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Antimicrobial Effect of Rosemary Extracts

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ABSTRACT

A rosemary extract commercially exploited (Oxy'less) as an antioxidant of lipids in foods was dissolved in ethanol (100 mg/ml), and the solution was tested against foodborne microorganisms. For gram-positive bacteria, the MIC of the ethanolic solution was 1% for *Leuconostoc mesenteroides,* 0.5% for *Listeria monocytogenes,* 0.5% for *Staphylococcus aureus,* 0.13% for *Streptococcus mutans,* and 0.06% for *Bacillus cereus.* It slowed the growth of *Penicillium roquefortii* and *Botrytis cinerea.* Up to 1% of the ethanolic solution had no activity on the gram-negative bacteria *Escherichia coli, Salmonella* Enteritidis, and *Erwinia carotovora* and on the yeasts *Rhodotorula glutinis* and *Cryptococcus laurentii.* Antibacterial activity of the rosemary extract was strongly influenced by the composition of the media. The MIC was reduced by low pH, high NaCl contents, and low temperatures. Low pH and high NaCl concentration had a synergistic effect on the MIC of the rosemary extract for *S. aureus.* Lipids, surface-active agents, and some proteins decreased its antibacterial activity, whereas pectin had no effect. The inhibitory effect was little modified by heat treatment (100°C). The natural microflora of pasteurized zucchini broth was inhibited by 0.5% of the rosemary extract. The antibacterial activity was linked to the compounds extracted with hexane, which are presumably phenolic diterpenoids.

Growth of microorganisms in foods may cause spoilage or foodborne diseases. Antimicrobial activities of plant essential oils have been known for centuries *(2, 8),* but their strong flavor limited their use in foods. In recent years, plant extracts have been developed and used in foods as antioxidants *(1, 10, 15, 16).* These extracts contain a broad range of phenolic compounds, such as abietane diterpenes *(13),* carnosol, and ursolic acid *(4),* and could have antimicrobial properties in addition to their antioxidant activity.

The objective of this work was to determine the antimicrobial activity of a commercial rosemary extract used as an antioxidant of lipids in various foods. Because the antimicrobial activity of phenolic compounds may be influenced by the physicochemical environment of foods *(7),* the rosemary extract was tested against some food spoilage and foodborne pathogenic microorganisms under various conditions of pH, water activity, and temperature and in the presence of major food components.

MATERIALS AND METHODS

Preparation of the rosemary extract. Oxy'less, produced by Naturex (Avignon, France), is a rosemary extract commercially sold as an antioxidant to prevent the deterioration of lipids in food products. It is prepared by organic extraction of rosemary leaves from which essential oil had been removed by steam distillation. For all the experiments, 10 g of Oxy'less (batch 155.6) was dispersed in 100 ml of 96% ethanol by a 10-min ultrasonic treatment in an ultrasonic bath (Bioblock Scientific, Illkirch, France). The residue not solubilized in ethanol amounted to 3 g. The suspension was centrifuged at 13,000 × *g* for 5 min, and the clear supernatant was collected and stored at −20°C for less than 6 months. This supernatant is referred to as ''the rosemary extract'' in this work and was always prepared with the same concentration of this commercial rosemary extract. High-performance liquid chromatography (HPLC) analyses showed that all the rosemary extracts prepared during this work had the same qualitative composition. Concentrations of the rosemary extracts tested in the various culture media ranged from 0.03 to 1% (vol/vol).

HPLC analysis. The rosemary extract was analyzed with a Varian 5,500 high-performance liquid chromatograph (Varian Associates Inc., Walnut Creek, Calif.) equipped with an Alltech Alltima (Alltech Associates, Deerfield, Ill.) RP-18 column (150 × 4.6 mm inside diameter; 5 μm) set at 35°C. Samples were injected in a 10-μl loop, and the flow rate was 0.8 ml/min. The mobile phase consisted of a gradient of three solvents: acetonitrile (A), water (pH 2.6) acidified with H_3PO_4 (B), and a mixture of methyl alcohol and water (pH 2.6) and acetonitrile (3:1:1) (C). The elution profile was 0 to 15 min, 95 to 85% B, and 5 to 15% C (linear gradient); 15 to 25 min, 85 to 70% B, and 15 to 30% C (linear gradient); 25 to 40 min, 70 to 62% B, and 30 to 38% C (linear gradient); 40 to 60 min, 62% B, and 38% C (isocratic); 60 to 110 min, 0 to 100% A, 62 to 0% B, and 38 to 0% C (linear gradient); 110 to 120 min, 100% A (isocratic). The total running time was 120 min, and the detection of phenolic compounds was done with a Waters 990 photodiode array detector (Waters, Milford, Conn.) in the range of 200 to 600 nm. Some compounds were characterized by their retention times and spectra and compared with standards, such as rosmarinic acid and apigetrin, purchased from Extrasynthèse, Genay, France.

