Available free online at www.medjchem.com

Mediterranean Journal of Chemistry

Mediterranean Journal of Chemistry **2020**, 10(7), 687-696

Chemical composition and biological evaluation of essential oils from some Moroccan plants

Badia Douhri [1], Ihssane Raissouni [2], Nadia Amajoud [1], Abdellah Farah [3], Nadia Skali Senhaji [1], Jamal Abrini [1] and Hikmat Douhri [2,*]

[1] Biology and Health Laboratory, Department of Biology, Faculty of Science, Abdelmalek Essaadi University, Po Box 2121, Tetuan, Morocco

[2] Materials and Interfacial Systems Laboratory, Department of Chemistry, Faculty of Science, Abdelmalek Essaadi University, Po Box 2121, Tetuan, Morocco

[3] Laboratory of Applied Organic Chemistry, Faculty of Sciences and Technology, Sidi Mohammed Ben Abdellah University, 30000 Fez, Morocco

Abstract: This study describes the chemical composition and biological activities of essential oils of the Moroccan plants *Thymbra capitata* (L.) Cav. and *Origanum elongatum* (Bonnet) Emb. & Maire on biofilms of *Staphylococcus aureus* strains. The qualitative and quantitative composition of the two essential oils was analyzed by gas chromatography and gas chromatography-mass spectrometry. The Minimum Inhibitory Concentration was determined by the broth microdilution method. The results showed that essential oils of *Thymbra capitata* and *Origanum elongatum* inhibit biofilm formation in biofilm inhibitory concentrations (0.5 and 2% v/v) and eradicate biofilm at biofilm eradication concentrations (1 and> 4% v/v), specific to each essential oil. Values were twofold or fourfold higher than the concentration required to inhibit or eradicate planktonic bacteria growth. The use of essential oils of *Thymbra capitata* and *Origanum elongatum* as natural antimicrobial agents may effectively inhibit the growth of biofilms of medical relevance. Results indicated that essential oils of *Thymbra capitata* and *Origanum elongatum* might be a potential alternative against Staphylococcus resistant strains.

Keywords: *Thymbra capitata*; *Origanum elongatum*; essential oils; chemical composition; biological activities; biofilm.

1. Introduction

The increase in the frequencies of multidrug-resistance due to the irrational use of antimicrobials or antibiotics has become a major public health issue rendering treatments against previously minor infections ineffective. Biofilm development could bring physiological changes that contribute to withstand the harsh environmental conditions and multiple antibacterial agents. These biofilms are involved in 60% of nosocomial infections [1] and severe food poisoning [2]. *Staphylococcus aureus* is Gram-positive bacteria, ubiquitous commensals of the skin and mucous membranes of humans and animals, reputed for their ability to form biofilms [3] and their genetic strategy to develop resistance to antibiotics. Infections caused by these bacteria are increasingly uncontrollable, and this because of the biofilm that protects them and allows them to survive in adverse environments [4,5], which makes them more challenging to eradicate and gives increased resistance to biocides and antibiotics. Therapeutically, the use of methicillin has been replaced by oxacillin [6,7]. Also, recently, there has been a growing interest in researching an alternative or adjacent method using essential oils or plant extracts [8]. Given its geographical position, Morocco is renowned for its botanical richness. Among the 7000 species and subspecies of the Moroccan plants, about 537 are endemic [9,10]. Ethnobotanical data on these plants have been indicated by several studies [9].

Oregano (*Origanum elongatum*, Lamiaceae), is an endemic species of Morocco [10]. It is an essential, versatile medicinal plant usually useful against respiratory infections, diarrhea, urinary tract infections, and it is also used for food preservation or as an aromatic plant for its flavor [9]. Thyme (*Thymbra capitata*, Lamiaceae), is one of the most common and widespread North African species [11]. It is mostly used to flavor food and culinary preparations and in folk medicine [10]. Many types of research have reported that there is a relationship between chemical compounds and antimicrobial activity of plants. Indeed, Nostro*et al* [12] have shown a significant antibacterial effect of the essential oil of *Origanum*

**Corresponding author: Hikmat Douhri*
Email address: douhri.enviro@gmail.com
DOI: http://dx.doi.org/10.13171/mjc10702007281485hd

Received May 27, 2020
Accepted June 30, 2020
Published July 28, 2020

vulgare L. against biofilm-grown *Staphylococcus aureus* and *Staphylococcus epidermidis* strains. Moreover, an intense antibacterial activity of *Origanum compactum* essential against *Escherichia coli K12* and *Bacillus subtilis 6633* were shown by Bouyahya*et al* [13]. On the other hand, Charfi*et al* [14] have also demonstrated that essential oil of *Thymbra capitata* had a significant antimicrobial effect on pomegranate juice natural flora (aerobic mesophilic bacteria, *Streptococcus thermophilus*, yeasts, and molds) allowing the preservation of the quality of the juice.

The aim of this study was to describe the chemical composition of two essential oils derived from Moroccan plants *Thymbra capitata* (L.) Cav. and *Origanum elongatum* (Bonnet) Emb. &Maire, and assess their antibiofilm effect.

2. Experimental

2.1. Plant material and essential oils extraction

The aerial part of the *Origanum elongatum* (Bonnet) Emb. & Maire and *Thymbra capitata* (L.) Cav. used in this study were collected in September 2014 from two different sites in the northwest of Morocco: Beni Aammart site, the Rif mountains, at an altitude of 1.240 meters (Al Hoceima, Morocco) and site Nekkata, the Rif mountains, at an altitude of 65 meters (Tetuan, Morocco). Plant material has been authenticated by the taxonomist, Professor Mohamed Kadiri (Laboratory of Algology and Mycology, Department of Biology, Faculty of Science, Abdelmalek Essaadi University, Tetuan, Morocco).

Aerial parts were dried at room temperature and under dark condition. After that, the extraction of essential oils was carried out by the hydrodistillation method using a Clevenger-type apparatus. Essential oils were then stored at a temperature between 2°C and 4°C until use.

2.2. Chemical composition

The oils were analyzed to determine the qualitative and quantitative composition using gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS).

