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Mode of Antimicrobial Action of Origanum Vulgare Essential Oil Against Clinical Pathogens.

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Abstract

Oregano essential oil (EO) was extracted via hydro-distillation from Origanum vulgare aromatic plant and tested for its mode of action against 16 clinically isolated strains of *Escherichia coli* and *Staphylococcus aureus* (Methicillin resistant and non-methicillin resistant). Initially, Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were estimated by the broth micro-dilution method. Additionally, the extracellular concentrations of total proteins were measured in bacterial suspensions with the presence of EOs near the MIC concentrations and without the presence of EOs by using the Bradford protein assay. Ampicillin was used as a positive control. Most of *S. aureus* and almost half of *E. coli* strains exhibited relatively low MIC values when tested with the EO of oregano. Based on the protein assay a 65% of *E. coli* strains but over 80% of *S. aureus* strains exhibited a clear dose-response curve indicating that the mode of action was the disruption of the cytoplasmic membrane and cell wall. Differences in sensitivities of Gram(-) and Gram (+) bacteria on the action of EOs are known with the later been more sensitive than Gram (-). However, MRSA strains were proven resistant to the EOs when compared with their non-MRSA counterparts.

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antibacterial action,
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Introduction

Overuse and abuse of antibiotics resulted in antibiotic resistance of many pathogens during the last decays[1-3]. As a result, to this growing resistance, many antibiotic agents has lost their efficacy with dramatic consequences in the treatment of infectious diseases. In the research for new alternative agents, phytochemicals and particularly essential

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oils (EOs) coming from aromatic and medicinal plants has drawn the scientific attention[4-6]. The majority of these compounds are natural defense mechanisms of plants against bacteria, other competitors, or herbivores[7]. They are complex mixtures of volatile and aromatic compounds classified primarily as terpenoids and according to the number of isoprene units as monoterpenes and sesquiterpenes[7]. More than 3000 EOs has been identified and despite their use in traditional medicine, a very small fraction of about 10% is approved for use in pharmaceuticals, cosmetics and food products. These natural compounds are generally recognized as safe by FDA. Biological activities attributed to EOs are antimicrobial, antiviral, antimycotic, antiparasitic, insecticidal, antidiabetic, antioxidant, and anticancer[8-9]. Previous reports have shown that EOs effectiveness is based among others on their ability to disrupt both the cell wall and the cytoplasmic membrane of bacteria, resulting to leakage of intracellular compounds such as proteins[6,10-11]. The aim of this work was to study this hypothesis by using oregano EO against clinically isolated strains of *Staphylococcus aureus* and *Escherichia coli*.

Materials And Methods

Bacterial Strains

The microorganisms utilized in the study composed of 26 *E. coli* strains and 24 *S. aureus* strains, were collected and identified in the microbiological departments of various hospitals. All strains were initially stored at -30 ^{0}C in cryovials then transferred to the laboratory and subcultured in Mueller-Hinton agar (37 ^{0}C/24h) before testing. Eight out of the 24 strains of S. aureus were identified as methicillin resistant (MRSA).

Essential Oils

Cultivated oregano was harvested in mid-summer. Drying was accomplished in a laboratory oven at 30 ŒC for 48h. Batches of 200 g from dried plant material (stems and leaves) were used for EOs extraction by hydrodistillation in a Clevenger apparatus[4]. After collection the EOs dehydrated with sodium sulfate and stored in amber glass vials at -30 ^{0}C until used.

Antimicrobial Action

The microdilution broth method was used for the determination of Minimum Inhibitory Concentration (MIC) of EOs[4-5]. In brief, bacterial suspensions equivalent to the 0.5 McFarland index were added in Muller-Hinton broth supplemented with 3% DMSO and increased concentrations of EOs (0.5-1024 μg/mL) in 96-well sterile microplates. Microplates were incubated at 37 ^{0}C for 24 h and MIC determined only by visual inspection in order to prevent the integrity of the culture. Minimum Bactericidal Concentration (MBC) was determined after MIC by plating 10 μL from each well equal and above the MIC on Muller-Hinton Agar plates. The absence of growth after incubation at 37 ^{0}C for 24 h, was considered as the Minimum Bactericidal Concentration value.

Assessment Of Cell Wall And Cytoplasmic Membrane Disruption

The Bradford method of protein assay (Pierce® Coomassie Protein Assay Kit, Thermo Scientific™) was used to detect any possible increase of the total proteins into the broth as a result of bacterial cell wall and cytoplasmic disruption occurred by the action of EOs. Therefore, a reference curve was constructed by using elevated concentrations of Total Bovine Serum Albumin (BSA) standards in MH broth and measured photometrically at 595nm (Fig 1). Negative controls (without EOs) and positive controls (with ampicillin) were also used (Fig 2). Ampicillin belongs to the b-lactam group of antibiotics which is known for their bacteriolytic mode of action against Gram (-) and Gram (+) bacteria[12].

