Journal of Food Protection, Vol. 69, No. 7, 2006, Pages 1579–1586

Antimicrobial Activity of Essential Oils on *Salmonella* Enteritidis, *Escherichia coli,* and *Listeria innocua* in Fruit Juices

ROSA M. RAYBAUDI-MASSILIA, JONATHAN MOSQUEDA-MELGAR, AND OLGA MARTÍN-BELLOSO*

Department of Food Technology, University of Lleida, UTPV-CeRTA, Avenida Alcalde Rovira, Roure, 191, 25198 Lleida, Spain

MS 06-009: Received 8 January 2006/Accepted 7 March 2006

ABSTRACT

The antimicrobial properties of essential oils (EOs) and their derivatives have been known for years. However, the information published about the minimal effective concentration of EOs against microorganisms in fruit juices is scarce. In this study, both MIC and MBC of six EOs (lemongrass, cinnamon, geraniol, palmarosa, or benzaldehyde) against *Salmonella* Enteritidis, *Escherichia coli,* and *Listeria innocua* were determined by the agar and broth dilution methods, respectively. All of the six EOs inhibited the microbial (*Salmonella* Enteritidis, *E. coli,* and *L. innocua*) growth at a concentration from 1 μl/ml (MIC). These studies led to choosing the three most effective EOs. Lemongrass, cinnamon, and geraniol were found to be most effective in inhibiting the growth of the microorganisms and thus were used for the MBC analysis. On this last point, significant differences ($P < 0.05$) among EOs, their concentrations, and culture media (apple, pear, and melon juices, or tryptone soy broth medium) were found after comparing the results on MBC for each microorganism. A concentration of 2 μl/ml from lemongrass, cinnamon, or geraniol was enough to inactivate *Salmonella* Enteritidis, *E. coli,* and *L. innocua* in apple and pear juices. However, in melon juice and tryptone soy broth medium, concentrations of 8 and 10 μl/ml from cinnamon, respectively, or 6 μl/ml from geraniol were necessary to eliminate the three microorganisms, whereas lemongrass required only 5 μl/ml to inactivate them. These results suggest that EOs represent a good alternative to eliminate microorganisms that can be a hazard for the consumer in unpasteurized fruit juices. The present study contributes to the knowledge of MBC of EOs against pathogenic bacteria on fruit juices.

Unpasteurized fruit juices have desirable flavor characteristics but short shelf life due to microbial and enzymatic spoilage *(27).* In addition, those foods may be vehicles of pathogenic microorganisms *(2).* Several outbreaks of foodborne disease caused by pathogenic bacteria have been associated with juices *(2, 7, 15, 19).* The widespread production and consumption of juices without thermal process suggest the need to develop new strategies to protect juices and consumers against pathogens. In many cases, the infective dose is very low, and thus only the presence of the microorganism is enough to cause illness. Traditional thermal process is used to inactivate enzymes and destroy pathogenic microorganisms in juices; however, it can produce important sensorial changes.

Antimicrobial agents have been used to inhibit foodborne bacteria and extend the shelf life of processed food *(4, 18).* Herbs and spices, widely used in the food industry as flavors and fragrances, have shown additional antimicrobial functions against foodborne pathogens *(16, 21).* Moreover, essential oils (EOs) or their constituents also have demonstrated antimicrobial activity *(3).*

The majority of the EOs are classified as generally recognized as safe according to the *Food Additive Status List (24).* However, their use in foods as preservatives is often limited due to flavor considerations, because effective antimicrobial doses might exceed organoleptically acceptable levels. Therefore, there is an increasing interest for an accurate knowledge of the MIC of EOs to enable a balance between the sensory acceptability and the antimicrobial efficacy *(17).*

Several previous studies have shown that EOs from cinnamon, geraniol, lemongrass, clove, and palmarosa have an antimicrobial effect in culture media *(1, 3, 6, 13, 14, 16, 20, 22–24).* However, there is little information available about the MIC or MBC of these oils in fruit juices *(5, 12).*

The first objective of this study was to investigate the effectiveness of selected EOs on the survival and growth of microorganisms, which can be used as indicators of the incidence of pathogens in food such as *Salmonella* Enteritidis, *Escherichia coli,* and *Listeria innocua* in culture media (tryptone soy agar [TSA] and tryptone soy broth [TSB]) and fruit juices with different characteristics (apple, pear, and melon). The second objective of this work was to find MIC and MBC of the EOs that were more effective against each microorganism and thus contribute to the knowledge of the needed concentrations of specific EOs for preventing the incidence of or eliminating pathogenic microorganisms present in fruit juices.

MATERIALS AND METHODS

Microorganisms. Strains of *Salmonella* Enteritidis 1.82 (NCTC 9001, Public Health Laboratory Service, Central Public Health Laboratory, London, UK), *E. coli* 1.107 (Laboratoire de Répression des Fraudes, Montpellier, France), and *L. innocua* (Laboratoire de Répression des Fraudes) were provided for the culture

* Author for correspondence. Tel: +34 973 702 593; Fax: +34 973 702 596; E-mail: omartin@tecal.udl.es.

TABLE 1. *Inhibitory activity of some EOs against* E. coli, Salmonella *Enteritidis, and* L. innocua

EO	Concn (μl/ml)	Microorganism (CFU/ml)[a]		
		E. coli	*Salmonella* Enteritidis	*L. innocua*
Lemongrass	0	6.20×10^2	9.85×10^2	5.90×10^3
	1	4.25×10^2	4.40×10^2	3.88×10^3
	3	<10	6.5×10^1	<10
	5	<10	<10	<10
	10	<10	<10	<10
C. zeylanicum (cinnamon)	0	6.20×10^2	9.85×10^2	5.90×10^3
	1	3.55×10^2	5.20×10^2	3.60×10^3
	3	<10	<10	3.08×10^3
	5	<10	<10	<10
	10	<10	<10	<10
Geraniol	0	6.20×10^2	9.85×10^2	5.90×10^3
	1	3.60×10^2	1.11×10^3	4.50×10^3
	3	<10	<10	<10
	5	<10	<10	<10
	10	<10	<10	<10
C. martini (palmarosa)	0	6.20×10^2	9.85×10^2	5.90×10^3
	1	5.00×10^2	1.07×10^3	4.48×10^3
	3	2.50×10^2	<10	<10
	5	1.00×10^1	<10	<10
	10	<10	<10	<10
E. caryophyllata (clove)	0	6.20×10^2	9.85×10^2	5.90×10^3
	1	4.65×10^2	3.85×10^2	4.06×10^3
	3	<10	1.00×10^2	2.1×10^2
	5	<10	<10	<10
	10	<10	<10	<10
Benzaldehyde	0	6.20×10^2	9.85×10^2	5.90×10^3
	1	5.95×10^2	4.25×10^2	5.72×10^3
	3	<10	1.55×10^2	3.68×10^3
	5	<10	<10	3.12×10^3
	10	<10	<10	<10

[a] <10, not detected.

collections of the Department of Food Technology, University of Lleida, Spain.

