



Antibacterial Effects of Oregano Essential Oil (OEO) and Its Potential Applications

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Authors' contributions

This work was carried out in collaboration among all authors. Author RCC designed the study, wrote the protocol, wrote the first draft of the manuscript and managed literature searches. Authors RMR managed experimental methods and wrote the methodology section of the study and literature searches. Authors JPQR and DMQ kindly contributed materials (EOs=OEO). All authors read and approved the final manuscript.

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ABSTRACT

Essential oils (EOs) are commonly used in food industry, due that they possess antioxidative and antimicrobial properties. There are few essential oils that have been used in medicine, due to its potent antibacterial activity against intrahospital pathogens. OEO has experimentally shown potent antibacterial effect on nosocomial Gram-positive bacteria, therefore it can be very useful in hospital environments, where there are many bacterial pathogens, which are the etiological agents of nosocomial infections and most of them are resistant to several antibiotics.

Objective: The aim of this study was to determine antimicrobial effect of OEO on most frequent bacterial intrahospital pathogens: MRSA, MRSE comparatively to selected ATCC bacterial reference strains.

Methods: This experimental study investigates the antibacterial action of oregano (*Origanum vulgare*) essential oil (OvEO) on two human pathogens: *Staphylococcus aureus* (SA) and

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Staphylococcus epidermidis (SE) Here, we used OEO against one of the most prominent antibiotic-resistant bacterial strains: methicillin-resistant SA (MRSA $_{mecA+}$ = Methicillin Resistant SA and $_{mecA-}$ = *Methicillin Resistance SA*), methicillin-resistant SE (MRSE $_{mecA+}$ = Methicillin Resistance *Staphylococcus epidermidis mecA+*) and reference strains: *S. aureus* ATCC 700699, *S. epidermidis* ATCC 359845 and *E. coli* ATCC 25922. Bactericidal effects of the OEO on these bacteria were mainly evaluated using undiluted and four serial dilutions in coconut oil (CCO) 1: 1:10, 1:100, 1:200, 1:400.

Results: OEO, undiluted and 4 serial dilutions showed potent antibacterial activity against all strains tested. In conclusion, this OEO could be used as an alternative in medicine. The ability of OEO to inhibit and kill clinical Multi-Drug-Resistant (MDR): MRSA and MRSE strains, highlights its potential for use in the management of drug-resistant MDR infections in hospitals wards.

Keywords: Antibacterial effects; essential oil; antibiotics; contaminant bacteria.

1. INTRODUCTION

OEOs in addition to having aromatic properties they also have outstanding therapeutic properties; therefore, they are widely used in the pharmaceutical, food, perfume industries and more recently in medicine. OEO has been described to have several benefits for human health, it may support gastrointestinal, respiratory, and skin health. These properties were once recognized in ancient Greece where they were often used for treating bacterial infections on the skin or in wounds, and it was also employed to protect food from contaminant bacteria. There have been recognized several disinfectant and antimicrobial properties of OEO: antioxidant, antiviral, antibacterial, antifungal, antiparasitic, anti-inflammatory, digestive, emmenagogue and anti-allergenic substance [1, 2,3,4]. The OEO consisted mainly of phenol compounds (80%): carvacrol (75%) and thymol (5%) as the main components and the rest corresponded to oxygenated monoterpenes and sesquiterpenes (20%). Due to its high concentration of phenols compounds, that OEO possesses relevant disinfectant properties [1,5,6,7].

Antimicrobial resistance is a major health concern worldwide. A narrowing of the antibiotic development pipeline and resurgence in public opinion towards 'natural' therapies have renewed the interest in using essential oils as antimicrobial agents [8,9,10].

