

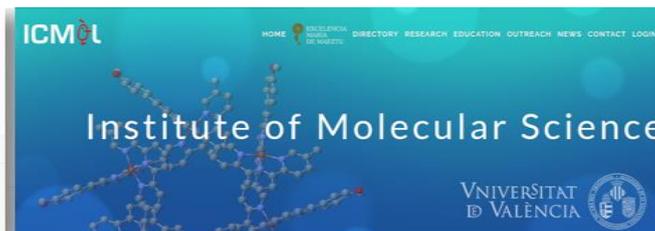


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Essential oils as natural antimicrobial and antioxidant products in the agrifood industry

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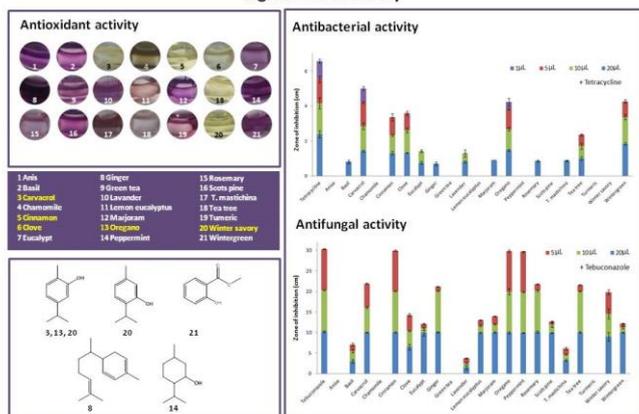
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Graphical Abstract

Essential Oils as Natural Antimicrobial and Antioxidant Products in the Agri-Food Industry



Abstract.

Consumers are aware of the dangers arising from the use of synthetic antioxidants and antimicrobials in the agrifood industry, demanding safer and “greener” alternatives. In this study, the antioxidant activity of commercial essential oils through DPPH method, their antimicrobial effects against the bacterium *Pseudomonas syringae* and the phytopathogenic fungus *Fusarium oxysporum* by means of the standardized disk method were determined. Clove along with winter savory, cinnamon and oregano essential oils as well as carvacrol showed the highest antioxidant activity comparable to reference standards. Wintergreen essential oil was the most potent inhibitor

against *P. syringae* growth at the highest doses (20 and 10 μL). Oregano essential oil and its main component carvacrol were able to stop the bacterium growth even at the lowest treatment (1 μL). Cinnamon, oregano and peppermint essential oils inhibited *F. oxysporum* development at all doses (20, 10 and 5 μL) assayed. In general, most of the essential oils displayed more antifungal than antibacterial and antioxidant activities.

Introduction

Nowadays, consumers are increasingly aware of the synthetic and harmful products used as antimicrobials and preservatives in the agrifood industry. In response to this, there is an increasing interest in natural products, which could extend shelf-life and avoid pest attack in harvests, the emergence of food spoilage pathogens which cause detrimental effects on the physical characteristics and quality of the products and serious economic losses. In particular, essential oils arise as harmless and “greener” alternatives to the synthetic antioxidant and antimicrobial products, whose safety towards environment and human health is still debated.

Essential oils have demonstrated their usefulness in overcoming storage losses and enhancing shelf-life [1], being often incorporated in films and coatings for food protection. In this respect, oregano essential oil has been included in edible pectin coatings, thus increasing the antioxidant activity in coated tomatoes without negative effects on aroma acceptability of the food [2,3]. Similarly, *Zataria multiflora* Boiss’ essential oil was incorporated into chitosan nanoparticles and its antioxidant activity was evaluated on treated cucumbers, resulting in higher levels of DPPH (2,2-Diphenyl-1-picrylhydrazyl), radical scavenging activity and longer shelf-life during storage [4]. Furthermore, the antioxidant activity of essential oils has been compared with that exerted by commonly used antioxidant standards [5]. In this sense, the antioxidant activity of *Xylopiya sericea* A. St.-Hil. essential oil was evaluated by DPPH scavenging, ferric reduction antioxidant power (FRAP), β -carotene/linoleic acid bleaching and phosphomolybdenum and thiobarbituric acid-reactive substance (TBARS) assays, showing a higher antioxidant effect (80 % of inhibition) in the TBARS assays as compared to BHT (13.7 % of inhibition) [6]. Likewise, the essential oil of other species such as *Lantana camara* L. showed better radical scavenging power than quercetin, ascorbic acid and BHT [7].