Preparation of bacterial cultures for inhibitory tests. The strains of *Salmonella* Enteritidis and *E. coli* were maintained in nutrient agar (NA; Biokar Diagnostics, Beauvais, France) slants at 5°C, whereas the strain of *L. innocua* was in TSA (Biokar Diagnostics) slants at 5°C. Stock cultures of *Salmonella* Enteritidis and *E. coli* were grown in TSB (Biokar Diagnostics) at 37°C for 11 h and 120 rpm (cell in early stationary phase), whereas stock culture of *L. innocua* was grown in TSB plus 0.6% of yeast extract (Biokar Diagnostics) at 35°C for 15 h and 200 rpm (cell in early stationary phase). The maximum level for each microorganism was 4.8×10^9, 4.0×10^9, and 4.9×10^9 CFU/ml for *Salmonella* Enteritidis, *E. coli,* and *L. innocua,* respectively. Concentrations were then adjusted to 10^2 to 10^3 or 10^6 CFU/ml using saline peptone water (Scharlau Chemie, Barcelona, Spain) in agar dilution method and broth dilution assay, respectively.

EOs. EOs of herbaceous portions of palmarosa (*Cymbopogon martini*) whole plant, clove (*Eugenia caryophyllata*) leaf, cinnamon (*Cinnamomum zeylanicum*) leaf, and lemongrass (*Cymbopogon citrates*) were obtained from Aceites Esenciales Dicana (Barcelona, Spain); benzaldehyde (98%) was obtained from Acros Organics (Morris Plains, N.J.), and geraniol (approximately 98%) was obtained from Sigma (Steinhein, Germany).

Antibacterial assay. The MIC of each EO against *Salmonella* Enteritidis, *E. coli,* and *L. innocua* were determined by the agar dilution method reported by Davidson and Parish *(9)* in TSA with the following modifications: A short heating (5 min at 100°C) was applied after adding the EO to the agar to facilitate the dissolution of the oil in the media. In addition, 5 ml/liter of 2,3,5-triphenyltetrazolium chloride (TTC; 0.05%; Merck, Darmstadt, Germany) was incorporated into the agar after cooling until 50°C to facilitate colony recount. The final concentrations of EOs in the agar were 1, 3, 5, and 10 μl/ml. One hundred microliters of culture stock was diluted until 10^2 to 10^3 CFU/ml of each microorganism was spread on the plates. A control of each microorganism was made in TSA without EO. Plates were incubated at 35°C for 24 h. The MIC was considered to be the lowest concentration to maintain or reduce the inoculum level.

Based on the results of the first part of this work, lemongrass, cinnamon, and geraniol were selected as more effective to be assayed during the determination of the MBC. MBC were determined by the broth dilution method reported by Davidson and Parish *(9)* in TSB and fruit juices (apple, pear, and melon) with the following modifications: a final concentration of 2% (vol/vol) Tween 80 (Scharlau Chemie) was incorporated into the broth or the juices before autoclaving to facilitate miscibility of EOs in each liquid medium. Nevertheless, Lambert et al. *(17)* reported that MIC or MBC tend to be a function of dispersion agent used, because the MIC or MBC could be lower when this agent either is not used or is used scarcely.

The fruits were washed, peeled, cut into pieces, and blended (model BP 4512, Ufesa, Victoria, Spain). The obtained juices were centrifuged (Avanti J-25 centrifuge, Beckman Coulter, Fullerton, Calif.) at 12,500 rpm for 15 min at 4°C. The supernatant was filtered and autoclaved. A 500-μl aliquot of bacterial suspension (*Salmonella* Enteritidis, *E. coli,* or *L. innocua*) at 10^6 CFU/ml and each of the EOs giving a final concentration of 2, 3, 5, 6, 8, or 10 μl/ml were added to each tube containing 450 μl of sterile TSB, apple, pear, or melon juices with 2% Tween 80. A control of each medium (TSB, apple, pear, or melon juices with 2% Tween 80) without EOs was made. These experiments were conducted twice and in duplicate for each medium, EO, and concentration.

The tubes were incubated at 35°C for 24 h to simulate abuse conditions by consumers in the handling of these kinds of products, and then a 100-μl aliquot was spread on TSA plates to check bacterial survival and growth. Before establishing the MBC, an examination of injured and dead cells was made by adding a 500-μl aliquot of medium from the tubes incubated previously to tubes with 450 μl of fresh medium (TSB) without EOs and incubating at 35°C for 24 h. The MBC was considered to be the EO concentration where growth in the plate or fresh media was not detected.

Determination of pH. The pH values of apple, pear, and melon juices and of TSB medium were determined with a Microprocessor pH meter (PH210, Hanna Instruments, Vernon Hills, Ill.).