There have been several reports of antimicrobial effects of OEO on several human and animal pathogens, mainly Gram-positive and Gram-negative bacteria, such as: *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, *Bacillus cereus*, *Clostridium perfringens*, *Salmonella typhimurium*, *Klebsiella pneumoniae*, *Shigella sonnei*, *S. flexneri*, *Yersinia enterocolitica*, *Enterobacter cloacae*; *Vibrio sp.*

Listeria monocytogenes and *Bacillus subtilis*, [1,3,5,6]. There are scarce reports on the mechanisms of action of OEO, most of the active antimicrobial compounds have been identified as: thymol and carvacrol components. It seems that the main initial carvacrol action is on the integrity of both layers, cytoplasmic membrane and cell wall, therefore altering cell permeability, the thymol mode of action is as growth inhibitor [3,5,6] Antimicrobial activity of OEO have also been documented against other microorganisms: virus, molds and parasites [1,3].

1.1 Objective

The aim of this study was to determine *in vitro* the potential antimicrobial effect of commercial OEO on most frequent bacterial intrahospital pathogens: MRSA, MRSE comparatively to selected ATCC bacterial reference strains.

2. METHODS [5,6,8,11]

To perform this study, three clinical staphylococci strains were isolated from hospitalized patients with Nosocomial Infection (NI) and three reference strains were chosen from the American Type Culture Collection "ATCC" (*S. aureus* ATCC 700699, *S. epidermidis* ATCC 359845 and *E. coli* ATCC 25922). All three clinical staphylococci strains (MRSA $_{mecA+}$, MRSA $_{mecA-}$ and MRSE $_{mecA+}$) and all bacterial reference strains were characterized in the laboratory by conventional (phenotypic analyses) and by molecular methods (genotypic analyses) by Duplex PCR detection of *coa* and *mecA* genes. These clinical strains were isolated in a period since 2010 to 2013 from 3 newborns, with nosocomial infection at the National Institute of Perinatology "INPer" (Instituto Nacional de Perinatología "Dr. Isidro Espinosa de los Reyes", SSA). The isolates came from two different infection sites: blood (2 strains) and membrane conjunctive (1 strain) (Table 1).

The SA and SE strains used in this work were obtained by the donation from the microbiology collection of the INPer. A consent form was not required. Original identification keys and clinical data concerning the isolates are maintained under control of INPer.

INPer strain identifiers were substituted by common names to prevent further interpretations of the data. Authors do not have access in any form to the specific clinical information of strains and patients. Staphylococci strains were isolated at the INPer under the ethical standards of the Medicine Faculty of the UNAM.

Conventional phenotypic analyses, including identification of bacterial strains by colony morphology, Gram stain characteristics, coagulase test (COT, Remel Coagulase Plasma), manitol fermentation (MT, Salt-Mannitol Agar, OXOID LTD., England) and Cefoxitin resistance (Cfx by Disk Diffusion "DD" Assay, OXOID Ltd., England), were performed on the SA and SE, clinical and reference strains. The biotype was first determined using the automatic VITEK equipment at the Microbiology Laboratory of the INPer. The second phenotypic confirmatory tests: COT, MT and Cfx, were performed at the Bacteriology Laboratory of the Faculty of Medicine at UNAM.

The COT assay was performed with amounts of 0.5 mL of the rabbit plasma in assay tubes (13 x100 mm, Kimax). Several colonies of a 24 old culture of each staphylococci strain, were inoculated in each tube containing plasma. All tubes were incubated in a water bath at 35°C. Results were observed at 4 and 24 hours. Coagulation of plasma was considered as positive test and plasma without coagulation was considered as negative test. Positive and negative control tubes were used containing plasma without bacteria and bacteria alone with sterile saline solution. Reference strains used here were as follows: *Staphylococcus aureus* (Mu50) ATCC 700699 Coagulase positive and *Staphylococcus epidermidis* (RP62A) ATCC 35984 Coagulase negative.

MT assay was performed using Petri dishes (100 x10 mm) containing Salt-Mannitol Agar. Staphylococci strains were inoculated onto these plates and were incubated at +35°C for 24 hours. Change of the original pink color of the media showing yellow colonies, was considered as positive result of fermentation of mannitol by SA strains. Culture media without change of initial

pink color was considered as negative result of fermentation of mannitol by SE strains. Reference strains used here were as follows: *Staphylococcus aureus* (Mu50) ATCC 700699 Mannitol positive and *Staphylococcus epidermidis* (RP62A) ATCC 35984 Mannitol negative.

