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Antimicrobial Effects of Antibiotics in Combination with Oregano Essential Oil against *Staphylococcus aureus*

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Posted Date: 16 August 2024

doi: 10.20944/preprints202408.1155.v1

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Antimicrobial Effects of Antibiotics in Combination with Oregano Essential Oil against *Staphylococcus aureus*

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Abstract: The antimicrobial resistance to clinically approved antibiotics is rapidly increasing worldwide. *Staphylococcus aureus* is a critical contributor to deaths associated with antibiotic resistance. In this regard, the search for natural compounds is quite active, that alone or in combination with other agents can be effective as new antibacterial agents. The antibacterial activity of essential oils has presented an increasing interest during the last years and was effective even on multidrug-resistant strains. The present study aims to investigate the interaction effects of oregano essential oil (OEO) with some conventional antibiotics against *Staphylococcus aureus* tested by agar methods. For this purpose, we selected *S. aureus* ATCC 29213 and five clinical isolates representative of the species. The essential oil was analyzed using gas chromatography coupled with mass spectrometry (GC-MS). The analysis of the Oregano essential oil revealed the main component to be carvacrol (81,20%). Concentration 2.5% (v/v) OEO was combined with drugs proposed by EUCAST, 2024, specifically penicillin, cefoxitin, erythromycin, gentamycin and tetracycline. The research results showed that the combination of OEO with penicillin, cefoxitin, erythromycin, and tetracycline enhanced the antimicrobial activity of the antibiotics against *S. aureus* in almost all cases. The area of suppression remained identical (but not smaller) in the unaffected cases. When comparing the effects of the GEN-OEO combination and GEN against most *S. aureus* strains, a decrease in antimicrobial activity was reported by the diffusion agar method.

Keywords: antimicrobial effects; antibiotics; oregano essential oil; combination; *S. aureus*

1. Introduction

The global increase in antimicrobial resistance to widely used antibiotics and chemotherapeutic agents highlights the urgent need to identify alternative antimicrobial compounds for the prevention and treatment of infectious diseases in both human and veterinary medicine [1,2]. *Staphylococcus aureus* is a major contributor to mortality associated with antibiotic resistance. Although it is a commensal organism naturally present in the human body, it has the potential to cause a wide range of diseases, from skin and soft tissue infections to life-threatening conditions such as bacteremia [3,4]. *S. aureus*, especially methicillin-resistant *S. aureus* (MRSA), has emerged as a major cause of healthcare-associated infections. In 2019, MRSA was identified as the most significant pathogen-drug combination in antimicrobial resistance, leading to an estimated 13,800 deaths in Europe and 121,000 deaths worldwide [5–8].

In this context, efforts are ongoing to discover natural compounds that alone or in combination with other agents can serve as effective new antibacterial therapies [9,10]. The antibacterial properties of essential oils (EOs) have attracted considerable interest in recent years, showing efficacy even against multidrug-resistant strains [11–13]. The antifungal and antiviral properties of some essential

 doi:10.20944/preprints202408.1155.v1

oils are also widely documented in the scientific literature [12,14,15]. The antimicrobial activity of essential oils is mainly due to their ability to generate reactive oxygen species (ROS), which cause genetic damage, and their ability to disrupt microbial metabolic processes. In dealing with antibiotic-resistant bacteria, the essential oils of *O. compactum* and *O. elongatum*, especially those rich in thymol and (E)-caryophyllene, show significant potential when used in combination with antibiotics [16]. Xiao S et al., 2020 reported that among the nine essential oils demonstrating the strongest activity against *S. aureus* at a concentration of 0.25% (v/v) were oregano, cinnamon bark, white thyme, Bandit "Thieves", Lemongrass, Health Shield, Spice, Palmarosa and Amyris. Their effectiveness surpasses that of the well-known persistent-acting drug tosufloxacin [4].

