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Application of thyme essential oil for biofilm prevention and water treatment by photosensitization

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ABSTRACT

In order to improve the water disinfection (waste and drinking water) by photosensitization, thyme (*Thymus vulgaris*) essential oils were investigated in this study. A synthetic water samples including bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus cereus* and *Salmonella typhi*) were irradiated by an artificial ultraviolet lamp of brand UVA at 365 nm in the presence and in the absence of added concentration of thyme (*Thymus vulgaris*) essential oils (at nearly 5% w/v). After irradiation of an hour, samples were taken for bacteriological analyses. The exploitation of the results shows an enhancement of bacterial inactivation for irradiated samples containing a volume of thyme essential oil (TEO). This result could be interesting in further application for water treatment by photosensitization. In addition, the added volume of thyme essential oils affects distinctly the expression of virulence factors expressed by tested bacteria namely *P. aeruginosa* such as motility, enzyme production and biofilms formation. For instance, results reveal biofilm inhibitor effects of TEO despite the low added concentration compared to the irradiated bacteria without any addition. This fact could used to enhance water treatment without chemical addition and, also to overcome the disadvantages of water disinfection process such the non-remnant effect of UV irradiation and the post-treated bacterial resuscitation.

Keywords: Water; Biofilm; *Thymus vulgaris*; Photosensitization

1. Introduction

Water distribution system particularly drinking water may act as a reservoirs for biofilms. These latter represent a resistant structure formed by complex communities of bacterial cells within an hydrated matrix composed of extracellular polymeric substances (EPS) that they produce [1].

Such species include *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, etc. Biofilms are problematic in cause of their aptitude to adapt to respective environment and their higher resistance to environmental conditions than free-floating cells (planktonic cells). It is well known that biofilms protect microbes against disinfectants. Among the most effective and easily implementable system for water disinfection, ultraviolet light (UV) is cited [2]. Unlike chemical biocides, UV does not create any appearance disinfection by-product DBPs. However, some post-irradiated microorganisms can develop mechanisms

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to appropriately respond to UV stress. Most of them can repair UV-DNA lesions.

From this background, it makes sense to consider other products such as essential oils as possible anti-biofilm agents and for enhancing the ultraviolet water disinfection process.

Nowadays, essential oils (EOs) and other extracts from plants, herbs and spices have shown interesting antimicrobial activities against a number of different food pathogens and spoilage microorganisms [3,4]. Extracts from thyme are among the most studied natural antimicrobials for food applications [5–7]. More specifically, the activity of these molecules against biofilm formation and already formed biofilm has been described [8,9].

Phenolic compounds such as thymol are present in thyme and have been shown to be the most active EO components of these species [7,10–12]. From this point of view, this makes thyme essential oils (TEOs) an interesting research subject when looking for new anti-biofilm and anti-virulence agents in water disinfection operation.

Furthermore, TEO has recently been investigated as natural photosensitizer in few studies [13,14]. It was found, a better antimicrobial activity of TEO among some others essential oils in presence of visible light. In fact, photosensitization is a process having three main components with nontoxic nature, namely, photosensitizer (light-sensitive substance), light, and oxygen [15]. The photosensitizer absorbs the light, produced photoreactions leading the production of various active oxygen species, resulting in apoptosis and microorganisms death [16].

Therefore, the aim of this work is to (1) evaluate the effect of TEO on biofilm prevention and (2) to enhance the effectiveness of UV water disinfection process by the use of TEO to prevent an eventual recontamination of treated water without addition of chemical reagents.

2. Material and methods

2.1. Bacterial strains and growth media preparation

Each bacterial strains *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 4114, *Salmonella typhi* ATCC 560, and *Bacillus cereus* (14759) were grown overnight in nutrient broth NB medium under aerobic conditions at 37°C with constant shaking at 160 rpm. The bacterial growth was monitored by measurement of optical density at 600 nm. Then, for photocatalytic experiments bacteria were cultured overnight, collected by centrifugation at 4,000 rpm for 4 min and washed twice with sterile demineralized water. Cell pellets were finally re-suspended in a sterile saline buffer (NaCl 0.85%). The required cell density corresponded to approximately 5×10^6 CFU/mL.