The DD assay for cefoxitin "Cfx" was performed with 30µg Cfx disks. Plates of Brain Heart Infusion Agar (Oxoid Ltd., England) were inoculated by swabbing the surfaces with a 0.5 McFarland standardized suspension of *S. aureus* ATCC25923 as a negative control strain, HGC3 as an internal positive control strain and clinical or reference samples of SA and SE strains, the disks were placed on the BHI plates and were incubated at +35°C for 24 h. Strains were considered resistant when the diameter of growth inhibition was ≤ 14 mm, in accordance with the manufacturer's recommendations.

Genotypic analyses: Duplex PCR for *coa* and *mecA* genes. A 458 bp fragment of the *mecA* gene (GenBank accession number NP_370565) was amplified using the primers 5'-ATGGCAAAGATATTCAACTA-3' (upstream) and 5'-GAGTGCTACTACTCTAGCAAAGA-3'(downstream). A second set of primers 5'-AACTTGAAAT-AAAACCACAA-3' (upstream) and 5'TACCTGTACCAGCATCTCTA-3' (downstream); specific for *S. aureus*, was included in each reaction to amplify a 177 bp fragment of the *coa* gene (GenBank accession number AP009324.1). Both set of primers were designed in our laboratory with the nucleotide sequences from gen bank accession number mentioned above and after the free software primer3. Ten to twenty colonies of each SA and SE isolates to be tested were resuspended in 250 µL of lysis solution (Buffer Tris-EDTA plus lysozyme plus lysostaphyne), this bacterial suspension then was incubated at $\pm 37^\circ\text{C}$ during 90 minutes, when the incubation period elapsed, the sample was warmed up at 95°C for 10 minutes and finally the sample was centrifuged at 8000 x g, the supernatant was recovered under aseptic conditions and semipurified with isopropanol 99.5% (Tecsiquim, México D.F.) The PCR was performed in a Thermal Cycler 100 BIORAD equipment. After amplification, the PCR products were separated by electrophoresis through 1% agarose gels in 1X TAE buffer (Invitrogen Life Technologies Carlsbad, CA, USA) at 95 V for 40 minutes. Gels were then stained with 0.08 µL/mL of ethidium bromide (Invitrogen Life Technologies Carlsbad, CA, USA. 10 mg/mL) and visualized under UV light.

Antimicrobial activity of OEO was tested against three clinical staphylococci strains (MRSA*mecA*+, MRSA*mecA*- and MRSE*mecA*+) comparatively to three reference selected strains from the ATCC (*S. aureus* ATCC 700699, *S. epidermidis* ATCC 359845 and *E. coli* ATCC 25922). Effects of the OEO on bacteria were mainly evaluated using undiluted and 4 serial dilutions in duplicates of the OEO in CCO diluent (1 mL of OEO + 9 mL of CCO= 1:10; 1 mL of 1:10 + 9 mL of CCO= 1:100; 5 mL of 1:100 + 5 mL of CCO = 1:200 and 5 mL of 1:200 + 5 mL of CCO = 1:400). The CCO diluent without OEO was used as positive control. The assay was performed with 0.5 mL of the bacterial suspension of each strain, adjusted to a 0.5 McFarland Standard (~1.5x10⁸ Colony Forming Units, "CFU"). This standardized bacterial suspension of each strain tested, clinical and reference, was inoculated in each tube (16x100 mm, Kimax) containing duplicates of undiluted and 4 serial dilutions of OEO. The mixture was homogenized for 60 seconds on tube Vortex device (Stuart Scientific, CO., LTD., UK.) and the OEO was allowed to act for 15 minutes. Immediately after, an aliquot of 1 mL of each tube mixture was inoculated on a sterile Petri dish (100 x10 mm) duplicates and a 20 mL of melted Brain Heart Infusion (BHI) culture media (Oxoid Ltd., England), maintaining at 50-55 °C in a water bath, was poured on a duplicate sterile dish (100 x10 mm). These mixtures with melted culture media were homogenized by gentle circle movements to the right and to the left. Petri dishes with solidified media were incubated at 36 ± 1 °C for 24 hours. CFU were estimated using a Colony Counter (Stuart Scientific, CO., LTD., UK.)

