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Antibacterial Activities of Essential Oils from Three Medicinal Plants in Combination with EDTA against Methicillin-resistant *Staphylococcus aureus*

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Authors' contributions

This work was carried out in collaboration between all authors. Author JRA designed the study, wrote the protocol, supervised the research and wrote the first draft of the manuscript. Authors LHV and ECR collected the samples and the microorganisms used. Authors JRQ and RAR were involved with the microbial analysis. Authors RCA and AA performed the statistical analysis. Authors JOS and MPP managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To determine the antibacterial activity of essential oils (EOs) from *Thymus vulgaris* L, *Origanum vulgare* L and *Minthostachys mollis* (Benth.) Griseb in combination with

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ethylenediaminetetraacetic acid (EDTA) on methicillin-resistant *Staphylococcus aureus* (MRSA) from clinical isolates.
Study Design: Collection of plant material, extraction, phytochemical analysis and evaluation of antimicrobial activity of the essential oils.
Place and Duration of Study: Clinical Research Institute, Faculty of Medicine, Universidad Nacional Mayor de San Marcos, Department of Microbiology, Faculty of Pharmacy and Biochemistry, and Department of Microbiology, Hospital Dos de Mayo, Lima, Peru, between February 2014 and February 2015.
Methodology: The EOs' chemical composition was determined by gas chromatography coupled with mass spectroscopy (GC-MS). Clinical isolates of MRSA were obtained from the "Dos de Mayo" National Hospital in Lima, Peru. The inhibitory activity on MRSA was determined by disk diffusion method and Minimum inhibitory concentration (MIC) by the microdilution colorimetric method in 96-well plates. All statistical analysis was performed using SPSS V 21.0 software.
Results: The main components of *Thymus vulgaris* EO were thymol (46.47%), γ-terpinene (20.27%) and p-cymene (15.80%); from *Origanum vulgare* EO were γ-terpinene (21.17%), (-)-4-terpineol (12.61%) and cis-β-terpineol (12.18%); and from *Minthostachys mollis* EO were pulegone (33.48%) and menthone (26.68%). The *Thymus vulgaris* EO presented inhibition halo diameters of 32.23 ± 1.70 mm, and *Thymus vulgaris* + EDTA had inhibition halo diameters of 32.47 ± 2.06 mm, both significantly higher compared to 30 mcg of cefoxitin, which had inhibition halo diameters of 17.60 ± 0.68 mm ($p < .01$). The MIC of *T. vulgaris* against MRSA was 0.57 µg/mL. *Origanum vulgare* and *Minthostachys mollis* EOs were resistant to MRSA.
Conclusion: This study showed that the *Thymus vulgaris* essential oil had antibacterial activities against MRSA and indicates their potential application in the control of this pathogen commonly known for their resistant activities to most conventional antibiotics.

Keywords: Essential oils; Thymus vulgaris; Origanum vulgare; Minthostachys mollis; MRSA clinical isolates.

1. INTRODUCTION

Bacterial resistance to antibiotics is increasing day by day both in the community and in the hospital. It has a significant impact on morbidity and mortality rates and on the financial burden that is associated with it [1]. According to the World Health Organization (WHO), antimicrobial resistance is a global problem that requires urgent action [2]. In addition, the increased commercial trades and the international travels allow resistant microorganisms to spread quickly to other continents in which they were not resistant previously [3].

Staphylococcus aureus causes a variety of infections ranging from skin and soft tissue infections to blood stream infections, pneumonia, meningitis, endocarditis and toxic shock syndrome [4]. Highly resistant bacteria such as methicillin-resistant Staphylococcus aureus (MRSA) or vancomycin-resistant enterococci (VRE) cause a high percentage of infections contracted in hospitals. Last years, MRSA has appeared out of hospitals more frequently than previous times, and it has been considered as a major public health concern in the world [5].