2.2. Virulence factors essays

2.2.1. Protease expression

Qualitative estimation of the extracellular secretion of proteases was determined by inoculating 10 μL of the bacteria cells on an agar medium (1.5% agar + 0.75% casein) treated by TEO at different concentrations of thyme oil (0.5, 1, 2 and 4 MIC concentrations). After incubation for 24 to 36 h at 30°C, the proteolytic activity of the casein present in the culture medium was revealed by the presence of a halot of inhibition.

2.2.2. Lipase expression

Extracellular secretion of lipase was also estimated by inoculating 10 μL of the treated bacteria cells on an agar medium (1.5% agar) supplemented with 2% Tween 20 and treated by TEO at different concentrations of thyme oil (0.5, 1, 2 and 4 MIC concentrations). The lipase activity was reflected by the presence of a halot of inhibition after 2–3 d of incubation at 30°C.

2.2.3. Motility essays

In order to investigate bacterial motility, different samples were carried out at different concentrations of thyme oil (0.5, 1, 2 and 4 MIC concentrations) and according to the method of Rashid and Kornberg [17], three kinds of motility were followed as outlined below.

- *Swimming motility*: Tryptone broth [10 g/L tryptone (DIFCO)/5 g/L NaCl] containing 0.3% (w/v) agarose (GIBCO/BRL) and 0.3% (w/v) agar was prepared and used as media. Swim plates were inoculated with bacteria cultured overnight on LB agar (1.5%, w/v) plates at 37°C. The plates were then wrapped with Saran Wrap to prevent dehydration and incubated at 30°C for 12–14 h.
- *Swarming motility*: For swarming mobility assays, a media was prepared from 0.5% (w/v) agar and 8 g/L NB medium, to which 5 g/L glucose was added. Swarm plates were firstly dried at room temperature overnight then inoculated with cells from swim agar (0.3%, w/v) plates incubated overnight at 30°C.
- *Twitching motility*: LB broth (10 g/L tryptone/5 g/L yeast extract/10 g/L NaCl) solidified with 1% (w/v) agar was prepared for twitching assays. Twitch plates were briefly dried and strains with a sharp toothpick to the bottom of the Petri dish from an overnight-grown LB agar (1.5%, w/v) plate. After incubation at 37°C for 24 h, the zone of motility at the agar/Petri dish interface was measured.

2.2.4. Biofilm formation

Biofilm disinfecting susceptibility was studied into their free-cells or planktonic form and with biofilm community. Biofilms formation was quantified as described by O'Toole et al. [18]. An overnight of bacterial culture was diluted 100-fold in fresh TSB both and 200 μL was added to each well of a 96 wells of Microtiter plate. Cells were grown for 3 d at 30°C (preformed biofilm) before treatment by disinfectant agents (EO, UV-A irradiation and UV-A/EO). Then, they were stained with crystal violet (CV) and quantified. Biofilm formation for different samples was determined by an increase or decrease in optical density at 600 nm, compared to the control test (media only).

2.3. Thyme essential oil to boost UVA water treatment

To test the ability of tested thyme essential oil to improve water treatment, we were monitored the bacteria reduction under different water treatment conditions.

For UV irradiation, an artificial UVA lamp was used (Philips Actinic BL 15W/10SLV under intensity of 1.7 mW/cm^2) with constant magnetic stirring during 1 h.

3. Results and discussion

3.1. Effect of TEO on biofilm formation by bacteria on planktonic form

The exploitation of the curve (Fig. 1) showed biofilm formation by tested strains after treatment by different concentrations of thyme essential oils.

After treatment bacteria in their planktonic form with thyme oils at concentrations ranging from 1.5 µg/mL to 100 µg/mL, biofilm production presents a gradual decrease varying with bacterial strain in a dose dependent manner [19].

These results were consistent with previous reports of Kerekes et al. [20] who founded a strong biofilm-reducing effect of TEO on *E. coli* and *L. monocytogenes* at a concentration of 0.5–4 mg/mL. The destructive effect of thyme EO and thymol on *E. coli*, *S. aureus*, *L. monocytogenes* and *P. putida* was also observed in a study using the scanning electron microscope (SEM). Thyme oil revealed a concentration-dependent biofilm inhibition in various studies. The maximum reduction of biofilm formation at 12.8% thyme oil (highest concentration tested) was 88.7% [9,21].

