Blended formulations of oregano-sage essential oils: antimicrobial, phytotoxic, and anti-quorum sensing investigations

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Abstract

There is a growing interest in the potential use of essential oils (EOs) as a possible alternative to synthetic pesticides. The formulation of bioinsecticides with different EOs could improve their bioactivities through synergic mechanisms. This study aimed to evaluate the biological activities of three blended oil formulations (BOFs) derived from oregano (*Origanum vulgare* L.) and sage (*Salvia officinalis* L.). The chemical composition of the individual EOs was investigated using GC-MS analysis. The BOFs were prepared as follows: i) 25% oregano EO + 25% sage EO (BOF-I); ii) 25% oregano EO + 5% sage EO (BOF-II); iii) 5% oregano EO + 25% sage EO (BOF-III). The BOFs were tested for their phytotoxic effects on *Lepidium sativum*, *Solanum lycopersicum*, and *Lactuca sativa* as well as their antimicrobial activity against some phytopathogens. The tested BOFs were evaluated for their possible anti-quorum sensing activity against *Chromobacterium violaceum* Schröter. GC-MS analysis revealed that the oregano EO is mainly composed of thymol (76%), p-cymene (5.7%) and carvacrol (3.2%). Whereas the dominant constituents of sage EO were transthujone and camphor. The results demonstrated that all tested BOFs possess an antimicrobial effect higher than each parent EO. In particular, BOF-II showed the highest effect against all tested bacteria and fungi. In addition, the three BOFs showed notable phytotoxic effects against all tested plants, particularly BOF-I. Whereas, the single sage EO at 25% showed the lowest significant phytotoxic effect, indicating its possible use as a natural herbicide. All examined BOFs showed promising quorum quenching activity against *C. violaceum*, especially at a concentration of 100%.

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Introduction

Synthetic pesticides and herbicides demonstrate, generally, a high contamination risk for the environment, soil, water, and for human health as well.[1-3] Therefore, there is a huge interest in discovering natural substances based on plant or microbe origin with pesticidal and/or herbicidal effects.[3,4] It is well-known that the utilization of synthetic pesticides has increased microorganism resistance. The use of natural substances based on medicinal plants or microbial origins can be useful for decreasing environmental hazards and avoiding microbial resistance to synthetic pesticides,[5-7] hence the search for alternative natural substances or new, efficient formulations against serious phytopathogens is necessary.[8]

Among the most important natural substances with promising biological effects are essential oils (EOs), which are concentrated hydrophobic liquids containing volatile compounds extracted from plants.[9,10] Plant EOs are complex mixtures of mostly terpenoids as plant secondary metabolites,[10-13] which can be used as a possible alternative to conventional microbicides and/or herbicides.[11,14,15]

Origanum vulgare L. (family Lamiaceae) is a widespread aromatic plant, commonly known as oregano, that is particularly appreciated in the Mediterranean region for its biological, nutritional, cosmetic, and pharmaceutical activities.[4] Oregano EO has

recently been considered a natural herbicide against several harmful weeds and a promising substitute for synthetic herbicides.[4] *Salvia officinalis* L. (Lamiaceae), commonly called sage, native to the Mediterranean region and naturalized in many places around the world, has a long history of medicinal and culinary uses and is considered one of the most important sources of plant EOs.[16-18]

The composition of EOs from the same plant species can vary considerably, depending on growth conditions, variety, environmental factors, etc.[19-21] The widespread use of EOs has decreased due to several issues, including high costs. Therefore, many researchers are eager to discover novel formulations of two or more EOs that could have a synergistic biological effect while also being more affordable. However, the new formulations should be accurately evaluated to avoid any possible negative health impact or phytotoxic effect.[22]

The main objective of the current study was to prepare, characterize, and assess the efficacy of three novel blended essential-oil formulations (BOFs) between *O. vulgare* and *S. officinalis* that might have a synergistic biological effect to enable a reduction in effective dose at a lower cost. In particular, this research was carried out to i) chemically characterize the main single constituents of both tested EOs using Gas Chromatography-Mass Spectroscopy (GC-MS); ii) evaluate the antimicrobial activity of the three novel BOFs against some phytopathogens; iii) determine the minimum inhibitory concentration (MIC) of the most bioactive tested formulation; and iv) evaluate the possible phytotoxic effect of new BOFs on the seed germination and radical elongation of *Lepidium sativum* L., *Solanum lycopersicum* L. and *Lactuca sativa* L.

Materials and Methods

Plant materials, EOs extraction and formulation

The EOs used for BOFs were extracted from oregano (*O. vulgare*) and sage (*S. officinalis*) which were cultivated in the greenhouse of the School of Agricultural, Forestry, Food, and Environmental Sciences (SAFE), University of Basilicata, Potenza (Italy). The aerial parts, used for EOs extraction, were collected in Spring 2021 and dried in an oven at 65°C for 48 hrs. One hundred g of dried materials were ground in a Waring blender (MX1050XTPEE, Noventa Padovana, Italy) and subjected to hydrodistillation for 3 hrs using a Clevenger-type apparatus (HFY-CDY, Shenzen China) according to the standard procedure described in the European Pharmacopoeia.[23] The extracted EOs were solubilized in *n*-hexane, filtered using anhydrous sodium sulfate (Na_2SO_4), and stored under liquid nitrogen (N_2) at 4°C in darkness.

The studied BOFs were prepared in 0.7% dimethyl sulfoxide (DMSO) + Tween 20 (0.2%) using a reflux apparatus in stirred conditions overnight at 60°C in a water bath at the following concentrations: A) [BOF-I] oregano EO (250 µg/mL) + sage EO (250 µg/mL); B) [BOF-II] oregano EO (250 µg/mL) + sage EO (50 µg/mL); C) [BOF-III] oregano EO (50 µg/mL) + sage EO (250 µg/mL). In addition, the two EOs have been tested, individually, at 250 µg/mL for biological assays compared to the prepared formulations.

