Use of Essential Oils as Natural Food Preservatives: Effect on the Growth of *Salmonella* Enteritidis in Liquid Whole Eggs Stored Under Abuse Refrigerated Conditions

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Abstract

The steam distillation-extracted essential oils (EOs) of three aromatic plants from the Kabylie region of Algeria (*Eucalyptus globulus*, *Lavandula angustifolia*, and *Satureja hortensis*) were analyzed by gas chromatography coupled with mass spectrometry (GC/MS). The primary compounds from these EOs were 1,8-cineole (81.70%) for *Eucalyptus globulus*, 1,8-cineole (37.80%) and β-caryophyllene (20.90%) for *Lavandula angustifolia*, and carvacrol (46.10%), p-cymene (12.04%), and γ-terpinene (11.43%) for *Satureja hortensis*. To test the antibacterial properties of the EOs, agar diffusion and microdilution methods were used for *Salmonella enterica* serovar Enteritidis CECT 4300. The results revealed that all of the EOs possessed a significant anti-Salmonella activity. The inhibition diameters for *Lavandula angustifolia* and Eucalyptus globulus were 41.30 and 35.26 mm, respectively, whereas the essential oil (EO) of *Satureja hortensis* showed a stronger anti-Salmonella activity (51.15 mm) when compared to the two other EOs. The minimum inhibitory concentration values ranged from 1 to 8 µL/mL, and the MIC value of the *Lavandula angustifolia* EO was the lowest (1 µL/mL). Moreover, the anti-Salmonella activity of the EOs added at various concentrations to liquid whole eggs was investigated, and the results showed that the antibacterial effect is proportional to the quantity of EO added to the product. Based on the observed anti-Salmonella activity, the EOs tested are promising natural alternatives for the preservation of liquid whole eggs stored at 7 ± 1ºC to simulate Algerian refrigeration conditions.

Keywords: essential oils, *Eucalyptus globulus*, *Lavandula angustifolia*, *Satureja hortensis*, antibacterial activity, liquid eggs, *Salmonella* enteritidis

1. Introduction

Eggs are a staple diet for many populations throughout the world and represent an important source of proteins and phospholipids of high nutritional value. Egg and the derived products are used in the preparation of a large number of foods, such as mayonnaise, pastries, sauces, tortillas, and pastas. In the case of industrial applications, the egg is exploited for its various functional properties, such as its aromatic, hydrating, viscous, coloring, and emulsifying properties. However, changes in the consumption habits, modes of consumption, and the development of fast-food restaurants have changed the demand for egg products to include more elaborated products.

The infectious diseases transmitted by foods have remained a major concern of public health and an important economic problem in many countries over the past two decades, and Salmonella poisoning is one of the most important examples of a foodborne disease. The link between the increase in infection from *Salmonella enterica* serovar Enteritidis and the consumption of food preparations that use raw eggs or eggs that are not fully cooked is demonstrated by several studies. The risk associated with egg-derived infections remains high, but this risk

also varies as a function of the production conditions of the egg product and the technologies applied to its transformation and usage (European Commission, 2003). The importance of egg products in the human diet requires methods that ensure the safety of these products. The presence of Salmonella in eggs and its derived products, particularly the presence of *Salmonella* Enteritidis, is closely related to the prevalence of this bacterium during the entire process of primary production (Guard-Petter, 2001). Although sanitary plans, good hygienic and fabrication practices, and the application of hazard analysis-critical control points (HACCP) are imposed during food processing by competent authorities in developed countries, the success of these tools for the reduction of the incidence of human Salmonella infection always requires improvement. In the case of less-developed countries, these measures are inefficient because of their inadequate implementation. Consequently, it is important to focus on efforts to identify the best strategies to decrease the risk of Salmonella infection throughout the food chain to reduce the incidence of *S*. Enteritidis in egg products.

One of the modern ways to improve the hygienic safety of manufactured food products is to exploit the antimicrobial properties of natural plant extracts, allowing for the reduction of the use of chemical antimicrobial agents, which constitute a potential human health hazard. In this regard, the antimicrobial properties of EOs have been known for a long time and continue to be the subject of several studies that evaluate their microbial potential as alternatives to chemical agents in Food industries (Hammer, Carson, & Riley, 1999; Djenane et al., 2009; Djenane et al., 2012b). Thus, the objective of this study was to determine the in vitro anti-Salmonella activity of the EOs extracted from the leaves of *E. globulus* (Eucalyptus), *L. angustifolia* (Lavender), and *S. hortensis* (Summer savory) in relation to their chemical compositions and to evaluate the antibacterial activity of these EOs when added to liquid whole eggs stored at 7 ± 1ºC (to simulate Algerian abuse refrigeration conditions) under aerobic conditions for 6 days.

2. Materials and Methods

2.1 Essential Oils

2.1.1 Plant Material and Extraction of Essential Oils

The EOs was extracted from the aerial parts of *E. globulus*, *L. angustifolia*, and *S. hortensis* specimens collected in Tizi-Ouzou Province (Algeria) from March to May of 2009. These plant materials were deposited at the Herbarium of the Faculty of Biological Sciences and Agronomical Sciences of the University Mouloud Mammeri of Tizi-Ouzou (Algeria). The material was dried for 10 days at room temperature (25-30°C) in a dark room. The EOs were extracted using water steam distillation with a semi-pilot Clevenger apparatus (SAIDAL Pharmaceutical Group, Biotic Company, Algeria) and stored in the dark at 2°C in sealed tubes.