Gram-positive and Gram-negative bacteria were treated under planktonic phase cells by TEO to demonstrate its effectiveness to disinfect and to inhibit the initiation of biofilm formation under sun irradiation (Fig. 1).

To compare the response of tested bacteria and their behaviour to form a sessile community in interaction with TEO, kinetic parameters were determined according to the first-order model with modification as follows:

$$Y_T = A \exp\left(-K^{nC}\right) \quad (1)$$

where Y_T: biofilm reduction in function of additional concentration of essential oil. This reduction represents M_C: bacterial biomass involved in biofilm formation after treatment with essential oils to a determined concentration (C) determined by the measure of OD at 600 nm compared with; $M_{0:}$ the bacterial biomass involved in biofilm formation prior to treatment with essential oils; A: the rate of inhibiting biofilm formation at first contact with EO; K: the inactivation coefficient of biofilm formation (min^{-1}) and n: dilution ratio. In our case n = 1.

The comparison of different kinetics parameters determined according to the first-order model with modification mainly the coefficient of biofilm inhibition (k) (Table 1) showed a difference in the response of tested bacteria to form biofilm after treatment. This difference in bacterial behaviour was probably related to an inter-specific difference concerning the bacteria shape, cell growth strategy in response to the environmental stress, the metabolism activity, the genetic regulation and flexibility, etc.