On the other hand, the broad spectrum and potent antimicrobial activity of certain essential oils has already been highlighted [8]. Specifically, eucalyptus (*Eucalyptus globulus* L.), peppermint (*Mentha x piperita* L.) and rose-scented geranium (*Pelargonium graveolens* L’Hér) essential oils are some examples exhibiting antimicrobial efficiency in the control of pre- and post-harvest rot. They have also shown a remarkable *in vitro* antimicrobial effect against Gram positive food-spoiling bacteria, such as *Bacillus subtilis* and *Staphylococcus aureus*, as well as against fungi and yeasts like *Aspergillus flavus*, *A. niger*, *Mucor* spp., *Fusarium oxysporum* and *Candida albicans*. This activity is especially noticeable in vapour phase, thus being suitable alternatives for use in the food industry as natural antimicrobial agents [9-11]. Many other essential oils like those obtained from *Z. multiflora*, *Thymus vulgaris* L. and *T. kotschyanus* Boiss. & Hohen. displayed antimicrobial activity that completely inhibited the growth of the phytopathogenic fungi *Pythium apanidermatum*, *Rhizoctonia solani*, *F. graminearum* and *Sclerotinia sclerotiorum* at 200 $\mu\text{L/L}$ [12]; *Thuja occidentalis* essential oil inhibited

the growth of the most detrimental plant pathogenic bacteria *Agrobacterium tumefaciens* and *Erwinia carotovora* var. *carotovora* with Minimum Inhibitory Concentrations (MIC) values of 400 and 350 mg/L, while *Artemisia monosperma* Delile essential oil showed effective concentrations (EC₅₀) values between 106-148 mg/L against the phytopathogenic fungi *Alternaria alternata*, *Botrytis cinerea*, *F. oxysporum* and *F. solani* [13]. Recently, *in vivo* antifungal activity of peppermint essential oil has been demonstrated against the spoilage yeasts *C. albicans*, *C. tropicalis*, *Pichia anomala* and especially *Saccharomyces cerevisiae* in cashew, guava, mango and pineapple juices, affecting the pathogen's membrane permeability and potential, enzymatic activity and efflux pump at the minimum dose assayed (1.875 µL/mL) [14]. Finally, other *in vivo* tests showed significant improvement of potato slices infected with *A. niger*, *Mucor wutungkiao*, *Penicillium funiculosum* and *Rhizopus oryzae* after the application of 2.0 µL/mL of the essential oil obtained from navel orange peel (*Citrus sinensis* (L.) Osbeck) [15].

All these promising results reinforce the need to delve in the research for antioxidant activity of other commercial essential oils, as well as in their antimicrobial activities against different species. Therefore, the aim of this work is firstly to evaluate the antioxidant activity of selected commercial essential oils using DPPH method and its comparison *versus* the established antioxidant activity of the natural antioxidants quercetin, ascorbic acid, and the synthetic one BHT. Secondly, this study tries to determine the antibacterial and antifungal capacity of the essential oils by means of the disk diffusion technique on phytopathogenic bacteria and fungi affecting several food crops.

Materials and Methods

Essential Oils and Reference Standard

Commercial essential oils and reference standard were purchased from Guinama, Planalto Dourado, Pranarôm, Plantis and Carobels (table 1), and stored at 4 °C until biological studies were carried out.

