the scientific literature including bacterial cell wall degradation, enzyme and membrane protein destruction, and cell contents leakage after disruption of the cytoplasmic membrane. Therefore, positive effects against bacteria may occur with joint implementation of antibiotics and EOs [8, 9].

Many studies have reported that the synergistic combination of EOs with conventional antimicrobials is an effective solution for developing preparations with increased antimicrobial properties and low toxicity to the microorganisms [7].

One of them is thyme essential oil (TEO), obtained from the plant *Thymus vulgaris* L. belonging to the genus *Thymus* (thyme) which consists of about 215 species of herbaceous perennials and shrubs. *Thymus vulgaris* L. is a low-growing herbaceous plant native to Southern Europe and for centuries widely used as herbal tea, spice, perfume, and insecticide [10]. The EOs of *T. vulgaris* have antiseptic, antiviral, and antimicrobial properties, and according to some studies [11], they have a better antimicrobial effect on Gram-positive strains. Other studies reported a higher antimicrobial activity of TEO against *Escherichia coli* compared to the tested Gram-positive strains [12, 13].

In our previous study [14], the antimicrobial activity of two (for external and internal use) commercial essential oils from *Thymus vulgaris* against *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25922 was demonstrated.

The aim of the current study was to evaluate the use of TEO to improve the effectiveness of the standard antibiotics against *S. aureus*, *E. coli*, and *K. pneumoniae*.

MATERIALS AND METHODS

The study was conducted at the Medical College – Varna, Bulgaria. We used the statistical software IBM SPSS, version 25, to present and analyze the data.

Thyme essential oil

The thyme essential oil used in this study was purchased from the commercial market and is 100% pure with certified organic ingredients.

Gas chromatography-mass spectrometry

For the purposes of the analysis, the equipment consisting of gas chromatograph 7890A, flame ionization detector, and mass spectrometer 5975C (Agilent Technologies) was used. A Stabilwax column (Restek) with the following parameters was employed: length 30 m, diameter 0.25 mm, and film thickness 0.25 μm. The temperature program was as follows: initial temperature 65°C, ramped to 170°C at 1.5°C/min; total analysis time 70 min; injector and detector temperatures 250°C, FID temperature: 250°C; carrier gas hydrogen with a flow rate of 0.8 ml/min; carrier gas helium with a flow rate of 0.8 ml/min; mass spectrometer scan range m/z = 40-450; sample injection volume 1.0 μl in split mode 100:1. The compounds were identified by comparing the retention times and Kovats retention indices (RI) with those of standard substances and mass spectral data from the NIST'08 (National Institute of Standards and Technology, USA) and Adams Libraries.

Tested bacterial strains

The antimicrobial activity of combinations between antibiotics and TEO was studied against *Staphylococcus aureus*, *Escherichia coli*, and *Klebsiella pneumoniae*, presented in Table 1. Nine of the tested strains were clinical isolates and three were reference strains of microorganisms. The bacterial strains were stored at -18°C in glycerol medium in a microorganism bank at the Varna Medical College.

Table 1. The strains of *S. aureus*, *E. coli*, and *K. pneumoniae* tested in a study of the antimicrobial activity of conventional antibiotics with TEO.

Bacterial species	Clinical material/Source
Staphylococcus aureus	throat swab
Staphylococcus aureus	wound
Staphylococcus aureus	wound
Staphylococcus aureus	wound
Staphylococcus aureus	wound
Staphylococcus aureus ATCC 29213	reference strain
Escherichia coli	urine
Escherichia coli	urine
Escherichia coli ATCC25922	reference strain
Klebsiella pneumoniae	sputum
Klebsiella pneumoniae	wound
Klebsiella pneumoniae ATCC13883	reference strain

For the purposes of the research in this work, they were cultured initially in brain heart infusion broth for 24 h and then on blood agar for another 24 h. The

referent strains were provided from MicroSwap, Ridacom, Bulgaria.