2.2.1. Gas chromatography analysis (GC)

GC analyses were performed on a Hewlett-Packard (HP 6890) gas chromatography (FID), equipped with an HP-5 capillary column (30 m x 0.25 mm x 0.25 µm). The temperature was programmed from 50°C after 5 min initial hold to 250°C at 4°C/min. The conditions for Gas Chromatography were as follows: N2 as carrier gas (1.8 ml/min); the split mode was used (Flow: 72.1ml/min, ratio: 1/50); the temperature of injector and detector was 275°C. The machine was managed by a computer system of the « HP ChemStation » type, administering the machine operation and allowing to follow the progress of the chromatographic analyses. Diluted samples (1/50 in hexane) of 1.2 µl were injected manually.

2.2.2. Gas chromatography-mass spectrometry (GC-MS)

GC/MS analyses were performed on a Hewlett-Packard equipped with an HP-5MS (Crosslinked 5% PHME Siloxane) capillary column (30 m x 0.25 mm i.d, 0.25 µm film thickness) and coupled with a mass spectrometer (HP 5973). The temperature has been set from 50 to 250°C at 2°C/min. The carrier gas was He (1.5 ml/min) and used split mode (Flow: 112 ml/min, ratio: 1/74.7). The different compounds were confirmed by reference to their MS identity (Library of NIST98 Spectra). MS operating parameters were: ionization voltages 70eV, ion source temperature 230°C, scan mass range 35-450 amu.

2.3. Cell culture

Three different *S. aureus* strains were used in this study: *S. aureus* MBLA, *S. aureus* CECT 976, and *S. aureus* ATCC 25923. The bacterial strains were stored in glycerol at - 80°C, grown and maintained in Tryptone Soy Agar medium (TSA), and in Tryptone Soya Broth (TSB), then incubated at 37°C in a shaking incubator for 18 hours before use.

2.3.1. Determination of minimum inhibitory and minimum bactericidal concentrations

The effectiveness of essential oils was estimated by determining The Minimum Inhibitory Concentration (MIC) on planktonic cells using the microdilution technique following the guidelines of the Clinical Laboratory Standards Institute [15] and the micro-broth dilution method according to Nostro *et al.* [12]. Bacterial culture was grown in TSB medium at 37°C.

The wells, with a clear bottom, of a 96-well polystyrene microtiter plate (Costar, # 3603, Corning Acton, MA) were filled with 100µl of TSB medium and EO which concentrations ranging from 0.0625% to 4%. Tween 80 was used to facilitate the solubility of EO in the medium. TSB medium served as a positive control, and TSB supplemented with EO served as a negative control. The plate was incubated at 37°C for 24 h. All tests were performed in triplicate. The MIC is defined as the lowest concentration of oil able to inhibit the growth of bacterial inoculum. After incubation, the optical densities of the wells were determined at 570 nm. Aliquots from wells with no growth were plated onto TSA and incubated at 37°C for 24 h. The Minimum Bactericidal Concentration (MBC) was determined to be the lowest concentration that showed no growth on TSA.

2.3.2. Biofilm formation

Quantification of the biofilm was according to the method described by Christensen et *al.* [16]. Bacterial

culture was prepared in TSBG medium.100 µl of this suspension was used to inoculate a sterile well polystyrene microtiter plate with a transparent bottom (Costar, # 3603, Corning Acton, MA). Then the microplate was incubated at 37°C to allow the development of the biofilm. During the incubation time, the medium was replaced every 8 to 10 h with fresh TSBG. After 24 h, the wells were washed gently with phosphate-buffered saline (PBS). The plates were air-dried, and the individual wells were stained with 125 µl of 0.2% crystal violet for 10 minutes at room temperature then rinsed three times with sterile distilled water. An ethanol/acetone mixture (80:20 v/v) was added to dissolve the rest of the dye. Absorbance was measured with a micro-ELISA auto reader at 492 nm. Each assay was performed in quadruplicate.

2.3.3. Determination of inhibitory and eradication concentrations

The assay for the formation of biofilm in a sterile 96-well clear bottom (Costar # 3603, Corning Acton, MA) was carried out according to the method described by Christensen *et al.* [16] and by Kärpänen *et al.* [17]. A serial dilution of EO was added in TSB medium with inoculum. TSB medium served as a positive control, and TSB supplemented with EO served as a negative control. After incubation at 37°C, the medium was gently removed, and the wells were washed three times with PBS at physiological pH. Biofilm inhibitory concentration (BIC) was determined to be the lowest concentration without visually apparent growth occurring in wells after 24 h incubation at 37°C, confirmed by the absence of an increase in optical density compared with the initial reading. Growth was estimated by reading absorbance at 492 nm. Biofilm samples from the bottom of the wells were scraped using a sterile scraper, spread over the surface of TSA previously poured into sterile Petri dishes and incubated for 24 h at 37°C. This test was carried out in five repetitions. The Biofilm Eradication Concentration (BEC) was determined to be the lowest concentration at which no bacterial growth occurred on the TSA plates. All tests were performed in duplicate.

2.4. Effect of essential oils application

The effect of different concentrations of EO (MIC/16; MIC/8; MIC/4; MIC/2 and MIC) on the biofilm formation capacity was tested on flat-bottomed polystyrene microtitre plates as described by Cramton *et al.* [18] and Nostro et *al.* [12] with some modifications. The culture was used to inoculate TSB medium supplemented with 0.25% glucose and 0.1% Tween 80 (TSBG), incubated at 37°C for 24 h. Diluted to 10%, were distributed in each well of 96-well flat-bottomed polystyrene microtitre plates containing HE or just medium (for the control).