2.2 Essential Oil Analyses

2.2.1 Chromatography

The EOs were analyzed using a Hewlett-Packard 6890 chromatograph equipped with a Stabilwax capillary column (polyethylene glycol, PEG) (length of 30 m, internal diameter of 0.32 mm), with a film thickness of 1 μm (polysilphenylene-siloxane, by SGE), a split/splitless injector, and a flame ionization detector (FID) (Center for Technical and Scientific Research in Physical-Chemical Analysis, Alger, Algeria). The carrier gas was helium, and the auxiliary gas was hydrogen; gases were filtered to remove organic impurities. The analytical conditions were as follows: an injector temperature of 250°C; a detector temperature of 280°C; and an oven temperature of 60°C (8 min), increasing from 60 to 280°C (2°C/min), with a constant temperature of 280°C for 30 min.

2.2.2 Gas Chromatography (GC)/Mass Spectrometry

The EOs were analyzed using a Hewlett-Packard 6890 gas chromatograph system (Agilent Technologies) equipped with a capillary column (5% methylsiloxane phenyl, length of 30 m, internal diameter of 0.25 mm, film thickness of 0.25 μm) coupled to a Hewlett Packard 5973 quadrupole mass spectrometer with an electron impact detector, 70 eV, scanning 30-550 units of atomic mass (CRAPC, Algeria). The analytical conditions were as follows: an injector temperature of 250°C; a detector temperature of 280°C; and an oven temperature of 60°C (8 min), increasing progressively to 280°C at 2°C/min, with a constant temperature maintained for 30 min. The carrier gas was helium at a flow rate of 1.5 mL/min. For all of the analyses, a mixture of oil-hexane was injected (0.2 μL) in split mode. Each EO sample was injected three times, as was the internal calibration solution containing a mixture of C7-C29 n-alkanes. The various compounds of the EOs were identified by comparing their mass spectra to those of the compounds in the Willet and National Institute of Standards and Technology-NIST 98 databases for gas-phase chromatograph/mass spectrometry (CPG/MS) and the Adams spectral databases. The identification of the molecules was confirmed by comparing their retention indices to

those from the literature (Adams, 2001). The retention indices of the compounds were calculated using the time of retention of an n-alkane series with a linear interpolation.

2.3 Antibacterial Activity

2.3.1 Bacterial Strain and Culture Conditions

The *Salmonella enterica* serovar Enteritidis CECT 4300 strain used in this study was obtained from the Colección Española de Cultivo Tipo - CECT (Spanish Type Culture Collection). This strain was cultivated for 12 h at 37 ± 1°C on Mueller Hinton agar (MHA, Oxoid, Basingstoke, UK). Two successive inoculations were performed for 24 h at 37 ± 1°C in tubes containing 9 mL of Brain Heart Infusion Broth (BHIB; Oxoid, Basingstoke, UK). After 48 h, 100 μL of the bacterial suspension was used to inoculate BHI broth. The cultures were then incubated at 37 ± 1ºC for 12 h to obtain a fresh bacterial solution of approximately $2\text{-}3 \times 10^5$ colony-forming units (cfu)/mL, as determined by the transmission at 600 nm (Spectrophotometer: Spectronic 20 Bausch & Lomb). The *S*. Enteritidis strain was stored at -80ºC and re-isolated before each test.

2.3.2 Test of Agar Diffusion

The assessment of the antibacterial activity of the EOs was determined using the technique of agar diffusion described by Hazzit, Baaliouamer, Veríssimo, Faleiro, and Miguel (2009). Petri dishes containing 15 mL of molten Mueller Hinton agar were placed under a vertical laminar flow hood and were allowed to solidify and dry at 25 ± 1°C for 30 min. A 0.1 mL aliquot of the standardized inoculum suspension ($2\text{-}3 \times 10^5$ cfu/mL) was applied and uniformly spread on each plate. All of the plates were allowed to dry for 5 min. The EOs were dissolved in a 0.5 % (v/v) solution of dimethyl sulfoxide (DMSO) (Sigma Aldrich®-Química, S.A.), and 5 μL of the EO was applied to sterile paper disks (6 mm in diameter, Filter LAB ANOIA, Barcelona, Spain) using a capillary micropipette (Finnpipette®, Thermo Fischer Scientific Inc.). The Petri dishes were incubated for 15 min at 25 ± 1°C and then at 37 ± 1°C for 24 h. The antibacterial activity was evaluated using a caliper (Wiha dialMax® ESD-Uhrmessschieber, CH) by measuring the diameter of the clear zones (mm) that developed around the disks (∅ of the disk is included: 6 mm). The sensitivity of *S*. Enteritidis to the different EOs was classified based on the diameter of the inhibition halos (Ponce et al., 2003): ∅ < 8 mm, insensitive; 9 < ∅ < 14 mm, sensitive; 15 < ∅ < 19 mm, very sensitive; and ∅ > 20 mm, highly sensitive. A negative control was used with the same solvent in which the EOs was dissolved. A standard antibiotic, chloramphenicol (10 μg/disk), was used as a positive control. Each test was repeated three times.

2.3.3 Minimum Inhibitory Concentration: Microdilution Test

The minimum inhibitory concentration was also determined for *S*. Enteritidis. The inoculum of *S*. Enteritidis was obtained from a pre-culture incubated for 12 h, and the microbial charge was adjusted to 5×10^3 cfu/mL using the McFarland 0.5 turbidity standard. The EOs were dissolved in DMSO (0.5%), and ½ serial dilutions were prepared in a concentration range of 32 to 0.3125 μL/mL in sterile test tubes containing Mueller Hinton (MH) broth. The MICs of the various EOs against *S*. Enteritidis were determined using the well microdilution method: 96-well plates (Iwaki brand, Asahi Techno Glass, Japan) were prepared by adding 95 μL of MH broth and 5 μL of inoculum to each well. A 100-μL aliquot of each EO solution previously prepared at a concentration of 32 μL/mL was added to the first well of each plate, and 100 μL of each serial dilution was then added to each successive well. The last wells were used as the negative controls and contained 195 μL of nutritive broth without EO and 5 μL of inoculum. The final volume of each well was 200 μL.