Microorganisms. Bacterial strains were kept at −20°C in 30% glycerol in distilled water (vol/vol). They were streaked on Trypticase soy agar plates (Biotrypticase, Biomérieux, Marcy l'Etoile, France, 17 g; Biosoyase, Biomérieux, 3 g; NaCl, 5 g;

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K_2HPO_4, 2.5 g; glucose, 2.5 g; granulated agar, Biomérieux, 15 g; distilled water, 1 liter) and subcultured twice at 30°C in Trypticase soy broth (TSB). *Staphyloccocus aureus* 57.10, *Bacillus cereus* 51.27, *Salmonella* Enteritidis 82.97, *Lactobacillus plantarum* 103151 T, and *Streptococcus mutans* 103220 T were purchased from the Collection Institut Pasteur (Paris, France). *Erwinia carotovora* 1336 was purchased from the Collection Française des Bactéries Phytopathogènes (Angers, France). A strain of *B. cereus* (Z4234) and a strain of *Leuconostoc mesenteroides* had been isolated from vegetables in the laboratory. *Listeria monocytogenes* strain Scott A and *Escherichia coli* were kindly supplied by, respectively, Dr. B. Lund (Institute of Food Research, Norwich, UK) and Professor J. Guiraud (Université Montpellier II, Montpellier, France).

Molds were kept at 4°C on potato dextrose agar slants (Biomérieux) and grown 5 days at room temperature on potato dextrose agar plates. Plugs of 3 mm diameter, cut out of the external margin of the mycelium, were used as inoculum. One strain of *Penicillium roquefortii* from blue cheese and one strain of *Botrytis cinerea* from decayed strawberry had been isolated in the laboratory. Yeasts were kept in sterile distilled water at 4°C, streaked on potato dextrose agar plates to check purity, and grown in yeast glucose broth (yeast extract, 5 g, Biomérieux; glucose, 5 g; distilled water, 1 liter) 3 days at 25°C to produce inoculum. One strain of *Rhodotorula glutinis* and one strain of *Cryptococcus laurentii* had been isolated from strawberry fruit in the laboratory.

Measurement of inhibitory activity of the rosemary extract. Serial, twofold dilutions from 2^0 to 2^{-5} of the rosemary extract were done in 96% ethanol. One percent of each dilution (vol/vol) was added to the culture media. The addition of the dilutions 2^0 and 2^{-1} made the culture media cloudy, so turbidity could not be used to measure microbial growth. In the case of agar media, dilutions of the rosemary extract were added to the melted agar at 50°C. Ethanol alone diluted 100-fold in the medium was used as control. The addition of the rosemary extract did not affect the pH of the culture media. Concentrations were expressed as percentage of the rosemary extract in the culture media (vol/vol). Concentrations tested varied from 0.03 to 1%. Unless otherwise specified, the inhibitory effect was measured in TSB for bacteria, in yeast glucose broth for yeast, and in potato dextrose agar for molds. Media were incubated at temperatures of 4, 10 (two temperatures of refrigerated foods), 25, or 30°C (two temperatures of nonrefrigerated foods). Sodium chloride concentrations in TSB or potato dextrose agar varied from 5 to 100 g/liter, and pH was adjusted with hydrochloric acid (1 N) to values of 3.5 to 7.0. Hydrochloric acid was added to melted potato dextrose agar kept at 50°C. TSB was adjusted to the desired pH and sterilized by filtration through 0.22-μm MITEX filter units (Millipore, Saint-Quentin Yvelines, France). Microorganisms were selected for their ability to grow over the range of conditions tested: *S. aureus*, *L. plantarum*, and *L. monocytogenes* were used to test the effect of high sodium chloride concentrations, low pH, and low temperature, respectively.

Broths were inoculated with approximately 10^5 CFU/ml of bacteria and approximately 100 CFU/ml of yeasts. Yeast and bacteria were enumerated at regular intervals by serial dilution and plating in duplicate on yeast glucose agar and Trypticase soy agar, respectively, with a spiral plater apparatus (Interscience, St Nom-la-Breteche, France). Plates were incubated at 30°C for 24 h. The MIC was determined as the mean of the lowest concentration of the rosemary extract that prevented the growth of inoculum during the incubation period. The minimum lethal concentration (MLC) was the mean of the lowest concentration that killed the inoculum to a level close to the detection limit (20 CFU/ml). For molds, potato dextrose agar plates were inoculated in triplicate with one plug of mycelium placed in the center of the plate. The diameter of the three disks of mycelium was measured regularly, and the result was the mean of the three measures.

Effect of heat on the inhibitory activity. Hungate tubes (Bellco Glass, Flobio, Courbevoie, France) containing 9.9 ml of sterile TSB were equilibrated at 70, 75, 80, 90, and 100°C in a water bath. One hundred microliters of the rosemary extract was injected through the septa of each tube. Tubes were removed at regular intervals during 30 min at 70°C, 20 min at 75°C, and 15 min at 80, 90, and 100°C and cooled in melting ice. Serial twofold dilutions were done in TSB to obtain concentrations ranging from 0.13 to 1% of the rosemary extract. Tubes containing various dilutions of the heated rosemary extract were inoculated with 10^5 *B. cereus* (CIP 51.27) per ml to measure the inhibitory activity.

Effect of various ingredients on the inhibitory activity. The following compounds were added to TSB before testing the inhibitory activity of the rosemary extract on bacteria: surface-active agent diacetyltartric ester of monoglyceride (Datem, Naturex, Avignon, France); high methylated pectin (Ruban brun, Unipectine, Redon, France); sterilized dairy cream (Elle & Vire, Condé-sur-Vire, France), containing 15 g of fat per 100 g of dried weight; and bovine serum albumin (Sigma, l'Isle d'Abeau Chesne, France). The inhibitory activity was also measured in skim milk (Regilait, St-Martin Belle Roche, France).