The plate was incubated without agitation to allow the cells to attach to the surface. After incubation at 37°C, each well was washed twice with sterile PBS, dried, and then crystal violet was added to each well. After washing with distilled water, the optical density (OD) at 492 nm was measured using ELISA microplate reader. The positive control corresponds to TSB medium, and negative control corresponds to TSB supplemented with EO. Each test was performed in duplicate and repeated at least three times. The relative biofilm formation was determined by the percentage rate using the following equation:

$$\%\mathrm{RBF} = \frac{\text{Experimental well absorbance } OD_{492\,\mathrm{nm}}}{\text{Control well absorbance } OD_{492\,\mathrm{nm}}} \times 100$$

Effect of the essential oils of *T. capitata* and *O. elongatum* on established biofilms was tested as described by Nostro *et al.* [12], with some modifications. All strains were incubated in each well of 96-well polystyrene flat-bottomed microtitre plates at 37°C. After 24 h, the bacterial suspensions were carefully aspirated, and the wells were rinsed three times with PBS to remove non-adherent bacteria, then filled with a serial dilution f EO from 8 MIC to MIC / 4 even for negative control. For positive control, EO was replaced by TSBG. The OD was measured at $t_{0,}$ and the microplates were incubated at 37°C for 24 h. Biofilm inhibitory concentration (BIC) was determined to be the lowest concentration without visually apparent growth occurring, and it is confirmed by no increase in OD compared with the initial reading. Two biofilm controls consisting of TSB medium for positive control and TSB medium added of EO for negative control were used. The percentage of biofilm eradication (% BE) in comparison with untreated wells (positive control) was calculated using the following equation:

$$\%\mathrm{BE} = \frac{\mathrm{OD}_{\text{at t0}} - \mathrm{OD}_{\text{(after tre atment)}}}{\mathrm{OD}_{\text{(at t0)}}} \times 100$$

2.5. Statistical analysis

The data were analyzed by one-way ANOVA followed by Duncan's test using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, USA). All experiments were performed in duplicate and repeated three times. The statistical significance was set at 0.05, and the results are expressed as the mean± SEM.

3. Results and Discussion

3.1. Identification of compounds

The chemical composition of essential oils of *T. capitata* and *O. elongatum* analyzed with GC and summarized in Table 1. In the *T. capitata* EO a higher percentage of carvacrol (70.92%) than thymol (0.38 %) was noted. However, these percentages were reversed in the *O. elongatum* EO (35.51% of thymol and 6.52% of carvacrol, respectively). Several studies have reported the chemical composition of *T. capitata* and *O. elongatum* EO.

Our results agree with those found by Bakhy et *al.* [11] that highlighted carvacrol (68.2%-85.9 %) as a major component and thymol (0.1-0.3%) in the essential oil of *Thymbra capitata*. Furthermore, Salas et *al.* [19] have also demonstrated that carvacrol was a significant component with percentages higher than or equal to 66% in the essential oil of Spanish populations of *Thymbra capitata.* Similar results were found by Moukhles et *al.* [20,] who reported that carvacrol (85.35%) was a major component in the pure essential oil of *Thymbra capitata*.

Table 1. Chemical composition of essential oils of *T. capitata* and *O. elongatum.*

KI	Compounds	*O. elongatum* %	*T. capitate* %
926	α-Thujene	0.43	1.92
939	α-Pinene	0.42	0.78
953	Camphene	-	0.14
978	1-Octene-3-ol	0.12	-
980	β-Pinene	0.32	2.48
986	p-Menthene - 3	0.04	-
991	β-Myrcene	2.12	-
1005	α-Phéllandrene	0.31	0.31
1010	δ-3-Carene	0.03	1.39
1018	α-Terpinene	2.82	-
1026	**p-Cymene**	**11.13**	**6.34**
1031	Limonene	0.51	-
1033	1.8-Cineole	0.02	-
1050	E-β-Ocimene	0.12	-
1062	**γ-Terpinene**	**26.72**	**4.92**
1074	Cis- oxyde de linalol	0.29	-
1088	Terpinolene	0.09	0.14
1098	Linalool	1.46	3.38
1116	Exo-Fenchol	1.65	-
1165	Borneol	0.08	0.93
1177	Terpin-4-ol	0.27	-
1189	α-Terpineol	0.08	-
1202	Trans-Dihedron carvone	0.18	-
1287	p-Cymen-7-ol	0.07	-
1290	**Thymol**	**35.51**	**0.38**
1298	**Carvacrol**	**6.52**	**70.92**
1418	β-Caryophyllene	3.21	3.57
1439	Aromadendrene	0.23	-
1454	α-Humulene	0.18	-
1513	γ -Cadinene	0.12	-
1524	δ-Cadinene	0.03	-
1556	Germacrene	0.04	-
1581	Oxyde de caryophyllene	0.23	-
1583	Globulol	0.12	-
	Total	**95.47**	**98.08**

KI: Identification based on kovats indices

On the other hand, our results are not in accordance with those found by Oualili et *al.* [21], who reported that carvacrol (60.42%) was a major component in the *O. elongatum* EO and thymol (0.60%) only. In

addition, Boukhira et *al.* [22] also highlighted carvacrol (63.06%) as a primary compound in the *O. elongatum* EO, while no thymol was present. These variations in the results concerning the chemical composition of *O. elongatum* EO are probably due to the variability of ecological conditions between the areas of the collection.

3.2. Bacterial biofilm formation

Staphylococci are frequently the most responsible bacteria for infections initiated by foreign bodies (intravascular catheters, bone, and joint prostheses, prosthetic heart valves, urinary catheters, or even contact lenses). These infections are a major cause of morbidity for public health and also one of the leading reasons for nosocomial bloodstream infections, causing significant mortality [23, 24].

Attachment of bacteria to solid surfaces is the initial stage of biofilm formation. Sessile bacteria exhibit physiological characteristics that are distinct from those of their planktonic counterparts in a liquid medium. Sessile bacteria form colonies overlapping organic polymers (composed of proteins and polysaccharides), which tend to cluster in communities. Then they form biofilms found on the mucosal surface, on the dental plaque, on the prosthesis, and various medical devices. The inhibition of attachment of these cells can be the starting point to eradicate the biofilm and combat phenomenon of antimicrobial resistance [25].