Levofloxacin, a standard antibiotic, was used as the positive control, with Levofloxacin concentrations (32-0.3125 μL/mL) prepared in MH broth. The same protocol as that for the EOs was used. All contents of each well were then homogenized (300 rpm/20 s), and the plates were placed at 37 ± 1°C for 18-24 h in an incubator with shaking. After the incubation, each well was examined, and the MIC (μL/mL) was determined by taking into account the lowest concentration of EO that inhibits all bacterial growth (no turbidity). The DMSO solution (0.5%) used to dissolve the EOs was used as the negative control. Each test was repeated twice.

2.4 Application to Liquid Whole Eggs

2.4.1 Preparation of Liquid Whole Eggs

Industrial chicken eggs were purchased from a local producer in Tizi-Ouzou (Algeria) and stored at 4 ± 1°C; eggs with blood spots or that were dirty or cracked were discarded. Before the beginning of the experiment, the egg shells were rinsed in 70% ethanol (Monfort et al., 2010) and air-dried. The disinfected eggs were then broken and placed in sterile Stomacher bags (Tekmar Co. Cincinnati, Ohio, U.S.A). The bags were then homogenized for 2 minutes at 300 rpm using a Stomacher 400 circulator (Tekmar Co. Cincinnati, Ohio, U.S.A).

The liquid whole eggs obtained were centrifuged at $10^2 \times g$ for 2 min using a Heraeus Megafuge 1.0R to eliminate any residual air.

2.4.2 Treatments of Liquid Whole Eggs

Before the inoculation with *S*. Enteritidis, samples of the liquid whole eggs were analyzed for the presence/absence of *S*. Enteritidis. A 50-g sample of the liquid whole eggs was placed in a sterile glass container and inoculated with approximately 5×10^3 cfu of *S*. Enteritidis/g. Prior to the inoculation; various concentrations of the studied EOs were added to the samples, except for the negative controls. A total of 128 samples of 50 g each were prepared to examine the growth of *S*. Enteritidis in the presence of various EO concentrations (1 × MIC, 4 × MIC, 8 × MIC, and 16 × MIC). All of the samples were placed at $7 \pm 1°C$ under aerobic conditions for 6 days. The counting of *S*. Enteritidis was performed every other day for the duration of the storage period.

2.5 pH Measurement

The pH of the liquid whole eggs was measured using a micro pH-meter model 2001 (Crison Instruments, Barcelona, Spain) after homogenizing 3 g of the product in 27 mL of distilled water for 10 s at 1300 rpm using a Ultra-Turrax T25 macerator (Janke & Kunkel, Staufen, Germany). Each value was the average of three measurements.

2.6 Sensory Analysis

Samples of liquid whole eggs were evaluated for freshness odor by a sixth-member trained panel. Panelists were selected among students and staff of the department and trained according to the guidelines of Djenane et al. (2001, 2012).

Three open-discussion sessions were held to familiarise panelists with the attributes and the scale to be used. The attribute "EO odor" referred to the intensity of perceptible EO odor after sample opening: 1 = none; 2 = slight; 3 = small; 4 = moderate; and 5 = extreme. A score of 3 or higher in any of the attributes denoted that liquid whole eggs were unacceptable for sale or consumption.

2.7 Bacteriological Analysis

In between each analysis, 25 g of samples was placed in sterile bags, and 225 mL of buffered peptone water was added aseptically. The bags were homogenized in a Stomacher for 1 min at 25ºC. Serial decimal dilutions were prepared in sterile peptone water (0.1%), and 0.1 mL of each dilution was inoculated onto Petri dishes containing the appropriate medium for *S*. Enteritidis (Salmonella-Shigella Agar - SS Agar, BD, 274500). The dishes were then incubated at 37ºC for 24 h. The results are expressed in $\log_{10}$ cfu/g.

2.8 Statistical Analysis

The variance was evaluated to determine significant differences between the treatments using the software SPSS (SPSS for Windows, 6.1.2., SPSS Inc., Chicago, IL). The differences between means were tested using the least significant difference (LSD). The values were considered significantly different when $p < 0.05$.

3. Results and Discussion

3.1 Chemical Composition of the Essential Oils

Hydro-distillation is currently the preferred industrial method to extract EOs. However, despite the fact that the extraction by supercritical carbon dioxide (CO_2) under high pressure produces EOs with natural organoleptic profiles, the use of this method in industry is limited due to its low economic profitability. The chemical analysis (Table 1) revealed a set number of compounds for the three EOs, representing 94.63%, 96.35%, and 95.57% for *E. globulus*, *L. angustifolia* and *S. hortensis*, respectively.

Table 1. Chemical composition (%) of the EOs obtained from the leaves of *E. globulus*, *S. hortensis* and *L. angustifolia*