The resistance of *Staphylococcus aureus* to antibiotics disables available drugs in the market and it produces high morbidity and mortality rates, both in hospitals and community. It is a significant health problem worldwide because it is estimated that MRSA kills more people than the acquired immunodeficiency syndrome (AIDS) and tuberculosis together [6]. There are few antibiotics that are available to treat MRSA infections, they are very expensive. It has a strong impact on poor people for whom such treatment is inaccessible. For these reasons, the searching of active agents against MRSA is justified, and natural resources are a potential alternative for the solution of the problem.

Mintostachys mollis (Lamiaceae) is a sub aromatic shrub restricted to Andes. In Peru, it is known with the popular name of "muña" [7]. Its species' essential oil showed significant inhibitory effect against gram positive and gram negative bacteria [8] and it had also inhibitory effect against yeast and fungal pathogens [9]. The Thymus vulgaris [10] and *Origanum vulgare* essential oils *(*EO) [11] also showed antimicrobial activity. However, there is very few information about the antimicrobial effect of these plants against MRSA's patient samples. Therefore, this study aims to evaluate the *Minthostachys mollis*, *Thymus vulgaris* and *Origanum vulgare* EOs effect against 17 MRSA clinical isolates and to

determine the influence of its association with EDTA, in an *in-vitro* model.

2. METHODOLOGY

2.1 Plant Samples and Microbial Cultures

Thymus vulgaris L, *Minthostachys mollis* (Benth) Griseb and *Origanum vulgare L.*, were purchased from spice vendors in Lima, Peru. The plants were identified in the Natural History museum of "Universidad Nacional de San Marcos" (No. 184-HSM-USM-2015, No. 185-HSM-USM-2015 and No. 186-HSM-USM-2015, respectively).

Clinical strains of MRSA were collected from the Microbiology Laboratory of the National Hospital Dos de Mayo in Lima, Peru. The strains were isolated some from bronchial aspirate, blood samples and sputum, but most of them from patients with skin infections. Initial identification was performed in the hospital laboratories by using their routinely techniques [12,13]. Strains were transported to the Microbiology Institute's laboratory in the Pharmacy and Biochemistry Faculty of Universidad Nacional Mayor de San Marcos. Direct colony suspension was used for the inoculum preparation. It was grown overnight in physiological saline solution at a McFarland standardized turbidity density of 0.5 (about 1-2 x 10^8 colony forming units per ml, CFU/mL).

2.2 Extraction of Essential Oils

Leaves of *Thymus vulgaris L*, *Minthostachys mollis* (Benth) Griseb and *Origanum vulgare L.* were subjected to a 3 hr of hydrodistillation, using a Clevenger type apparatus [14]. The resulting oils were separated and dried over anhydrous sodium sulphate, filtered and stored in an amber glass bottle under refrigeration at a temperature of 4°C until used.

2.3 Phytochemical Determination

The major compounds of the EOs were determined by gas chromatography coupled to mass spectrometry (GC-MS) analysis on Agilent Technologies 5975 C mass spectrometer system. They were diluted 20 µL of the sample in 1 mL of dichloromethane and the injection volume was 1 µl using a DB-5ms column, 325°C: 60 cm x 250 microns x 0.25 microns; ramp temperature was 50°C for 5 min, 10°C/min to 100°C, 5°C/min to 150°C, 15°C/min to 200°C for 1 minute and finally 5°C/min up to 270°C maintained for 2 minutes. The run time was 40.333 min, Split 50: 1, the carrier gas was helium, 1 mL/min. The detector was mass spectrometer.

2.4 Evaluation of Antistaphylococcal Activity

The disk diffusion assay was carried out according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [15] using Mueller Hinton agar which was prepared according to the manufacturer's instructions, adding 25 mL of the agar into plates of 90 mm of diameter. A sterile cotton swab was introduced into the inoculum suspension and it was rotated against the inner wall of the tube to remove the liquid excess. The inoculum was extended over the entire plate's surface by rubbing around 3 directions. 10 µL of oil were placed on sterile filter paper disks of 6 mm, and subsequently, the paper discs were placed firmly on the surface of the plates that were inoculated and dried. Discs with dimethyl sulfoxide (DMSO) and 30 µg of cefoxitin were used as controls. Fifteen minutes after the application of the discs at 35 ± 1°C, plates were inverted and incubated for 16-20 hours. The diameters of the zones of inhibition were measured in millimeters with Vernier.