Figures 1 and 2 show the formation of biofilms by the three reference strains: S. aureus MBLA, S. aureus CECT 976, and S. aureus ATCC 25923. All these tested strains were able to form biofilms with different OD. S. aureus MBLA is the most strain biofilm-producing with an OD 0.994, S. aureus ATCC 25923 with an OD 0.804 whereas S. aureus CECT 976 with an OD 0.701 against OD 0.051 of the negative control. Arguably S. aureus MBLA is the most biofilms forming bacteria compared to the other two staphylococci**.**

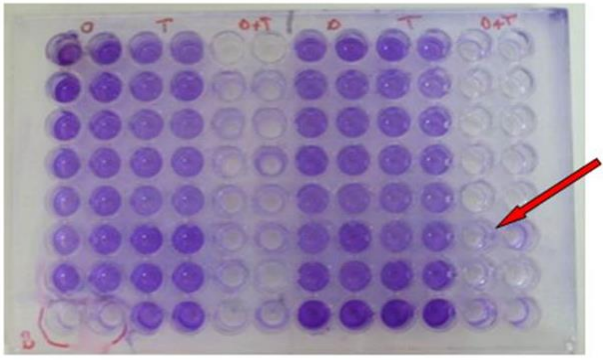

Figure 1. Biofilm formed in flat-bottom microtiter plate

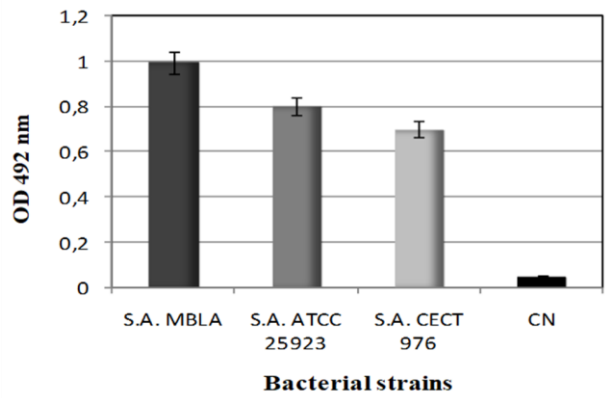

Figure 2. Absorbance (OD 492 nm) of the biofilm of the three reference strains (*S. aureus* MBLA, *S. aureus* ATCC 25923, *S. aureus* CECT 976 and CN = Negative Control)

3.3. Determination of MIC, MBC, BIC, and BEC

In this study, we evaluated the effect of two essential oils, essential oil of *T. capitata* and *O. elongatum* on Staphylococci and biofilms. From the results obtained (Table 2), we note that the MIC of the two tested EO varies between 0.25 and 0.5% v/v for the three strains, while the BIC biofilm varies between 0.5 and 2% v/v. Ditto for MBC of the two tested EO, which vary between 0.5 and 1% v/v, while the BEC varies between 1 and >4% v/v. These concentrations were determined on average over two repetitions.

Table 2. Sensitivity of planktonic cells and biofilm to the essential oils of *T. capitata* (TC) and *O. elongatum* (OE).

Bacterial strains	Essential oils	MIC (% v/v)	BIC (% v/v)	MBC (% v/v)	BEC (% v/v)
S. aureus MBLA	TC	0.25	0.50	0.50	1.00
	OE	0.50	2.00	1.00	>4.00
S. aureus CECT 976	TC	0.25	0.50	0.50	1.00
	OE	0.50	1.00	0.50	2.00
S. aureus ATCC 25923	TC	0.50	1.00	1.00	2.00
	OE	0.50	2.00	1.00	>4.00

Remarkably, the essential oils tested interfered with the formation of biofilms and planktonic growth, but these biofilms were significantly more resistant to the action of EO (Table 2). As it is known, the minimum inhibitory concentration (MIC) is defined as the lowest concentration of the EO, which will inhibit the growth of planktonic cells, specific for each oil. On the other hand, biofilm formation was inhibited with a minimum biofilm inhibition concentration (MBIC), which is higher than the MIC (BIC> MIC).

For the bactericidal effect, bacteria were eradicated in a specific MBC for each EO, which is lower than the concentration of the biofilm eradication (BEC) BEC>MBC (Table 2).To calculate the BEC, we had to scrape the biofilm samples from the bottom of wells plates when there were more bacteria in suspension or the culture medium.

Spreading on the surface of a solid medium and incubated for 24 hours at a temperature of 37°C reveals bacterial colonies, hence the idea of a survival linked to biofilm and not only to floating plankton. These results are consistent with other studies that have also reported the increased biofilm resistance to antimicrobial every day [26-30]. We can deduce that at an MBC, the essential oils were less effective in eradicating bacterial biofilms.

3.4. Effect on biofilm formation

For tested strains, a reduction in biofilm formation is observed in the treated wells compared to the positive control. Even at concentrations below the MIC, a reduced level of biofilm formation has been found, which results in a final OD below the positive control. We note that the biofilm formation was significantly reduced in a dose-dependent manner with a significant difference ($P < 0.05$, Duncan test) (Table 3). The inhibitory effect of biofilm formation is more visible with *T. capitata* EO then with *O. elongatum* EO (Table 3). The bacteria that were most capable of producing biofilm (*S. aureus* MBLA) were less sensitive to the effect of EO (OD: 0.145 for EO *T. capitata*) by contributing to bacteria that produce less biofilm such as *S. aureus* CECT 976 (OD: 0.075 for EO *T. capitata*). Or in other words, it is more resistant to the strong inhibitory effect of the essential oil on its biofilm formation than other bacteria. If we have already mentioned that the inhibition of biofilm by EO is dose-dependent, it can be expressed differently by the relative composition of a biofilm depending on the concentration of EO. Thus, with the reduction in the concentration of EO, it was a decrease in the inhibition of biofilm formation or an increase in the relative biofilm formation (Table 3 and 4). Therefore, this percentage is dose-dependent.

Table 3. Effect of EO of *T. capitata* (TC) and *O. elongatum* (OE) on biofilm formation.