Activity of the rosemary extract in pasteurized zucchini broth. Zucchinis were steam cooked, homogenized, and centrifuged 10 min at 10,000 × *g*. The supernatant was mixed with the water used to wash vegetables to increase the contamination with spore-forming bacteria and with sterilized dairy cream, an ingredient frequently added to recipe dishes made with vegetables in France. A total of 3 × 10 ml of zucchini broth with 1% (vol/vol) ethanolic dilutions of the rosemary extract was dispensed in vials. Vials were closed with a rubber stopper and heated at 80°C for 15 min in a water bath (a temperature probe was placed in one vial with zucchini broth to measure the internal temperature) to destroy all nonsporulated bacteria. The vials were placed at 10°C to simulate storage in a domestic refrigerator. After 5, 10, and 14 days, vials were opened, and aerobic and facultative anaerobic spore-forming bacteria from zucchini broth were enumerated by serial dilution and plating in duplicate on Trypticase soy agar with a spiral plater. Plates were incubated 24 h at 30°C.

Activity and composition of hydrophilic and hydrophobic fractions of rosemary extract. Hydrophobic compounds were extracted from 2 g of Oxy'less with 100 ml of hexane (CarloErba, Val de Reuil, France) for 2 h using a Kumagawa apparatus (Prolabo, Paris, France). Hexane was evaporated, and the dried fraction was collected in 10 ml of ethanol. This ethanolic suspension was centrifuged and collected to give fraction F_1. It contained apolar compounds. A small pellet, not soluble in alcohol, was dried and dissolved in ethyl acetate (Merck, Darmstadt, Germany). The HPLC analyses of this solution showed the same composition as F_1 but 10-fold less concentrated. The residue of the extraction with the Kumagawa apparatus, which contained more polar compounds, was dispersed in 12 ml of water and methanol (25/75, vol/vol) by a 10-min ultrasonic treatment in an ultrasonic bath (Bioblock Scientific, Illkirch, France), heated at 70°C for 5 min, and centrifuged 5 min at 10,000 × *g*. The supernatant was collected (fraction F_2), and a small insoluble residue was discarded. The dry weight of each fraction was measured, the inhibitory ac-

TABLE 1. *MIC and MLC of the rosemary extract (vol/vol) for seven gram-positive bacteria measured after 24 h at 30°C in TSB and numbers of bacteria (log CFU/ml ± SD) in broth containing MIC and MLC of rosemary extract*[a]

Strains	Initial number	Number in control after 24 h at 30°C[b]	MIC (%)	Number at MIC after 24 h at 30°C	MLC (%)	Number at MLC after 24 h at 30°C
B. cereus CIP 51.27	3.17 ± 0.08	7.43 ± 0.10	0.06	≤1.31[c]	0.06	≤1.31
B. cereus Z4234	3.32 ± 0.05	8.07 ± 0.16	0.06	≤1.31	0.06	≤1.31
S. mutans	4.24 ± 0.11	8.13 ± 0.11	0.25	2.88 ± 0.09	0.5	≤1.31
S. aureus	5.28 ± 0.19	8.57 ± 0.18	0.5	3.90 ± 0.36	1	≤1.31
L. monocytogenes	5.20 ± 0.00	9.46 ± 0.01	0.5	5.45 ± 0.09	1	≤1.31
L. mesenteroïdes	4.55 ± 0.09	8.55 ± 0.01	1	4.47 ± 0.05	>1	—
L. plantarum	4.19 ± 0.13	7.94 ± 0.00	>1	—	>1	—

[a] Standard deviation calculated over two to four replicates.
[b] Control was broth without rosemary extract.
[c] 1.31 = limit detection.

tivity was determined, and HPLC analyses were done as described above.

RESULTS

Activity of rosemary extract on various microorganisms. In TSB at 30°C, the rosemary extract had no effect on the gram-negative bacteria *E. coli, Salmonella* Enteritidis, and *E. carotovora,* whereas growth of the gram-positive bacteria *S. aureus, L. monocytogenes, B. cereus, L. mesenteroides,* and *S. mutans* was inhibited (Table 1). The two strains of *B. cereus* were the most sensitive bacteria among the gram-positive bacteria tested, with an MIC of 0.06% rosemary extract. *L. plantarum* was not inhibited by the concentrations tested. The MLCs were two- to fourfold higher than the MICs for *S. mutans, L. monocytogenes* and *S. aureus.* In contrast, for *B. cereus,* the MIC of the rosemary extract was also lethal. The growth of *P. roquefortii* and *B. cinerea* was slowed in the presence of the rosemary extract, but the fungi were not totally inhibited (Fig. 1). For 3 days at 25°C, the yeasts *R. glutinis* and *C. laurentii* were not affected by the rosemary extract on yeast glucose agar (pH 7). *R. glutinis* and *C. laurentii* grew, respectively, from 2.46 ± 0.30 to 6.62 ± 0.13 log CFU/ml and from 2.83 ± 0.01 to 7.14 ± 0.13 log CFU/ml in media regardless of the concentration in the rosemary extract tested from 0 to 1%.