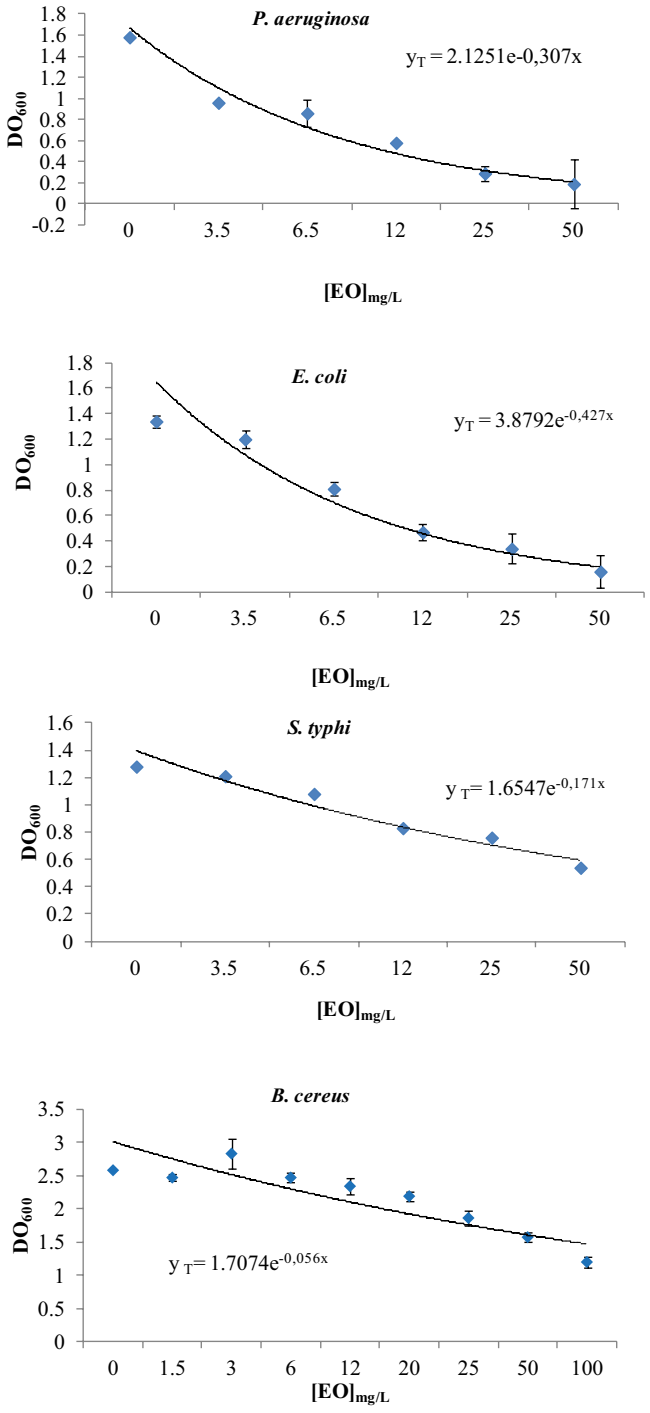

Fig. 1. Effect of different concentrations of EO on biofilm production (planktonic form) by planktonic cells. Data are average of three experiments.

The operating curves (Fig. 1) and the determination of inactivation parameters (K and A) for each bacterial strains tested: *E. coli*, *S. typhi*, and *P. aeruginosa* after treatment with various concentrations of essential oils, has provided the following results:

Table 1
Parameters of inactivation of biofilm production by tested bacteria on planktonic form

Bacterial strains	K_T	A_T
E. coli	0.253	2.129
S. typhi	0.087	1.423
P. aeruginosa	0.307	2.125
B. cereus	0.056	1.264

Higher was the coefficient of inactivation (K), more sensitive was the bacteria to the action of the stressor. On the basis of the kinetic parameter (K) (Table 1), we have estimated that the thyme oils have a relatively great power of inhibition. Example, for *E. coli*, the coefficient of inactivation (K) was equal to 0.253 min^{-1} after treatment with thyme oils.

The inhibition rate of biofilm production at the first contact with the essential oil (A) which was directly related to the bactericidal action of EO tested on the bacterium.

The kinetic parameter (A) determined for *E. coli* treated with TEO was respectively 2.13. This parameter showed a bactericidal power of thyme oil.

3.2. Effect of TEO on biofilm destabilization by bacteria on sessile form

To report the action of TEO on bacterial biofilm shown by Table 2, we used the model of the first order model with modification as follow:

$$Y'_T = A' \exp\left(-K'^{n}C\right) \quad (2)$$

with Y'_T: the biofilm reduction in function of additional concentration of essential oil. This reduction represent M'_C: bacterial biomass still involved in biofilm formation after treatment with the essential oil to a determined concentration (C) determined by the measure of OD at 600 nm compared to M'_0: the bacterial biomass involved in biofilm formation prior to treatment with the essential oil; A': the attenuation rate of biofilm at first contact with the essential oil; K': the coefficient of disruption or destruction of the biofilm (min^{-1}), n: the dilution ratio. In our case $n = 1$.

In this case of study, the result shows the partial ability of the TEO to perturbed resistant structure. Indeed, the biofilm is a very complex and resistant structure. Moreover, the intercellular communication system (QS) is the process by which bacteria produce and detect signal molecules to coordinate their collective behaviour and the expression of different virulence factors such as biofilm formation [22]. Biofilm formed by bacteria is defined as an adherent group of sessile communities surrounded by a matrix of EPS. This matrix promotes bacterial persistence by resisting disinfectant processes [23].

Thyme oils decreased significantly the preformed biofilm ($P < 0.05$) while increasing TEO concentration (Fig. 2). This is consistent with recent work of El-Tarabily et al. [24] who found that the biofilm formation of *E. faecalis* after 72 h of growth was decreased by 256 μg/mL of thyme oil. Khan et al. [25] studied the activity of *Thymus vulgaris* EO against formed biofilms of *Candida* spp., they have revealed a concentration dependent activity, indeed, biofilm formation was reduced progressively while increasing EO concentrations.

Table 2
Parameters of inactivation of biofilm production by tested bacteria on sessile form

Bacterial strains	K'_T	A'_T
E. coli	0.018	1.54
S. typhi	0.023	1.16
P. aeruginosa	0.03	1.72
B. cereus	0.012	1.11

However the lower value of K' (0.03 min^{-1}) in comparison with K (0.3 min^{-1}) express the higher efficacy of thyme oil against biofilm formation than against the eradication of already established biofilms. This result as suggested by Astani et al. [26] may be explained by the fact that the antimicrobial thyme essential oil may adversely affect the viability of bacteria in the biofilm but may not directly disrupt the biofilm matrix or EPS structure by chemical intervention. The observable and measurable destruction or eradication of existing biofilms by the use of EOs is most likely to be indirect, by killing the bacteria in the biofilms, which prevents further biofilm formation.