2.5 Determination of Minimum Inhibitory Concentration (MIC)

Essential oils' MIC against MRSA was performed using the colorimetric microdilution method in 96-wells, according to Sarker et al. [16] protocol. The EO were diluted in DMSO in serial two-fold dilutions. Ten doubled concentrated end dilutions were obtained between the ranges of 0.07 to 38.68 µg/mL. The final DMSO concentration was equal to or less than 5% (v/v). Each strain of clinical MRSA's isolate was seeded 24 hours before on trypticase soy agar and it was aerobically incubated at 37°C. On the assay's day, a small aliquot of the bacteria was transferred to a test tube containing sterile saline solution at 0.85%, until it reached a similar turbidity degree of 0.5 McFarland scale, which is equivalent to 1.5 x 10^8 CFU/mL (concentrated inoculum). Immediately, a double dilution of 1:50 and 1:20 was performed with Mueller Hinton agar to obtain an inoculum of 1-5 x 10^5 CFU/mL (2x inoculum). 0.1 mL 2x of the resazurin solution in 20 mg/mL was added per 20 mL of the inoculum suspension. In each microplate's well, we introduced 100 µL of EOs 2x dilution or the corresponding control, and then it was added 100 mL of 2x inoculum with resazurin indicator

placed. On the other hand, they were considered sterility control wells (Mueller Hinton broth only) and growth control wells (Mueller Hinton with the inoculum of MRSA). The microplates were incubated at 37°C for 24 hr under aerobic conditions. The reading of the results were done visually. Any color changes from purple to pink or colorless were recorded as positive result. The lowest concentration at which there was no color change was taken as the MIC value. The average of three values were calculated and reported as the MIC [16].

2.6 Statistical Analysis

Data from susceptibility testing were expressed as mean ± standard deviation. Normality and homogeneity of variance was determined by Shapiro-Wilk and Barlett tests, respectively. Comparisons between experimental groups were performed by one-way ANOVA followed by Tukey post-hoc test. A *P*-value < .05 was considered significant. All statistical analysis was performed using SPSS V 21.0 software.

3. RESULTS

3.1 Phytochemical Analysis of the Essential Oils

The *Thymus vulgaris* L essential oil components were identified in a previous study, with 27 compounds which included 100% from the total, and the main ones were thymol (44.19% of m-thymol, 2.28% p-thymol and 46.47% in total), γ-terpinene (20.27%) and p-cymene (15.80%) [17]. In this study, we found that the most abundant *Minthostachys mollis* essential oil compounds were pulegone (33.48%) and menthone (26.68%) as it is shown in Table 1. Moreover, we found that the most abundant compounds of the *Origanum vulgare* essential oil were γ-terpinene (21.17%), (-)-4-terpineol (12.61%) and *cis* -β-terpineol (12.18%) (Table 2).

3.2 Antistaphylococcal Activity Assessment

The essential oil of *Thymus vulgaris* and *Thymus vulgaris* + EDTA inhibited the growth of MRSA with inhibition halos of 32.23 ± 1.70 mm and 32.47 ± 2.06 mm, respectively. Both were significantly higher compared to the17.60 ± 0.68 mm of cefoxitin ($p < 0.01$) (Fig. 1, Table 3).

The MIC value was 0.57 µg/mL for the EO of *Thymus vulgaris* (Fig. 2).