Results

Inhibitory And Bactericidal Concentration.

In Table 1 the values of MIC and MBC obtained from oregano essential oil against *E. coli* and *S. aureus* are shown. All strains of both bacteria were inhibited by the oregano EO. Mean MIC value for *E. coli* was 256 μg/mL (128-1024 μg/mL) and mean MIC value for *S. aureus* was 128 μg/mL (64-512) indicating a stronger inhibiting action. Regarding the bactericidal effect, oregano EO was most effective against *S. aureus* with a mean MBC of 64 μg/mL, and 128 μg/mL, respectively, for *E. coli*.

Effect On Bacterial Membrane Integrity.

Figure 1 shows the reference curve of bovine serum albumin initial measurements. The reference curve could be adequately described by the below 4th degree polynomial equation.

$OD = 7E-08[C^4 - 1E-05[C^3 + 7E-05[C]^2 + 0.0116[C] + 0.4874$, where:

OD is the optical density of the photometer and C the concentration of the total proteins in the medium. R^2 value was 0.9997 which indicate a fully satisfactory fit.

Figure 2 shows the curve obtained by using ampicillin as a positive control. Based on the protein assay over 80% of *S. aureus* strains (19 out of 24) and 65% of *E. coli* strains (17 out of 26) exhibited a clear dose-response curve indicating that the target of action of the oregano EO was the disruption of the cell wall and cytoplasmic membrane. However, 5 out of 8 MRSA strains showed no indications of extracellular protein increase after exposure to the EO action (Fig 3).

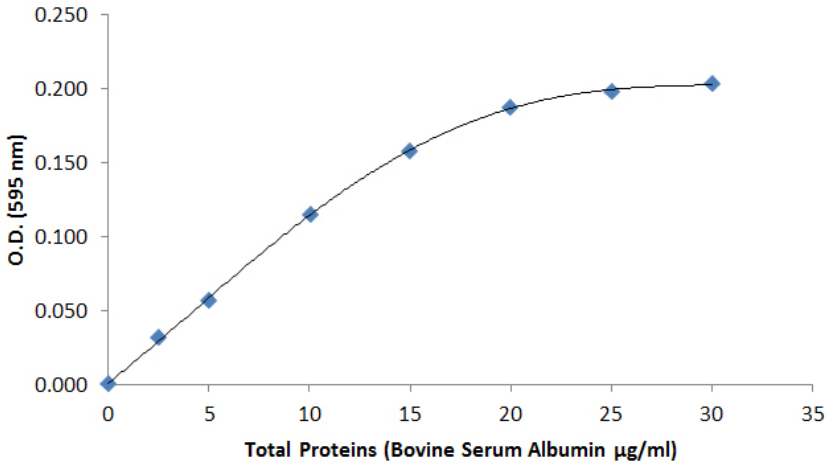

Fig.1: Reference curve of bovine serum albumin used in the experiment.

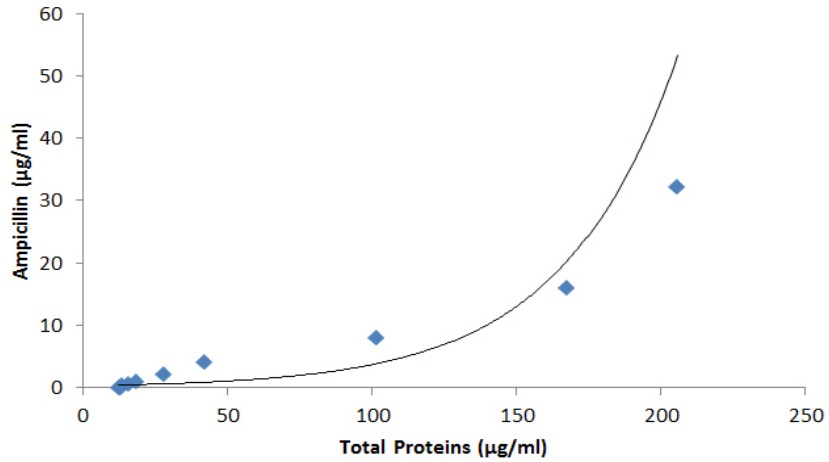

Fig.2: Total protein concentration in the culture media after exposure of E. coli strains to various concentrations of ampicillin.