3.3. Protease and lipase activity of the bacteria treated by thyme EO

The production of proteolytic enzymes such as proteases and exotoxin is indispensable for the colonization of tissue and for the development of the infectious power characteristic of certain bacterial species.

We investigated the production of proteases after treatment with thyme oil. The method is based on the presence of an inhibition halot by the producing strain on a culture medium (GN + 0.75% casein). Purified lipase, in synergy with hemolytic phospholipase C, also increases the release of inflammatory mediators by certain host cells and is known to induce an inflammatory reaction and cause cellular damage to the host. The results of the demonstration of Lipase activity (Tween 80-based medium) are illustrated in Fig. 3. Colonies of lipase-producing bacteria appear surrounded by an opaque halo due to the formation of calcium salt crystals. The results show the repressing effect of the lipase and protease activity after treatment with thyme oil (50 μg/mL). While Singh et al. [27] have found non-significant affect of thyme oil on protease and lipase activity of *Xanthomonas oryzae* a common bacterial plant pathogen.

3.4. Exploitation of bacterial mobility after treatment with EO

Both biofilm formation and bacterial motility are virulence factors of the utmost importance for the spread of bacteria throughout tissues and in producing high biofilm mass [28]. Hence, we investigated the effects of thyme oil on bacterial motility and biofilm formation (Table 3).

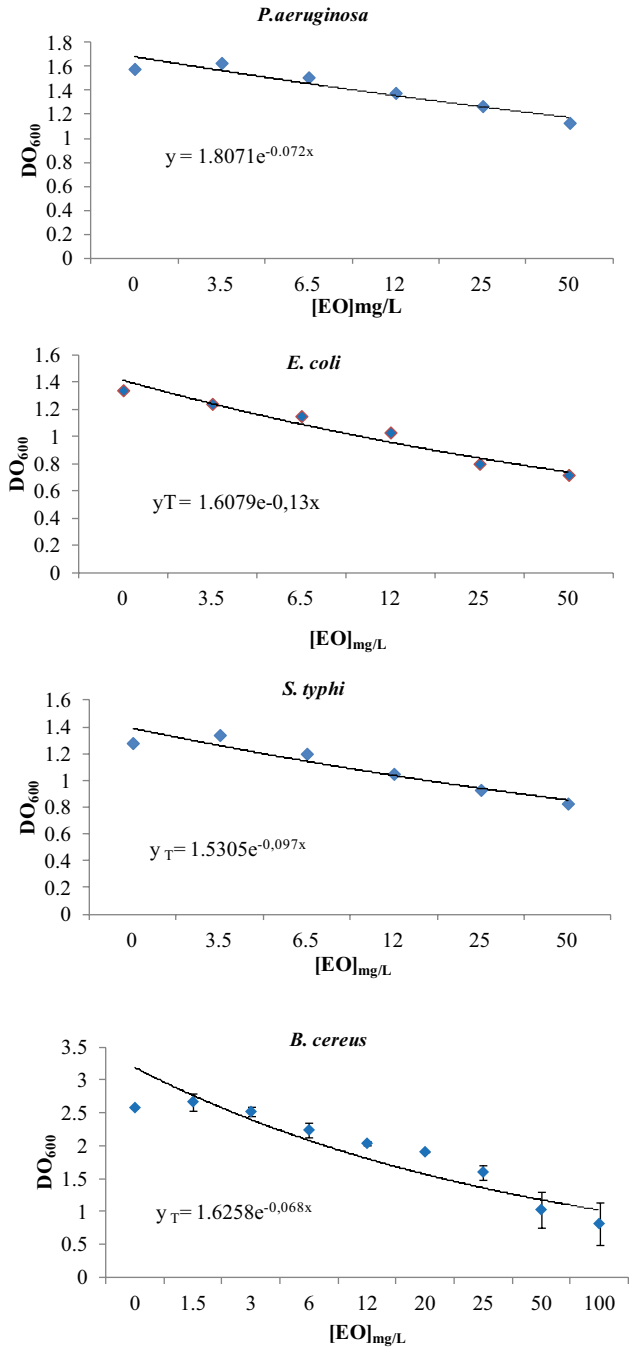

Fig. 2. Effect of different concentrations of TEO on sessile cells (biofilm associated cells). Data are average of three experiments.