Table 1. Chemical composition of volatiles in the *Minthostachys mollis* (Benth.) Griseb essential oil

Compound	tR (min.)	% of total
α-Pinene	15.56	0.67
β-Phellandrene	16.61	0.26
β-pinene	16.85	0.85
p-Cymene	18.01	0.25
trans-β-Ocimene	18.09	0.13
D-Limonene	18.15	1.00
cis-β–Ocimene	18.42	0.52
γ-Terpinene	18.9	0.36
β-Linalool	19.89	2.94
Unknown (C10H20O2)	20.25	0.43
Menthone	21.59	26.68
Isomenthone	21.8	2.43
Isopulegone	22.03	2.73
Dihydrocarvone	22.49	0.62
Pulegone	23.38	33.48
D-Carvone	23.44	3.20
Piperitone oxide	23.63	5.65
Piperitone	23.68	1.33
Unknown (C10H14O)	25.33	0.42
Unknown (C10H14O2)	25.67	3.98
Copaene	26.03	0.26
Unknown (C15H24)	26.19	0.20
β-Bourbonene	26.23	0.17
β-Caryophyllene	26.91	5.34
7-methoxy-2,2-dimethyl-2H-1-Benzopyran	27.43	0.24
α-Caryophyllene	27.51	0.78
Germacrene D	27.91	3.74
Bicyclogermacrene	28.15	1.13
δ-cadinene	28.38	0.24

tR = Retention Time

4. DISCUSSION

Essential oils are a complex mixture of many volatile aromatic secondary metabolites [18]. In this study, the most abundant compounds in the essential oil of *Minthostachys mollis* were pulegona and menthone (33.48% and 26.68%, respectively) (Table 1). These results are consistent with the findings from Cano et al. [9] who found 36.7% of pulegone and 24.24% of menthone in a sample from Junin, Peru. Our findings also correlate with other data of samples from Peru, such as Jauja (33.1% and 48.2%), Lima (47.4% and 25.3%) and southern Peru (45.0% and 18.0%) of pulegone and menthone, respectively [7]. However, in a sample from Venezuela it was found higher percentages: 55.2% of pulegone and 31.5% of trans-menthone [8].

Table 2. Chemical composition of volatiles in the *Origanum vulgare* L essential oil

Compound	tR (min.)	% of total
Origanene	15.26	0.72
α-Pineno	15.54	0.50
Sabinene	16.60	5.45
β-Myrcene	16.85	2.05
α-Phellandrene	17.51	0.21
α-Terpinolene	17.79	7.51
p-Cymene	18.00	4.78
β-*trans*-Ocimene	18.08	4.38
D-Limonene	18.14	1.52
β-Phellandrene	18.24	1.12
cis-β-Ocimene	18.40	0.59
12-Terpinene	18.91	21.17
(1α,2β,5α)-2-methyl-5-(1-methylethyl) bicyclo [3.1.0] hexan-2-ol	19.28	1.82
(+)-4-Carene	19.67	2.20
Linalool	19.89	2.54
cis-β-Terpineole	20.17	12.18
Unknown (C10H18O)	20.77	0.66
(-)-4-Terpineol	22.14	12.61
α-Terpineol	22.40	1.61
Thymol methyl ether	22.95	0.73
2-methoxy-4-methyl-1-(1-methylethyl)-Bencene	23.16	2.18
Linalool Anthranilate	23.25	3.24
Thymol	24.13	5.48
β-Caryophyllene	26.88	2.43
α-Caryophyllene	27.50	0.21
Germacrene D	27.89	1.21
Bicyclogermacrene	28.13	0.90