Oregano essential oil (OEO) is recognized for its potent antimicrobial properties, including antifungal [17,18], antibacterial [19,20] and antiparasitic effects [10,21]. However, as noted by Yoncheva et al., 2021, its application is limited by its low water solubility and potential instability [1]. Their study aimed to address these limitations by investigating the incorporation of OEO into an aqueous dispersion of chitosan-alginate nanoparticles and evaluating its impact on antimicrobial activity. The findings show that the nano-delivery system enhances the antimicrobial efficacy of oregano oil, making it a promising candidate for the development of nutritional supplements.

Recent research has shown that oregano essential oil (OEO) can function as an alternative to the prophylactic use of antibiotics in veterinary medicine [22] and in some cases can potentially replace antibiotics entirely in the treatment of pathological conditions [23]. Zhao et al., 2021 found that in cloth broilers, the addition of OEO to their diet changed the morphology of the small intestine, improved the structure of the gut microbiota, and improved the digestion and absorption of nutrients [22]. These changes contributed to better growth and reduced death rates. Therefore, OEO shows promise as a viable replacement for growth-promoting antibiotics in broiler broiler diets. Additionally, other studies support the effectiveness of oregano in managing pathogen levels in the gastrointestinal tract *in vivo* [24]. Oregano essential oil (OEO) has demonstrated inhibitory activity against pathogenic bacteria such as *Acinetobacter baumannii* and highly resistant *Klebsiella pneumoniae*, which are often implicated in healthcare-associated pneumonia [21]. Recent research has also highlighted that Gram-positive cocci, including methicillin-resistant Staphylococcus aureus, are particularly susceptible to OEO. Conversely, *Pseudomonas aeruginosa* showed the highest resistance to this essential oil [25].

Lu M et al., 2018 investigated the effectiveness of oregano essential oil (OEO) against pathogenic bacteria, including multidrug-resistant (MDR) strains from combat injuries, both in vitro and using a mouse burn model. OEO showed notable antibacterial activity against 11 MDR clinical isolates, including four strains of methicillin-resistant *Staphylococcus aureus* (MRSA), with minimal inhibitory concentrations (MICs) ranging from 0.08 mg/ml to 0.64 mg/ml. Furthermore, OEO effectively destroyed biofilms produced by all 13 tested pathogens at comparable MIC levels [26]. In addition to their antimicrobial properties, essential oils (EOs) and their components, such as carvacrol and thymol, have been shown to enhance the antimicrobial effects of some antibiotics. Carvacrol (2-methyl-5-(1-methyl ethyl)-phenol) is a monoterpene phenol isomeric with thymol and is present in various aromatic plants, including *Origanum dictamnus, Origanum majorana, and Origanum vulgare.*

There is increasing scientific evidence that oregano essential oil (OEO), especially its component carvacrol, exhibits synergistic effects when used together with various antibiotics [27–29]. Carvacrol and other oregano-derived compounds can exhibit cytotoxicity at elevated concentrations [30,31], highlighting the importance of optimizing forage levels of oregano to maximize beneficial effects while minimizing cytotoxicity [24] . Thymol, a monoterpenoid phenol found in oils extracted from species of the genera *Origanum* L. and *Satureja* L., among others, has been shown to potentially increase the efficacy of antibiotic treatment [32–36]. The present study aimed to investigate the interaction effects of OEO with various conventional antibiotics against Staphylococcus aureus tested by agar methods.

 doi:10.20944/preprints202408.1155.v1

2. Materials and Methods

The study was conducted at Medical College, Varna, Bulgaria in July 2024.

2.1. Gas Chromatography-Mass Spectrometry

For the purposes of the analysis, an apparatus consisting of a gas chromatograph 7890A, a flame ionization detector and a mass spectral detector 5975C (Agilent Technologies) was used; column Stabilwax (Restek) with parameters: length 30m, diameter 0.25mm and film coating thickness 0.25µm at the following temperature program: initial temperature 65°C, increase to 170°C with 1.5°C/min; analysis duration 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; scanning range of the mass spectral detector m/z=40-450; injected sample volume 1.0 µl in 100:1 flow split mode. Compounds were identified by comparing retention times and relative Kovach 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.