Mobility is the ability to allow some bacteria to move and thus to find more favorable environmental conditions by getting nutrients or escape from an hostile environment. For pathogenic bacteria, mobility allows to colonize the host and disperse in the environment in search of new prey. The mobility of a bacterium is due to a polar flagellum insertion. This flagellum gives it the ability to swim in an aqueous environment or containing a small amount of agar (0.4% lower), it's the swimming type of mobility. The flagellum

Table 3
Motility represented as the average of three separate experiments conducted ± SEM in the presence of thyme oil

	E. coli				*Salmonella*				*B. cereus*				*P. aeruginosa*			
	*CMI				*CMI				*CMI				*CMI			
	0.5	1	2	4	0.5	1	2	4	0.5	1	2	4	0.5	1	2	4
Swimming	30 ± 3.5	0	0	0	6.5 ± 3.2	6.5 ± 1.2	0	0	35 ± 1.2	0	0	0	21 ± 1.9	20 ± 0.5	14 ± 0.5	7 ± 1.3
Swarming	3 ± 3.2	0	0	0	3	0	0	0	40	6.5 ± 1.3	0	0	24 ± 1.2	22 ± 1.2	12 ± 1.9	5 ± 1.7
Twitching	12.5 ± 1.3	12.5 ± 1.3	6.5 ± 1.3	6.5 ± 1.9	12.5 ± 1.9	12.5 ± 1.9	6.5 ± 2.8	6.5 ± 1.3	35 ± 2.2	0	0	0	35 ± 1.2	32 ± 1.3	30 ± 1.9	25 ± 1.3

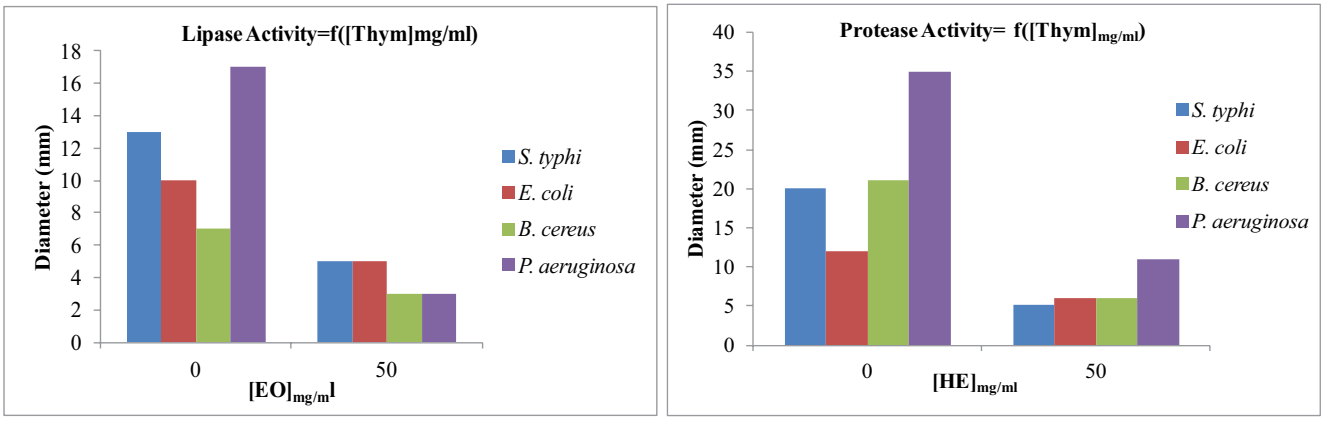

Fig. 3. Monitoring of lipase/protease activity of different bacterial strains in interaction with tested essential oil.

is also involved in swarming type of mobility or twitching mobility that allows development in semi-solid surface.