Confocal scanning laser microscopy. The analysis was performed according to the methodology of Wierzchos et al. *(26)* and De los Ríos et al. *(10).* This methodology allows the distinguishing between living and dead cells through the application of the L-13152 kit (Molecular Probes, Eugene, Oreg.), which has two

TABLE 2. *Effects of different concentrations of cinnamon, lemongrass, and geraniol against* Salmonella *Enteritidis in apple, pear, and melon juices or TSB after 24 h of incubation at 35°C*

EO	Concn (μl/ml)	Medium[a]: Apple juice	Pear juice	Melon juice	TSB
Cinnamon	0	3.01 ± 0.29 Abα	4.13 ± 0.03 Abα	7.88 ± 0.18 Abβ	8.96 ± 0.06 Abγ
	2	<1 Aaα	<1 Aaα	<1 Aaβ	<1 Aaγ
	3	<1 Aaα	<1 Aaα	<1 Aaβ	<1 Aaγ
	5	<1 Aaα	<1 Aaα	<1 Aaβ	<1 Aaγ
	6	<1 Aaα	<1 Aaα	<1 Aaβ	<1 Aaγ
	8	<1 Aaα	<1 Aaα	<1 Aaβ	<1 Aaγ
	10	<1 Aaα	<1 Aaα	<1 Aaβ	<1 Aaγ
Lemongrass	0	3.01 ± 0.29 Abα	4.13 ± 0.03 Abα	7.88 ± 0.18 Bbβ	8.96 ± 0.06 Bbγ
	2	<1 Aaα	<1 Aaα	2.78 ± 0.01 Bcβ	3.41 ± 0.02 Bcγ
	3	<1 Aaα	<1 Aaα	1.50 ± 0.71 Bdβ	3.42 ± 0.02 Bcγ
	5	<1 Aaα	<1 Aaα	<1 Baβ	<1 Baγ
	6	<1 Aaα	<1 Aaα	<1 Baβ	<1 Baγ
	8	<1 Aaα	<1 Aaα	<1 Baβ	<1 Baγ
	10	<1 Aaα	<1 Aaα	<1 Baβ	<1 Baγ
Geraniol	0	3.01 ± 0.29 Abα	4.13 ± 0.03 Abα	7.88 ± 0.18 Abβ	8.96 ± 0.06 Abγ
	2	<1 Aaα	<1 Aaα	<1 Aaβ	<1 Aaγ
	3	<1 Aaα	<1 Aaα	<1 Aaβ	<1 Aaγ
	5	<1 Aaα	<1 Aaα	<1 Aaβ	<1 Aaγ
	6	<1 Aaα	<1 Aaα	<1 Aaβ	<1 Aaγ
	8	<1 Aaα	<1 Aaα	<1 Aaβ	<1 Aaγ
	10	<1 Aaα	<1 Aaα	<1 Aaβ	<1 Aaγ

[a] Values are means ± standard deviations of plate counts from two experiments, each in duplicate ($n = 4$), expressed as log CFU per milliliter. Different capital letters (A, B) represent significant differences ($P < 0.05$) among EO types by each medium. Different lowercase letters (a, b, c, d) represent significant differences ($P < 0.05$) among EO concentrations by each EO and medium. Different Greek letters (α, β, γ) represent significant differences ($P < 0.05$) among culture media by each EO.

proprietary nucleic acid stains that differ in their ability to penetrate bacterial cell membranes. The green fluorescence nucleic acid stain, SYTO 9, labels all cells, and the red fluorescence nucleic acid stain, propidium iodide, only penetrates cells with damaged membranes and quenches the green SYTO 9 stain. Green cells are alive and red cells are dead. This technique may be used to know in a direct and rapid way the bactericidal effect of any substance against the microorganisms.

Transmission electron microscopy. For transmission electron microscopy, *Salmonella* Enteritidis cells were cultured for 24 h in TSB medium, apple juice, and apple juice with lemongrass (5 μl/ml). Afterward, they were fixed in glutaraldehyde (2.5% in 0.1 M phosphate buffer, pH 7.4) for 1 h, rinsed three times for 10 min with 0.1 M phosphate buffer (pH 7.4), and postfixed with 1% osmium tetraoxide for 2 h at 4°C. After fixation, the cells were rinsed three times for 10 min with 0.1 M phosphate buffer (pH 7.4) and then dehydrated using 30, 50, 70, and 95% acetone sequentially for 15 min each. Next, the cells were dehydrated three times for 30 min with 100% acetone. After dehydration, the cells were treated with propylene oxide twice for 10 min at 4°C. The cells were sequentially infiltrated with a mixture of propylene oxide: Durcupan's ACM Epoxy Resin (3:1, 1:1, and 1:3) for 45 min. Polymerization of the resin to form specimen blocks was performed in an oven at 60°C for 72 h. The specimen blocks were hand trimmed with a razor blade and sectioned with a diamond knife in a Reichert Ultracut R ultramicrotome (Leica, Wetzler, Germany). Thin sections (70 to 80 nm) were placed on 300-mesh copper grids. The sections were stained for 15 to 20 min in uranyl–ethyl alcohol (1:1), washed three times for 2 min, and then incubated in a drop of Reynold's lead citrate and examined using an EM 910 Zeiss transmission electron microscope.

Statistical analysis. The results of the MBC were analyzed by multifactor analysis of variance (ANOVA), using STATGRAPHICS Plus, 5.1. MBC obtained for each microorganism were analyzed independently, and the evaluated factors were the EO type (lemongrass, cinnamon, and geraniol), EO concentration (0, 2, 3, 5, 6, 8, and 10 μl/ml), and the type of culture media (apple, pear, and melon juices and TSB).

RESULTS AND DISCUSSION

MIC. The evaluated EOs were shown to have antibacterial activity against the three tested microorganisms but in different degrees. Lemongrass, cinnamon, geraniol, clove, and benzaldehyde were more effective than palmarosa in inhibiting the growth of *E. coli.* The growth *of Salmonella* Enteritidis was better inhibited by lemongrass, cinnamon, geraniol, and palmarosa than by clove or benzaldehyde, whereas the greatest inhibition of the growth of *L. innocua* was found when using lemongrass, geraniol, and palmarosa (Table 1).