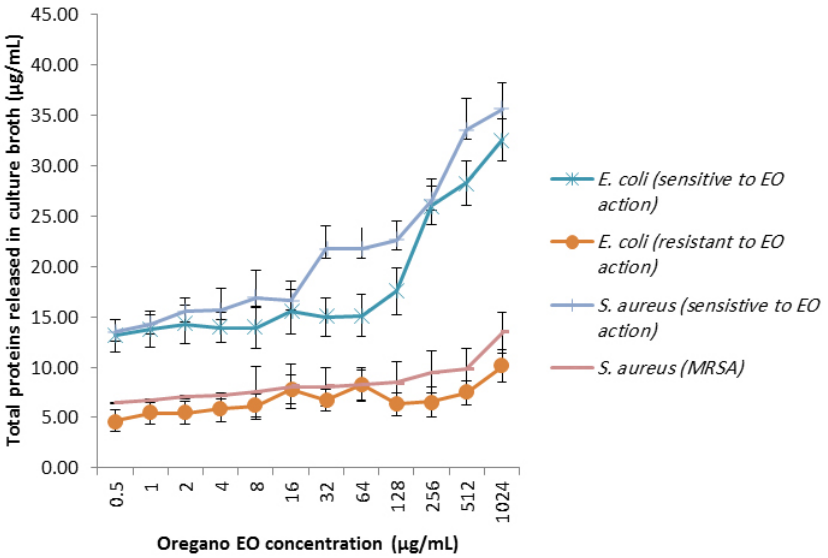

Fig.3: Mean total protein concentration in the culture media after exposure of E. coli (n=26), S. aureus (n=19) and S. aureus MRSA strains (n=5) to various concentrations of oregano essential oil.

Table 1: MIC and MBC of oregano Essential oil against clinical strains of E. coli (n=26) and S. aureus (n=24).

Strain	MIC (µg/mL)			MBC (µg/mL)		
	min	mean	max	min	mean	max
E. coli	128	256	1024	128	128	1024
S. aureus	64	128	512	32	64	512

Discussion

Essential oils as well as their major components are having a variety of targets on the bacterial cell where exhibiting their action. Various reports indicate that EOs can act by degrading the cell wall[13] or can damage the cytoplasmic membrane, causing cytoplasm coagulation[14]. Their detrimental effect on membrane proteins increase membrane permeability and ends up to leakage of the cell contents[15]. The reduction of both the proton motive force and the intracellular ATP content have also reported[16]. However the various reports, information on the specific type of action of various EOs is limited and the particular mechanisms involved are still not completely clarified[17]. In this study, the mode of action of oregano EO is investigated by using a simple approach which is the estimation of the total proteins in the culture media before and after the addition of increasing concentrations of EO. If the protein content of the micro plate cells contained the exposed to EOs bacteria increases more than in those cells contained the unexposed bacteria then it can be concluded that these proteins were the result of membrane disruption. It is also known that EOs or other components of the experimental mixture – besides the culturing media, does not contain any proteins[7-8,20] in order to consider any interferences or bias during the analysis.

The inhibition study showed that the EO of oregano tested was effective at various concentrations against *E. coli* and *S. aureus*. It was observed that the MIC and also the MBC values obtained are in accordance with most of other reports which found MIC values between 200-300 µg/mL[18-20], although lower values (31-40 µg/mL) are also reported as by Sahin *et al.,* [21] and Penalver *et al,*.[22] In the present study, Gram-negative bacteria were more resistant than the Gram-negative ones to the EO. This behavior is also

observed in previous reports and it is attributed to the more complex cell-wall of Gram-negative bacteria. According to Burt & Reinders[23] the Gram-negative cell-wall is impermeable to hydrophobic molecules as is the Gram-positive bacteria and in that manner the EOs are less able to easily affect the cell growth of the Gram-negative bacteria. However, MRSA strains in our study were proven resistant to the EO when compared with their non-MRSA counterparts (Fig 3). This could be the result of an analogous mechanism by which methicillin-resistant *Staphylococcus aureus* changed the proteins to which ɒlactam antibiotics binds and therefore decreasing their effectiveness at disrupting cell wall synthesis[24]. The most active compound of oregano essential oil is carvacrol, a natural phenol which can increase the permeability of cytoplasmic membrane[25]. As demonstrated by previous reports, carvacrol causes a structural and functional damage on microbial cells targeting on their membranes which results in an increased permeability. Studies on carvacrol's effectiveness on membrane permeability have confirmed such an action by monitoring either the efflux of H^+, K^+ and carboxyfluorescein or the ATP pool and the influx of nucleic acid[26-27]. In their experiments Gill and Holley (2006)[13] showed the release of ATP from carvacrol exposed *E. coli* strains and considered these results as an indication of non-specific permeabilization of the cytoplasmic membrane while other effects can be anticipated as a result of secondary membrane interactions. However, the mode EOs action depends mostly on their chemical composition (i.e chemotype), and their activity against bacteria is not attributed on a single mechanism but is the result of numerous reactions concerning the bacterial cell[25]. Based on this observation it is apparent the various sensitivity levels exhibited by our pathogens.

In conclusion, oregano EO is effective against clinical pathogens as *E. coli* and *S. aureus* with a mode of action targeting the bacterial membrane permeability or integrity. However, bacteria resistant to chemical antibiotics seem to be capable of overcoming the action of EOs which requires further investigation before the

EOs or their components could be utilized either in medicine or in food safety.

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Conflict Of Interest

All authors disclose any conflict of interest that can influence their work.

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