tR = Retention Time

We found that the most abundant compounds of the *Origanum vulgare* essential oil were γ-terpinene (21.17%), (-)-4-terpineol (12.61%) and *cis* -β-terpineol (12.18%) (Table 2). A great variability in the composition of this essential oil is observed. In a Brazilian sample, the 33.3% of the total was composed of terpin-4-ol and 38% of thymol [18]; 57.71% was composed of carvacrol, 10.91% was composed of p-cymene and 7.18% was composed of γ-terpinene [19]. In Iran, 50.1% of the composition was beta-caryophyllene [20]. In Greece, the 43.6% of the composition was carvacrol, the 23% was cymene and 13.9% was γ-terpinene [21] as well as carvacrol and thymol [22]. In Jordan, 27.19% of the composition was trans-sabineno [23]. In Serbia, 64.5% of the composition was carvacrol, 10.9% was p-cymene and 10.8% was γ-terpinene [24]. In Portugal, 55% of the composition was thymol and 8.66% was γ-terpinene [25], 14.5% was carvacrol, 12.8% was β-fenchyl alcohol, 12.6% was thymol and 11.6% was γ-terpinene [26]. In Italy, there were found three chemotypes: carvacrol/thymol, thymol/α-terpineol, and linalyl acetate/linalool [10]. Chemotypes are "biochemical variety" or "physiological forms" of botanical species; each one of them has specific enzymatic equipment. These species are genetically encoded and they guide their biosynthesis to a defined compound's making. The variety of compounds could be explained because the samples are from different places worldwide. The most important factors on the chemotypes' differentiation are mainly related to intrinsic factors such as sexual polymorphism or genetic mechanism, but when it is about phenolic essences, environmental conditions such as altitude, temperature, season and remarkable stress are able to influence the biosynthetic pathway [10,14].

Table 3. Inhibition zones diameter (mm) produced by essential oils from *T. vulgaris*, *M. mollis* and *O. vulgare* in 17 clinical isolates of MRSA

Grupo	Inhibition zones (mm)	P value
Thymus vulgaris	32.23 ± 1.70	0.001*
Origanum vulgare	12.88 ± 1.13	0.872
Minthostachys mollis	8.71 ± 0.57	0.202
Thymus vulgaris + EDTA	32.47 ± 2.06	0.001*
Origanum vulgare + EDTA	14.47 ± 1.10	0.982
Minthostachys mollis + EDTA	12.65 ± 1.70	0.843
Cefoxitin 30 µg	17.60 ± 0.68	
DMSO (negative control)	0	

**Significant from cefoxitin 30 µg*
Mean ± S.E.M = Mean values ± Standard error of means of three experiments

The alarming *Staphylococcus aureus* resistance to antibiotics has led to a permanent searching for new antibiotics. We aimed explore the potential use of *Thymus vulgaris*, *Origanum vulgare* and *Mintosthachys mollis* essential oils against MRSA. The only essential oil that showed significant inhibitory effect on MRSA when tested by disk diffusion was the one from *Thymus vulgari* (Fig. 1 and Table 3). The MIC of the *Thymus vulgaris* essential oil was 0.57 µg/mL with the dilution method (Fig. 2). Similarly,

an Iran study showed that *Thymus vulgaris* EO had inhibitory effect against MRSA clinical isolates [27]. The same result was found in Poland [28], Spain [9] and Brazil [18]. It is likely that the significant effect of *Thymus vulgaris* against MRSA, was due to its main component, thymol, whose chemical composition was determined in a previous paper [17]. Like in other studies that observed inhibitory effect against *Staphylococcus aureus,* thymol was the major compound of the essential oils [10,18, 27,28]. In addition, pure thymol has showed inhibitory effect on *Staphylococcus aureus* [29,30]. The antimicrobial activity of most terpenoids, as thymol, is related to their functional group; the hydroxyl group of the phenolic terpenoid and delocalised electrons are important elements for their antimicrobial action [31]. Diverse mechanisms have been described to explain the activity of EO on bacterial cells. It has been shown that EOs targets include the cell wall and membrane of bacterial cells, thereby disturbing ATP production and pH homeostasis. EOs can affect the cellular transcriptome, proteome, and the quorum-sensing system [32]. The activity of EO can affect both the external envelope of the cell and the cytoplasm. The hydrophobicity that is typical of EOs is responsible for the disruption of bacterial structures that leads to increased permeability with leak of metabolites and ions due to an inability to separate the EOs from the bacterial cell membrane [31]. Besides, diminished enzymatic activity and the appearance of coagulated material have been reported to result from EO activity [32].