Compound	E. globulus	S. hortensis	L. angustifolia
α-Pinene	2.32	0.07	0.51
Camphene	-	2.06	-
Sabinene	-	0.08	0.72
β-Myrcene	-	-	2.83
p-cymene	1.07	**12.04**	3.81
Limonene	-	-	**11.20**
β-Phellandrene	-	-	2.30
1,8- Cineole	**81.70**	-	**37.82**
Cis-β-Ocimene	-	-	0.50
β-Pinene	-	0.06	0.22
α-Phellandrene	0.08	0.20	-
α-Terpinene	0.02	3.70	-
γ-Terpinene	8.50	**11.43**	-
α-Terpinolene	-	0.34	1.11
Linalool	-	-	0.53
Borneol	-	0.37	0.60
2-Méthylbuterate	-	-	-
Terpinene-4-ol	0.08	0.90	-
Bornyl acetate	-	-	0.40
Linalyle acetate	-	-	0.22
α-terpinyl acetate	0.10	-	-
Carvacrol acetate	-	9.57	-
Carvacrol	-	**46.10**	-
α-Caryophyllene	-	5.06	-
β-Caryophyllene	0.04	-	**20.90**
Allo-aromadandrene	-	0.08	-
β-Cubebene	-	0.38	-
Ledene	-	0.09	-
β-Bisabolene	-	2.65	-
Cadinene	-	0.27	7.14
Spathulenol	-	0.07	-
Caryophyllene Oxyde	-	0.05	2.00
Camphre	-	-	1.73
Bergamotenes	-	-	1.81
Total identified(%)	94.63%	95.57%	96.35%

The EO of *E. globulus* was characterized by a high percentage of 1,8-cineole (81.70%) and γ-terpinene (8.50%). In contrast, the EO of *L. angustifolia* displayed a notable quantity of 1,8-cineole (37.82%), β-caryophyllene (20.90%), and limonene (11.20%), followed by cadinene (7.14%), p-cymene (3.81%), and β-myrcene (2.83%). The results from the analysis also showed that the EO of *S. hortensis* is rich in carvacrol (46.10%), p-cymene (12.04%), γ-terpinene (11.43%), and carvacrol acetate (09.57%), followed by β-caryophyllene (5.06%), γ-terpinene (3.70%), β-bisabolene (2.65%), and camphene (2.06%). However, the percentages of most of the identified compounds in the EOs vary significantly in the literature. These differences in composition could be due to seasonal variations in the different compounds of the plant's EO that are linked to changes throughout the life cycle of the plant. The environmental conditions in the Mediterranean region could also have an impact.

Indeed, environmental factors, such as geography, temperature, day length, and nutrients, have been examined and are thought to play a role in the chemical composition of EOs (Masotti, Juteau, Bessière, & Viano, 2003; Angioni, Barra, Coroneo, Dessi, & Cabras, 2006; Gardeli, Vassiliki, Athanasios, Kibouris, & Komaitis, 2008). These factors influence the biosynthetic pathways of the plant and, consequently, play a role in the relative proportions of the major characteristic compounds, leading to the existence of different chemotypes that distinguish EOs from different regions. Our data analysis showed that the EO chemical profiles were different from those of the same EOs derived from plants from different areas. Moreover, quantitative differences in individual compounds exist. For example, the EO of *E. globulus* from the municipality of Sobral in Brazil has been characterized by 1,8-cineole (83.89%), limonene (8.16%), and α-pinene (4.15%) (Maciel et al., 2010), whereas in the central area of the Congo in Africa, the chemotype of eucalyptus EO was reported to be mostly 1,8-cineole (44.30%), with high amounts of camphene (23.10%) (Cimanga et al., 2002). However, *E. globulus* from Portugal contains 1,8-cineole (63.80%) and α-pinene (14%) as the major compounds of its EO (Silvestre, Cavaleiro, Delmond, Filliatre, & Bourgeois, 1997).

According to the presence and quantity of the major compounds, the EO of L. angustifolia tested in the present study produced results that are different from those previously published. The EO of *L. angustifolia* from Iran contains 1,8-cineole (65.40%), borneol (11.50%), and camphor (9.50%) as the most abundant compounds (Hajhashemi, Ghannadi, & Sharif, 2003). However, the EO derived from *L. angustifolia* grown in Greece is characterized by linalool (20.10%), linalyl acetate (13.30%), and eucalyptol (12.40%) (Hassiotis, Tarantilis, Daferera, & Polissiou, 2010), whereas 1,8-cineole is the primary compound (38.40%), followed by cis-verbenol (4.30%) and cymene-8-ol (3.80%) in the EO of *L. angustifolia* from the Cherchel region (North Algeria) (Dob, Dahmane, Tayeb, & Chelghoum, 2005). Finally, the oil of *L. angustifolia* from the South of France is characterized by a high percentage of linalool (42.52%), 1,8-cineole (14.40%), and borneol (9.38%) (Sahraoui, Vian, Bornard, Boutekedjiret, & Chemat, 2008).

Several analyses were undertaken to determine the chemical profile of the EOs extracted from species of savory from various sources. Based on the presence and quantity of the major compounds, the EO of *S. hortensis* from our region contains the same percentage of carvacrol (46%) as the EO from an Iranian source (Sefidkon, Abbasi, & Khaniki, 2006). However, qualitative and quantitative differences were noted relative to other compounds. In a recent study, Hadian, Ebrahimi, and Salehi (2010) analyzed the EO of *S. hortensis* from several areas in Iran, and the primary compounds were found to be carvacrol (42-83.30%), γ terpinene (0.50-28.50%), and p-cymene (1-17.10%). Güllüce et al. (2003) determined the chemical composition of the EO of *S. hortensis* obtained by hydro-distillation using gas chromatography/flame ionization detection (GC/FID) and GC/MS, and 22 compounds representing 99.9% of the EO were identified, with thymol (29.0%), carvacrol (26.50%), γ-terpinene (22.60%), and p-cymene (9.30%) being the major compounds. Bakkali, Averbeck, Averbeck, and Idaomar (2008) noted that EOs is a very complex natural mixture that can contain approximately 20-60 compounds at very different concentrations. EOs are generally characterized by a few major compounds at relatively high concentrations (20-70%), with other compounds present in minute quantities, and the major compounds determine the biological properties of the EO. In conclusion, the various chemical compositions of the EOs could be correlated with the geographic origin of the plants and the ecological conditions under which the plants develop. Indeed, several authors suggest the possible existence of new chemotypes.