Table 1. Information about commercial essential oils and reference standard used

Essential oils				
Name	Plant part	Batch	Sell-by-date	
Guinama				
Anise (<i>Pimpinella anisum</i> L.)	Ripe dried fruit	0059857	06/2017	
Cinnamon (<i>Cinnamomum verum</i> J. Presl)	Leaves	0072188	30/11/2018	
Clove (<i>Syzygium aromaticum</i> L. Merr. & Perry)	Leaves	0065709	22/08/2018	
Eucalypt (<i>Eucalyptus globulus</i> Labill.)	Leaves & stems	0065901	28/02/2019	
Marjoram (<i>Origanum majorana</i> L.)	Leaves & flowers	0042773	13/11/2016	
Oregano (<i>Origanum vulgare</i> L.)	Flowers	0042451	31/05/2016	
Peppermint (<i>Mentha piperita</i> L.)	Leaves	0058567	25/11/2017	
Rosemary (<i>Rosmarinus officinalis</i> L.)	Leaves	0037337	30/04/2016	
Scots pine (<i>Pinus sylvestris</i> L.)	Needles	0065144	08/08/2016	
Tea tree (<i>Melaleuca alternifolia</i> Maiden & Betche ex Cheel)	Leaves	0051451	30/09/2019	
Winter savory (<i>Satureja montana</i> L.)	Whole plant	0054366	18/02/2017	
Planalto Dourado				
Spanish marjoram (<i>Thymus mastichina</i> L.)	Leaves & Flowers	TM010711	07/2017	
Pranarôm				
Basil (<i>Ocimum basilicum</i> L. ssp. <i>basilicum</i>)	Flowering top	0F22144	08/2020	
Ginger (<i>Zingiber officinale</i> Rosc.)	Rhizome	0F26093	04/2022	
Lavender (<i>Lavandula angustifolia</i> Mill.)	Flowers	0082842	30/11/2020	
Lemon eucalyptus (<i>Eucalyptus citriodora</i> Hook)	Leaves	0F25830	02/2022	

Turmeric (<i>Curcuma longa</i> L.)	Root	0F27683	10/2021
Wintergreen (<i>Gaultheria procumbens</i> L.)	Leaves	0F18989	11/2020
Plantis			
Chamomile (<i>Matricaria chamomila</i> L.)	Flowers	725	11/2017
Carobels			
Green tea (<i>Camelia sinensis</i> (L.) Kuntze)	Leaves	26903	09/2015
Reference standard			
Compound	Company	Batch	Sell-by-date
Carvacrol	Sigma-Aldrich	MKBN3724V	01/2018

Antioxidant Assay

The antioxidant activity of the essential oils was evaluated by the DPPH method with some minor modifications [16]. Briefly, 1 mL of ethanol was taken as blank and 750 μ L added to 250 μ L of 0.5 mM DPPH solution was taken as control (A_0). Reaction mixture (A_1) was prepared by taking 740 μ L of ethanol mixed with 250 μ L of 0.5 mM DPPH and 10 μ L of essential oils, or reference standard or positive controls. After incubation of the mixture at 25 °C for 30 min in the dark, the absorbance at 517 nm was measured using a Pharmacia Biotech 1000E UV–VIS (Pharmacia Biotech, Piscataway, NJ, USA) spectrophotometer. The results were compared with the positive controls: the natural flavonoid quercetin and the synthetic antioxidant BHT (0.5, 5 and 25 mM), as well as ascorbic acid (1 and 2.5 mM). The antioxidant activity (%) was expressed as percentage of inhibition of the DPPH radical by using the following formula:

$$\text{DPPH scavenging effect (\% inhibition)} = A_0 - A_1 / A_0 \times 100$$

Where A_0 is the absorbance of the control reaction (without test compound), and A_1 is the absorbance in presence of all of the essential oils or positive control. All the tests were performed in triplicates and the results were averaged.

Antibacterial Assay

Pseudomonas syringae pv. *tomato* Δ AvrPto was grown during 48 h at 28 °C in LB agar medium with the antibiotics rifampicin (10 mg/mL), kanamycin (0.25 mg/mL), and spectinomycin (2.5 mg/mL) according to López Gresa et al., 2017 [17]. When colonies were grown, they were transferred into 15 mL of King's B liquid medium supplemented with rifampicin and were grown overnight at 28 °C under continuous stirring (200 rpm).

After 24 h, 1 mL of the bacterial culture was mixed with 14 mL of King's B agar and poured into Petri dishes. Once solidified, 5 mm-diameter Whatman paper disks (GB005 Blotting Paper, Schleicher & Schuell) were placed on top of the agar and then different volumes (1, 5, 10 and 20 μ L) of each compound were applied into the disks. Methanol was used as negative control and the antibiotic tetracycline was chosen for the positive one at the concentration of 0.3925 % (w/v). After incubation during 48 h at 28 °C, the inhibition zone (cm) was measured using a slide gauge according to the standard disk method.