2.2. Studied Bacterial Strains

The antimicrobial activity combinations between OEO and antibiotics were investigated against *S. aureus* ATCC 29213 and five clinical strains of *S. aureus* isolated from throat swab (1 specimen) and wounds secretions (4 specimens).

2.3. EO Origanum Vulgare, Antibiotics, and Agar Media

In this experiment, the used EOs are 100% pure with certified organic ingredients and are commercially available. Antibiotic discs (HiMedia) used for testing the antimicrobial activity of the compounds were provided by Ridacom, Bulgaria. The antimicrobial activity assay was performed on Mueller-Hinton agar (HiMedia), also provided by Ridacom, Bulgaria.

2.4. Antimicrobial Activity Evaluation Test

The antimicrobial activities of the combinations of antibiotics with OEO were investigated by the Kirby-Bauer disk diffusion susceptibility test. We also studied the action of each studied antimicrobial agent separately. The selection of antibiotics against each of the *S. aureus* test strains was made according to the EUCAST, 2024 instructions [37].

For the study, we initially plate a densitometer standardized (Grant Bio DEN-1, UK) 0.5 MF microbial culture of the respective strain on the surface of Mueller-Hinton agar. We left the media for approximately 15 minutes at room temperature until their surfaces were completely dry. Then, in each culture medium with the corresponding microbe culture, we placed three disks (d=6 mm) in the following combinations and concentrations of active agents:

- a disc soaked with 100 µl 2.5% (v/v) OEO;
- factory-prepared antibiotic disc in concentration, according to EUCAST standards, 2024;
- a second identical antibiotic disc, additionally soaked with 100 µl 2.5% OEO.

OEO solutions were prepared with 1% DMSO solution. We performed all samples in triplicate, then incubated the test cultures for 24 hours at 37°C. The obtained inhibition zones were measured in mm and compared to each other, as well as for all antibiotics, the results were determined for their effect on the respective microbe: S – sensitive, or R – resistant, following the EUCAST, 2024 instructions. A list of antibiotics included in the study is presented in Table 1.

Table 1. List of antibiotics studied.

Antibiotics(ATBs)	Abbreviation	Chemical Family
Penicillin	p	Beta-lactams
Cefoxitin	FOX	Beta-lactams
Erythromycine	ERY	Macrolides

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Gentamycin	GEN	Aminoglycosides
Tetracyclin	TET	Tetracyclines

2.5. Statistical Processing of the Results

We applied a Paired samples t-test (t-test for dependent samples) to investigate whether there was a statistically significant difference between the mean values of antimicrobial effects between antibiotics and the combination of OEO with antibiotics (Table 3). For the significance level of the statistical test, we set α=0,05.

3. Results and Discussion

Natural products are increasingly recognized as sources of novel pharmacological molecules with antimicrobial potential [38]. In this context, various essential oils have been investigated, and the current study focuses on oregano essential oil (OEO). This oil was analyzed for its chemical constituents using retention times and Kovats retention indices (RI) compared to standard compounds, along with mass spectrometry data from the NIST'08 (National Institute of Standards and Technology, USA) and Adams Libraries, as detailed in Table 2. The analysis identified a total of 22 constituents in OEO, including p-Cymene, γ-Terpinene, β-Linalool, Carvacrol, and β-Bisabolene, among others. The results showed that carvacrol is the predominant compound, constituting 81.20% of the EO. This finding aligns with the study by Walasek-Janusz et al., which identified carvacrol as a major component in oregano essential oils, with percentages ranging from 76.64% to 85.70% as assessed by gas chromatography-mass spectrometry (GC-MS) [39]. Similarly, Penteado et al. reported carvacrol as one of the principal components in oregano essential oil, with a concentration of 69.1% [40], while Gomes et al. found a carvacrol percentage of 45.74% [41]. Silva et al. also demonstrated a carvacrol content of 40.52% in their analysis of OEO [2]. Carvacrol is identified as the primary compound responsible for the antibacterial activity of OEOs. Its mechanism of action involves depleting intracellular ATP (adenosine triphosphate) reserves and increasing the permeability of the cytoplasmic membrane to cations, which disrupts critical cellular processes and leads to bacterial cell death [42].