The MIC against *E. coli, Salmonella* Enteritidis, and *L. innocua* showed by the six studied EOs were always the same (1 μl/ml) (Table 1). Similar studies with *Salmonella* Enteritidis and *L. innocua* were not found in the literature, and thus other *Salmonella* and *Listeria* species have been selected as a reference for comparison. A behavior similar

TABLE 3. *Effects of different concentrations of cinnamon, lemongrass, and geraniol against* E. coli *in apple, pear, and melon juices or TSB after 24 h of incubation at 35°C*

EO	Concn (μl/ml)	Medium[a]: Apple juice	Pear juice	Melon juice	TSB
Cinnamon	0	3.39 ± 1.19 Abα	4.36 ± 0.88 Abα	8.70 ± 0.01 Abβ	9.14 ± 0.02 Abβ
	2	<1 Aaα	<1 Aaα	<1 Aaβ	<1 Aaβ
	3	<1 Aaα	<1 Aaα	<1 Aaβ	<1 Aaβ
	5	<1 Aaα	<1 Aaα	<1 Aaβ	<1 Aaβ
	6	<1 Aaα	<1 Aaα	<1 Aaβ	<1 Aaβ
	8	<1 Aaα	<1 Aaα	<1 Aaβ	<1 Aaβ
	10	<1 Aaα	<1 Aaα	<1 Aaβ	<1 Aaβ
Lemongrass	0	3.39 ± 1.19 Abα	4.36 ± 0.88 Abα	8.70 ± 0.01 Abβ	9.14 ± 0.02 Abβ
	2	<1 Aaα	<1 Aaα	<1 Aaβ	<1 Aaβ
	3	<1 Aaα	<1 Aaα	<1 Aaβ	<1 Aaβ
	5	<1 Aaα	<1 Aaα	<1 Aaβ	<1 Aaβ
	6	<1 Aaα	<1 Aaα	<1 Aaβ	<1 Aaβ
	8	<1 Aaα	<1 Aaα	<1 Aaβ	<1 Aaβ
	10	<1 Aaα	<1 Aaα	<1 Aaβ	<1 Aaβ
Geraniol	0	3.39 ± 1.19 Abα	4.36 ± 0.88 Abα	8.70 ± 0.01 Bbβ	9.14 ± 0.02 Bbβ
	2	<1 Aaα	<1 Aaα	3.40 ± 0.02 Bcβ	3.44 ± 0.01 Bcβ
	3	<1 Aaα	<1 Aaα	3.36 ± 0.02 Bcβ	3.41 ± 0.02 Bcβ
	5	<1 Aaα	<1 Aaα	2.36 ± 0.13 Bdβ	1.25 ± 0.07 Bdβ
	6	<1 Aaα	<1 Aaα	<1 Baβ	<1 Baβ
	8	<1 Aaα	<1 Aaα	<1 Baβ	<1 Baβ
	10	<1 Aaα	<1 Aaα	<1 Baβ	<1 Baβ

[a] Values are means ± standard deviations of plate counts from two experiments, each in duplicate ($n = 4$), expressed as log CFU per milliliter. Different capital letters (A, B) represent significant differences ($P < 0.05$) among EO types by each medium. Different lowercase letters (a, b, c, d) represent significant differences ($P < 0.05$) among EO concentrations by each EO and medium. Different Greek letters (α, β) represent significant differences ($P < 0.05$) among culture media by each EO.

to those of *E. coli* and *Salmonella* Typhimurium (strain VR-19) was reported by Pattnaik et al. *(22),* who found an MIC of 1.66 μl/ml of lemongrass and palmarosa against *E. coli,* whereas for *Salmonella* Typhimurium (VR-19), they encountered MIC of 1.66 and 0.80 μl/ml, respectively. However, Hammer et al. *(14)* needed a higher concentration of lemongrass (2.5 μl/ml), palmarosa (5 μl/ml), and clove (>20 μl/ml) to inhibit *Salmonella* Typhimurium (ATCC 13311). On the other hand, Kim et al. *(16)* found an MIC of geraniol similar to ours, reporting concentrations of 0.5 and 1 μl/ml of geraniol against *Salmonella* Typhimurium and *Listeria monocytogenes,* respectively. The differences found among the results shown by diverse researchers may be consequences of the spice's nature, the specific strains, or the microorganism resistances.

The methodology applied has great influence on the results. Different methods for detecting the MIC and inoculum volumes have been used. In this way, Hammer et al. *(14)* used 1- to 2-μl spots containing the microorganism and applied the agar dilution method, whereas Kim et al. *(16)* used a 200-μl aliquot of bacterial suspension and the broth dilution method. The inoculum is sometimes dotted and other times streaked onto the agar surface. On the other hand, different solvents to incorporate the EOs in the medium have been used; thus Pattnaik et al. *(22)* used sodium taurocholate, whereas Hammer et al. *(14)* used Tween 20 to facilitate the miscibility of EOs within the medium.

In addition to the specific strains, species resistances, and methodology applied, the MIC definition used by each research group is a factor that may complicate the comparison among the published data. The definition of MIC itself differs among publications. Some researchers consider MIC to be the lowest concentration resulting in maintenance or reduction of inoculum viability *(3),* whereas others define it as the lowest concentration resulting in a significant decrease (>90%) *(8)* or complete destruction of the inoculum viability *(25).* The application of different concepts and methodologies may influence significantly the results and, as a consequence, make comparison among the results more difficult.

MBC. Lemongrass, cinnamon, and geraniol were selected as more effective against the evaluated microorganisms based on the results of the MIC made previously.

Similarly to the MIC assay, differences among the effectivity of lemongrass, cinnamon, or geraniol against the three microorganisms were observed (Tables 2 through 4). Statistical analysis was made independently for each microorganism and showed significant differences ($P < 0.05$) among EOs, their concentrations, and culture media.