GC-MS analysis

The chemical composition of the studied two EOs was carried out using a Gas Chromatograph Shimadzu brand (GC 2010 Plus) coupled with a QP 2010 Ultra Mass Spectrometer (GC-MS). The separation of EO components was achieved by capillary column chromatography on 0.25 µm thick flash silica RTX-5MS (30 mm×0.25 mm), using Helium as eluting gas with a flow rate set of 1.2 mL min^{-1}. Samples (1 µL) were injected in split mode (leakage ratio: 1/50). The device was connected to a computer system managing a mass spectrum library (NIST 98) and driven by software to monitor chromatographic analyses. The identification of each single constituent of both EOs was made through the comparison of their retention indices with those of standard compounds presented in the database NIST 02 and Wiley 275 libraries (Wiley Registry of Mass Spectral Data).[24]

Antimicrobial activity

The tested bacterial strains were *Xanthomonas campestris* (Pammel) Dowson, *Clavibacter michiganensis* (Smith) Davis, and *Bacillus cereus* Frankland & Frankland. All tested bacteria were cultured on King B (KB) medium[25] and incubated at 37°C for 24 hrs. Whereas, the tested fungal strains were *Botrytis cinerea* Pers., *Penicillium italicum* Wehmer, and *Fusarium oxysporum* Schlecht. All tested fungal isolates were cultured on Potato Dextrose Agar (PDA) medium and incubated at 24°C for 96 hrs.

Bactericidal assay

The disc diffusion method has been carried out to evaluate the antibacterial activity of the parent EOs (250 µg/mL) and BOFs.[26,27] Briefly, the bacterial suspension of each strain was prepared in sterile distilled water (SDW) and incorporated in soft agar at 0.7% (9:1, *v/v*) adjusted by spectrophotometer (Amersham, Ultrospec 1100 pro/500 pro, UK) at 10^8 colony forming unit (CFU)/mL corresponding to 0.2 nm optical density (OD). Four mL of each bacterial suspension were poured singularly into a Petri dish (Ø 90 mm) containing 10 mL of KB. Blank Discs (Ø 6 mm) (OXOID, Milan, Italy) were pre-treated with each parent EO (250 µg/mL) or different BOFs and placed over inoculated plates and incubated at 37°C for 24 hrs. The bactericidal activity was evaluated by measuring the diameter of eventual inhibition zones (mm). The bacterial growth inhibition (BGI%) was calculated using the equation (1) compared to tetracycline at 1600 µg/mL as positive control (C+ve). The experiment was carried out in triplicate, and the standard deviations (SDs) were calculated.

$$\mathrm{BGI}\ (\%) = 100 - \left[\frac{(Gc - Gt)}{Gc} \times 100\right] \qquad \text{(Equation 1)}$$

where: BGI is the bacterial growth inhibition percentage; Gc is the average diameter of bacteria growth in the control plate (mm); Gt is the average diameter of the inhibition zone in inoculated plates (mm).

Fungicidal assay

The antifungal activity of the parent EOs and the prepared BOFs has been evaluated against the above-mentioned phytopathogenic fungi following the incorporation method.[14,28] Briefly, 14 mL of PDA supplemented with each single EO at 250 µg/mL or BOFs were poured into Petri dishes (Ø 90 mm). Single agar disks (Ø 0.5 cm) of fresh fungal cultures were inoculated in pre-treated PDA Petri dishes. Untreated PDA plates were inoculated only with tested fungi as negative control (C-ve). All plates were incubated at 24°C for 6 days in darkness, and the diameter of the mycelium was measured (mm).[14,15,29] The fungal growth inhibition (FGI%) was calculated following equation (2) compared to cycloheximide

at 100 µg/mL as a positive control (C+ve). The experiment was carried out in triplicate, and SDs were calculated.

$$FGI\ (\%) = \frac{(Gc-Gt)}{Gc} \times 100 \qquad \text{(Equation 2)}$$

where: FGI is the fungal growth inhibition percentage; Gc is the average diameter of fungal mycelium in control plates; Gt is the average diameter of fungal mycelium in treated plates.

Minimum bactericidal concentration (MBC)

The minimum bactericidal concentration (MBC) of the most bioactive BOF was carried out against all tested bacteria using 96-well microplates (Nunc MaxiSorp®, Vedbaek, Denmark) following the micro-dilution method.[16] The Minimal Mineral (MM) broth was used for the preparation of bacterial suspensions. The most bioactive EO formulation (BOF-II) was dissolved in the prepared medium at concentrations 10000, 8000, 4000, 2000, 1000, and 500 µg/mL according to the obtained results from the preliminary *in vitro* assay. One hundred microliters/well from each prepared concentration were added into the microplate pre-supplemented with 50 µL/well of each bacterial suspension. All plates were incubated at 37°C for 24 hrs. The absorbance was measured using a microplate reader instrument (DAS S.r.l., Rome, Italy) at λ = 540 nm. Tetracycline (1.6 mg/mL) was used as the positive control (Cont+ve), whereas wells only filled with MM broth were considered the negative control (Cont+MM). The MBC values for each tested strain were determined by monitoring the lowest tested concentration causing a significant growth reduction compared to the positive control. Whereas, the IC_{50} was calculated using the tendency-line formula provided by Microsoft Excel software.

Phytotoxicity assay

A bioassay based on seed germination (SG) and radical elongation (RE) was carried out to evaluate the possible phytotoxic effect of studied crude EOs and newly prepared BOFs were tested on the seeds of *L. sativum*, *S. lycopersicum*, and *L. sativa*.[30] Seeds were sterilized in 3% hydrogen peroxide (H_2O_2) for 1 min, rinsed twice with deionized SDW, and then placed either in each single BOF at 250 µg/mL for 2 hrs or SDW as a negative control (C-ve) under shaking condition (200 rpm/min). Fifteen seeds were transferred into Petri dishes (Ø 90 mm) containing two sterile filter papers (Whatman No.1), pre-moistened with 2 mL of deionized SDW, and sealed with Parafilm. All Petri dishes were incubated in a growth chamber at 28°C with relative humidity (RH) 80% in the darkness for 72 hrs. The number of germinated seeds was counted, and the radical elongation was measured in centimeters (cm). The experiment was carried out in triplicate, and the germination index (G.I.) was calculated with the following equation (3):

$$G.I.\ (\%) = \frac{(SGt \times REt)}{(SGc \times REc)} \times 100 \qquad \text{(Equation 3)}$$

Where: G.I. is germination index; SG_t is the average number of germinated treated seeds; RE_t is the average radical elongation of treated seeds; SG_c is the average number of germinated seeds of negative control; RE_c is the average radical elongation of negative control.