Bacteria	EO	Absorbance ($OD_{492\ nm}$)					
		MIC	0.5 MIC	0.25 MIC	0.125 MIC	0.0625 MIC	Control (+)
S. aureus MBLA	TC	0.145 ± 0.007	0.192 ± 0.002	0.306 ± 0.022	0.455 ± 0.007	0.560 ± 0.014	0.601 ± 0.001
	OE	0.255 ± 0.007	0.307 ± 0.018	0.422 ± 0.002	0.504 ± 0.013	0.595 ± 0.014	0.635 ± 0.005
S. aureus CECT976	TC	0.075 ± 0.007	0.122 ± 0.002	0.343 ± 0.004	0.474 ± 0.006	0.536 ± 0.005	0.540 ± 0.014
	OE	0.105 ± 0.006	0.245 ± 0.007	0.381 ± 0.001	0.498 ± 0.003	0.556 ± 0.006	0.603 ± 0.004
S. aureus ATCC25923	TC	0.083 ± 0.004	0.133 ± 0.004	0.240 ± 0.014	0.351 ± 0.001	0.454 ± 0.004	0.495 ± 0.007
	OE	0.142 ± 0.002	0.215 ± 0.007	0.400 ± 0.014	0.415 ± 0.007	0.489 ± 0.001	0.533 ± 0.004

Biofilm formation is estimated by measuring the absorbance at 492 nm

Values are expressed as the mean of two replicates ± standard deviation.

Table 4. Percentage of relative biofilm formation (% RBF) under the effect of two different concentrations of the tested EO.

Bacteria	EO	% Relative Biofilm Formation *				
		MIC	0.5 MIC	0.25 MIC	0.125 MIC	0.0625 MIC
S. aureus MBLA	TC	18.102 ± 0.883	23.908 ± 0.265	38.140 ± 2.737	56.804 ± 0.883	69.913 ± 1.766
	OE	31.308 ± 0.868	37.692 ± 2.257	51.750 ± 0.260	61.878 ± 1.563	73.051 ± 1.736
S. aureus CECT976	TC	10.186 ± 0.351	15.093 ± 0.264	42.547 ± 0.439	58.882 ± 0.703	66.522 ± 0.615
	OE	12.272 ± 0.747	28.773 ± 0.830	44.745 ± 0.166	58.485 ± 0.332	65.238 ± 0.747
S. aureus ATCC25923	TC	10.049 ± 0.431	16.139 ± 0.431	29.233 ± 1.723	42.692 ± 0.086	55.238 ± 0.431
	OE	16.936 ± 0.254	25.733 ± 0.846	47.876 ± 1.693	49.671 ± 0.846	58.528 ± 0.169

* The biofilm formation was calculated as follows:

RBF% = (mean of $OD_{492\ nm}$ of treated well / mean $OD_{492\ nm}$ of control wells) x100

Values are expressed as the mean of two replicates ± standard deviation (repeated three times)

3.5. Effect on established biofilms

According to the obtained results, there was a significant reduction in the biofilm already formed by the three bacterial strains as a result of EO of *T. capitata* and *O. elongatum* effect at different concentrations, ranging from 8 MIC to MIC/4. The results indicating this eradication of biofilm are illustrated in Figure 3, compared to the positive control. By increasing the concentration of essential oil, the percentage of biofilm eradication is increased, and vice versa. Based on these results, we note that for the three strains, EO of *T. capitata* has more power to eradicate biofilm than EO of *O. elongatum*.

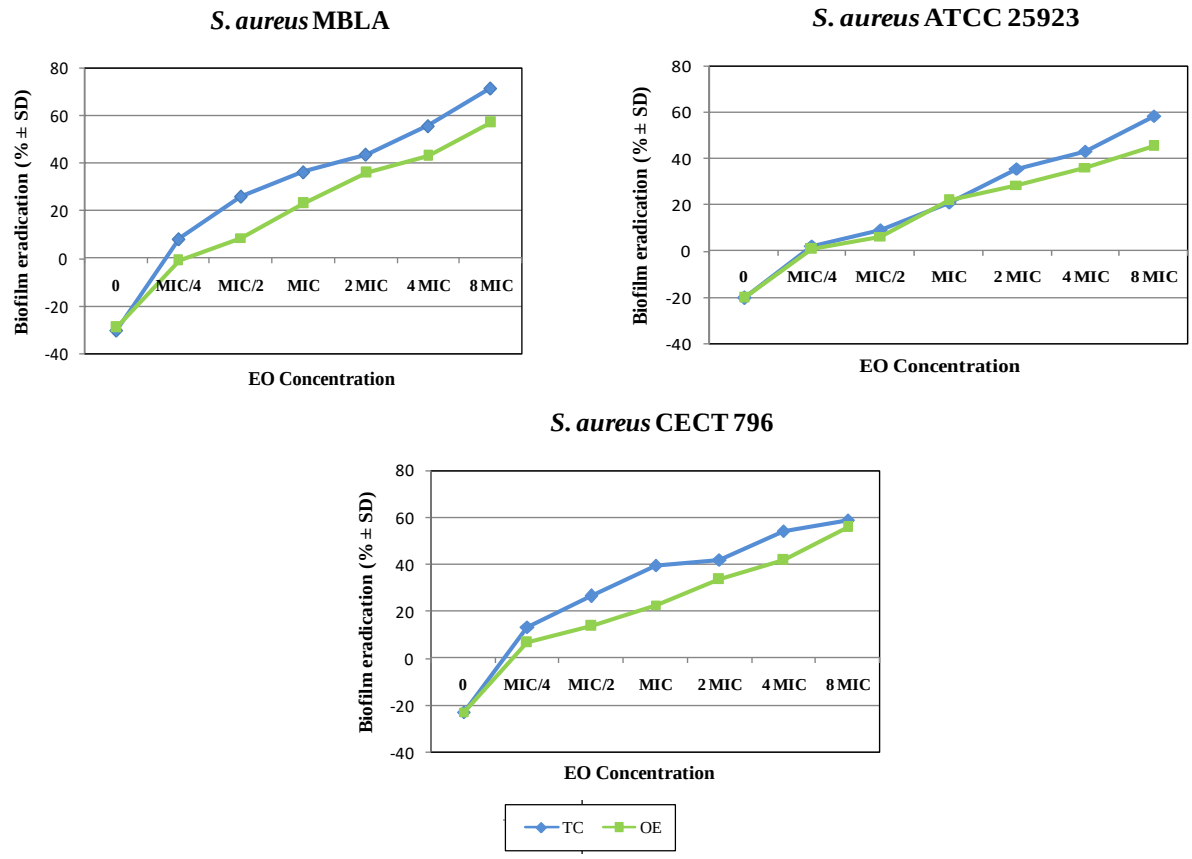

Figure 3. Eradication of biofilm formed by *S. aureus* MBLA, *S. aureus* ATCC 25923, and *S. aureus* CECT 976, after treatment with the essential oil of *T. capitata* and *O. elongatum* at different concentrations. Each value is the mean ± SD from three independent experiments performed as duplicate.