Bactericidal activity varied among culture media for the same strain. Higher concentrations of EOs were required to inactivate populations of each strain in melon juice and TSB than in apple and pear media (Tables 2 through 4). These results are supported by the statistical analysis, which show significant differences ($P < 0.05$) be-

TABLE 4. *Effects of different concentrations of cinnamon, lemongrass, and geraniol against* L. innocua *in apple, pear, and melon juices or TSB after 24 h of incubation at 35°C*

EO	Concn (μl/ml)	Medium[a]: Apple juice	Pear juice	Melon juice	TSB
Cinnamon	0	1.80 ± 0.37 Abα	2.01 ± 0.50 Abα	8.31 ± 0.19 Bbβ	9.02 ± 0.03 Bbβ
	2	<1 Aaα	<1 Aaα	3.40 ± 0.01 Bcβ	3.41 ± 0.01 Bcβ
	3	<1 Aaα	<1 Aaα	3.39 ± 0.03 Bcβ	3.40 ± 0.02 Bcβ
	5	<1 Aaα	<1 Aaα	3.38 ± 0.02 Bcβ	3.39 ± 0.02 Bcβ
	6	<1 Aaα	<1 Aaα	3.06 ± 0.19 Bcβ	3.39 ± 0.01 Bcβ
	8	<1 Aaα	<1 Aaα	<1 Baβ	1.85 ± 0.21 Bdβ
	10	<1 Aaα	<1 Aaα	<1 Baβ	<1 Baβ
Lemongrass	0	1.80 ± 0.37 Abα	2.01 ± 0.50 Abα	8.31 ± 0.19 Abβ	9.02 ± 0.03 Abβ
	2	<1 Aaα	<1 Aaα	<1 Aaα	<1 Aaα
	3	<1 Aaα	<1 Aaα	<1 Aaα	<1 Aaα
	5	<1 Aaα	<1 Aaα	<1 Aaα	<1 Aaα
	6	<1 Aaα	<1 Aaα	<1 Aaα	<1 Aaα
	8	<1 Aaα	<1 Aaα	<1 Aaα	<1 Aaα
	10	<1 Aaα	<1 Aaα	<1 Aaα	<1 Aaα
Geraniol	0	1.80 ± 0.37 Abα	2.01 ± 0.50 Abα	8.31 ± 0.19 Cbβ	9.02 ± 0.03 Cbβ
	2	<1 Aaα	<1 Aaα	3.40 ± 0.00 Ccβ	3.36 ± 0.01 Ccβ
	3	<1 Aaα	<1 Aaα	3.34 ± 0.05 Ccβ	3.37 ± 0.02 Ccβ
	5	<1 Aaα	<1 Aaα	1.30 ± 0.17 Cdβ	2.23 ± 0.39 Cdβ
	6	<1 Aaα	<1 Aaα	<1 Caβ	<1 Caβ
	8	<1 Aaα	<1 Aaα	<1 aβ	<1 Caβ
	10	<1 Aaα	<1 Aaα	<1 aβ	<1 Caβ

[a] Values are means ± standard deviations of plate counts from two experiments, each in duplicate ($n = 4$), expressed as log CFU per milliliter. Different capital letters (A, B, C) represent significant differences ($P < 0.05$) among EO types by each medium. Different lowercase letters (a, b, c, d) represent significant differences ($P < 0.05$) among EO concentrations by each EO and medium. Different Greek letters (α, β) represent significant differences ($P < 0.05$) among culture media by each EO.

tween the counts of microorganism obtained from different culture media (TSB and melon, pear, and apple juices). Nychas and Skandamis *(21)* reported that the antimicrobial activity of the naturally occurring antimicrobial compounds in plants or their constituents (EOs, for example) is influenced by the culture medium, the temperature of incubation, and the inoculum size.

EOs of cinnamon, lemongrass, and geraniol were shown to be effective at a 2-μl/ml concentration in apple and pear juices for the three microorganisms. Similar results were reported by Ceylan et al. *(5),* who evaluated the antimicrobial activity and synergistic effect of cinnamon with sodium benzoate or potassium sorbate in controlling *E. coli* O157:H7 in apple juice and demonstrated that cinnamon exhibited significant antimicrobial activity against *E. coli* O157:H7 in apple juice at 1, 2, and 3 μl/ml concentrations, and its antimicrobial activity increased with increasing concentrations of cinnamon in apple juice at both 8 and 25°C.

On the other hand, a bactericidal or, in some case, inhibitory effect on the microorganisms was observed in pear and apple juices without EO (control). In contrast, melon juice or TSB media did not show any effect. Differences among culture media could be consequence of the pH (apple, 4.20 ± 0.02; pear, 3.97 ± 0.01; melon, 5.91 ± 0.02; TSB, 7.51 ± 0.01), because the influence of pH on bacteria growth is well known. However, other factors such as some self-constituents of each fruit may play a role as inhibitory or bactericidal agent.

Lemongrass was different from cinnamon or geraniol in the multiple range tests for *Salmonella* Enteritidis, showing to be the least effective of those three EOs in melon and TSB media. A concentration of 2 μl/ml of geraniol or cinnamon was enough to inactivate the *Salmonella* Enteritidis population, whereas 5 μl/ml of lemongrass was required to obtain the same effect in both media (Table 2). Kim et al. *(16)* reported a lower concentration of geraniol (0.5 μl/ml) for *Salmonella* Typhimurium. It is important to highlight that they worked with different species, and this is a factor that may influence the results.

A concentration of 6 μl/ml of geraniol was needed to destroy the *E. coli* population inoculated in melon juice or TSB as growth media, whereas 2 μl/ml of lemongrass or cinnamon was sufficient to eliminate the microorganism (Table 3). Kim et al. *(16)* reported the lowest MBC of geraniol (0.5 μl/ml) for *E. coli* and *E. coli* O157:H7. These researchers used a turbidimetric analysis to determine MBC. The method is not sensitive enough, because the detection limit is high. A minimal concentration of 10^6 to 10^7 CFU/ml of a bacterial culture is required for detection by the spectrophotometer. Thus, a bacterial culture with a concentration below 10^5 CFU/ml, and actively growing, is undetected. In contrast to turbidimetric analysis, the plate count method, which was used in this work, has a level of 10^2 CFU/ml as detection limit.