Table 2. Chemical composition of Oregano essential oil.

No. compound	% of TIC	RT(min)	RI
1. α -Thujene	0,12	9,05	921
2. α -Pinene	0,42	9,27	928
3. Camphene	0,16	9,79	945
4. β- Pinene	0,08	10,71	976
5. β -Myrcene	0,64	11,15	988
6. α -Phelandrene	0,11	11,66	1004
7. α -Terpinene	0,90	12,02	1015
8. p-Cymene	3,69	12,28	1023
9. Limonene	0,13	12,40	1025
10. β -Phelandrene	0,21	12,45	1027
11. ɣ -Terpinene	3,52	13,37	1056
12. (Z) Sabinene hydrate	0,16	13,73	1068
13. Terpinolene	0,10	14,25	1386
14. β -Linalool	3,76	14,73	1099
15. Borneol	0,71	16,87	1170
16. α -Terpineol	0,24	17,60	1195
17. Thymol	1,19	20,36	1293
18. Carvacrol	81,20	20,75	1301
19. β -Caryophyllene	0,97	23,70	1420
20. Aromadendrene	0,15	24,18	1438

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21. β- Bisabolene	1,24	25,93	1506
22. Caryophyllene oxide	0,17	27,66	1581
	99,88 %		

In our study, we followed the antimicrobial effects of the combined in vitro action of antibiotics and OEO against *S. aureus*. When investigating synergistic interactions of antimicrobial agents, the most commonly used method is Checkerboard Assays. A large number of such studies have been conducted with combinations of different types of EOs with conventional antibiotics [43–45] and more specifically with OEO and antibiotics [9,16,45]. Several recent studies have been conducted to investigate the synergistic effects of OEO with antibiotics [2,9,16,42,45] against microbes of clinical importance. Lorenzo HB et al., 2024 reported results according to which the combination of oxytetracycline with OEO reduced the concentration of some multiresistant strains [46]. There have been also studies on the interactions between carvacrol and/or thymol with conventional antibiotics [47] – two of the compounds with the most powerful antimicrobial action in combination with OEO. Kissels W et al., 2017, investigated the potential synergistic effects of these compounds with antibiotics against some of the most common pathogenic isolates from the respiratory tract. According to their data, based on factorial inhibitory concentration checkerboard assay, an additive effect was observed when doxycycline was combined with thymol. Synergism was observed when carvacrol was combined with doxycycline or with thymol. Although the antibacterial effects of the tested EO components were observed at high concentrations for in vitro conditions, the additive and synergistic effects of carvacrol and thymol with antibiotics suggest the option to apply them as antibiotic adjuvants [47]. Thymol also leads to improving the effectiveness of gentamicin and neomycin when used against *S. aureus*, which leads to 16-fold and 32-fold reduction in MIC, respectively [35]. In our study, we compared some of the results obtained with the same and similar combinations tested with one of the standard techniques for susceptibility testing in clinical practice – the disk-diffusion method. For our research, we followed the antimicrobial action of OEO, of a given antibiotic, as well as the combination between the two agents. We performed the tests on Mueller-Hinton agar with measurement of the inhibition zone obtained. We set the concentrations of the antibiotics following the instructions of EUCAST, 2024, and for OEO – concentration 2.5% (v/v). The results are presented in Table 3.

Table 3. Effects of combined in vitro action of antibiotics and oregano essential oil (OEO).