The six curves (Figure 3) show the variation in the percentage of biofilm eradication formed by *S. aureus* MBLA, *S. aureus* ATCC 25923, and *S. aureus* CECT 976 under the effect of EO of *T. capitata* and *O. elongatum* at various concentrations relative to the positive control. From the results, we can see negative values (% <0). Thus, for *S. aureus* CECT 976 at a concentration 8 MIC, the percentages

of biofilm eradication of two EO *T. capitata*, and *O. elongatum* were respectively 71.42% and 57.25%, for a concentration MIC, the percentages are 36.23% and 23.21%. In contrast, for a concentration MIC/4, percentages are 8.12% and -0.72%. For S. *aureus* MBLA at a concentration 8 MIC, the percentages of biofilm eradication of two EO *T. capitata* and *O. elongatum* were respectively 58.74% and 55.88%, while for a MIC/4 concentration, the percentages of 13.30% and 6.52%. Similar results were also observed for *S. aureus* ATCC 25923.

In general, the percentage of biofilm eradication of *S. aureus* MBLA, the most secreting of biofilm, is lower than that of *S. aureus* ATCC 25923 followed by *S. aureus* CECT 976 for the two EO. Whereas, for the positive control (without EO) percentage varies between -30.20% and -23.24% on average for all three cultures. The percentage of biofilm eradication increases with the increase of the concentration of EO, and this percentage is the highest for the EO of *T. capitata*.

The essential oil of *T. capitata* showed a more significant effect on *S. aureus* ATCC 25923 and *S. aureus* CECT 976 on *S. aureus* MBLA biofilm, respectively, knowing that this EO is rich in Carvacrol (70.92%). This can only be explained by the fact that even if the three bacteria of the same species of staphylococci but of different types, their formative capacity of power varies. This form of resistance "biofilms" to EO has been attributed to the presence of an exopolysaccharide matrix, to the slow growth of bacteria and spatial heterogeneity and physiological specificity of the biofilm, but this is not a unique feature of biofilm cells [28-30]. Until Ashbey et *al.* [31] and Lewis [23] and many authors have been able to give more meaning to this bacterial lifestyle, this form of resistance has remained mysterious until nowadays. They explained that there are two bacterial lifestyles, a free-living planktonic and biofilm lifestyles. And this planktonic lifestyle is, in reality, only a transitional stage for the passage from a biofilm to another or from a planktonic life to a sedentary life, the life of "persistent bacteria," also called "sleeper cells". These form cellular clusters which have multi-resistance to bactericidal agents, such as in this case, essential oils. These bacteria are subject to phases of abundance and nutritional restriction. At this stage, they can neither multiply nor die. Still, they are responsible for an escape and resistance phenomenon, hence the reluctance to infections *in vivo* and their recurring nature caused by their established biofilms. This explains why the biofilm is responsible for several infections in general and for nosocomial infection, in particular [32].

All the resistance mechanisms essentially consist in preventing the bactericidal agent from reaching the target. However, this tolerance works by modifying these targets. Lewis, in 2004 [23], was able to isolate the persistent bacteria, study their actions and reveal their secret to escape the harmful environmental factors (desiccation, parasitism, presence of antibiotics, antiseptics or disinfectants). Following this work, he was able to deduce that even at very high doses of antibiotics, these cells seemed invulnerable while all the floating bacteria disappeared. Subsequently, the author was able to unveil a bacterial gene expression, which can lead the bacteria to block their own functions such as translation. One of the antibiotic actions is the interruption of the translation and, therefore, the inhibition of DNA replication hence its bactericidal power. If these bacteria are able to block translation by themselves, it will stop other cellular functions and remain asleep, such as how they can prevent the attack of antibiotics, corrupt their targets, and give rise to more persistent bacteria. These bacteria, also called "survivors", capable of regenerating the original population (planktonic cells produced by the biofilm), are therefore variants of wild type phenotypes and not mutant phenotypes.

In our study, *S. aureus* MBLA strain is the most productive biofilm with the highest OD, which means that it secretes more amount of exopolysaccharides than the other two bacteria (*S. aureus* CECT 976 and *S. aureus* ATCC 25923) which makes it more protected against the bactericidal effects of the two. It was noted that the EO of *O. elongatum*, which is rich in thymol (35.51%) has a less inhibitory effect on *S. aureus* MBLA than the two other bacteria (Tables 3 and 4). We could say that carvacrol, which is the major component of the essential oil of *T. capitata* with a percentage of 70.92% has a greater effect on the biofilm than thymol. This fact has been mentioned in other studies by Knowles et *al.* [33] and Nostro et *al.* [12]. But Lambert et *al.* [34] and Juven et *al.* [35] have shown in their research that thymol binds to membrane proteins and increases the permeability of bacterial cell membrane by ion leakage, ATP, and the same nucleic acid and therefore impending death. Other studies have also suggested that the volatile compound is responsible for the inactivation of enzymes, including those involved in the production of energy and synthesis of cellular components [36]. From a serial dilution f EO from 8 MIC to MIC/4, the percent reduction of biofilm decreases. Knowles et *al.* [33], have shown that carvacrol selectively inhibits *S. aureus* during the early stages of biofilm formation. In another study, Nostro et *al.* [37] reported that low concentrations of carvacrol have good antibacterial and anti-biofilm effects, which is correlated with our results. The work by Zodrow et *al.* [38] has demonstrated that carvacrol can be used at low concentrations as a natural additive in polymer coatings for indwelling devices to delay colonization by bacteria. However, it should be noted that carvacrol and thymol are only a fraction of all the compounds of essential oil, without forgetting the synergistic or competitive effect with the other compounds [34].