Lemongrass was useful against *L. innocua* in melon juice or TSB media at a concentration of 2 μl/ml, whereas

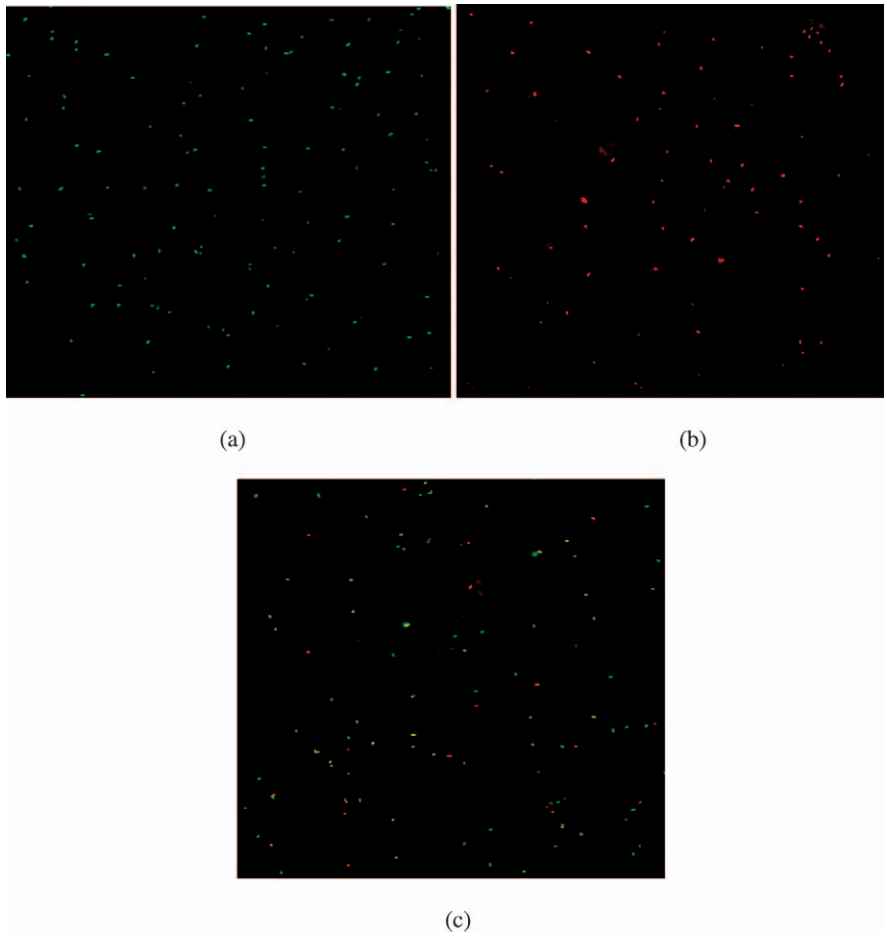

FIGURE 1. *Confocal scanning laser microscopy image of* Salmonella *Enteritidis cells from (a) live cells (green) of a pure culture in TSB grown by 11 h at 37°C and 120 rpm, (b) dead cells (red) in apple juice with lemongrass (5 μl/ml), and (c) live/dead cells in apple juice without addition of EO, incubated by 24 h to 37°C. The LIVE/DEAD BacLight bacterial viability kit (L-13152; Molecular Probes) and wavelengths of 515 to 545, 570, and 488 nm, respectively, were used.*

geraniol was effective at 6 μl/ml and cinnamon was successful at 8 μl/ml in melon juice and 10 μl/ml in TSB medium (Table 4). A lower concentration of geraniol (1 μl/ml) was indicated by Kim et al. *(16)* for killing *L. monocytogenes.*

Friedman et al. *(13)* found that strains of *E. coli, S. enterica,* and *L. monocytogenes* exhibited similar susceptibilities to inactivation by EOs or oil compounds. However, our results demonstrated differences among the behaviors of *Salmonella* Enteritidis, *E. coli,* and *L. innocua* in the presence of cinnamon, geraniol, or lemongrass oil, showing different MBC. These results show that the species or strain has a significant influence on the effect of different antimicrobial substances.

In general, lemongrass was shown to be more effective than cinnamon and geraniol in melon juice and TSB media for the three studied microorganisms, because a concentration of 5 μl/ml eliminated all of them, whereas higher concentrations of cinnamon (10 μl/ml) or geraniol (6 μl/ml) were required to obtain a similar effect.

The biggest differences in the bactericidal effects of EOs within culture medium were observed between the media with and without EO (Tables 2 through 4). A concentration of 2 μl/ml of lemongrass, cinnamon, or geraniol was shown to have a strong effect against the three studied microorganisms in apple and pear media, and hence an increase of oil concentration did not lead to a higher effectiveness.

To see the effects of the exposure to EOs on *Salmonella* Enteritidis, *E. coli,* and *L. innocua,* cells of each microorganism cultured for 24 h in TSB media, apple, pear, and melon juices were prepared to be observed by confocal scanning laser microscopy according to the methodology of Wierzchos et al. *(26)* and De los Ríos et al. *(10),* and transmission electron microscopy.

Figure 1 shows the effects of lemongrass (5 μl/ml) added to apple juice on the *Salmonella* Enteritidis cells using confocal scanning laser microscopy. The LIVE/DEAD BacLight kit (L-13152, Molecular Probes) permitted distinguishing between dead or damaged cells in red color and the alive cells in green color. This figure illustrates in a rapid and direct way the bactericidal effect of the EOs on microorganisms. A smaller percentage of fluorescent red cells was observed in the fruit juice sample without EO (Fig. 1c), whereas the addition of 5 μl/ml of lemongrass to the fruit juice sample resulted in 100% fluorescent red cells (Fig. 1b), indicating that the EO deteriorated the cellular membrane and converted intracellular nucleic acids approachable to propidium iodide for staining in red. These results are in agreement with those of Lambert et al. *(17),* who observed the same behavior in TSB medium in evaluating the MIC and mode of action of oregano EO, thymol, and carvacrol.