Bacterial strain Conc.	**OEO 2,5% (v/v)**	**P 1 unit**	**P-OEO 1 unit+ 2,5%(v/v)**	**FOX 30 µg**	**FOX-OEO 30µg+ 2.5%(v/v)**	**ERY 15µg**	**ERY-OEO 15µg+ 2.5%(v/v)**	**GEN 10µg**	**GEN-OEO 10µg+ 2.5%(v/v)**	**TET 30µg**	**TET 30µg+ 2.5%(v/v)**
EUCAST2024		**S≥18 R<18**		**S≥27 R<21**		**S≥21 R<21**		**S≥18 R<18**		**S≥22 R<22**	
S. aureus 1	15	16	26	16	18	28	32	28	28	14	34
S. aureus 2	15	14	24	18	19	28	30	26	30	12	42
S. aureus 3	16	26	26	28	29	24	26	20	20	22	22
S. aureus 4	15	28	28	29	32	24	26	20	20	26	26
S. aureus 5	17	30	32	30	30	26	26	26	18	26	26
S. aureus ATCC 29213	18	19	30	28	30	35	35	44	41	35	36

Antibiotics (ATBs): P – penicillin, FOX – cefoxitin, ERY – erytromycine, GEN – gentamycin, TET – tetracyclin.

We investigated the action of the combination of penicillin (P) and OEO against six strains of *Staphylococcus aureus*, compared to the action of the antibiotic alone. Two of the strains *(S. aureus 1* and *S. aureus 2*) were resistant to P, the others showed sensitivity (S≥18, R<18). Our results show that

 doi:10.20944/preprints202408.1155.v1

against 4 (out of 6) strains, antimicrobial activity is enhanced with P-OEO compared to P with an increase in the zone of inhibition between 2-11 mm (p=0,05, t=-2,515, CI [-11,122; 0,122]) . The most pronounced increase in the antimicrobial effect of P-OEO compared to P was observed against *S. aureus* ATCC 29213 (P-19 mm, P-OEO-30 mm zones of inhibition). Against two of the tested strains, the action of P did not change with the addition of OEO - the zone of inhibition remained identical. When OEO was applied independently, zones of inhibition were also reported – 15-18 mm. In other studies, we found data on combining EOs with aminopenicillins that corroborate our results. Certain antibiotics, including amoxicillin and ampicillin, have been shown to elicit synergistic effects with OEO. The strain that stands out the most is *Staphylococcus aureus* (*S. aureus 2220*), for which they found 14 additive and synergistic combinations. These synergistic effects could result from their ability to target the same bacterial proteins or facilitate access to target sites, as suggested by molecular docking simulations [16].

The combination of cefoxitin (FOX) with OEO also showed an increase in antimicrobial activity against *S. aureus* compared to FOX alone (p=0.017, t=-3,503, CI [-2,601; -0,399]). Although to a much lesser extent compared to P and P-OEO, this combination also reported an increase in zone of inhibition over 5 (of 6) strains by between 1-3 mm. Again, against *S. aureus* ATCC 29213 and another *S. aureus* strain (*S. aureus 4*) isolated from wound, the greatest enhancement of antimicrobial activity was reported. Against one of the isolates, the area upon application of FOX and FOX-OEO was identical. Two of the strains (*S. aureus 1* and *S. aureus 2*) were resistant to FOX, the others showed sensitivity (S≥27, R<27).We found no published reports to date investigating synergistic interactions between cefoxitin and OEO.