The negative values (% <0) of the percentage of biofilm eradication indicate that there is no more biofilm eradication if we reduce the EO concentration below the threshold. However, if we exceed this concentration, there is more production of biofilm between time t0 and t24 h (Figure 3). Whereas, for the positive control (without EO), the percentage is less than 0 (% <0) because there is no antibiofilm agent in the culture, and the growth is normal. So, if we take the CMB for one of the two oils tested, there has been bacterial cell death, but this value is still far from the CEB. The death and destruction that took place were of planktonic bacteria, while the persistent bacteria are still present in the biofilm matrix and await an opportunity to resume growth. It is the most common cause of recurrent infections after quantitatively and qualitatively adequate antibiotic therapy. If the antibiotics cannot penetrate the biofilm depth and do not act on these cells in question, even at increasing doses, we can reach toxic and even fatal doses before hitting the target.

Nosocomial infections are nowadays a major public health problem and one of the main causes of death in hospitalized patients. They are mainly due to biofilms, which are a lifestyle and a bacterial resistance mode. However, current therapies to treat and prevent chronic mediation in biofilm infections are limited, hence the recent interest in looking for a natural, non-invasive alternative, which minimizes the risk of increased bacterial resistance.

4. Conclusion

Our results revealed that the essential oils of *T. capitata* and *O. elongatum* are rich in many phytochemical components with bioactive effects. They can eradicate the established biofilm and to interfere with the formation of biofilms during the growth of planktonic bacteria. These results are encouraging and underline the promising role of essential oils of *T. capitata* and *O. elongatum* as alternatives and/or adjuvants to common antibiotics often used, such as new natural antibacterial agents in general and antibiofilm agents in particular. Therefore, other additional experiments must be carried out in this way.

References

1- J.W. Costerton, P.S. Stewart, E.P. Greenberg, Bacterial biofilms: a common cause of persistent infections. Science, **1999**, 284, 1318-1322.
2- A.L. Spoering, K. Lewis, Biofilms and planktonic cells of *Pseudomonas aeruginosa* have similar resistance to killing by antimicrobials, J. Bacteriol., **2001**, 183, 6746-6751.
3- A.S. Fauci, E. Braunwald, K.J. Issekbacher, J.D. Wilson, J.B. Martin, D.L. Kasper, S.L. Hauser, D.L. Longo, Editors. Harrison's Principles of Internal Medicine, McGraw-Hill New York, **1998**.
4- M. Chen, J. Cheng, Q. Wu, J. Zhang, Y. Chen, H. Zeng, Q. Ye, S. Wu, S. Cai, J. Wang, Y. Ding, Prevalence, potential virulence, and genetic diversity of *Listeria monocytogenes* isolates from edible mushrooms in Chinese markets, Front. Microbiol., **2018**, 9, 1711.
5- M. Simões, L.C. Simões, M.J. Vieira, A review of current and emergent biofilm control strategies, Food Sci. Technol., **2010**, 43, 573-583.
6- V. Sharma, S. Sharma, D.K. Dahiya, A.Khan, M. Mathur, A. Sharma, Coagulase gene polymorphism, enterotoxigenecity, biofilm production, and antibiotic resistance in *Staphylococcus aureus* isolated from bovine raw milk in northwest India, Ann Clin. Microbiol. Antimicrob. **2017**, 16, 65.
7- K.K. Kumarasamy, M.A. Toleman, T.R. Walsh, J. Bagaria, F. Butt, R. Balakrishnan, U. Chaudhary, M. Doumith, C.G. Giske, S. Irfan, P. Krishnan, A.V. Kumar, S. Maharjan, S. Mushtaq, T. Noorie, D.L. Paterson, A. Pearson, C. Perry, R. Pike, B. Rao, U. Ray, J.B. Sarma, M. Sharma, E. Sheridan, M.A. Thirunarayan, J. Turton, S. Upadhyay, M. Warner, W. Welfare, D.M. Livermore, N. Woodford, Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study, Lancet Infect. Dis., **2010**, 10, 597-602.
8- N. El Moussaoui, G. Sanchez, K. El Ouardy, M. Idaomar, A. Ibn Mansour, J. Abrini, R. Aznar, Antibacterial and antiviral activities of essential oils of northern Moroccan plants, Br. Biotechnol. J., **2013**, 3, 318-331.
9- J. Bellakhdar, Ibis Press. Paris, **1997**, 764.
10- A. Benabid, Édition Ibis press, Paris, France, 2000, 236.
11- K. Bakhy, O. Benlhabib, C. Al Faiz, A. Bighelli, J. Casanova, F. Tomi, Wild *Thymbra capitata* from Western Rif (Morocco): essential oil composition, chemical homogeneity and yield variability, Nat. Prod. Commun., **2013**, 8, 1155-1158.
12- A. Nostro, A. Sudano Roccaro, G. Bisignano, A. Marino, M.A. Cannatelli, F.C. Pizzimenti, P.L. Cioni, F. Procopio, A.R. Blanco, Effects of oregano, carvacrol and thymol on *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms, J. Med. Microbiol., **2007**, 56, 519-523.
13- A. Bouyahya, J. Abrini, N. Dakka, Y. Bakri, Essential oils of *Origanum compactum* increase membrane permeability, disturb cell membrane integrity, and suppress quorum-sensing phenotype in bacteria, J. Pharm. Anal., **2019**, 9, 301-311.
14- S. Charfi, N Boujida., N. El Moussaoui, J. Abrini, N. Skali Senhaji, *Thymbra capitata* Essential Oil Use to Preserve Physicochemical and Microbiological Qualities of Pomegranate Juice, *Food Sc. Technol. Res.*, **2019**, 2, 257-263.