Anti-quorum sensing activity

The Agar Disc diffusion method has been carried out to evaluate the anti-quorum sensing of the three tested BOFs as described by Rajivgandhi *et al.*[31] *Chromobacterium violaceum* Schröter was first cultured on lysogeny broth (LB) medium and kept at 28°C for five to seven days before being streaked onto LB-agar plates and the blank discs (6 mm, Oxoid, Milan, Italy) were deposited over the plates. Twenty µL from each studied BOF at three different concentrations (100, 50 and 25%) were then transferred to the discs and the plates were incubated for 24 hrs at 28°C. A positive control consisting of streptomycin 10 mg/mL and a negative control consisting of DMSO 1%, were employed. The reduction of violacein formation around the discs allowed for the detection of anti-quorum sensing activity. The standard deviations (SDs) were determined for each of the tested treatments, which were all performed in triplicate.

Statistical analysis

The obtained results of the biological assays were statistically analyzed using one-way ANOVA using Statistical Package for the Social Sciences (SPSS) version 13.0 (Prentice Hall: Chicago, IL, USA, 2004). Tukey B Post-Hoc multiple comparison tests were applied to evaluate the significance level with a probability of $p<0.05$.

Results

GC-MS analysis

The analysis of the chemical composition of *O. vulgare* EO allowed the identification of 42 components, which represent 96.4% of the total oil (*Table S1*). In particular, the predominant constituents are thymol (76.0%), p-cymene (5.7%), carvacrol (3.2%), linalool (2.6%), and γ-terpinene (2.5%). Based on the dominance of thymol, the tested oregano EO is identified as a thymol chemotype, in agreement with Mancini *et al.*[32] On the other hand, the chemical analysis of *S. officinalis* EO allowed the identification of 64 compounds, accounting for 98.7% of the total oil (*Table S2*). Monoterpenes are the most abundant compounds found in sage EO. In particular, the most abundant single components are: *trans*-thujone (37.9%), camphor (13.9%), and borneol (7.6%) in agreement with Elshafie *et al.*[16]

Bactericidal activity

The studied BOFs exhibited promising antibacterial effects against all three tested bacteria (Figure 1). In particular, BOF-II showed the highest significant effect against *B. cereus* and *X. campestris*, and moderate against *C. michiganensis*. In addition, tetracycline showed significant activity against *C. michiganensis*. than BOF-II and lower activity against *B. cereus* and *X. campestris*.

Fungicidal activity

The three studied BOFs exhibited promising antifungal effects against the tested fungi compared to the tested crude EOs (Figure 2). In particular, the BOF-II formulation showed complete inhibition of the mycelium growth of the three tested pathogenic fungi. Additionally, BOF-I exhibited a higher antifungal effect against *P. italicum* compared to BOF-III, however BOF-I was less effective

against *B. cinerea*. In addition, both formulations (BOF-I and BOF-III) showed a moderate effect against *F. oxysporum*.

MBC analysis

This assay was carried out to determine the minimum bactericidal concentration (MBC) which is defined as the lowest concentration of the most bioactive BOF that can significantly inhibit the growth of bacteria compared to the negative control. The MBC values of the tested BOF-II against *B. cereus*, *C. michiganensis*, and *X. campestris* were 4000, 2000, and 1000 µg/mL, respectively (Table 1). The results of the MBC of BOF-II are illustrated in Figure 3 A, C, E, whereas the IC_{50} was calculated using the tendency-line formula of the chart in Microsoft Excel (Figure 3 B,D,F), where the BOF-II showed 4462.5, 6219.9, and 7715.6 µg/mL, corresponding to the inhibition of 50% visible growth of bacterial colonies of *B. cereus*, *C. michiganensis*, and *X. campestris*, respectively (Table 1).

Phytotoxic activity

The studied BOFs exhibited high phytotoxic effects against all tested plants (Table 2). In particular, BOF-I showed the highest

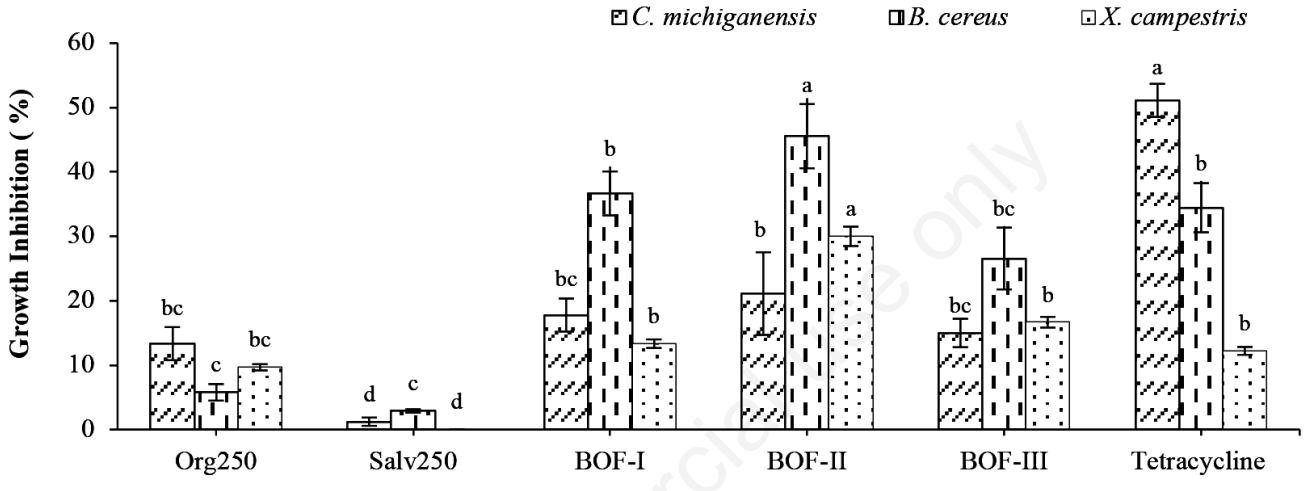

Figure 1. Antibacterial activity of crude EOs and BOFs. Org250: oregano EO at 250 µg/mL; Salv250: sage EO at 250 µg/mL; BOF-I: formulation between oregano EO at 250 and sage EO at 250 µg/mL; BOF-II: formulation between oregano EO at 250 and sage EO at 50 µg/mL; BOF-III: formulation between oregano EO at 50 and sage EO at 250 µg/mL. Tetracycline was used as positive control at 1.6 mg/mL. Bars with different letters for each tested bacteria indicate mean values significantly different at $p<0.05$ according to one-way ANOVA combined with Tukey B post hoc multiple comparison tests. Data are expressed as the mean of three replicates ± SDs.