Antifungal Assay

Fungicidal activity against the tomato phytopathogenic fungus *Fusarium oxysporum* f. sp. *lycopersici* was evaluated in accordance with Hernández, 2018 [18]. The fungus was grown in a liquid sporulation medium during 72 hours at 25 °C under permanent light and continuous stirring (200 rpm). Then, the solution was filtered through a sterile double gauze to remove the mycelium and the sediment spores were obtained by centrifugation at 3600 rpm for 5 minutes. A solution of purified

spores was prepared at a concentration of 105 spores/mL by using a hemocytometer, mixed in PDA agar medium and distributed in Petri dishes. Once solidified, 5 mm-diameter Whatman paper disks (GB005 Blotting Paper, Schleicher & Schuell) were placed on top of the agar and then different volumes (1, 5, 10 and 20 μ L) of each compound were applied into the disks. Methanol was used as negative control and the fungicide tebuconazole was chosen for the positive one at the concentration of 0.3925 % (w/v). After incubation of 72 h C in darkness at 28 $^{\circ}$ C, the inhibition zone (cm) was measured using a slide gauge according to the standard disk method.

Statistics

Experiments were performed *in vitro* with three replicates. Data were subjected to statistical analysis using a Kruskal-Wallis test (non-parametric test equivalent to the one-way ANOVA). Different letters indicate significant differences ($p < 0.05$) between the essential oils at the same dose. The IBM SPSS v.19 package was used for all the statistical analyses.

Results and Discussion

Antioxidant Activity of Essential Oils

The antioxidant properties of certain essential oils through different methods have been established [19]. In this study, the ability of 21 essential oils to donate hydrogen atoms or electrons was evaluated spectrophotometrically by DPPH method.

The results were compared with those obtained using the standards quercetin, BHT and ascorbic acid at different concentrations to estimate the antioxidant potency (figure 1).

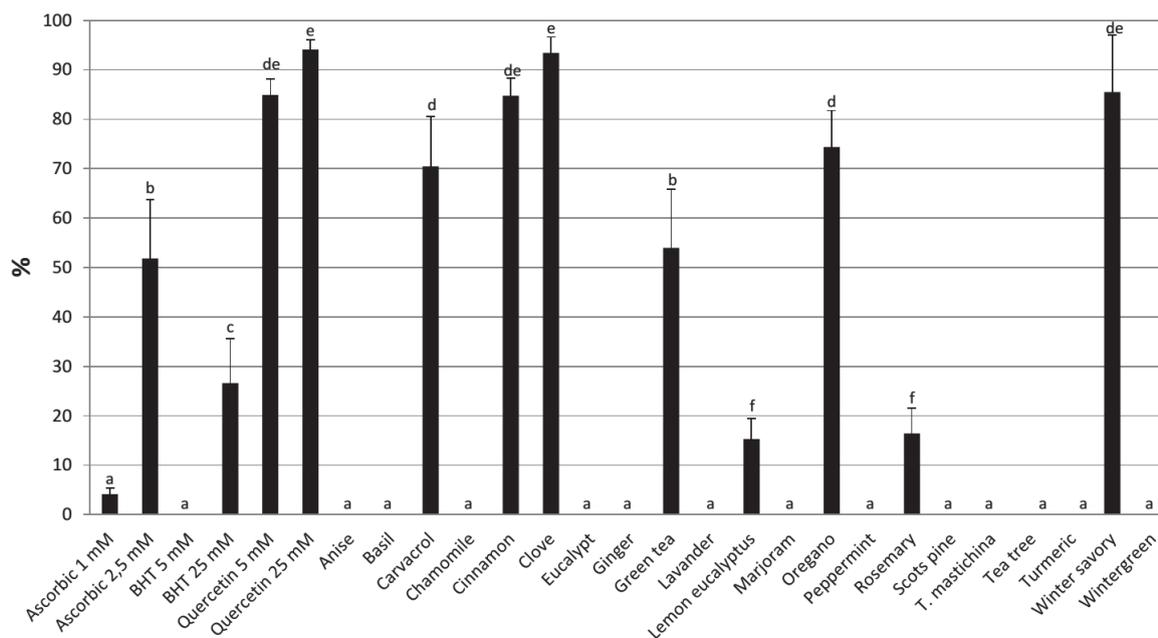


Fig. 1. Antioxidant activity of essential oils studied compared to the standards quercetin, ascorbic acid and BHT. A Kruskal-Wallis analysis was performed and different letters indicate significant differences ($p < 0.05$) between essential oils.