Effect of incubation temperature, sodium chloride, pH, and heating temperature on the activity of the rosemary extract. The activity of the rosemary extract was tested at three incubation temperatures (30, 10, and 4°C) on *S. aureus* and *L. monocytogenes.* The inhibitory effect of the rosemary extract was more pronounced at low temperatures (Fig. 2). A total of 0.5% rosemary extract was necessary to inhibit *S. aureus* at 30°C, whereas 0.13% inhibited the bacteria at 10°C. At 4°C, *L. monocytogenes* was inhibited by 0.25% rosemary extract, whereas 0.5 to 1% was necessary at 30°C. On the contrary, the lethal effect observed at 30°C with 1% rosemary extract was not noticed at 10 and 4°C. The inhibitory and lethal effects on *L. monocytogenes* were not permanent with most concentrations of the rosemary extract at the three temperatures tested, whereas the effects lasted during the whole incubation times for *S. aureus.*

High contents in sodium chloride increased the inhibitory and bactericidal activities of the rosemary extract at 30°C. With 10% sodium chloride, 0.13% rosemary extract was sufficient to kill *S. aureus* (Fig. 3), whereas 1% was required in normal TSB containing 0.5% sodium chloride (Fig. 2). *Salmonella* Enteritidis was inhibited with 0.13% rosemary extract in the presence of 5% sodium chloride (Fig. 3), whereas 1% rosemary extract had no effect in normal TSB. However, the inhibition lasted for only 24 h regardless of the concentration in the rosemary extract tested at 30°C. The inhibitory effect of the rosemary extract at different pH was measured for *S. aureus, Salmonella* Enteritidis, and *L. plantarum* in TSB at 30°C. A concentration of 0.13% rosemary extract was bactericidal for *S. aureus* at pH 4.5 and inhibitory for *L. plantarum* at pH 3.7 (Fig. 3). At pH 7, 1% rosemary extract did not inhibit *L. plantarum* (Table 1). The growth of *Salmonella* Enteritidis at pH 4.5 reached about 8 log CFU/ml regardless of the concentration in the rosemary extract tested (0 to 1%).

The combined effect of pH and sodium chloride at 10 and 30°C on the antibacterial effect of the rosemary extract was studied for *S. aureus* because of its capacity to grow in high concentrations of sodium chloride, low pH, and relatively low temperatures. In addition, this strain was not as sensitive as *B. cereus* or as resistant as *L. mesenteroides* to the inhibitory effect of the rosemary extract, allowing the observation of both an increase and a reduction in the inhibitory activity. At 30°C, the MIC was eightfold lower at pH 6 and 2% sodium chloride than in normal TSB, whereas it was not reduced significantly by either a reduction of pH to 6 or an increase in sodium chloride to 2% (Table 2). The MLC was fourfold lower at pH 6 and 2% sodium chloride and 16-fold lower at pH 5 and 3% sodium chloride than in normal TSB (Table 3). At 10°C after 21 days, the MIC was equal to or lower than 0.03% rosemary extract in all conditions tested. However, the MLC was fourfold lower at pH 6 and 2% NaCl (0.06% rosemary extract) and eightfold lower at pH 5.5 and 3% NaCl (0.03%) than in normal TSB (0.25%).

At 10 and 4°C, the growth of *P. roquefortii* and *B. cinerea* was reduced compared with 25°C, but the inhibitory effect of the rosemary extract was the same at all tem-