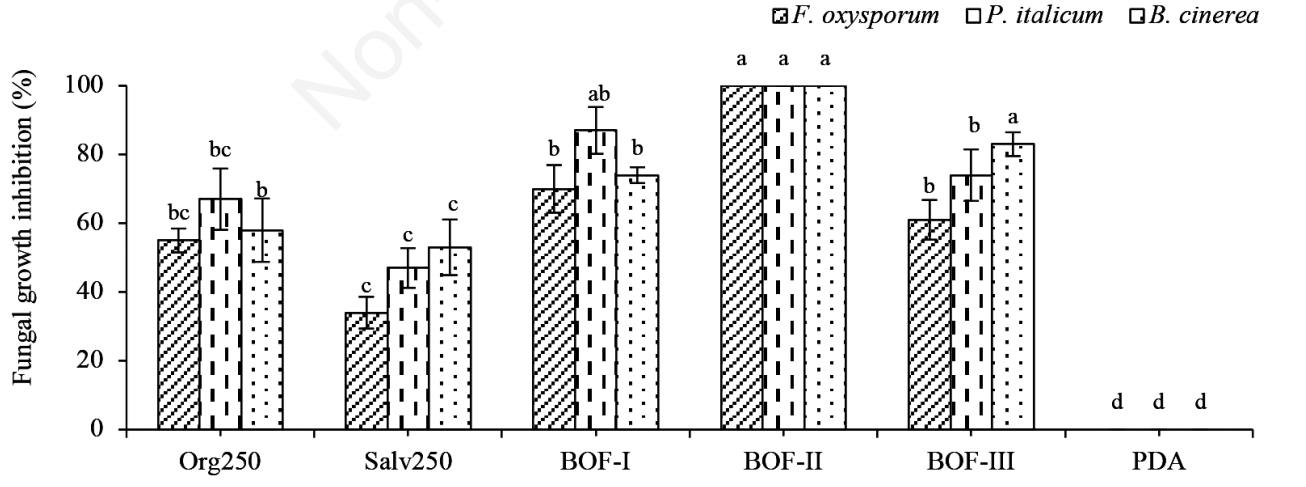

Figure 2. Antifungal activity of crude EOs and BOFs. PDA: plates containing untreated PDA (C-ve); Org25: oregano EO at 250 µg/mL; Salv25: sage EO at 250 µg/mL; BOF-I: formulation between oregano EO at 250 and sage EO at 250 µg/mL; BOF-II: formulation between oregano EO at 250 and sage EO at 50 µg/mL; BOF-III: formulation between oregano EO at 50 and sage EO at 250 µg/mL. Bars with different letters for each tested fungi indicate mean values significantly different at $p<0.05$ according to one-way ANOVA combined with Tukey B post hoc multiple comparison tests. Data are expressed as the mean of three replicates ± SDs.

significant effect on the seed germination of *L. sativum* and a moderate effect on *S. lycopersicum*. In addition, the three tested BOFs showed the most significant phytotoxic effect against the seeds of *L. sativa*. Furthermore, sage crude EO (250 µg/mL) showed the lowest significant phytotoxic effect on both *L. sativum* and *S. lycopersicum* compared to all other treatments.

Anti-quorum sensing activity

Positive results were observed for all three examined BOFs, particularly at a concentration of 100%. Each formulation's inhibition performance displayed a range of activity (Figure 4). However, quorum quenching activity against *C. violaceum* was present in all