Outstandingly, as little as 10 μ L of clove essential oil were able to reduce the DPPH reagent in 93.4 %, being the essential oil with the highest antioxidant activity of all the assayed. Indeed, this result was comparable to the most potent antioxidant reference, quercetin (94.11 %) at 25 mM. The fact that clove essential oil had analogous antioxidant activity to quercetin is especially interesting, as this

natural flavonoid is considered to be a powerful free radical scavenger, even more potent than others such as curcumin, commonly used in foods and widely-known for its many beneficial effects on health [20-22]. In previous studies, clove essential oil also displayed the highest percentage of inhibition of DPPH radical, above other essential oils like oregano, thyme, rosemary and sage [23], and even higher than the combinations of them [24]. Furthermore, clove essential oil not only showed DPPH radical reduction, but also worked on ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) and superoxide anion, besides hydrogen peroxide scavenging, and metal chelating activities compared with reference antioxidants, including α -BHA, BHT, tocopherol and trolox [25]. As a consequence, clove essential oil represents an alternative, and natural preservative of foodstuff, such as meat products, without affecting organoleptic properties [26]. In fact, clove essential oil incorporated in an active packaging for sausages showed an antioxidant effect at the end of a 15-day refrigeration period without causing significant variations in pH and water content of the product [27].

In our study, winter savory and cinnamon essential oils also exhibited a relevant antioxidant activity, with values of 85.49 and 84.77 %, respectively. Both essential oils have previously shown significant higher persistent antioxidant activity [28]. Winter savory essential oil has already demonstrated a retardation of lipid oxidation in cured meat [29,30]. Cinnamon essential oil revealed remarkable *in vitro* DPPH radical scavenging activity in comparison to α -tocopherol, BHA and BHT mainly attributed to cinnamaldehyde and eugenol [31,32]. Due to this activity, cinnamon essential oil has been included in packaging containing pre-harvest and resulting foodstuff with the purpose of extending food shelf-life. In this way, biodegradable polyester nets with cinnamon were observed to maintain the quality of tomatoes during storage [33], and films containing cinnamon and based on carboxymethyl cellulose (CMC)-polyvinyl alcohol (PVA) demonstrated a great improvement of the antioxidant properties for bread preservation [34].

Furthermore, the antioxidant potential of oregano essential oil was compared to that exerted by its main compound, carvacrol. Analogous antioxidant activity was found between both (74.39 % and 70.49 %, respectively), being higher than that obtained by BTH and ascorbic acid. Previous studies also reported a higher activity of oregano as compared to carvacrol probably due to a synergistic effect between the different compounds present in the essential oil [35]. In relation to this, oregano essential oil has been combined with BHT showing a synergistic effect and consequently a higher antioxidant activity, preventing lipid oxidation in food, more specifically sunflower oil [36]. Similar to our findings, oregano essential oil and carvacrol have also demonstrated their remarkable antioxidant effect by other means, such as the chelating effect [37]. Specifically, carvacrol has already been incorporated in materials like gelatin edible films and potato starch nanofibers for its application in food preservation [38,39]. Nevertheless, different isolated components of other essential oils have improved the food preservation more than carvacrol, for instance *trans*-anethole and eugenol [40]. Combined with other components such as thymol, carvacrol has had an additive effect at lower doses but antagonistic at higher concentrations (2.50 mM) [41].

On the other hand, the antioxidant activity of green tea essential oil with *cis*-methyl dihydrojasmonate (15.82 %) as the main compound [42] was 53.99 %, analogous to that displayed by 2.5 mM ascorbic acid with a percentage of 51.83 %.

Finally, essential oils from either rosemary –whose main compounds are 1,8-cineole (25.0 %) and camphor (20.5 %)– or lemon eucalyptus with citronellal as major compound (88.0 %) showed low DPPH reducing power (16.46 and 15.35 %, respectively). These values were higher than those obtained with 1 mM ascorbic acid. Previous studies confirmed the moderate antioxidant activity of rosemary essential oil [43] with a wide range of DPPH radical scavenging activity (8.16-51.80 %) [44].

Other studies, however, indicated that lemon eucalyptus essential oil exhibited moderate to strong antioxidant activity in terms of TAA, FRAP, Fe⁺² chelating, DPPH and H₂O₂ scavenging [45,46].