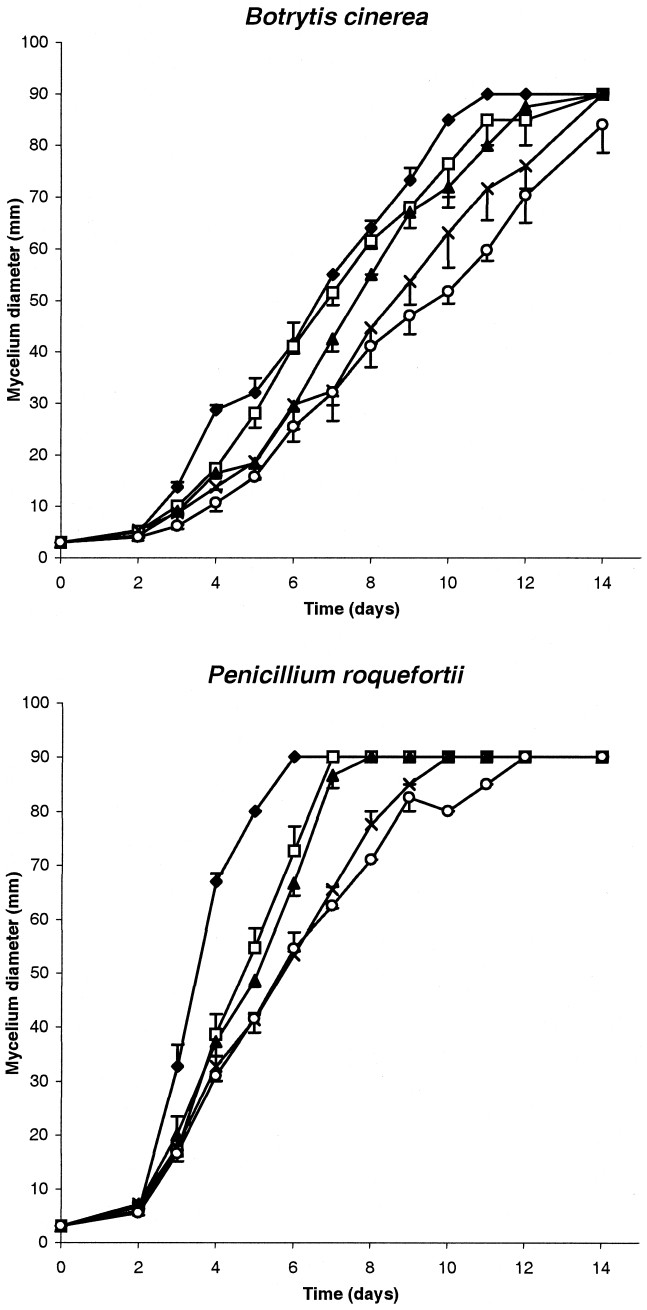


FIGURE 1. *Effect of the rosemary extract (♦: 0%; □: 0.13%; ▲: 0.25%, ×: 0.5%; ○: 1%) on the growth at 25°C of* P. roquefortii *and* B. cinerea. *Growth was measured by the diameter of the mycelium. Standard deviation was calculated for each point from three replicates.*

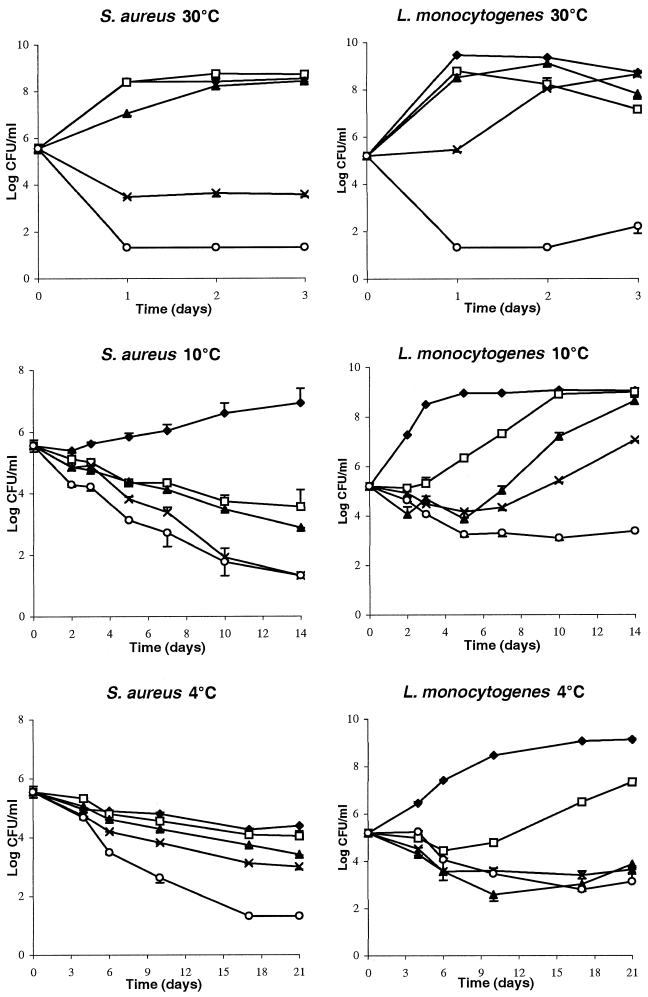
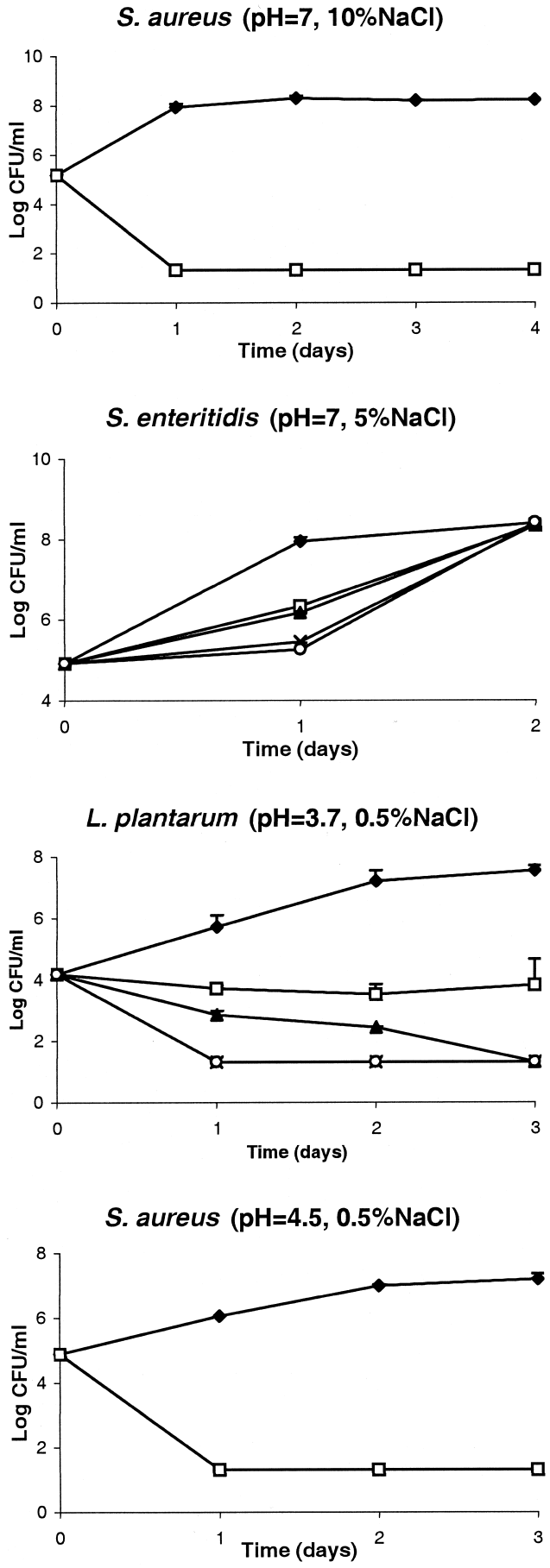