In the present study, the *Mintosthachys mollis* and *Origanum vulgare* essential oils showed little inhibition halos (Fig. 1), which indicated resistance to MRSA. This result is consistent to the information provided by Bussmann et al. [33] who used ethanolic extract of *Mintosthachys mollis* and did not found activity against *Staphylococcus aureus.* In Turkey, Karakaya et al. [34] reported that *Origanum vulgare* had no influence on *Staphylococcus aureus* survival; however, De Souza et al. [35] showed total suppression of *Staphylococcus aureus* enterotoxin production at concentrations of 0.3 to 0.15 µL/mL. De Souza also showed alteration on membrane permeability, the smearing formation on cell surface, and loss of cytoplasmic material. Likewise, other researchers also showed the significant effect of *Origanum vulgare* essential oils on *Staphylococcus aureus* [19,24,29]. This difference could be due to the essential oil chemical composition, because in the articles in which it was found antimicrobial effect against *Staphylococcus aureus,* carvacrol was the major component (about 60%), while in this study, the presence of carvacrol was zero (Table 2).

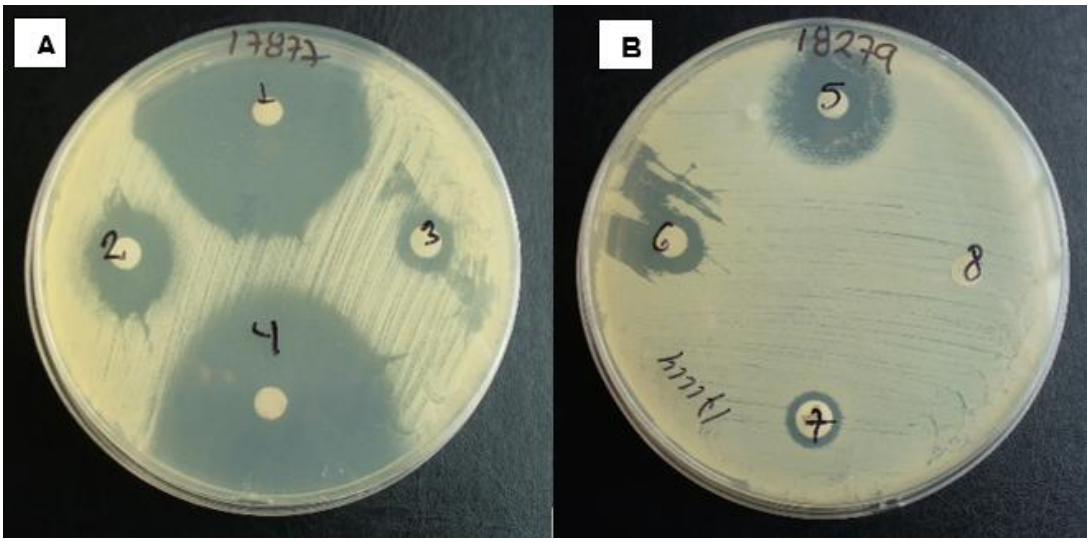

Fig. 1. Plates with Inhibition zones produced by essential oils on MRSA

A: 1 = Thymus vulgaris, 2 = Origanum vulgare, 3 = Mintosthachys mollis, 4 = Thymus vulgaris + EDTA. B: 5 = Origanum vulgare + EDTA, 6 = Mintosthachys mollis + EDTA, 7 = Cefoxitin 30 µg, 8 = DMSO (negative control)