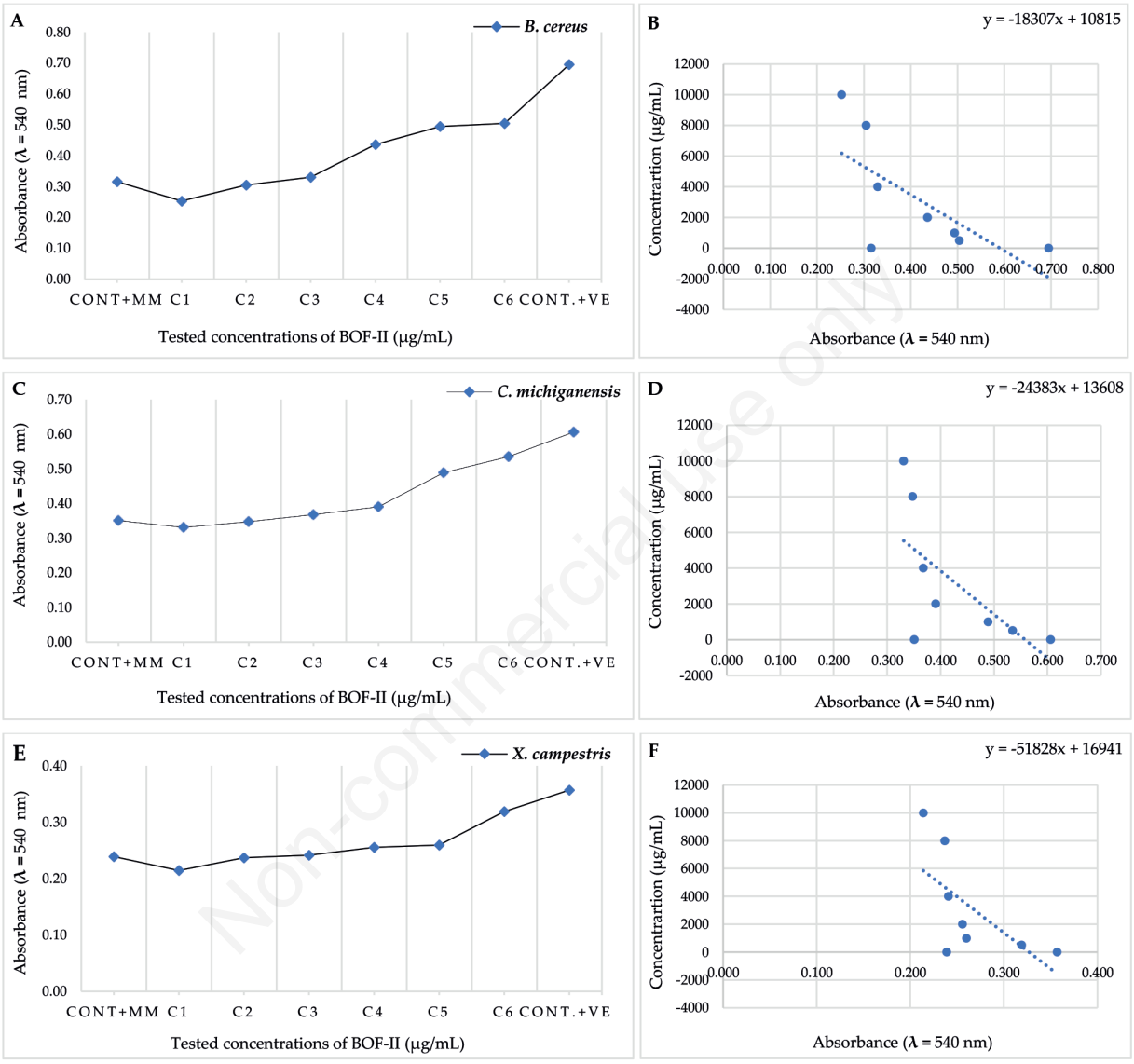

Figure 3. MBC (left) and IC_{50} (right) of BOF-II formulations against (A, B) *B. cereus*, (C, D) *C. michiganensis*, (E, F) *X. campestris*. C1, C2, C3, C4, C5 and C6 are the tested concentrations of BOF-II at 10000, 8000, 4000, 2000, 1000 and 500 µg/mL, respectively. Cont+MM: negative control (only MM broth). Cont+ve: positive control (tetracycline 1600 µg/mL).

Table 1. MBC values of BOF-II formulation against the three tested bacteria.

Tested bacteria	Abs. (540 nm)		MIC (µg/mL)	50% Colony Inhibition	
	Cont. MM	BOF-II		Abs. (540 nm)	IC_{50} (µg/mL)
B. cereus	0.316	0.330	4000	0.347	4462.5
C. michiganensis	0.351	0.368	2000	0.410	6219.9
X. campestris	0.239	0.260	1000	0.231	7715.6

Abs., absorbance.

examined BOFs, as evidenced by the lack of violacein pigment surrounding the wells. In particular, BOF-I at 100% demonstrated the maximum inhibition activity (18 mm), whereas BOF-III at 100% demonstrated the lowest inhibition activity (5 mm).

Discussion

Several studies reported that different species of oregano, such as *O. heracleoticum*, *O. majorana*, *O. vulgare*, *O. acutidens*, and *O. onites* have been known for their biological activity due to their main single constituents such as thymol, carvacrol, citral, linalool, γ- or cis-terpinene and trans-sabinene hydrate.[33,34] In particular, *O. vulgare* EO showed promising antibacterial, antifungal, and antiviral activities against several phytopathogens, as reported by different research.[3,4] Oregano EO was able to inhibit significantly some fungal and bacterial phyto- and human pathogens such as *Botrytis cinerea*, *Penicillium expansum*, *Phytophthora citrophthora*, *Rhizopus stolonifer*, *Aspergillus niger*, *Fusarium oxysporum*, *Sclerotinia sclerotiorum*,

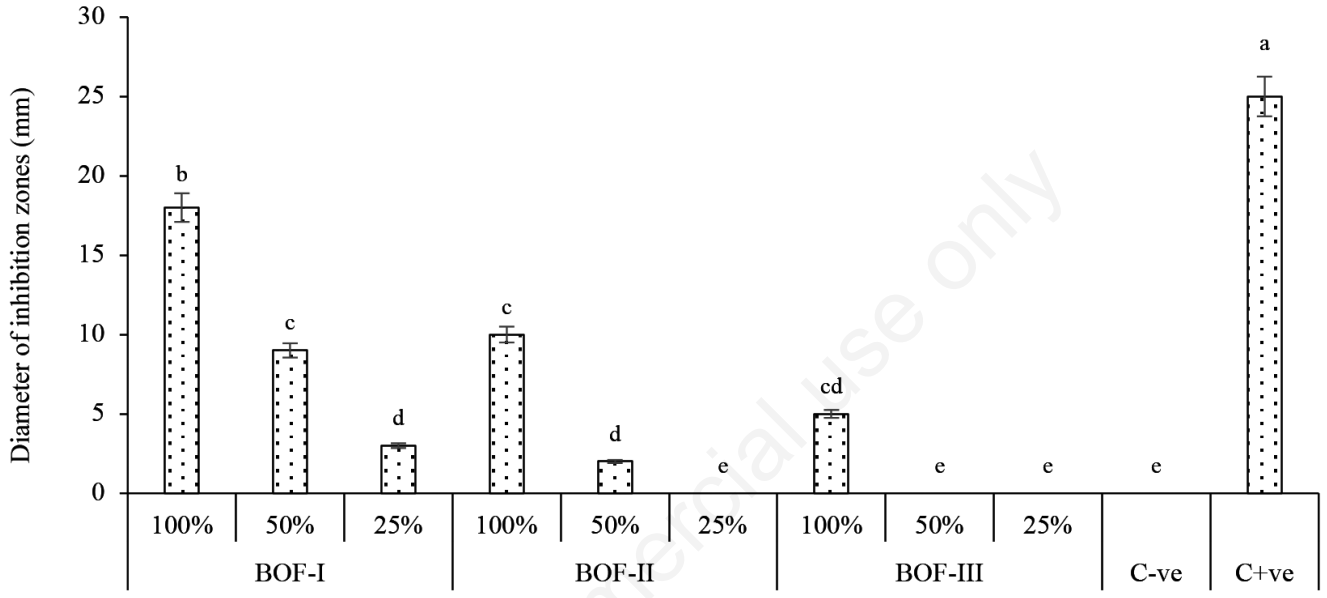

Figure 4. Anti-quorum sensing activity of studied BOF-I, BOF-II, and BOF-III against *C. violaceum*. Where 100, 50, and 25% are the tested concentrations of the studied BOFs; C-ve: negative control DMSO 1%; C+ve: positive control streptomycin 10 mg/mL.