All of *S. typhi*, *B. cereus* and possess peritrichous flagella which generate their main motility force and greatly enhance bacterial biofilm formation [18]. Bacterial motility is also involved in the early stages of surface colonization by bacteria [28]. Observations showed a decrease in flagellar swimming, swarming and twitching motility for all tested bacteria (except *B. cereus*) when treated with thyme oil at super-inhibitory concentrations in comparison to the control. Lee et al. [19] also found that thyme oil reduced swarming motility but not swimming motility of uropathogenic *E. coli* at sub-inhibitory concentration. This quiet discordance may be attributed to differences in the composition of thyme oil; in fact, [19] have used the red thyme oil. To our best knowledge, there is leakage of literature studying the effect of pure thyme oil on bacterial motility.

Furthermore, these results suggest a new alternative to enhance UV disinfection water treatment without addition of chemical reagents, also to overcome the disadvantages of water disinfection process such the non-remnant effect of UV irradiation basing on the repression of virulence factors expressed by bacteria such as production of biofilm, bacterial motility and protease activity [22].

3.5. Thyme oil to boost UVA water treatment: use of thymol oil as photosensitizer

In this work, we will focus on the application of thymol oil in the field of water disinfection and, especially to boost UVA water treatment using natural photosensitizer.

Table 4 shows the improvement of bacteria reduction under UVA radiation in presence of thyme essential oil. This result could be used to enhance bacterial inactivation by solar radiation without chemical addition.

The bacterial abatements obtained in presence of both TEO and UVA were significantly larger than those obtained by UVA radiation without TEO. Therefore, bacterial inactivation by UVA radiation was improved by the presence of the tested EO. This natural extract could be used consequently as photosensitizer. The synergy activity of TEO and UVA light could have many benefic applications in different field of researches [14,15]. However, to our knowledge, no paper has investigated the photosensitivity of TEO on the disinfection of water.

Synergistically, essential oils together with light rapidly sterilized acute infected or biofilm-associated wounds [29]. The photosensitization-induced damage can result in substantial morphological and functional changes in the microbial cells. Direct damage to the cell membrane leads to leakage of cellular contents and following inactivation of the membrane transport system. Functional damage results from loss of enzymatic activities, protein oxidation, and formation of protein–protein cross-links and inhibition of different metabolic processes. Morphological alterations include destruction of the mesosome structure [30].

Besides, Marqués-Calvo et al. [14], concluded that some components of essential oils particularly thymol could act as photosensitizers generating reactive oxygen species (ROS). These later cause oxidative cellular damage on bacteria in addition to the well-known antimicrobial effect of both light and essential oils. The detailed mechanism of action by thymol has been discussed recently by Liu et al. [29]. In fact, light react with thymol to produce sensitizers, thymoquinone and thymohydroquinone. Photo-excitations of thymoquinone and thymohydroquinone augmented reactive oxygen species production and initiated a torrent of cytotoxic events in bacteria while completely sparing the host tissue.

3.6. Comparison of TEO effect and TEO-UVA effect on biofilm produced by P. aeruginosa (planktonic and sessile form)

The use of natural extract, in our case thyme essential oil and particularly thymol could have many virtues such as, the natural antibacterial activity and, the use of this natural extract as photosensitizer to boost bacterial inactivation by UV irradiation and ameliorate water disinfection stage without use of chemical or synthetic addition and also, the ability of this natural extract to inhibit or prevent biofilm formation. Indeed the monitoring of biofilm formation by *P. aeruginosa* under different water treatment conditions (Fig. 4) shows the enhancement of the inactivation coefficient of biofilm formation (K) when we treated the bacterial model (under planktonic form) by UVA radiation in presence of TEO (at 1%). As shown in