The other essential oils here studied showed negligible antioxidant activity, such as eucalyptus essential oil with 1,8-cineole as the main compound (76.4 %), whose weak antioxidant capacity has also been reported by other authors [47]. In contrast, other essential oils like wintergreen essential oil with methyl salicylate (99.60 %) that have shown scarce antioxidant activity in this research, exhibited (having a similar composition, methyl salicylate 96.90 %) moderate antioxidant activity in DPPH method [48]. Likewise, turmeric essential oil with higher percentage of α -turmerone (42.6 %) than ar-turmerone (12.9 %) possessed antioxidant activity using both ABTS and DPPH methods [49] while in our study no antioxidant activity was detected in turmeric essential oil with α -turmerone and ar-turmerone values of 14.2 and 38.7 %, respectively.

Antimicrobial Activity of Essential Oils

In this study, antibacterial and antifungal activities of 21 essential oils were evaluated at different doses (1, 5, 10 and 20 μ L) against the Gram-negative bacterium *P. syringae* pv. *tomato*, the causative agent of the bacterial speck disease [50], and the fungus *F. oxysporum* f. sp. *lycopersici*, causal agent of the vascular wilt [51], both producing important economical losses on tomato plants (figure 2).

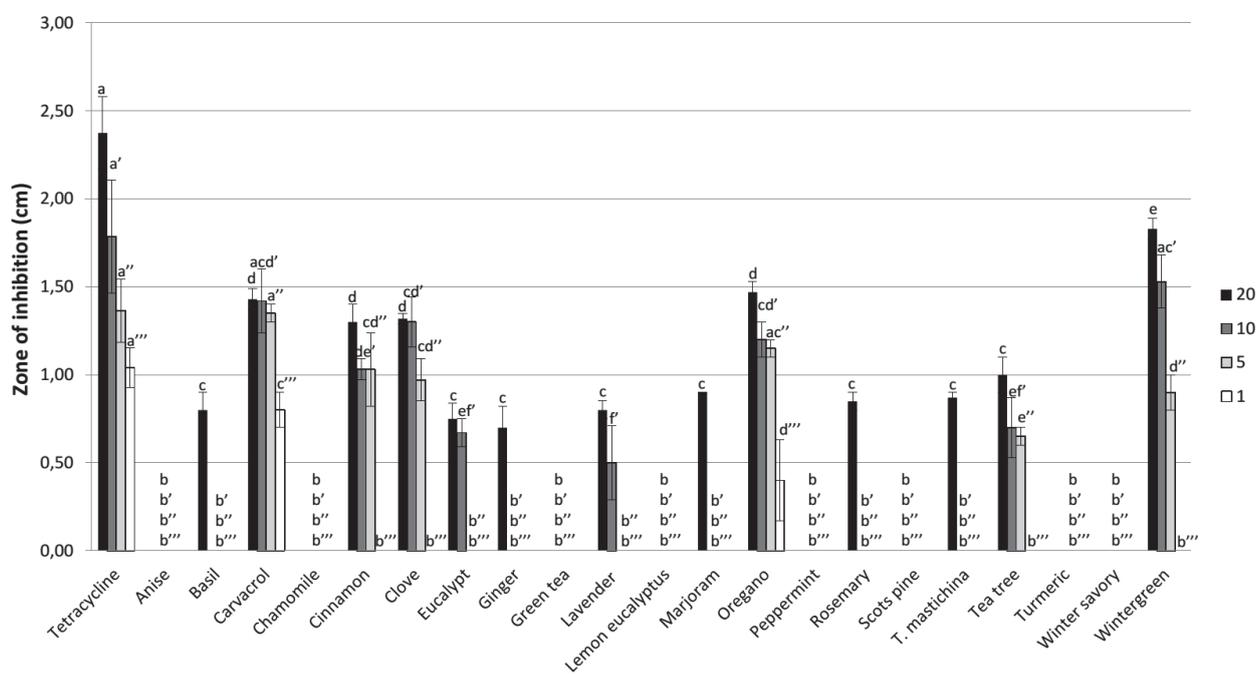


Fig. 2. The inhibition zone (cm) of the *P. syringae* growth by the essential oils at several doses (1, 5, 10 and 20 μ L) as compared to the standard (tetracycline). A Kruskal-Wallis analysis was performed and different letters indicate significant differences ($p < 0.05$) between essential oils at the same dose (same super index).

Wintergreen essential oil showed the major values of *P. syringae* growth inhibition at the highest doses (20 and 10) tested. In fact, this essential oil has been previously reported by its broad-spectrum against Gram-positive and Gram-negative food spoiling bacteria and fungi [48].