3. RESULTS

Phenotypic and genotypic laboratory results of all three clinical staphylococci strains (MRSA*mecA*+, MRSA*mecA*- and MRSE*mecA*+) and all three reference strains (*S. aureus* ATCC 700699, *S. epidermidis* ATCC 359845 and *E. coli* ATCC 25922) are shown in Table 1.

All positive control Petri dishes without OEO but with CCO were shown countless CFUs (Table 2). Undiluted and serial dilutions (1:10, 1:100, 1:200 and 1:400) were shown no bacterial growth at all (Table 2). All bacterial strains, clinical and reference ATCC, were grown in the presence of CCO diluent (positive control) but in the absence of OEO. All bacterial strains, clinical and reference ATCC, were inhibited in the presence

of undiluted and 4 serial dilutions of OEO. The OEO showed an outstanding bactericidal effect (higher than a 1:400 dilution) against a wide spectrum of microorganisms, such as Gram-positive (*S. aureus*, *S. epidermidis*, MRSA and MRSE) and Gram-negative (*E. coli*) bacteria. All clinical bacterial strains (*S. epidermidis mecA*+, *S. aureus mecA*+ and *mecA*-) were completely inhibited by undiluted and serial dilutions of OEO, since 1:10 until 1: 400. All ATCC reference bacterial strains (*S. aureus* # 700699, *S. epidermidis* # 359845 and *E. coli* # 25922) were completely inhibited by undiluted and serial dilutions of OEO, since 1:10 until 1: 400. Further experiments are needed to precisely determine the ending point of bactericidal activity (>1:400 dilution).

4. DISCUSSION

There are several new compounds from natural products, such as OEO, to which antimicrobial activities so far have been detected [2]. There are many scientific reports on antimicrobial effects of EOs on diverse bacterial species, but scarce reports specifically on MRSA and MRSE strains [3,5,6,12]. *Staphylococcus aureus* and *S. epidermidis*, specifically MRSA and MRSE strains are the most frequently strains isolated from NI worldwide [4,9]. Biofilm formation is one of the pathogenic mechanisms involved in medical device-related infections and is also responsible for antimicrobial resistance [6,8,13,14]. There are some scientific reports that show that clinical MRSE*mecA*+ and MRSA*mecA*+ strains carry a higher number of resistance determinants in biofilm-producer strains [8,14,11]. Some EOs have shown antibacterial activity against gram-positive bacteria that cause community or nosocomial infection, and some of these EOs have been tested for skin antiseptics. Even more when an EO (*Melaleuca alternifolia*) is combined with an antimicrobial agent (tobramycin), there is a synergistic effect against multi-drug-resistant *S. aureus* [15]. Therefore, here we confirmed antibacterial activity of OEO against two specifically clinical staphylococci strains, having methicillin resistance genes (*S. epidermidis mecA*+, *S. aureus mecA*+ and *mecA*-), further more we comparatively demonstrated antibacterial effect of OEO to three ATCC reference bacterial strains (*S. aureus* # 700699, *S. epidermidis* # 359845 and *E. coli* # 25922). The antibacterial outstanding effect of OEO against these nosocomial bacterial pathogens

Table 1. Phenotypic and genotypic analysis of all clinical Staphylococci strains and all ATCC reference strains used

Hospital*/ Strains	ID ¹ -code	Infection site	Hospital ward	Staphylococci/ <i>E. coli</i> (Clinical & ATCCstrains)	Genotype		Phenotype ⁵		
					³ coa	⁴ mecA	COT	MT	Cfx
INPer 550	FEH-RN**	Conjunctive membrane	ICU ²	<i>Staph. aureus</i>	+	-	+	+	-
INper 722	ANA-RN	Blood	ICU	<i>Staph. epidermidis</i>	-	+	-	-	+
INPer 883	URM-RN	Blood	ICU	<i>Staph. aureus</i>	+	+	+	+	+
ATCC ⁶	-700699	----	----	<i>Staph. aureus</i>	+	+	+	+	+
ATCC	-359845	----	----	<i>Staph. epidermidis</i>	-	-	-	-	-
ATCC	-25922	----	----	<i>Escherichiacoli</i>	-	-	-	-	-

*INPer= Instituto Nacional de Perinatología "Dr. Isidro Espinosa de los Reyes, SSA".