Table 2. Phytotoxic effect of crude EOs and BOFs

EOs formulations		Seed germination (%)	Radical elongation (cm)	Growth index (%)
L. sativum	Org250	4.3±0.8b	0.8±0.6ab	1.4±0.0b
	Salv250	80.0±1.8d	7.4±0.5bc	43.1±6.1d
	BOF-I	0.0±0.0a	0.0±0.0a	0.0±0.0a
	BOF-II	6.7±0.5b	1.8±0.5b	1.0±0.1b
	BOF-III	53.3±0.5c	5.4±0.7bc	17.9±2.6c
	Cont. SDW	100.0±0.0d	14.7±1.3d	97.5±3.9e
S. lycopersicum	Org250	3.0±0.4a	4.6±0.3b	8.9±0.0bc
	Salv250	73.3±1.0c	9.9±0.4b	56. 2±7.7d
	BOF-I	6.7±0.5a	0.0±0.0a	0.0±0.0a
	BOF-II	13.3±0.5b	0.7±0.2a	1.1±0.6b
	BOF-III	60.0±1.4c	6.3±1.4b	22.2±8.2c
	Cont. SDW	100.0±0.0d	11.6±0.5bc	100.0±0.0e
L. sativa	Org250	2.0±0.4b	0.8±0.5ab	0.9±0.2ab
	Salv250	12.4±1.0bc	5.2±0.4c	33.4±7.7c
	BOF-I	0.0±0.0a	0.0±0.0a	0.0±0.0a
	BOF-II	0.0±0.0a	0.0±0.0a	0.0±0.0a
	BOF-III	0.0±0.0a	0.0±0.0a	0.0±0.0a
	Cont. SDW	100.0±0.0d	10.5±0.3d	100.0±0.0d

Where: Org25: oregano EO at 250 µg/mL; Salv25: sage EO at 250 µg/mL; BOF-I: formulation between oregano EO at 250 and sage EO at 250 µg/mL; BOF-II: formulation between oregano EO at 250 and sage EO at 50 µg/mL; BOF-III: formulation between oregano EO at 50 and sage EO at 250 µg/mL; Cont. SDW is C-ve. Values followed by the different letters in each vertical column for each tested plant are significantly different according to Tukey B test at $p<0.05$. Data are recorded as mean values of three replicates (±SDs).

Staphylococcus aureus, *C. michiganensis* and *Xanthomonas vesicatoria*.[3,4,35,36]

The antimicrobial activity of *S. officinalis* EO has also been reported by several studies, particularly against some phytopathogenic bacteria in a dose-dependent manner such as *C. michiganensis, X. campestris*, and *Pseudomonas savastanoi*.[16,35] In addition, sage EO showed antifungal activity against *P. citrophthora* and *R. stolonifer* as reported by Camele et al.[35]

On the other hand, it has been recognized that various EO components act as multi-target molecules exerting several modes of action in the target organisms.[29] In particular, single EO-molecules can penetrate the microbial cell wall and directly interact with the plant plasma membrane, which is one of the potential cellular targets of EOs.[10,14,37] Monoterpenes, one of the main constituents of EOs, can alter the lipid organization, domain formation, and phenylpropanoid, which could interact with membrane receptors.[38] However, some research found that EOs had little effect on fungal development due to the physiological resistance mechanisms in fungi that neutralize the fungicides and use the liberated molecules as secondary nutrition, which may be responsible for this phenomenon. As an alternative, fungi might accelerate their reproductive processes in a toxic nutrient medium, which may increase the production of conidia.[39]

Certain issues with EOs-based microbicides, like volatility, solubility, and oxidation, considerably affect their applications and activities; therefore, the new formulations can solve these issues. In this situation, EOs are released under controlled conditions through blended formulations and may hold significant potential as available natural biopesticides.[40] The synergistic interactions of various crude EOs or their single constituents have been investigated in numerous studies.[41-43] However, the synergistic effects of more than two EOs or their constituents have previously received limited research attention.[44]

Because the antimicrobial actions of various EOs depend mainly on one or more primary constituents, combining different EOs or their constituents can enhance their efficacy by expanding the range of their sites of action. In consequence, this combination may enhance the EOs effectiveness against different microbial pathogens even at lower doses, as opposed to the use of a single EO or compound. This indicates that a particular chemical component of each tested EO may display a definite biological activity when present in a natural combination but may not do so when present as a single compound. Thus, when natural substances are present together, they may have a mutually beneficial impact (synergism).[41] In other cases, the combination of two or more single substances could have an adverse effect.[41]

It's relevant to note that phenolic monoterpenes like thymol and carvacrol, as well as phenylpropanoid compounds like eugenol and chavicol, have been shown to increase bioactivities such as antimicrobial and other biopharmaceutical properties.[45] The composition of bioactive single substances like thymol, carvacrol, linalool, etc. is primarily responsible for the antimicrobial activity of the two investigated EOs. These bioactive compounds exhibit a potent ability to penetrate microbial cells, damage their cell walls, and increase the permeability of the cells. Thus, following treatment, the cells shrink and thin more, ultimately resulting in total cell death.

The obtained results of the phytotoxic effect demonstrated that the studied BOFs have a clear effect against all tested plants, especially the BOF-I against the seeds of *L. sativum*. In addition, the three tested BOFs showed the most significant phytotoxic effect against the seeds of *L. sativa*. Furthermore, sage crude EO (250 µg/mL) showed the lowest significant phytotoxic effect on both *L. sativum* and *S. lycopersicum* compared to all other treatments.