FIGURE 2. *Effect of incubation temperatures (30, 10, and 4°C) on the antimicrobial activity of the rosemary extract (♦: 0%; □: 0.13%; ▲: 0.25%, ×: 0.5%; ○: 1%) on* S. aureus *and* L. monocytogenes *in TSB. Bars indicate standard deviation calculated from two replicates. Standard deviations lower than 0.15 log CFU/ml were smaller than the symbols.*

peratures (Fig. 4). Adding 6% sodium chloride to potato dextrose agar and lowering pH to 3.5 reduced the growth of *P. roquefortii* and *B. cinerea* but did not modify the action of the rosemary extract.

B. cereus (CIP 51.27) was used to study the effect of heat on the inhibitory effect of the rosemary extract, because it was the most sensitive microorganism and, therefore, the most appropriate to measure a decrease in the inhibitory activity. The MLC of the heated rosemary extract measured after 3 days at 30°C for *B. cereus* was 0.25% irrespective of the heating time and temperature tested. The MLC of unheated rosemary extract for the same strain of *B. cereus* was fourfold lower (Table 1).

Activity of the rosemary extract in presence of food ingredients. *B. cereus* (CIP 51.27) and *S. aureus* were chosen to study the effect of some food ingredients on the activity of the rosemary extract at 30°C. *B. cereus* (CIP 51.27) was the most sensitive microorganism and, therefore, the most appropriate to measure a decrease in the inhibitory activity. The effect of the rosemary extract on *S. aureus* was intermediate in the range of concentration tested, and *S. aureus* was chosen to detect an increase or a decrease in the inhibitory and bactericidal activity in the presence of food ingredients. With 1 mg/ml of Datem, 1% rosemary extract was necessary to inhibit *S. aureus* instead of 0.5% in normal TSB. A concentration of 0.06% rosemary extract was bactericidal for *B. cereus* in TSB but had no effect with 0.1 and 1 mg/ml of Datem (Table 4). Bovine serum albumin (1 mg/ml) reduced the inhibitory and the bactericidal effects of the rosemary extract on both *S. aureus* and *B. cereus.* A concentration of 0.5% rosemary ex-



tract had no effect on *S. aureus*, and 0.25% was necessary to kill *B. cereus* (Table 4). Pectin (1 and 5 mg/ml) had no effect on the inhibitory action of the rosemary extract on *B. cereus*, but slightly increased it on *S. aureus* (Table 4). The addition of sterilized dairy cream to TSB reduced significantly the inhibitory and bactericidal effects of the rosemary extract. A concentration of 1% rosemary extract had no effect on *S. aureus* in media containing 50 and 100 μl/ml of dairy cream (Table 4). Concentrations greater than 0.5% rosemary extract were necessary to kill *B. cereus* in the presence of 50 μl/ml of dairy cream. When 100 μl/ml of dairy cream was added, only 1% rosemary extract had a bactericidal effect.

The rosemary extract lost most of its activity in skimmed milk against *S. aureus* and *B. cereus*. One percent rosemary extract had no effect on *S. aureus* and delayed growth of *B. cereus* for only 24 h at 30°C (Table 4).

Inhibitory effect of the rosemary extract in zucchini broth. Because the rosemary extract lost its effect in the presence of proteins and fats but was not affected by pectin, it was tested as a preservative in a model food made with vegetables. Zucchini was chosen because it was the vegetable supporting the highest microbial growth among a range of pasteurized vegetables according to Carlin et al. *(3)*. The aerobic mesophilic bacteria grew in zucchini broth to reach 10^7 CFU/ml in 7 days at 10°C. The two concentrations of the rosemary extract (0.5 and 1%) had an inhibitory effect on this microflora. This inhibition lasted only 10 days with 0.25% rosemary extract. The presence of sterilized dairy cream in zucchini broth reduced the inhibitory effect of the rosemary extract. One percent rosemary extract was still inhibitory with 10% sterilized dairy cream but only for the first 5 days. No inhibition occurred in the presence of 50% sterilized dairy cream (Fig. 5).