Figure 2 provides images in detail of the EO effects on cellular structure using transmission electron microscopy. Changes were observed in the cytoplasm of *Salmonella*

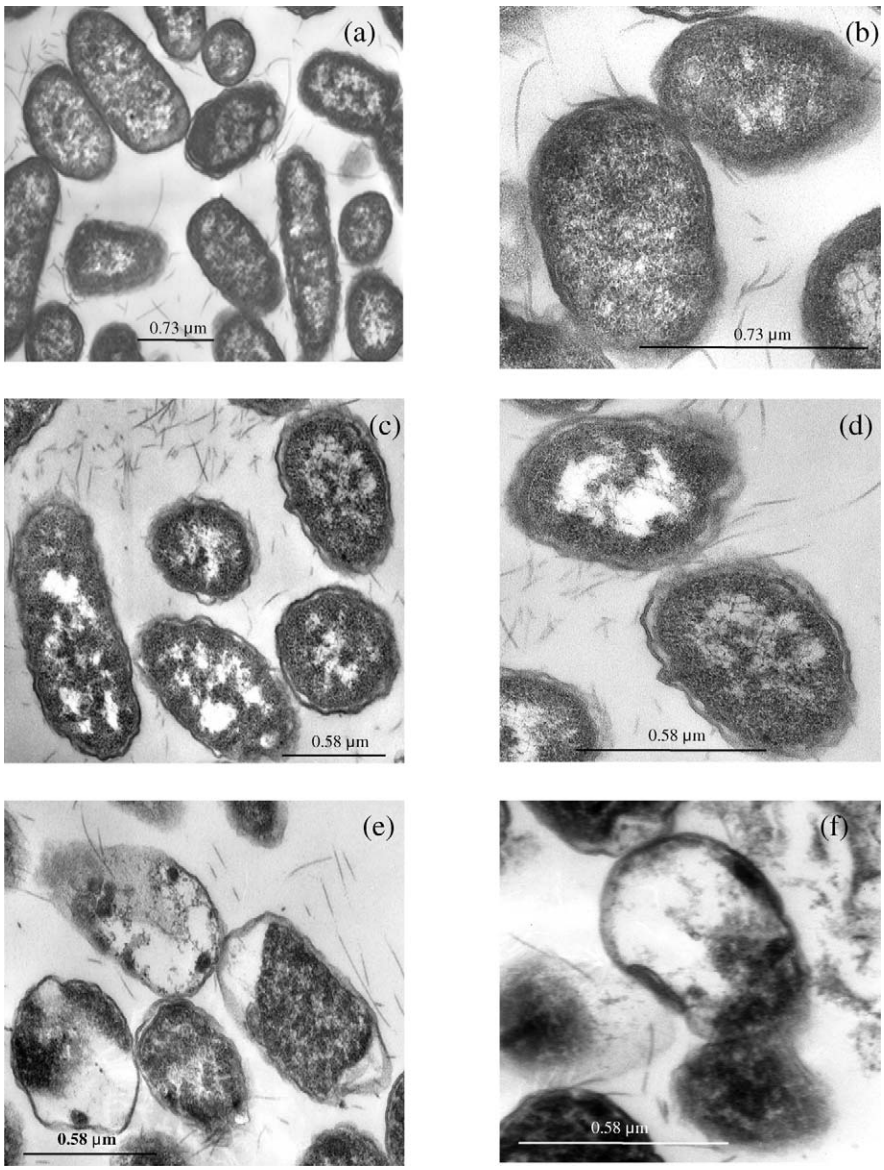

FIGURE 2. *Transmission electron microscopy micrographs of* Salmonella *Enteritidis cells from pure culture in TSB (a and b) grown by 11 h at 37°C and 120 rpm (undamaged cells; ×20,000), apple juice without addition of EO (c and d) (cells with alteration of cytoplasmic content and intact cells; ×25,000), and apple juice with lemongrass (5 μl/ml) (e and f) incubated for 24 h at 37°C (cells with structural damage and leakage of cellular content; ×25,000).*

Enteritidis cells inoculated in apple juice without lemongrass, which show clear zones caused by the action of the pH or some constituent of juice, whereas damages in the cellular membrane, including disruption of the same and leakage of cell content, were found in cells cultivated in apple juice with lemongrass. Results may be explained by the mechanisms of EOs actions proposed by different researchers who consider the hydrophobicity of EOs and their components to be an important characteristic. This characteristic enables them to spread through the lipids of the bacterial cell membrane and mitochondria, disturbing the structures and rendering them more permeable, causing leakage of ions and other cell contents, thus bringing about an extensive loss of cell contents or the exit of critical molecules and ions, leading to death *(3).*

EOs of lemongrass, geraniol, cinnamon, clove, palmarosa, and benzaldehyde effectively inhibited the *Salmonella* Enteritidis, *E. coli,* and *L. innocua* growth at a concentration of at least 1 μl/ml. These previous studies were useful in choosing the EOs more effective against the evaluated microorganisms. Hence, lemongrass, cinnamon, and geraniol were chosen for MBC analysis. A concentration of 2 μl/ml from these EOs was enough to inactivate *Salmonella* Enteritidis, *E. coli,* and *L. innocua* in apple and pear juices. However, lemongrass oil was shown to be more effective than cinnamon and geraniol in melon juice and TSB media for the studied microorganisms because a concentration of 5 μl/ml was enough to eliminate the three microorganisms, whereas higher concentrations of cinnamon (8 and 10 μl/ml) or geraniol (6 μl/ml) were required to obtain the same effect.

This work offers a contribution to the knowledge of MBC of lemongrass, cinnamon, and geraniol necessary to avoid the incidence of or eliminate pathogenic bacteria such as *Salmonella, Listeria,* and *E. coli* present in unpasteurized fruit juices, whose contamination originates directly or indirectly from animals or insects, soil, water, dirty equipment, and human handling.

Results suggest that EOs represent a good alternative to eliminate microorganisms that can be a hazard for the

consumer of unpasteurized fruit juices and be useful to prevent the risk of foodborne illness caused by these kinds of products.

Additional studies should be conducted to evaluate the sensory aspects of using these natural compounds.

ACKNOWLEDGMENTS

The Spanish Ministry of Science and Technology supported this work through Project AGL 2003-09208-C01. The Council of Scientific and Humanistic Development of University Central of Venezuela, Caracas-Venezuela, provides a grant for doctoral studies to R. M. Raybaudi-Massilia. We also thank Dr. Wierzchos for providing technical assistance in electronic microscopy.