On the other hand, various studies have reported that *O. vulgare* EO is effective in counteracting biofilm formation and quorum sensing mechanisms, primarily due to its main bioactive constituent, carvacrol.[46] Moreover, sage EO has been studied in the food industry and demonstrated an effective anti-quorum sensing effect by preventing biofilm formation, particularly against many foodborne pathogens.[47,48]

The obtained results from the current research underlined the potential antimicrobial and phytotoxic effects of the new BOFs, which indicate their possibility of controlling both serious phytopathogens and harmful weeds. Nevertheless, it seems speculative that the tested concentrations, which were potentially efficient *in vitro*, could be also achieved *in vivo* with appropriate applications. Therefore, further research is needed to explore the potential of these new formulations as green, plant-based EOs to replace traditional synthetic alternatives for pathogen and weed control in various crops grown in open fields. Furthermore, to better understand the synergistic interactions between various EOs and the precise contributions of certain single constituents, more investigations are required. A systemic examination of the synergy among various elements should be also conducted to examine the mode of action of both single and multiple EOs.

Conclusions

In conclusion, the results of the antimicrobial activity assays are promising, highlighting the feasibility of using the new BOFs at lower concentrations as potential natural microbicides in agriculture and the agro-pharmaceutical industry. On the other hand, the achieved results underscore the necessity for further exploration of the potential utilization of the novel EOs formulations as natural herbicides, especially in the organic farming. The synergistic effects of different EOs such as the new formulations or encapsulation can retain the higher efficacy of the EOs by reducing their volatility which is considered a major problem facing their large diffusion in agriculture, medical, and food industries. Furthermore, the potential synergistic effect resulting from the novel formulations might enhance the effectiveness and selectivity of each single EO, as well as optimize the individual constituents' single-acting potential against severe phytopathogens, even at lower dosages. The utilization of these alternative formulations for biocontrol can considerably lower the higher cost relative to using a single EO from an economic perspective.

References

1. Duke SO. Why have no new herbicide modes of action appeared in recent years? Pest Manag Sci 2012;68: 505–12.
2. Nicolopoulou-Stamati P, Maipas S, Kotampasi C, et al. Chemical pesticides and human health: the urgent need for a new concept in agriculture. Front Public Health 2016;4:148.
3. Gruľová D, Caputo L, Elshafie HS, et al. Thymol chemotype *Origanum vulgare* L. essential oil as a potential selective bio-based herbicide on monocot plant species. Molecules 2020; 25:595.
4. Della Pepa T, Elshafie HS, Capasso R, et al. Antimicrobial and phytotoxic activity of *Origanum heracleoticum* and *O. majorana* essential oils growing in Cilento (Southern Italy). Molecules 2019;24:2576.
5. Anyanwu MU, Okoye RC. Antimicrobial activity of nigerian