Antimicrobial activity evaluation test

The antimicrobial activity of antibiotic combinations with TEO was investigated by the Kirby-Bauer disk diffusion test [15]. The action of each of the studied antimicrobial agents was tested separately. The antibiotics selection against each of the test strains, presented in Table 1, was made according to the EUCAST, 2024 instructions *(NCIPD, 2024)* [16].

Standardized bacterial culture was prepared (0.5 MF) and spread on the surface of Mueller-Hinton agar media (*HiMedia,* provided by Ridacom, Bulgaria). We previously prepared a stock solution of 2.5% (v/v) TEO in DMSO (1% (v/v)). After that, in each culture medium with the culture of the corresponding microbe, three disks (d=6 mm) in the following combinations and concentrations of active agents were added:

- Disc soaked in 100 µl of 2.5% (v/v) TEO;
- Factory-prepared antibiotic disk in concentration according to EUCAST, 2024 standards;
- A second identical antibiotic disk additionally soaked in 100 µl of 2.5% (v/v) TEO.

Controls were set for the solvent used to prepare suspensions for the active substances – DMSO. All samples were triplicated, after which, we incubated the nutrient media for 24 h at 37°C. The obtained inhibition zones were measured in mm and compared to each other; for all antibiotics, results were determined for their effect on the respective microbe: S – sensitive or R – resistant, following the EUCAST, 2024 instructions [16].

A list of antibiotics included in the study is presented in Table 2.

Table 2. List of antibiotics studied

Antibiotics (ATBs)	Abbreviation	Chemical Family
penicillin G	P	penicillins
cefoxitin	FOX	2nd generation cephalosporins
erythromycin	E	macrolides
gentamycin	GEN	aminoglycosides
tetracyclin	TET	tetracyclines
ampicillin	A	aminopenicillins
ceftriaxone	CRO	3rd generation cephalosporin
meropenem	MEM	carbapenems
ciprofloxacin	CIP	quinolones

Statistical analysis

The statistical software IBM SPSS Statistics 25 was used to present and analyze the data. A Paired samples t-test was applied to investigate whether there was a statistically significant difference between the mean antimicrobial effects of antibiotics and of the combination of TEO with antibiotics. We used the level of significance for the 2-tailed test $\alpha = 0,05$.

RESULTS AND DISCUSSION

The chemical components of TEO, which were identified, are reported in Table 3. 24 components were identified in the Thyme EO representing 99.7% of the total, the major components being thymol (45.74%), p-cymene (21.05%), ɣ-terpinene (12.37%).

Table 3. Chemical composition of *Thymus vulgaris* essential oil.

No. Compound	Retention time (min)	% of total ion current
	Thyme EO	
1. α-Thujene	9.11	1.34
2. α-Pinene	9.32	1.26
3. Camphene	9.84	1.11
4. β-Pinene	10.75	0.15
5. 1-Octen-3-ol	10.97	0.20
6. β-Myrcene	11.23	0.88
7. α-Terpinene	12.06	1.13
8. p-Cymene	*12.31*	*21.05*
9. Limonene	12.47	0.50
10. ɣ-Terpinene	*13.41*	*12.37*
11. Sabinene hydrate	13.79	0.57
12. β-Linalool	14.74	2.03
13. Camphor	16.12	1.01
14. Borneol	16.90	1.79
15. Terpinen-4-ol	17.16	0.87
16. Thymol methyl ether	18.58	0.14
17. Carvacrol, methyl ether	18.84	1.33
18. Bornyl acetate	20.20	0.66
19.Thymol	*20.37*	*45.74*
20. Carvacrol	20.75	2.01
21.β-Caryophyllene	23.77	2.79
22.ɣ-Cadinene	26.07	0.12
23. δ-Cadinene	26.19	0.18
24. Caryophyllene oxide	27.73	0.48

Table 4. Antimicrobial activity of combining TEO with conventional antibiotics against *S. aureus*, *Escherichia coli*, and *Klebsiella pneumoniae* tested by disk-diffusion method (diameter of inhibition zones - mm).