3.2 Antibacterial Activity (Agar Diffusion Method)

The in vitro tests of the antimicrobial activity of the different EOs against *S.* Enteritidis, a common pathogenic bacteria associated with foodborne diseases, were assessed qualitatively and quantitatively by the presence or absence of inhibition zones and the determination of the MICs. According to the results, the different EOs showed strong anti-Salmonella activities that were comparable to those obtained with chloramphenicol, the antibiotic used as the positive control.

The inhibition diameters obtained (for *S.* Enteritidis) were 51.15 ± 2.20, 41.30 ± 0.90, and 35.26 ± 3.20 mm, for the EOs of *S. hortensis*, *L. angustifolia*, and *E. globulus*, respectively; the inhibition zone of chloramphenicol was 33.25 ± 0.80 mm. The strong antimicrobial activity of these three EOs was confirmed by the microdilution method, showing MIC values against *S.* Enteritidis close to 1, 2, and 8 μL/mL for the EOs of *L. angustifolia*, *S. hortensis*, and *E. globules*, respectively.

Vilela et al. (2009) reported similar results for the EO of *E. globulus* tested against *Aspergillus flavus* and *Aspergillus parasiticus*. The authors pointed out that the EO of *E. globulus*, applied either by direct contact or by using the microatmosphere method, provided a total inhibition of these two fungi. The antimicrobial effects observed in the present study are for the most part comparable to those reported in the literature. Vagionas,

Graikou, Ngassapa, Runyoro, and Chinou (2007) recorded MIC values for the EO of savory against certain pathogenic bacteria that are very similar to our observations (0.04 to 0.10%). Many authors also reported that the EO of savory plants was among the most powerful EO in terms of its antimicrobial properties (Ciani et al., 2000), as was confirmed in the present study. The EOs of many species of the Satureja genus (savory) are known to have antibacterial and antifungal properties, such as *S. montana*, *S. hortensis*, *S. thymbra*, *S. biflora*, *S. masukensis*, and *S. pseudosimensis* (Ciani et al., 2000; Özcan & Boyraz, 2000; Özcan & Erkmen, 2001). In a recent study, Hanamanthagouda et al. (2010) evaluated the antimicrobial activity of lavender EO against certain bacteria (Gram positive and Gram negative) and molds and reported that lavender oil has a considerable antibacterial activity against Gram-positive bacteria, with MIC values ranging from 0.5 to 2 µg/µl and from 2 to 4 µg/µl for bacteria and molds, respectively. According to the same authors, lavender EO is characterized by an antibacterial activity that is stronger than its antifungal activity. The antibacterial activity attributed to these EOs could be due their elevated levels of compounds known for their antimicrobial activity, such as linalool (Sonboli, Eftekhar, Yousefzadi, & Kanani, 2005), p-cymene (Bagamboula, Uyttendaele, & Debevere, 2004), β-myrcene (De-Oliveira, Ribeiro-Pinto, & Paumgartten, 1997), 1,8-cineole (Sonboli, Babakhani, & Mehrabian, 2006), carvacrol (Botelho et al., 2007), and thymol (Wendakoon & Sakaguchi, 1995). Confirming previous reports, it has been noted that the power and spectrum of the antimicrobial activity differ among the species of Satureja and type of bacteria (Gram positive or Gram negative) studied. However, Gram-positive bacteria are generally more sensitive to the effects of EOs, whereas Gram-negative bacteria have a higher overall resistance because of their external phospholipid membrane that is almost impermeable to lipophilic compounds. The absence of this barrier in Gram-positive bacteria allows the direct contact of the hydrophobic compounds from EOs with the phospholipid bilayer of the bacterial cellular membrane. Thus, these compounds can be effective and lead to an increase in the ion permeability and the release of vital intracellular constituents or an alteration of the bacterial enzyme systems (Wendakoon & Sakaguchi, 1995).

Furthermore, the antimicrobial activity of EOs is difficult to correlate to a specific compound because of their complexity and variability. Nevertheless, some researchers have mentioned that there is a tight relationship between the chemical composition of the most abundant elements and the antimicrobial activity. For example, 1,8-cineole (abundant in the *E. globulus* and *L. angustifolia* EOs tested in this study) is well known for its antimicrobial potential (Sonboli, Eftekhar, Yousefzadi, & Kanani, 2005). Lis-Balchin and Deans (1997) showed that EOs with high levels of 1,8-cineole are better antibacterial agents than EOs that have no 1,8-cineole. Accordingly, strong antimicrobial activities of the EOs found in this study could be attributed to their chemotypes. Moreover, numerous reports have mentioned that carvacrol and thymol and their precursors (p-cymene and γ-terpinene) are biologically and functionally tightly associated (Ultee, Bennik, & Moezelaar, 2002). Within this context, p-cymene was more abundant in the EO of *S. hortensis* (12.04%) than in the EOs of *E. globulus* and *L. angustifolia*. The MIC values show that the EOs of *L. angustifolia* and *S. hortensis* were more efficient than that of *E. globulus*. Kim, Marshall, and Wei (1995) indicated that, because of the variation in the diffusion and solubility properties of the various EOs in different media, the results obtained using the disk method are not directly comparable to those obtained using the microdilution method. Nevertheless, our results showed that the EOs that resulted in large inhibition zones of *S*. Enteritidis is those that generated the lowest MIC values.