REFERENCES

1. Aureli, P., A. Costantini, and S. Zolea. 1992. Antimicrobial activity of some plant essential oils against *Listeria monocytogenes. J. Food Prot.* 55:344–348.
2. Besser, R. E., S. M. Lett, J. T. Weber, M. P. Doyle, T. J. Barrett, J. G. Wells, and P. M. Griffin. 1993. An outbreak of diarrhea and hemolytic uremic syndrome from *Escherichia coli* O157:H7 in fresh-pressed apple cider. *JAMA* 269:2217–2220.
3. Burt, S. 2004. Essential oils: their antibacterial properties and potential applications in foods—a review. *J. Food Microbiol.* 94:223–253.
4. Buta, J. G., H. E. Moline, D. W. Spaulding, and C. Y. Wang. 1999. Extending storage life of fresh-cut apples using natural products and their derivatives. *J. Agric. Food Chem.* 47:1–6.
5. Ceylan, E., D. Y. C. Fung, and J. R. Sabah. 2004. Antimicrobial activity and synergistic effect of cinnamon with sodium benzoate or potassium sorbate in controlling *Escherichia coli* O157:H7 in apple juice. *J. Food Sci.* 69:102–106.
6. Chao, S. C., and D. G. Young. 2000. Screening for inhibitory activity of essential oils on selected bacteria, fungi and viruses. *J. Essent. Oil Res.* 12:639–649.
7. Cody, S. H., M. K. Glynn, J. A. Farrar, K. L. Cairns, P. M. Griffin, J. Kobayashi, M. Fyfe, R. Hoffman, A. S. King, J. H. Lewis, B. Swaminathan, R. G. Bryant, and D. J. Vugia. 1999. An outbreak of *Escherichia coli* O157:H7 infection from unpasteurized commercial apple juice. *Ann. Intern. Med.* 130:202.
8. Cosentino, S., C. I. G. Tuberoso, B. Pisano, M. Satta, V. Mascia, E. Arzedi, and F. Palmas. 1999. In vitro antimicrobial activity and chemical composition of Sardinian Thymus essential oils. *Lett. Appl. Microbiol.* 29:130–135.
9. Davidson, P. M., and M. E. Parish. 1989. Methods for testing the efficacy of food antimicrobials. *Food Technol.* 43:148–155.
10. De los Ríos, A., J. Wierzchos, L. G. Sancho, and C. Ascaso. 2004. Exploring the physiological state of continental Antarctic endolithic microorganisms by microscopy. *FEMS Microbiol. Ecol.* 216:1–57.
11. Farag, R. S., Z. Y. Daw, and S. H. Abo-Raya. 1989. Influence of some spice essential oils on *Aspergillus parasiticus* growth and production of aflatoxins in synthetic medium. *J. Food Sci.* 54:74–76.
12. Friedman, M., P. R. Henika, C. E. Levin, and R. E. Mandrell. 2004. Antibacterial activities of plant essential oils and their components against *Escherichia coli* O157:H7 and *Salmonella enterica* in apple juice. *J. Agric. Food Chem.* 52:6042–6048.
13. Friedman, M., P. R. Henika, and R. E. Mandrell. 2002. Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni, Escherichia coli, Listeria monocytogenes,* and *Salmonella enterica. J. Food Prot.* 65:1545–1560.
14. Hammer, K. A., C. F. Carson, and T. V. Riley. 1999. Antimicrobial activity of essential oils and other plant extracts. *J. Appl. Microbiol.* 86:985–990.
15. Harris, L. J., J. N. Farber, L. R. Beuchat, M. E. Parish, T. V. Suslow, E. H. Garrett, and F. F. Busta. 2003. Outbreaks associated with fresh produce: incidence, growth, and survival of pathogens in fresh and fresh-cut produce. *In* Comprehensive reviews in food science and food safety, vol. 2 (supplement), chap. III. Available at: http://www.ift.org. Accessed 20 December 2004.
16. Kim, J., M. R. Marshall, and C. Wei. 1995. Antibacterial activity of some essential oil components against five foodborne pathogens. *J. Agric. Food Chem.* 43:2839–2845.
17. Lambert, R. J. W., P. N. Skandamis, P. J. Coote, and G. J. Nychas. 2001. A study of minimum inhibitory concentration and mode of action of oregano essential oil, thymol and Carvacrol. *J. Appl. Microbiol.* 91:453–462.
18. Lanciotti, R., A. Gianotti, F. Patrignani, N. Belleti, M. E. Guerzoni, and F. Gardini. 2004. Use of natural aroma compounds to improve shelf-life of minimally processed fruits. *Trends Food Sci. Technol.* 15:201–208.
19. Millard, P. S., K. F. Gensheimer, D. G. Addiss, D. M. Sosin, G. A. Beckett, A. Houck-Jankoski, and A. Hudson. 1994. An outbreak of Cryptosporidiosis from fresh-pressed apple cider. *JAMA* 272:1592–1596.
20. Mishra, A. K., and N. K. Dubey. 1994. Evaluation of some essential oils for their toxicity against fungi causing deterioration of stored food commodities. *Appl. Environ. Microbiol.* 60:1101–1105.
21. Nychas, G. J. E., and P. N. Skandamis. 2003. Antimicrobials from herbs and spices. *In* S. Roller (ed.), Natural antimicrobials for the minimal processing of foods. CRC Press, Boca Raton, Fla.
22. Pattnaik, S., V. R. Subramanyam, and C. Kole. 1996. Antibacterial and antifungal activity of ten essential oils in vitro. *Microbios* 86:237–246.
23. Sacchetti, G., S. Maietti, M. Muzzoli, M. Scaglianti, M. Stefano, M. Radice, and R. Bruni. 2005. Comparative evaluation of 11 essential oils of different origin as functional antioxidants, antiradicals and antimicrobials in foods. *Food Chem.* 91:621–632.
24. U.S. Food and Drug Administration. 2005. Food additive status list. Available at: http://www.cfsan.fda.gov/~dms/opa-appa.html. Accessed 7 November 2005.
25. Wan, J., A. Wilcock, and M. J. Coventry. 1998. The effect of essential oils of basil on the growth of *Aeromonas hydrophila* and *Pseudomonas fluoresc*ens. *J. Appl. Microbiol.* 84:152–158.
26. Wierzchos, J., A. De los Ríos, and L. G. Sancho. 2004. Viability of endolithic micro-organisms in rocks from the McMurdo Dry Valleys of Antarctic established by confocal and fluorescent microscopy. *J. Microscopy* 216:57–61.
27. Winniczuk, P. P., and M. E. Parish. 1997. Minimum inhibitory concentrations of antimicrobials against micro-organisms related to citrus juice. *Food Microbiol.* 14:373–381.