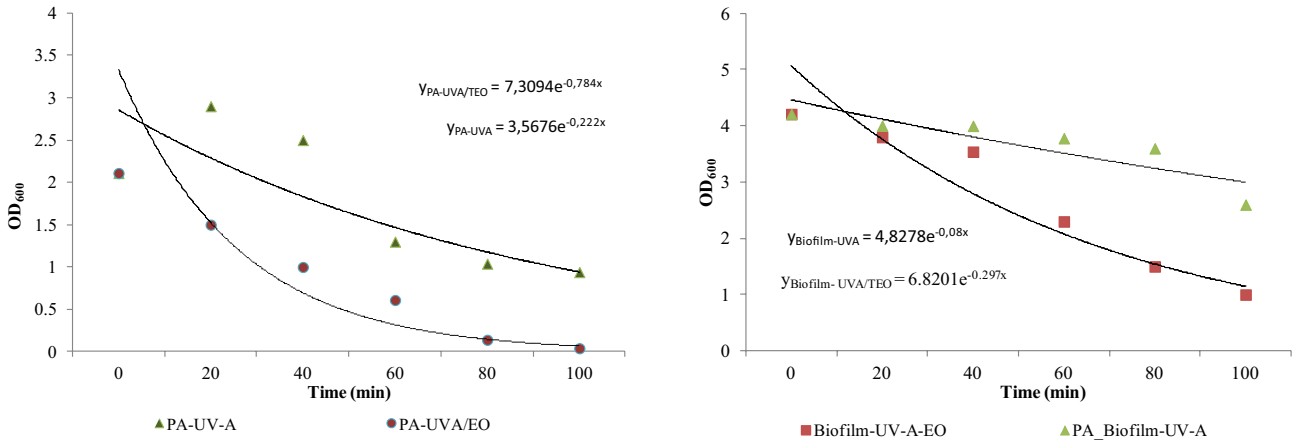

Fig. 4. (a) Monitoring of biofilm formation by *P. aeruginosa* under UVA and UVA-TEO (planktonic form). (b) Monitoring of biofilm formation by *P. aeruginosa* under UVA and UVA-TEO (sessile form). Data are average of three experiments where error bars are not shown; differences between duplicates were not detected

Table 4
Determination of bacterial abatement (U-log10(N/N_0)) under different water treatment conditions

Bacteria strains	UVA	TEO	UVA-TEO
E. coli	2.69	0.82	4.32
P. aeruginosa	1.69	0.98	4.49

Ab: Bacterial abatement equal to U-log10(N/N_0) with, N: Number of viable cultivable bacteria after treatment with essential oil whether or not exposed to ultraviolet radiations type A (352 nm); N_0: Number of viable bacteria cultivable before treatment (N_0 = 106 UFC/mL); LD: Limit of detection (absence of bacteria cultivable on usual culture medium).

first part of this study, the TEO is more effective to prevent biofilm formation but is less effective to fragile the resistant structure already established. In contrary, in the case where the biofilm is treated by UVA radiation in presence of TEO, the coefficient of disruption or destruction of the biofilm (k' = 0.3 min^{-1}) shows an enhancement in comparison with the value of k' when we treat only by UVA radiation (k' = 0.08 min^{-1}). This result is very pertinent and opens up perspectives to use EO for biofilm prevention and also biofilm eradication in different fields such as water treatment and agro food processes.

4. Conclusion

Bacterial motility is a virulence characteristic of bacteria necessary for the dissemination and initiation of production of bacterial biofilm. At high concentrations (more than CMI), complete attenuation of motility have been observed with thyme oil. We can conclude that concentration allowing the suppression of mobility is well above the minimum inhibitory concentration (MIC). As a result, the application of an essential oil to prevent recontamination of the water (pool water, spa water, treated water, etc.) should choose well the optimal concentration not necessarily the CMI.

Moreover, this is the first published study that uses thyme essential oils as photosensitizers for water disinfection. Results of preliminary experimental investigations demonstrate for the first time the efficacy of the photosensitization by TEO to enhance water disinfection by UVA. Furthermore, the results reveal a synergistic effect of both TEO and UVA together to combat already established biofilms expressed by the enhancement of the value of the coefficient of disruption or destruction of the biofilm.

This study opens perspectives to facilitate the disinfection or the conservation in other food industry as well as medical application. However, in this study only one essential oil has been sampled also for microorganisms, therefore additional studies with different essential oils and more range of microorganisms will be needed.

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