**RN=newborn. ¹ID= Identification. ²ICU= IntensiveCareUnit. Genotype: ³coa= coagulase gene; ⁴mecA= meticillinresistance gene. ⁶ATCC= American Type Culture Collection. Biochemical Tests⁵: COT=coagulase; MT= mannitolfermentation; Cfx= cefoxitinresistance (30 µg- disk)

Table 2. Antimicrobial effect of OEO on clinical and reference bacterial strains

Bacterial strain	Control + (CCO w/out OEO)	Serial dilutions of OEO				
		Undiluted (CCO with OEO)	1:10	1:100	1:200	1:400
<i>S. aureus</i> ATCC ¹ 700699	Countless UFC*	NO-growth	NO-growth	NO-growth	NO-growth	NO-growth
<i>S. epidermidis</i> ATCC 359845	CountlessUFC*	NO-growth	NO-growth	NO-growth	NO-growth	NO-growth
<i>E. coli</i> ATCC 25922	CountlessUFC*	NO-growth	NO-growth	NO-growth	NO-growth	NO-growth
INPer+ 550 <i>S. aureusmecA</i> ¹ (-)	CountlessUFC*	NO-growth	NO-growth	NO-growth	NO-growth	NO-growth
INPer+722 <i>S. epidermidis mecA</i> (+)	CountlessUFC*	NO-growth	NO-growth	NO-growth	NO-growth	NO-growth
INPer+ 883 <i>S. aureusmecA</i> (+)	CountlessUFC*	NO-growth	NO-growth	NO-growth	NO-growth	NO-growth

ATCC=American Type Culture Collection. UFC*= Colony Forming Units

INPer+ =Perinatology National Institute. ¹mecA =meticillinresistance gene, presence (+) or absence(-)

seems very potent (>1:400). EOs in combinations can produce additive antimicrobial activity, and EOs in combination with other antimicrobials can improve antimicrobial effectiveness. Bacterial resistance to antiseptic solutions has increased globally. Recent studies have shown the activity of EOs as penetration enhancers for antiseptics and as restorers of antimicrobial activity against resistant species [2,16]. The activity of EOs as penetration enhancers for antiseptics could be applied to prevent infections that are related to surgery and medical devices and to restore antimicrobial activity against resistant species [10,16,17]. EOs represent a source of natural antimicrobial substances and have the potential to be used in the hospital, in specific emergency wards such as intensive care units as a preservative to prevent bacterial growth [2,16,17].

EOs also possess bioactive properties with antibacterial activity that could be used directly for cleaning purposes. Natural antimicrobials could be used alone or in combination with other preservation technologies [18]. Many EOs have the ability to reduce bacterial numbers, as the majority of EOs tested exhibit a considerable inhibitory capacity against pathogenic microorganisms [19]. There are several useful biological properties shown by EOs, since potent antimicrobial activities, synergistic, antiseptic and bioactive activities, that could be used alone or in combination with other antibiotics or chemical agents, to inhibit pathogenic microorganisms and several resistant species in several hospital wards [15,16,17,18,19].

5. CONCLUSIONS

The analyzed OEO showed strong activity on the bacterial growth of all tested reference and clinical strains of Gram-positive *Staphylococcus* spp. and Gram-negative *Escherichia coli*.

EOs could also be evaluated in combination with disinfectants on contaminated surfaces.

It is likely that it will be more difficult for bacteria to develop resistance to the multi-component EOs than to common antibiotics that are often composed of only a single molecule.

ETHICAL APPROVAL

This study was carried out following the recommendations of the ethics review committee of the Faculty of Medicine at the National

Autonomous University of Mexico City "UNAM" (Facultad de Medicina de la UNAM).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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