3.3 Application to Liquid Whole Eggs and Antibacterial Efficacy

Because of the use of such plants as eucalyptus, lavender, and savory in the cuisine and traditional medicine of the Kabylie region, the aim of this study was first to determine the in vitro antibacterial efficacy of the EOs obtained from these plants in this region of Algeria. Second, this study aimed at assessing the application of EOs to liquid whole eggs to test their anti-Salmonella efficacy. Figures 1-4 show the results of the *S*. Enteritidis growth in liquid whole eggs stored at 7 ± 1ºC in the presence of various concentrations of the EOs from *E. globulus*, *L. angustifolia*, and *S. hortensis*. These results show that the antibacterial effect generated was proportional to the concentration of each EO added to the product. Figures 1-4 also show that the number of Salmonella in the liquid whole eggs used as the controls (no EO added) reached 4.8 $\log_{10}$ cfu/g after the 4th day of storage; two days later (6th day), this concentration reached a value of 5.4 $\log_{10}$ cfu/g. These results show that a significant antibacterial effect ($p < 0.05$) resulted from the EOs being applied to the product at a concentration equal to 1 × MIC (Figure 1), as compared to the samples with no treatment (controls). This effect was obvious from the 4th day of storage. Indeed, a decrease of 2.40, 2.55, and 1.96 $\log_{10}$ cfu/g (decreases of 44.50, 47.20, and 36.30%) was recorded on the last day of storage for the EOs of eucalyptus, lavender, and summer savory, respectively.
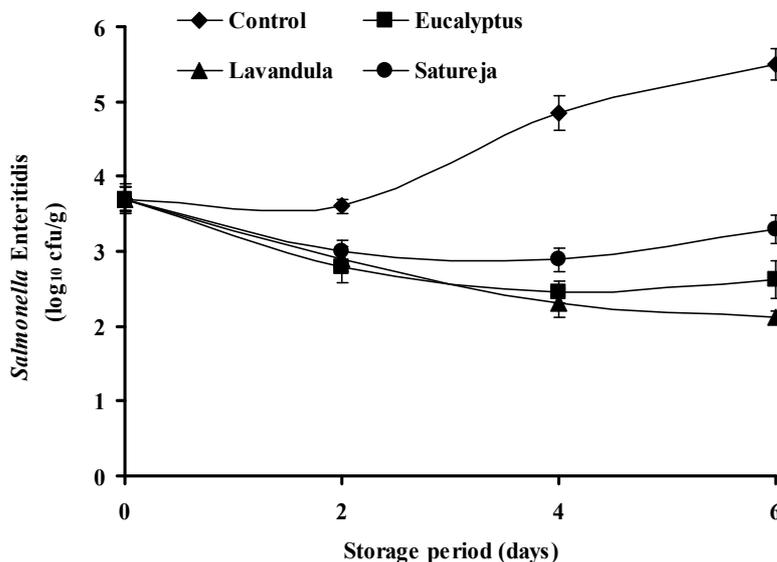
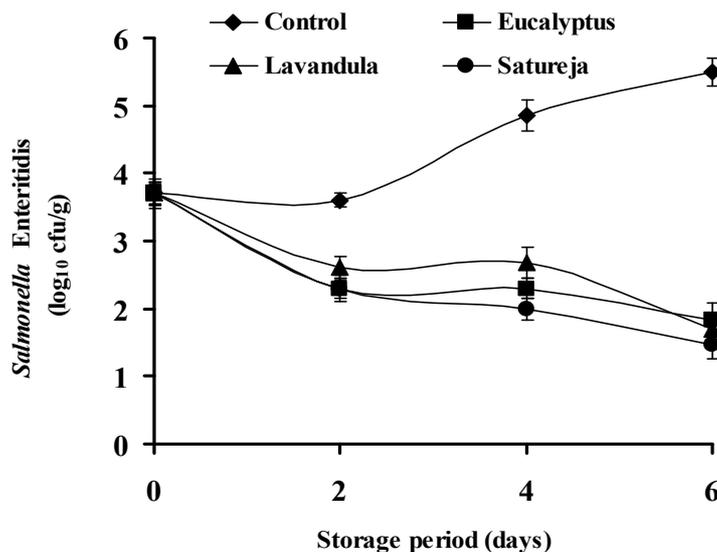Figure 1. Inhibition of *S. Enteritidis* by the EOs added at a concentration equal to the MIC value in liquid whole eggs stored at $7 \pm 1°C$. (♦) Control; (■) *E. globulus*; (▲) *L. angustifolia*; (●) *S. hortensis.* The error bars represent standard deviation

Figure 2 summarizes the antibacterial effect of the EOs applied to the liquid whole eggs at a concentration of $4 \times$ MIC. According to the results, these EOs were all potent against Salmonella ($p < 0.05$), but the effect was dependent on the concentration.

Figure 2. Inhibition of *S. Enteritidis* by the EOs added at a concentration equal to 4 times the MIC in liquid whole eggs stored at $7 \pm 1°C$. (♦) Control; (■) *E. globulus*; (▲) *L. angustifolia*; (●) *S. hortensis.* The error bars represent standard deviation