Carvacrol, the main component of oregano essential oil, also showed antibacterial activity at all of the studied doses (1.43 cm at 20 μ L, 1.42 cm at 10 μ L, 1.35 cm at 5 μ L and 0.80 cm at 1 μ L). However, in other studies, carvacrol exhibited negligible activity against *P. syringae* in comparison to other components of essential oils, such as eugenol, when it was incorporated in films at doses between 1 and 4 mg/cm² [52]. Anyway, the antimicrobial properties of carvacrol have been generally confirmed, being considered a natural alternative antimicrobial agent for future application in food preservation [53]. In this sense, oregano essential oil displayed antibacterial effect against *P. syringae*, analogous to

its main component carvacrol, achieving an inhibition halo of 0.4 cm even at 1 μ L. An antibacterial activity against different strains of *P. syringae* had also been described, being stronger than the antibiotic streptomycin [54].

The antibacterial activity of cinnamon and clove essential oils having eugenol as the principal component (56.34 and 89.37 %, respectively) was also noticeable and comparable to each other, with no bacterial growth inhibition at the lowest dose assayed (1 μ L). Several studies have demonstrated that both essential oils have antimicrobial effect against plant pathogenic bacteria [55] and even cinnamon essential oil, which has cinnamaldehyde as the main compound (~70 %), improved the effectivity and specificity against *P. syringae*, when encapsulated into mesoporous silica nanoparticles (MSNPs), eliminating 99.9 % of the bacterial growth [56].

Similarly, tea tree essential oil, whose antimicrobial properties have been extensively described [57], showed antibacterial activity at the three major doses (20, 10 and 5 μ L) applied, reaching reduction levels equivalent to half of the tetracycline ones.

Eucalyptus and lavender essential oils only showed antibacterial effect at the highest doses assayed with an inhibition zone of 0.75 and 0.80 cm at 20 μ L, and 0.67 and 0.50 cm at 10 μ L, respectively. In previous reports, eucalyptus essential oil also displayed a strong antibacterial effect against *P. syringae* pv. *tomato* even at a concentration of 1 % [58,59].

Finally, ginger, basil, rosemary, *T. mastichina* and marjoram essential oils only exhibited antibacterial activity at the maximum dose (20 μ L) tested. The reduction of activity of both rosemary and ginger essential oils against *P. syringae* was previously reported [60].

Regarding the antifungal properties, cinnamon, oregano and peppermint essential oils showed the most remarkable antifungal activity against *F. oxysporum* f. sp. *lycopersici* with no significant differences between all doses (20, 10 and 5 μ L) assayed (figure 3). Tea tree, ginger and rosemary essential oils displayed similar antifungal activity showing strong inhibition and no significant differences between the two higher doses (20 and 10 μ L) applied, but a low activity at 5 μ L. The antifungal activity of cinnamon, oregano, peppermint and rosemary against *F. oxysporum* and other micro- and phytopathogens has been recently corroborated [61,62]. Regarding tea tree essential oil, several studies reported activity against a broad spectrum of phytopathogenic fungi such as *Ascochyta rabiei*, *Colletotrichum lindemuthianum*, *F. graminearum*, *F. culmorum*, *Drechslera avenae*, *A. radicina*, *A. dauci* and *Aspergillus ochraceus* [63,64], being the antifungal activity of ginger essential oil against *F. oxysporum* also noticeable at lower doses of only 0.3 % (v/v) [65].