Composition and activity of hydrophilic and hydrophobic fractions. Among the phenolic compounds of the rosemary extract separated by HPLC (Fig. 6), rosmarinic acid was identified by comparison with a commercial standard (retention time = 60 min), and flavones were identified by their spectra previously reported by Scott *(17)* and eluted between 60 and 80 min. The fraction F_1 (1,384 mg), obtained by hexane extraction, contained only the most apolar phenolic compounds, with retention times longer than 80 min (Fig. 6). Fraction F_2 (309 mg), obtained after extraction by hexane, contained more polar phenolic compounds, with retention times lower than 80 min (Fig. 6). The fraction F_1 had an MIC for *S. aureus* of 79 μg/ml,

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FIGURE 3. Effect of pH and NaCl concentration on the antimicrobial activity of the rosemary extract (♦: 0%; □: 0.13%; ▲: 0.25%, ×: 0.5%; ○: 1%) at 30°C, on S. aureus *in modified TSB (pH 7, 10% NaCl),* L. plantarum *in modified TSB (pH 3.7, 0.5% NaCl), and* Salmonella *Enteritidis in modified TSB (pH 7, 5% NaCl). Bars indicate standard deviation calculated from two replicates. Standard deviations lower than 0.15 log CFU/ml were smaller than the symbols.*

TABLE 2. *MIC of the rosemary extract (vol/vol) for* S. aureus *measured after 24 h at 30°C in TSB for various pH and NaCl contents*[a]

	NaCl (g/liter)				
pH	5	20	30	40	50
7	0.5/0.5	0.5/0.5	0.06/0.13	0.06/0.06	0.06/0.06
6	0.5/0.5	0.06/0.06	0.06/0.06	0.06/0.06	0.06/0.06
5.5	0.5/0.5	0.06/0.06	0.06/0.06	—[b]	—
5	0.13/0.13	0.06/0.06	0.06/0.06	—	—
4.5	0.06/0.06	0.06/0.06	0.06/0.06	—	—

[a] Values are expressed in percents. Two MICs as the MIC obtained from two replicate experiments are shown.

[b] No growth of *S. aureus* at these values of pH and concentration in NaCl without rosemary extract.

whereas the fraction F_2 had no activity at the concentrations tested (MIC >129 μg/ml).

DISCUSSION

The most apolar phenolic compounds from the rosemary extract are presumably responsible of the antibacterial activity. Cuvelier et al. *(5)* characterized the phenolic composition of 24 rosemary extracts and classified them into three phenolic groups of compounds. In order of decreasing polarity, they distinguished phenolic acids, such as ferrulic acid, cafeic acid, and rosmarinic acid; flavonoids, such as apigenin and rutin; and phenolic diterpenoids, such as carnosic acid and carnosol *(5)*. The HPLC profiles obtained by the same authors are similar to ours, and we could reasonably assume that phenolic diterpenoids are the main compounds of the apolar fraction of our rosemary extract. However, some flavonoids are present in the apolar fraction of the rosemary extract. Parmar et al. *(14)* noted that among 102 natural and synthetic flavonoids tested at concentrations up to 100 μg/ml, only two exhibited moderate bacteriostatic, but not bactericidal, activity against the gram-positive bacteria *B. cereus*, *S. aureus*, and *Streptococcus pyogenes*. The MIC for *S. aureus* of the apolar fraction of the rosemary extract (79 μg/ml) was lower than the tested concentration of flavones in the work of Parmar et al. *(14)*. The compounds responsible for the antibacterial action seemed presumably not to be the flavones but rather the phenolic diterpenoids.

TABLE 3. *MLC of the rosemary extract (vol/vol) for* S. aureus *measured after 24 h at 30°C in TSB for various pH and NaCl contents*[a]

	NaCl (g/liter)				
pH	5	20	30	40	50
7	1/1	0.5/0.5	0.5/0.25	0.06/0.13	0.13/0.06
6	0.5/0.5	0.25/0.25	0.06/0.13	0.06/0.06	0.06/0.06
5.5	0.5/0.25	0.25/0.25	0.06/0.06	0.06/0.06	0.06/0.06
5	0.25/0.25	0.06/0.06	0.06/0.06	0.06/0.06	0.06/0.06
4.5	0.5/0.25	0.06/0.06	0.06/0.06	0.06/0.06	0.06/0.06

[a] Values are expressed in percents. Two MLCs as the MLC obtained from two replicate experiments are shown.