Bacterial strain	TEO	P	P+ TEO	FOX	FOX+ TEO	ERY	ERY+ TEO	GEN	GEN+ TEO	TET	TET+ TEO
EUCAST, 2024		S≥18		S≥27		S≥21		S≥18		S≥22	
		R<18		R<27		R<21		R<18		R<22	
S. aureus 1	15	16	18	16	18	28	26	28	34	14	18
S. aureus 2	16	14	16	18	19	28	28	26	30	12	18
S. aureus 3	17	26	26	28	29	24	26	20	22	22	22
S. aureus 4	19	28	28	29	32	24	29	20	22	26	28
S. aureus 5	19	30	30	30	30	26	26	26	30	26	28
S. aureus ATCC 29213	20	19	20	28	30	35	37	44	44	35	35
Bacterial strain		A	A+ TEO	CRO	CRO+ TEO	MEM	MEM+ TEO	CIP	CIP+ TEO	GEN	GEN+ TEO
EUCAST, 2024		S≥14		S≥25		S≥22		S≥25		S≥17	
		R<14		R<22		R<16		R<22		R<17	
E. coli 1	21	12	17	20	26	32	35	16	16	12	24
E. coli 2	20	12	26	20	29	34	37	14	22	18	26
E. coli ATCC 25922	23	12	32	34	39	38	42	45	49	25	34
K. pneumoniae 1	18	0	15	24	24	35	37	19	23	22	22
K. pneumoniae 2	17	0	16	22	27	35	38	29	30	22	23
K. pneumoniae ATCC 13883	20	14	21	35	35	38	42	45	45	25	25

TEO – Thyme essential oil; P - penicillin G; FOX – cefoxitin; ERY – erythromycin; GEN – gentamycin; TET – tetracyclin; A – ampicillin; CRO – ceftriaxone; MEM – meropenem; CIP – ciprofloxacin.

Table 4 shows the effect of TEO on the antibacterial action of antibiotics for clinical use. The influence of TEO on the antibacterial power of the antibiotics used on the tested strains of microorganisms can be determined by comparing the diameters of the inhibition zones provoked from the antimicrobial agents alone and in combination with essential oils. In our study, the reference strain *Escherichia coli ATCC25922* was the most sensitive to TEO, showing the largest (23 mm) zone of inhibition. TEO demonstrated stronger inhibitory effects against *E. coli* and weaker against *S. aureus*.

In the study, we investigated the antimicrobial effects of combining TEO with antibiotics using agar media techniques. The greatest increase in the zone of growth inhibition in *S. aureus* was observed with the combined use of TET-TEO (up to 6 mm) *(p=0.058, t=-2.445)* and ERY-TEO (5 mm) *(p=0.287, t=-1.190, CI [-3.687-1.353])*. In the remaining three combinations of TEO with penicillin (P), cefoxitin (FOX) and gentamycin (GEN), an increase in the zone of inhibition was also observed in a large proportion of strains - respectively P-TEO in 3/6 strains *(p=0.093, t=-2.076)*, FOX-TEO in 5/6 strains *(p=0.017, t=-3.503)* and GEN-TEO - 5/6 strains *(p=0,017, t=-3.503)*.

The zone of growth inhibition of tested *E. coli* increased between 3 and 20 mm in almost all studied combinations. Two strains of *E. coli* were resistant to ceftriaxone, but when it was combined with TEO, the growth inhibition zones increased to 26-29 mm. Studies were conducted on the synergistic interactions of active components of thyme essential oil, but we did not find any other studies conducted that investigated the interaction of TEO with different antibiotic groups.

In *Klebsiella pneumoniae*, administration of a combination of ampicillin and TEO resulted in a decrease in the zone of inhibition of neat oil, possibly due to an antagonistic effect of ampicillin on TEO *(p=0.003, t=-5.508)*. In the combinations of TEO with ceftriaxone *(p=0.035, t=-2.879)* and gentamycin *(p=0.069, t=-2.315)*, enhancement of antimicrobial activities was reported in 1/3 strain each, ciprofloxacine with TEO - 2/3 *(p=0.077, t=-2.221)* and TEO combined with meropenem *(p=0.001, t=-10,304)* - 3/3 strains of *K. pneumoniae*.