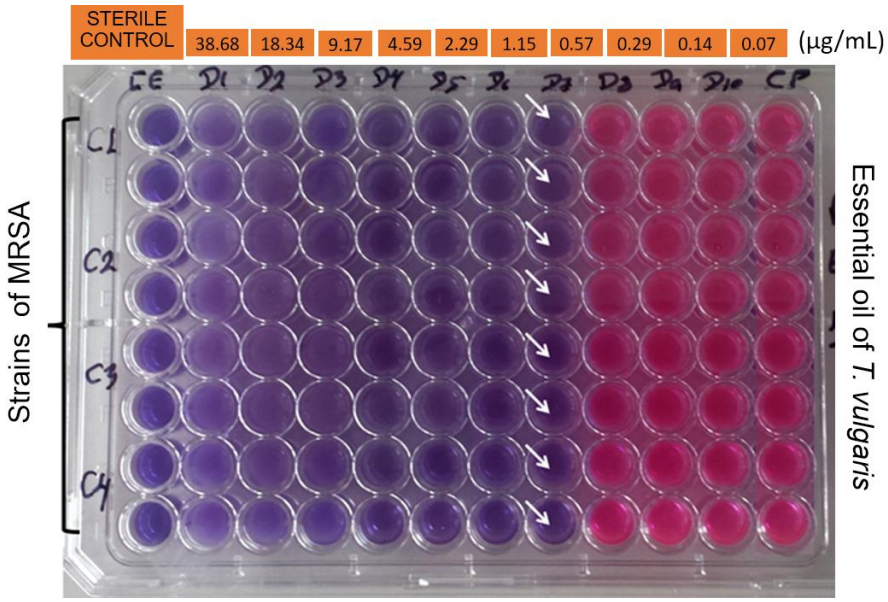

Fig. 2. Minimum inhibitory concentration assay of *T. vulgaris* against MRSA clinical isolates
C1-C4 = Strains of MRSA, CE = sterility control, D1-D10 = dilutions of essential oil, CP = positive control. Arrows indicates the MIC value

The influence of EDTA in association with EOs was explored. A slight increase in the inhibition zone diameter of the three plants compared to the effect of EOs alone with none association was observed (Fig. 1). Although EDTA is a chelating agent with widely known anticoagulant activity [36], it was also introduced into endodontics to chemically soften the root canal dentine and dissolve the smear layer, as well as to increase dentine permeability [37]. Some researchers like Judah et al. [38] showed that the *Staphylococcus epidermidis* adhesion and formation on Nelaton biofilaments and central venous catheters (CVC) was inhibited by EDTA at low concentrations (1-2 mmol/L); to eradicate *Staphylococcus epidermidis*, higher concentrations (> 32 mmol / L) were required, which showed it acts as a bacteriostatic agent. Root et al. [39] showed that a solution of 20 mg/mL of disodium EDTA, has an *in vitro* bactericidal effect against an initial inoculum of 10^3 CFU/ml of *Staphylococcus epidermidis* in a period of 24 hours. Percival et al. [40] found that the application of tetrasodium EDTA during 21 to 25 hours at a concentration of 40 mg/ml reduces the colonization of *Staphylococcus epidermidis, Pseudomonas aeruginosa, Klebsiella pneumoniae*, *Escherichia coli* and *Candida albicans* on central venous catheters segments. EDTA also disrupts ionic crosslinking mediated by lipid A in the cell membrane, producing that the lipidic bilayer becomes more permeable, this fact was demonstrated in *Klebsiella pneumoniae* and *E. coli* [41].

It is affirmed that EOs can be used as effective antiseptics against many species, including multidrug-resistant bacteria (MDR), such as MRSA, vancomycin-resistant *Staphylococcus aureus* (VRSA), and vancomycin resistant enterococci (VRE). Also, they can enhance antibiotics effectiveness against MDR bacteria, with the advantage that it allows to reduce the antibiotic doses and consequently contribute to the presentation of fewer side effects [30,42].

5. CONCLUSION

The *Thymus vulgaris* essential oil had *in-vitro* antibacterial activities against MRSA from clinical isolates and indicates its potential application in the control of this pathogen commonly known for their resistant activities to most conventional antibiotics. The association with EDTA had not a synergistic effect.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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