15- R. F. Cockerill, M.A. Wikler, J. Alder, M.N. Dudley, M. Eliopoulos, M. J. Ferraro, J. Hardy, D.W. Hecht, J. A. Hindler, Clinical and Laboratory Standards Institute. Method for dilution antimicrobial susceptibility tests for bacteria that grow aerobically approved standard-seventh edition. Clinical and Laboratory Standards Institute document M7-A7, **2006**, 26, 1-49.
16- G.D. Christensen, W.A. Simpson, J.J. Younger, L.M. Baddour, F.F. Barrett, D.M. Melton, D.H. Beachey, Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices, J. Clin. Microbiol., **1985**, 22, 996-1006.
17- T.J. Kärpänen, T. Worthington, E.R. Hendry, Antimicrobial efficacy of chlorhexidine digluconate alone and in combination with eucalyptus oil, tea tree oil and thymol against planktonic and biofilm cultures of *Staphylococcus epidermidis*, J. Antimicrob. Chemother., **2008**, 62, 1031-1036.
18- S.E. Cramton, C. Gerke, N.F. Schnell, W.W. Nichols, F. Götz, The intercellular adhesion (ica) locus in *Staphylococcus aureus* and is required for biofilm formation, Infect. Immun., **1999**, 67, 5427-5433.
19- B.J. Salas, R.T. Tellez, P.J.M. Alonso, V.F.M. Pardo, M. Capdevila, G. Rodriguez, Chemical composition and antioxidant activity of the essential oil of *Thymbra capitata* (L.) Cav. in Spain, Acta Bot. Gallica., **2010**, 157, 55-63.
20- A. Moukhles, A. Ibn Mansour, A. Ellaghdach, J. Abrini, Chemical composition and *in vitro* antibacterial activity of the pure essential oils and essential oils extracted from their corresponding hydrolats from different wild varieties of Moroccan thyme, J. Mater. Environ. Sci., **2018**, 9, 1, 235-244.
21- H. Oualili, R. Nmila, F. Chibi, M. Lasky, A. Mricha, H. Rachid, Chemical composition and antioxidant activity of *Origanum elongatum* essential oil, Phcog. Res., **2019**,11, 283-289.
22- S. Boukhira, F. Bousta, S. Moularat, A. Abdellaoui, Z. Benziane Ouaritini, D. Bousta, Evaluation of the Preservative Properties of *Origanum elongatum* Essential Oil in a Topically Applied Formulation Under a Challenge Test, Phytothérapie, **2018**, 1-7.
23- K. Lewis, Persistor Cells, and the Riddle of Biofilm Survival, Bioch. (Moscow), **2004**, 70, 267-274.
24- P. Cos, K. Toté, T. Horemans, L. Maes, Biofilms: an extra hurdle for effective antimicrobial therapy, Curr. Pharm. Design., **2010**, 16, 2279-2295.
25- I. Kolouchová, O. Maťátková, M. Paldrychová, Z. Kodeš, E. Kvasničková, K. Sigler, A. Čejková, J. Šmidrkal, K. Demnerová, J. Masák, Resveratrol, pterostilbene, and baicalein: plant-derived anti-biofilm agents, Folia Microbiol., **2018,** 63, 3, 261-272.
26- T. Coenye, H.J. Nelis, *In vitro* and *in vivo* model systems to study microbial biofilm formation, J. Microbiol. Methods, **2010**, 83, 89-105.
27- H. Ceri, M.E. Olson, C. Stremick, R.R. Read, D. Morck, A. Buret, The Calgary Biofilm Device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms, J. Clin. Microbiol., **1999**, 37, 1771-1776.
28- P.S. Stewart, J.W. Costerton, Antibiotic resistance of bacteria in biofilms, Lancet, **2001**, 358, 135-138.
29- P. Gilbert, D.G. Allison, A.J. Mc Bain, Biofilms *in vitro* and *in vivo*: do singular mechanisms imply cross-resistance? Symp, Ser. Soc. Appl. Microbiol., **2002**, 92, 98-110.
30- K. Poole, *Pseudomonas aeruginosa*: resistance to the max, Front. Microbiol., **2011**, 2, 65.
31- M.J. Ashby, J.E. Neale, S.J. Knott, I.A. Critchley, Effect of antibiotics on non-growing cells of biofilms of *Escherichia coli*, J. Antimicrob. Chemother., **1994**, 33, 443-452.
32- K. Lewis, Persister cells, dormancy, and infectious disease, Nat. Rev. Microbiol., **2007**, 5, 48-56.
33- J.R. Knowles, S. Roller, D.B. Murray, A.S. Naidu Kong, Antimicrobial action of carvacrol at different stages of dual-species biofilm development by *Staphylococcus aureus* and *Salmonella typhimurium*, Appl. Environ. Microbiol., **2005**, 71, 797-803.
34- R.J. Lambert, P.N. Skandamis, P.J. Coote, G.J. Nychas, A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol, J. Appl. Microbiol., **2001**, 91, 453-462.
35- B.J. Juven, J. Kanner, F. Schved, H. Weisslowicz, Factors that interact with the antibacterial action of thyme essential oil and its active constituents, J. Appl. Bacteriol., **1994**, 76, 626-631.
36- F.M. Schempp, K.E. Hofmann, J. Mi, F. Kirchner, A. Meffert, H. Schewe, J. Schrader, M. Buchhaupt, Investigation of monoterpenoid resistance mechanisms in Pseudomonas putida and their consequences for biotransformations, Appl. Microbiol. Biotechnol., 2020, 1-15.
37- A. Nostro, M.A. Cannatelli, I. Morelli, A.D. Musolino, F. Scuderi, F. Pizzimenti, V. Alonzo, Efficiency of Calaminthaofficinalis essential oil as a preservative in two topical product types, J. App.l Microbiol., 2004, 97, 395-401.
38- K.R. Zodrow, J.D. Schiffman, M. Elimelech, Biodegradable Polymer (PLGA) Coatings Featuring Cinnamaldehyde and Carvacrol Mitigate Biofilm Formation, Langmuir, **2012**, 28, 13993-13999.