In comparison with the previous results ($1 \times$ MIC), an increase in the inhibition rate on the order of 35, 35.08 and 53.20% (decreases of 1.05, 1, and 1.83 $\log_{10}$ cfu/g) was recorded on the last day of storage for the EOs of eucalyptus, lavender, and summer savory, respectively. Nevertheless, an application on the order of $8 \times$ MIC (Figure 3) generates a stronger decrease in the bacterial growth when compared to that of the previous two treatments. Indeed, growth reductions of 0.98, 1.13 and 1.04 $\log_{10}$ cfu/g were recorded on the 4th day of storage for the $4 \times$ MIC treatment for the EOs of eucalyptus, lavender, and summer savory, respectively.
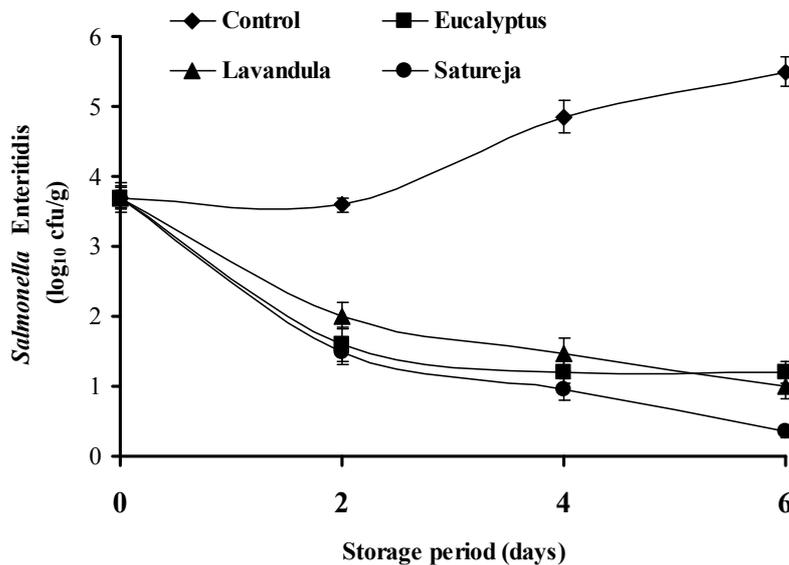
Figure 3. Inhibition of *S. Enteritidis* by the EOs added at a concentration equal to 8 times the MIC in liquid whole eggs stored at 7 ± 1ºC. (♦) Control; (■) *E. globulus*; (▲) *L. angustifolia*; (●) *S. hortensis*. The error bars represent standard deviation

However, the treatment of 8 × MIC generated a stronger growth reduction when compared to that of the 1 × MIC treatment. In this case, the growth reductions were 1.71, 1.54 and 2.30 $\log_{10}$ cfu/g and 1.96, 2.01 and 2.97 $\log_{10}$ cfu/g on the 4th and 6th storage days, respectively, for the same EOs. From our results, it is clear that an application on the order of 16 × MIC allowed a stronger bacterial reduction when compared to that of the other treatments. Figure 4 shows that, from day 4 of storage, the bacterial concentration reached values less than 1 $\log_{10}$ cfu/g for all of the EOs tested. Indeed, in control samples, inhibition rates of 81.55, 88.90 and 62.60% (reductions of 4.40, 4.80 and 5 $\log_{10}$ cfu/g) were recorded on the last storage day for the eucalyptus, lavender, and summer savory EOs, respectively.
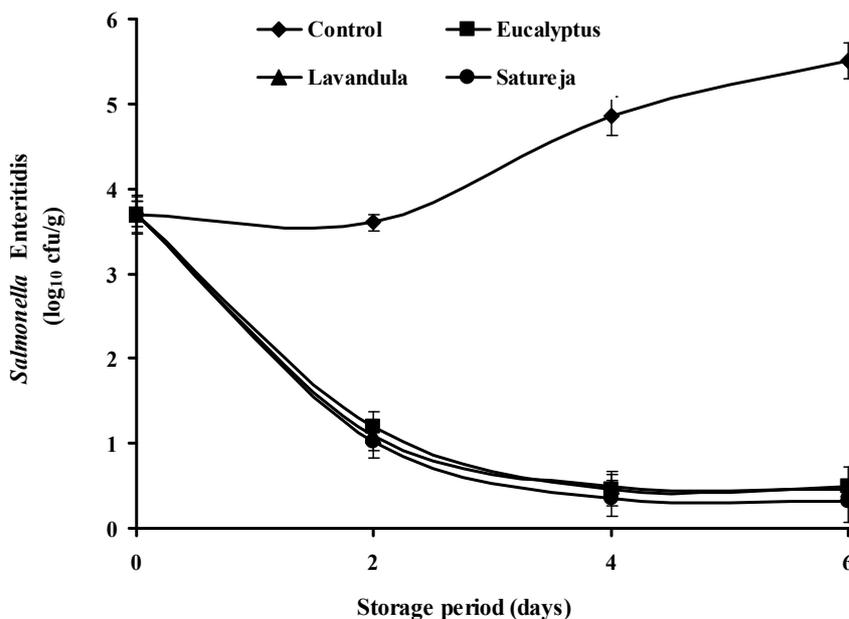

Figure 4. Inhibition of *S. Enteritidis* by the EOs added at a concentration equal to 16 times the MIC in liquid whole eggs stored at 7 ± 1 º C. (♦) Control; (■) *E. globulus*; (▲) *L. angustifolia*; (●) *S. hortensis*. The error bars represent standard deviation

This study shows that, when EOs are applied to a product at a low concentration, the observed effect is bacteriostatic. However, the bactericide activity was pronounced for all of the EOs, particularly for *S. hortensis*, when they are applied at higher concentrations. Several recent studies have successfully shown the potential application of EOs to reduce or control the presence of pathogenic agents in food products, such as milk (Karatzas, Kets, Smid, & Bennik, 2001), fish (Mejlholm & Dalgaard, 2002), fruits (Roller & Seedhar, 2002), and meat (Tsigarida, Skandamis, & Nychas, 2000). The antimicrobial activity of *S. hortensis* observed in this study could be attributed to the presence of higher concentrations of carvacrol, γ-terpinene, and p-cymene, three monoterpenes considered to be potential antibacterial (Oussalah, Caillet, Saucier, & Lacroix, 2007) and antifungal (Sefidkon, Abbasi, & Khaniki, 2006) compounds. Lis-Balchin and Deans (1997) showed that EOs with high quantities of 1,8-cineole are good antibacterial agents. The high antimicrobial activity of the EOs of eucalyptus and lavender could also be associated with their high levels of 1,8-cineole.