DISCUSSION

Thyme essential oil is characterized by a high concentration of thymol (45.74%), p-cymene (21.05%), and γ-terpinene (12.37%) (Table 3). Through chemical composition analysis, we have confirmed that the tested essential oil belongs to the thymol chemotype. Similar findings have been reported by other authors, who also classified their thyme essential oil samples as belonging to the thymol chemotype. Galovičová *et al*. observed a thymol content comparable to that found in our sample, at 48%. However, the levels of γ-terpinene and p-cymene were approximately half of those observed in our analysis [17]. In a similar study, Al-Asmari *et al.* identified the essential oil of *T. vulgaris* as thymol-type, with furan (12.19%) and p-cymene (2.78%) as additional key components [18]. The Food and Drug Administration (FDA) recognizes thymol as safe for consumption, which further supports its potential use as a natural antimicrobial agent [19].

Thymol exhibits a broad range of pharmacological properties including antioxidant, anti-inflammatory, antimicrobial, analgesic, and antitumor activities. Its antimicrobial action is particularly notable, as it effectively inhibits the growth of various pathogens, including *Salmonella spp.* and *Staphylococcus aureus*, which are capable of forming biofilms that enhance their adhesion to surfaces. This ability poses a significant risk to food safety, underscoring thymol's potential as an effective agent against a variety of infectious pathogens, in line with its FDA-approved safety for consumption [20, 21].

There are numerous studies on the antimicrobial activity of TEO against Gram-positive and Gram-negative microorganisms [11-13]. The main compounds in TEO are thymol and carvacrol, which are active against *Staphylococci*, *Streptococci*, and *Salmonella sp*. TEO has higher activity against *E. coli* compared to Gram-positive microorganisms, according to some studies [12, 13, 22, 23]. In our study, the reference strain *Escherichia coli* ATCC25922 was also the most sensitive to TEO, showing the largest (23 mm) zone of inhibition and smaller against *S. aureus*.

Investigating potential synergic interactions of TEO with antibiotics against *E. coli*, the zone of growth inhibition increased between 3 and 20 mm in almost all tested combinations. Two strains of *E. coli* were resistant to ceftriaxone, but when it was combined with TEO, the growth inhibition zones increased to 26-29 mm. In the study of Moussaoui and Alaoui [24], a synergistic effect was observed with a combination of *Thymus willdenowii* Boiss and gentamycin against *E. coli*. Another study by Amassmoud *et al*. demonstrated a strong synergistic effect between EO of *T. broussonnetii* and *T. pallidus* and ciprofloxacin against *S. aureus, S. enterica,* and *E. coli* [7].

Combining TEO with antibiotics, the greatest increase in the zone of growth inhibition in *S. aureus* was observed with the combined use of TET-TEO and ERY-TEO. In a study conducted by Pancu *et al*., the positive influence of TEO in combination with tetracycline against *S. aureus* was also found [25], and Rosato *et al.* proved the synergistic effects between gentamycin and TEO against *S. aureus* [9]. In the remaining three combinations of TEO with P, FOX and GEN, an increase in the zone of inhibition was also observed in most of the samples.

For *Klebsiella pneumoniae*, administration of a combination of ampicillin and TEO resulted in a decrease in the antimicrobial activity. This effect is probably due to the innate resistance of *Klebsiella pneumoniae* to ampicillin. In the combinations of TEO with FOX, GEN and CIP, antimicrobial activity enhancement was reported in 6 from 9 samples. Studies were conducted that investigate synergistic interactions of individual active components of thyme essential oil and antibiotics against *K. pneumoniae*, but we did not find any other studies that investigated the interaction of TEO with different antibiotic groups.

CONCLUSION

The present study highlights the potential of TEO to enhance the antimicrobial effects of conventional antibiotics. The combination of TEO with antibiotics exhibited a synergistic effect, indicating that this approach may contribute to mitigating the emerging issue of bacterial resistance and reducing the adverse effects associated with antibiotic therapies. Further investigation is warranted to fully elucidate the therapeutic potential of TEO in combination therapies and its broader clinical applicability.

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