medicinal plants. J Intercult Ethnopharmacol 2017;6: 240–259.
6. Cazella LN, Glamoclija J, Soković M, et al. Antimicrobial activity of essential oil of *Baccharis dracunculifolia* DC (Asteraceae) aerial parts at flowering period. Frontiers Plant Sci 2019;10:27.
7. Rossolini GM, Arena F, Pecile P, Pollini S. Update on the antibiotic resistance crisis. Curr Opin Pharmacol 2014;18:56-60.
8. Elshafie HS, Racioppi R, Bufo SA, Camele I. *In vitro* study of biological activity of four strains of *Burkholderia gladioli* pv. *agaricicola* and identification of their bioactive metabolites using GC–MS. Saudia J Biol Sci 2017;24:295–301.
9. Filho JG, Silva G, de Aguiar AC, et al. Chemical composition and antifungal activity of essential oils and their combinations against *Botrytis cinerea* in strawberries. J Food Meas Charact 2021;15:1815–25.
10. Elshafie HS, Camele I. An overview of the biological effects of some Mediterranean essential oils on human health (review article). Biomed Res Int 2017;9268468.
11. Elshafie HS, Caputo L, De Martino L, et al. Biological investigations of essential oils extracted from three *Juniperus* species and evaluation of their antimicrobial, antioxidant and cytotoxic activities. J Appl Microbiol 2020;129:1261-71.
12. Tomazoni EZ, Pauletti GF, da Silva Ribeiro RT, et al. *In vitro* and i*n vivo* activity of essential oils extracted from *Eucalyptus staigeriana*, *Eucalyptus globulus* and *Cinnamomum camphora* against *Alternaria solani* Sorauer causing early blight in tomato. Sci Hortic 2017;223:72–7.
13. Elshafie HS, Camele I, Mohamed AA. A comprehensive review on the biological, agricultural and pharmaceutical properties of secondary metabolites based-plant origin. Int J Mol Sci 2023;24:3266.
14. Camele I, Gruľová D, Elshafie HS. Chemical composition and antimicrobial properties of *Mentha piperita* cv. 'Kristinka' Essential Oil. Plants 2021;10:1567.
15. Ardakani MS, Mosadeggh M, Shafaati A. Volatile constituents from the aerial parts of *Verbena officinalis* L. (vervain). Iran J Pharm Res 2003;2:39-42.
16. Elshafie HS, Sakr S, Mang SM, et al. Antimicrobial activity and chemical composition of three essential oils extracted from Mediterranean aromatic plants. J Med Food 2016;19:1096-103.
17. Tundis R, Leporini M, Bonesi M, et al. *Salvia officinalis* L. from Italy: A comparative chemical and biological study of its essential oil in the Mediterranean context. Molecules 2020;10:5826.
18. Aćimović M, Pezo L, Čabarkapa I, et al. Variation of *Salvia officinalis* L. essential oil and hydrolate composition and their antimicrobial activity. Processes 2022;10:1608.
19. Glisic S, Ivanovic J, Ristic M, Skala D. Extraction of sage (*Salvia officinalis* L.) by supercritical CO_2: Kinetic data, chemical composition and selectivity of diterpenes. J Supercrit Fluids 2010;52:62–70.
20. Fatma EK, Ayse A, Caglar K. Extraction and HPLC analysis of sage (*Salvia officinalis*) Plant. Nat Prod Chem Res 2017;5:298.
21. Zinno P, Guantario B, Lombardi G, et al. Chemical composition and biological activities of essential oils from *Origanum vulgare* genotypes belonging to the carvacrol and thymol chemotypes. Plants 2023;12:1344.
22. Keifer MC, Firestone J. Neurotoxicity of pesticides. J Agromed 2007;12:17–25.
23. Council of Europe. European Pharmacopoeia, 5th ed. Council of Europe: Strasbourg Cedex, France, I, 217.
24. Wiley Registry of Mass Spectral Data, with NIST Spectral Data CD Rom, 7th ed. John Wiley & Sons: New York, NY, USA, 1998.
25. King EO, Ward MK, Raney DE. Two simple media for demonstration of pyocyanin and fluorescin. J Lab Clin Med 1954; 44:301–7.
26. Elshafie HS, Viggiani L, Mostafa MS, et al. Biological activity and chemical identification of ornithine lipid produced *by Burkholderia gladioli* pv. *agaricicola* ICMP 11096 using LC-MS and NMR analyses. J Biol Res 2017;90:96-103.
27. Elshafie HS, Sakr SH, Sadeek SA, Camele I. Biological investigations and spectroscopic studies of new Moxifloxacin/Glycine-Metal complexes. Chem Biodivers 2019;16:e1800633.
28. Zygadlo JA, Guzman CA, Grosso NR. Antifungal properties of the leaf oils of *Tagetes minuta* L. and *Tagetes filifolia* Lag. J Essent Oil Res 1994;6:617-21.
29. Buriani A, Fortinguerra S, Sorrenti V, et al. Essential Oil phytocomplex activity, a review with a focus on multivariate analysis for a network pharmacology-informed phytogenomic approach. Molecules 2020;16:1833.
30. Ceglie F, Elshafie HS, Verrastro V, Tittarelli F. Evaluation of olive pomace and green waste composts as peat substitutes for organic tomato seedling production. Compost Sci Util 2011;19:293–300.
31. Rajivgandhi G, Vijayan R, Maruthupandy M, et al. Antibiofilm effect of *Nocardiopsis* sp. GRG 1 (KT235640) compound against biofilm forming gram negative bacteria on UTIs. Microb Pathog 2018;118:190.
32. Mancini E, Camele I, Elshafie HS, et al. Chemical composition and biological activity of the essential oil of *Origanum vulgare* ssp. *hirtum* from different areas in the southern apennines (Italy). Chem Biodiver 2014;11:639-51.
33. Mirmostafaee S, Azizi M, Fujii Y. Study of allelopathic interaction of essential oils from medicinal and aromatic plants on seed germination and seedling growth of lettuce. Agronomy 2020; 10:163.
34. Kim JY, Lee SY. Application of food-grade natural antimicrobials for the control of crop disease caused by phytopathogens. Food Sci Biotechnol 2022;31:275-84.
35. Camele I, De Feo V, Altieri L, et al. An attempt of postharvest orange fruit rot control using essential oils from Mediterranean plants. J Med Food 2010;13:1515–23.
36. Bakkali F, Averbeck S, Averbeck D, Idaomar M. Biological effects of essential oils—a review. Food Chem. Toxicol 2008;46:446–75.
37. Bouyahya A, Abrini J, Dakka N, Bakri Y. Essential oils of *Origanum compactum* increase membrane permeability, disturb cell membrane integrity, and suppress quorum-sensing phenotype in bacteria. J Pharmaceut Anal 2019;9:301-11.
38. Dhifi W, Bellili S, Jazi S, et al. Essential oils' chemical characterization and investigation of some biological activities: a critical review. Medicines 2016;22:25.
39. Lak F, Zandi-Sohani N, Ghodoum Parizipour MH, Ebadollahi A. Synergic effects of some plant-derived essential oils and Iranian isolates of entomopathogenic fungus *Metarhizium anisopliae* Sorokin to control *Acanthoscelides obtectus* (Say) (Coleoptera: Chrysomelidae). Front Plant Sci 2022;13: 1075761.
40. Martin Á, Varona S, Navarrete A, Cocero MJ. Encapsulation and co-precipitation processes with supercritical fluids: applications with essential oils. Open Chem Eng J 2010;4:31–41.
41. Bunse M, Daniels R, Gründemann C, et al. Essential oils as multicomponent mixtures and their potential for human health and well-being. front. Pharmacol 2022;13:956541.
42. Govaris A, Solomakos N, Pexara A, Chatzopoulou P. The antimicrobial effect of oregano essential oil, nisin and their combination against *Salmonella enteritidis* in minced sheep

meat during refrigerated storage. Int J Food Microbiol 2010; 137:175-80.
43. Gutierrez J, Rodriguez G, Barry-Ryan C, Bourke P. Efficacy of plant essential oils against food-borne pathogens and spoilage bacteria associated with ready to eat vegetables: antimicrobial and sensory screening. J Food Protect 2008;71:1846-54
44. Basavegowda N, Baek KH. Synergistic antioxidant and antibacterial advantages of essential oils for food packaging applications. Biomolecules 2021;2:1267.
45. García-García R, López-Malo A, Palou E. Bactericidal action of binary and ternary mixtures of carvacrol, thymol, and eugenol against *Listeria innocua.* J Food Sci 2011;76:95–100.
46. Camele I, Elshafie HS, Caputo L, De Feo V. Anti-quorum sensing and antimicrobial effect of Mediterranean plant essential oils against phytopathogenic bacteria. Front Microbiol 2019;10: 2619.
47. Kerekes EB, Deák É, Takó M, et al. Anti-biofilm forming and anti-quorum sensing activity of selected essential oils and their main components on food-related micro-organisms. J. Appl Microbiol 2013;115:933–942.
48. Luciardi MC, Blázquez MA, Cartagena E, et al. Mandarin essential oils inhibit quorum sensing and virulence factors of *Pseudomonas aeruginosa*. LWT Food Sci Technol 2016;68: 373–380.

Online supplementary material:

Table S1. GC-MS of the total identified components in O. vulgare *EO.*

Table S2. GC-MS of the total identified components in S. officinalis *EO.*