With regard to their antibacterial properties, EOs are considered a complex mixture of numerous chemicals. Thus, their antibacterial effects could be the result of a synergy among all of the molecules or due the major molecules that are present. The mechanisms by which microorganisms are inhibited by EOs seem to imply that there are different pathways involved. The phenolic compounds present in EOs have been recognized by several authors for their antimicrobial activity (Tajkarimi, Ibrahim, & Cliver, 2010). In addition, the necessity of increasing the EO concentrations used in food is linked to the more complex nature of food matrices (the presence of fat and proteins). The pH of liquid whole eggs is an important factor that affects the activity of EOs: at a low pH, the hydrophobicity of some EOs increases, and, consequently, they distribute properly in the lipid phase of the product. The EOs can also more easily dissolve in the lipid phase of the bacterial membrane, thus reinforcing their antimicrobial activity. The initial pH of the liquid whole eggs was 7.4 ± 0.2 and decreased to approximately 7.2 after the treatment with the EOs. The pH values were not significantly different ($p > 0.05$) among all of the samples during storage. The fact that the initial pH of the product slowly decreased in the presence of the EOs and that there was no significant difference ($p > 0.05$) between the samples could be explained by the buffering power of the product. As a general rule, the sensitivity of bacteria to the antimicrobial effect of EOs seems to increase when a decrease is noted in the pH of the food, the storage temperature, and the amount of oxygen (O_2) present around the product (Tsigarida, Skandamis, & Nychas, 2000). Concerning the nature of the food matrix, Holley and Patel (2005) found that some EOs were more efficient in products containing lower amounts of lipids. In agreement with these findings, Smith-Palmer, Stewart, and Fyfe (2001) showed that the active compounds present in different EOs generated a higher antimicrobial activity against *Listeria monocytogenes* in products low in fats when compared to products with high amounts of fats. The above-mentioned aspects should be considered for any optimized application of EOs in food products. Generally speaking, the major compounds in EOs are the reflection of their antibacterial activity. Thus, the synergic functions of the different molecules within EOs, as opposed to the action of one or two major compounds in the oil, seem arguable. However, it is possible that the activity of those major compounds is modulated by other minor molecules. In fact, the synergic effects of the diversity of the major and minor compounds present in EOs should be taken into consideration to understand their biological activity. According to studies investigating the mechanisms of the antimicrobial action of those molecules, it appears that, following the breakdown of the bacterial cellular membrane caused by these substances, the release of intracellular metabolites leads to cellular death (Rasooli, Rezaei, & Allameh, 2006; Cristani et al., 2007). Longara-Delamare, Moschen-Pistorello, Artico, Atti-Serafini, and Echeverrigaray (2007) attributed the high antibacterial activity of EOs to the presence of caryophyllene, a compound found in the lavender (20.90%) used in the present study. In our study, various EO concentrations were evaluated to test the effect of the concentration on the organoleptic properties of the product. The application of the three EOs to liquid whole eggs at the indicated concentrations seemed to improve the antimicrobial efficacy of the EOs. It is important to note that a panel of consumers did not detect any particular odor linked to the presence of the EOs in the liquid whole eggs (Table 2).

Table 2. Mean sensory scores (1–5) for EOs odor of liquid whole eggs stored for 6 days at $7 \pm 1°C$ (1 = none; 2 = slight; 3 = small; 4 = moderate; and 5 = extreme. A score of 3 or higher denoted that product was unacceptable for sale or consumption)

Parameter	Treatment	Days of storage			
		0	2	4	6
Eucalyptus odor	Control	1.00±0.00	1.00±0.00	1.00±0.00	1.00±0.00
	1×MIC	2.17±0.41	2.33±0.52	1.00±0.00	1.00±0.00
	4×MIC	2.33±0.52	2.17±0.41	2.00±0.00	1.00±0.00
	8×MIC	2.50±0.55	2.50±0.84	2.17±0.41	2.00±0.00
	16×MIC	3.00±0.00	2.33±0.52	2.17±0.41	2.17±0.41
Lavender odor	1×MIC	2.00±0.00	2.66±0.82	1.00±0.00	1.00±0.00
	4×MIC	2.17±0.41	2.17±0.41	2.00±0.00	1.00±0.00
	8×MIC	3.00±0.00	2.33±0.52	2.17±0.41	2.00±0.00
	16×MIC	4.00±0.00	2.50±0.55	2.17±0.41	2.33±0.52
Summer savory odor	1×MIC	2.33±0.52	2.17±0.41	1.00±0.00	1.00±0.00
	4×MIC	2.67±0.52	2.50±0.84	2.00±0.00	1.00±0.00
	8×MIC	2.83±0.75	2.17±0.41	2.17±0.41	2.00±0.00
	16×MIC	3.00±0.63	2.33±0.52	2.17±0.41	2.17±0.41

Consequently, the sensorial properties of the liquid whole eggs treated with these EOs were acceptable to the panelists. Preliminary studies showed that a mixture of various EOs , or EOs mixed with other compounds, had a stronger antimicrobial efficacy against food-borne pathogenic agents (Gutierrez, Barry-Ryan, & Bourke, 2008; Djenane et al., 2011a,b). However, additional studies are necessary to evaluate the efficacy of an EO mixture and the use of active packaging to assess their performance as natural antimicrobial agents for preservation and food safety (Ponce, Fritz, del Valle, & Roura, 2003; Monfort et al., 2010).

4. Conclusion

The results of the antibacterial tests and the very interesting chemical profile of the EOs support their potential use as natural preservation agents to help reduce *Salmonella* in liquid whole eggs. Sensory evaluation revealed that the aroma of liquid whole eggs treated with EOs was acceptable by panellists at the levels used.

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