The results are similar for the ERY-OEO combination against the tested isolates - again the antimicrobial activity is increased, compared to ERY, for almost all strains of *S. aureus* (p=0.042, t=-2,712, CI [-3,247; -0,087]). In one of them, isolated from wound, the zone remained unchanged, in 5 (out of 6) strains we reported an increase within 2-9 mm. As the strongest, OEO affects the action of erythromycin against *S. aureus* ATCC 29213, as well as with the other discussed combinations. All the tested strains were sensitive to ERY (S≥21, R<21). When examining the effects of combining GEN with OEO, the results were mixed. In one of the strains, we reported an enhancement of the growth inhibitory effect over GEN, but in two others, the zone of inhibition was reduced by up to 4 mm, including *S. aureus* ATCC 29213. In the remaining three, no change in antimicrobial activity was reported when GEN and GEN+OEO (S≥18, R<18) were administered (p=0,509, t=-0,711, CI [-3,053; 5,386]). Xiao S et al., 2020 conducted a large-scale study investigating the activity of 139 essential oils against *S. aureus*, as well as the antimicrobial effects of combining EOs with antibiotics. They also concluded that OEO had no apparent enhancement for gentamicin against S. aureus. Other studies have reported that this combination could be synergistic. According to Zaharieva et al., 2022 encapsulated OEO with gentamicin leads to a synergistic effect against methicillin-susceptible (MSSA) and methicillin-resistant (MRSA) strains of *Staphylococcus aureus*. It is expressed in a four-fold decrease in the effective concentration of gentamicin and 98% inhibition of the metabolic activity of bacteria [48]. Another study also reported that carvacrol demonstrated clinically relevant antibacterial activity for both gram-positive and gram-negative bacteria and a synergistic effect when combined with gentamicin [49].

We also investigated the effect of the TET-OEO combination and in three of the strains we reported an increase in the antimicrobial effect compared to TET, and in two of them, the inhibition zone increased by 20 and 30 mm. These differences showed that, in this case, the TET-OEO combination led to overcoming the threshold of sensitivity of the two clinical strains to TET (S≥22, R<22), which in the independent action of the antibiotic demonstrated resistance. In the remaining three strains of *S. aureus* when TET action was considered, the inhibition zone was not affected by the addition of OEO (p=0,175, t=-1,581, CI [-22,323; 5,323]). Malczak I and Gajda A, 2023 reported that OEO from *Origanum vulgare* and its main compounds, thymol, and carvacrol, enhanced the inhibitory effects of tetracycline against *S. aureus* [36]. The mechanism responsible for this action is probably inhibiting efflux pumps in bacterial membranes [50]. Another mechanism responsible for the synergistic action of thymol and carvacrol with the antibiotics mentioned above may be their ability

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to increase the cellular permeability of bacteria due to the lipophilic effect of these terpenes on the cell membrane [51].

4. Conclusion

The results of the conducted research showed that the combination of OEO with P, FOX and ERY, in almost all cases, enhanced the antimicrobial activity of the antibiotics against *S. aureus*. Examining the effects of TET and TET-OEO against half of the strains, there was no change in the antimicrobial effects of TET, but in some of them the antimicrobial activity was greatly increased. In the unaffected cases, the area of suppression remained identical, but not smaller. When considering the effects of the GEN+OEO combination against GEN against most *S. aureus* strains, an underestimation of antimicrobial activity was reported by the diffusion agar method. From the applied statistical analysis, we reported that there is a statistically significant difference between the action of OEO with the antibiotics P, FOX and ERY and their action alone.

Author Contributions: Conceptualization, N.E., G.T., G.N., S.S. and E.G.; methodology, N.E., G.T., G.N., S.S. and E.G.; software, K.L.; validation, N.E., G.T., G.N., S.S. and E.G ; formal analysis,N.E., G.T., G.N. and K.L..; investigation, N.E., G.T., G.N., S.S. and E.G.; resources, N.E., G.T., G.N., S.S. and E.G..; data curation, N.E., G.T., G.N., S.S., K.L. and E.G.; writing—original draft preparation, N.E., G.T., G.N., S.S. and K.L.; writing—review and editing, N.E., G.T., G.N., S.S., K.L. and E.G.; All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Medical University if Varna, Fund "Science", project number 21012.

Data Availability Statement: All research data are described in the present work.

Acknowledgments: Medical University of Varna, Fund "Science", project number 21012.

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

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