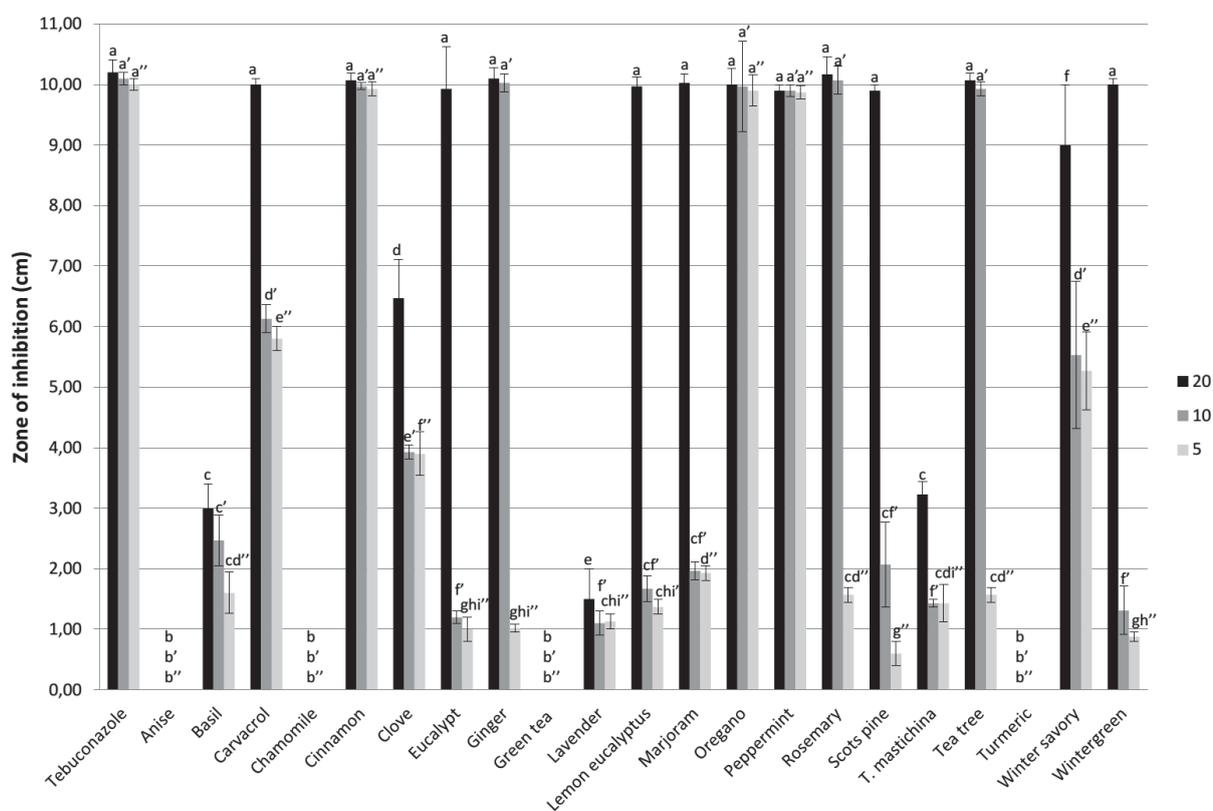


Fig. 3. The inhibition zone (cm) of *F. oxysporum* growth by the essential oils at several doses (5, 10 and 20 μ L) as compared to the standard (tebuconazole). A Kruskal-Wallis analysis was performed and different letters indicate significant differences ($p < 0.05$) between essential oils at the same dose (same superindex).

On the other hand, eucalyptus, lemon eucalyptus, marjoram, scots pine and wintergreen essential oils, as well as carvacrol, showed similar antifungal activity to that of tebuconazole at 20 μ L, although the fungal growth reduction was low at 10 μ L and 5 μ L (figure 3). Otherwise, basil, *T. mastichina* and lavender essential oils showed low antifungal potential at the three doses assayed. The lack of antifungal activity of lavender essential oil has been previously demonstrated against *F. oxysporum* f. sp. *lycopersici*, as well as other pathogens of agricultural interest such as *A. alternata*, *A. brassicae*, *B. spicifera*, *B. cinerea*, *R. solani*, *Cladobotryum mycophilum*, *C. gloeosporoides*, *Curvularia hawaiiensis*, *F. equiseti*, *F. graminearum*, *Phytophthora parasitica*, *Pythium aphanidermatum*, *P. expansum*, *P. italicum*, *S. sclerotiorum* and *Trichoderma aggressivum* f. sp. *europaeum* [61,66]. Finally, anise, chamomile, green tea and turmeric essential oils showed no detectable antifungal activity at neither dose assayed.

Conclusions

In general, the studied essential oils showed remarkable antioxidant, antibacterial or antifungal activity, being the latter one the most outstanding. Sixteen essential oils exhibited antifungal activity against *F. oxysporum* f. sp. *lycopersici* with respect to twelve and seven essential oils that showed antibacterial properties against *P. syringae* and antioxidant power, respectively. All the antibacterial essential oils also displayed antifungal activity, indicating that they could be excellent candidates as antimicrobiological agents. Particularly, three essential oils, cinnamon, wintergreen and oregano essential oil, as well as its main component carvacrol, were the most active antimicrobiological natural products.

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