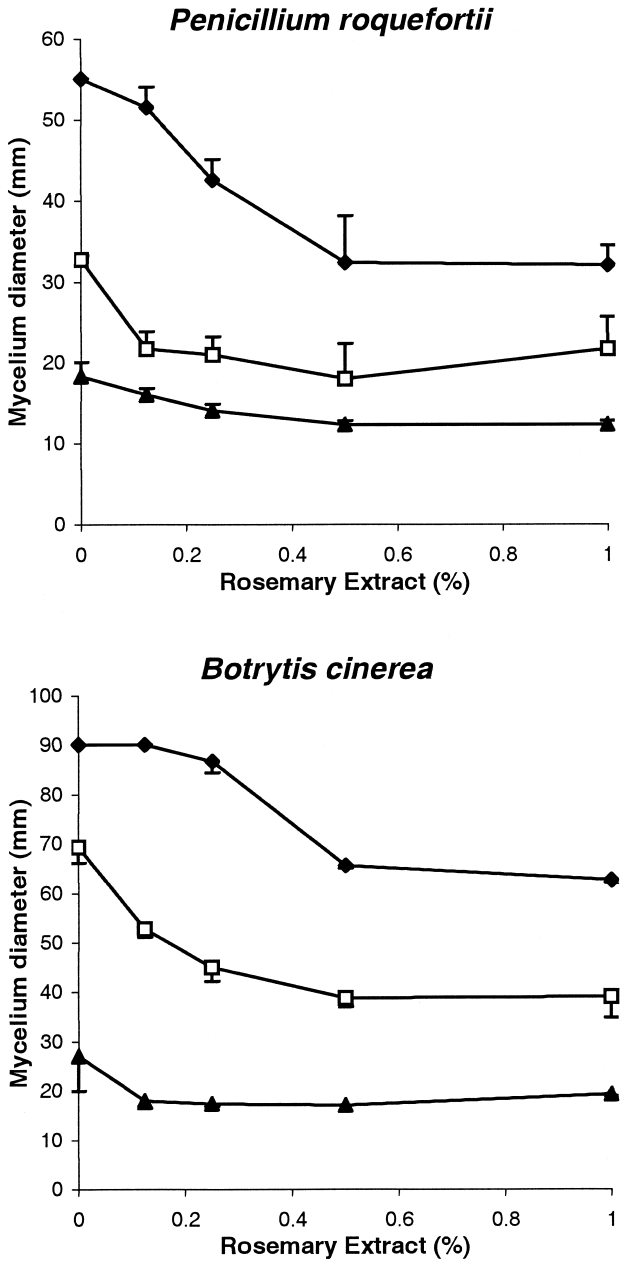

FIGURE 4. *Effect of incubation temperatures (♦: 25°C, □: 10°C, and ▲: 4°C) on the antimicrobial activity of the rosemary extract on* P. roquefortii *and* B. cinerea. *Growth of* P. roquefortii *and* B. cinerea *was measured by the diameter of the mycelium on potato dextrose agar after 7 days. Standard deviation was calculated for each point from three replicates.*

Among all the microorganisms tested, gram-positive bacteria were the most sensitive to the rosemary extract. Farbood et al. *(8)* and Shelef *(18)* also found that the extracts from rosemary and other *Lamiaceae* plants were inactive on gram-negative bacteria. According to Davidson *(6)*, the gram-positive bacteria are generally more susceptible to nonpolar phenolic compounds than gram-negative ones. Similarly, Moujir et al. *(13)* found that nine phenolic diterpenoids isolated from *Salvia* species inhibited the gram-positive bacteria *S. aureus*, *Staphylococcus albus*, and *Bacillus subtilis* at concentrations of 3 to 60 μg/ml and did not inhibit *E. coli* and *Pseudomonas aeruginosa* at 60 μg/

TABLE 4. *MIC of the rosemary extract (vol/vol) measured after 24 h at 30°C for* S. aureus *and* B. cereus *in the presence of food ingredients and numbers of bacteria (log CFU/ml ± SD) in broth containing MIC of rosemary extract*[a]

	Quantity of food ingredients in culture media (mg/ml and μl/ml for dairy cream)						
	0	0.1	1	5	50	100	Pure ingredient
Datem							
S. aureus							
MIC, %	0.5	0.5	1	NT	NT	NT	NT
log CFU[c]	5.28 ± 0.19	4.72 ± 0.12	4.04 ± 0.17				
B. cereus							
MIC, %	0.06	0.13	0.25	NT	NT	NT	NT
log CFU	3.17 ± 0.08	1.31[b]	1.31				
Bovine serum albumin							
S. aureus							
MIC, %	0.5	NT	1	NT	NT	NT	NT
log CFU	5.28 ± 0.19		4.41 ± 0.17				
B. cereus							
MIC, %	0.06	NT	0.25	NT	NT	NT	NT
log CFU	3.17 ± 0.08		1.31				
Pectin							
S. aureus							
MIC, %	0.5	NT	0.5	0.25	NT	NT	NT
log CFU	5.28 ± 0.19		4.12 ± 0.14	4.48 ± 0.08			
B. cereus							
MIC, %	0.06	NT	0.06	0.06	NT	NT	NT
log CFU	3.17 ± 0.08		1.31	1.31			
Dairy cream							
S. aureus							
MIC, %	0.5	NT	NT	NT	>1	>1	NT
log CFU	5.28 ± 0.19						
B. cereus							
MIC, %	0.06	NT	NT	NT	0.5	1	NT
log CFU	3.17 ± 0.08				1.31	1.31	
Skim milk							
S. aureus							
MIC, %	0.5	NT	NT	NT	NT	NT	>1
log CFU	5.28 ± 0.19						—
B. cereus							
MIC, %	0.06	NT	NT	NT	NT	NT	1
log CFU	3.17 ± 0.08						2.00 ± 0.69

[a] Initial numbers of *B. cereus* and *S. aureus* in broth were, respectively, 3.84 ± 0.26 log CFU/ml and 5.15 ± 0.09 log CFU/ml. Numbers of *B. cereus* and *S. aureus* in broths without rosemary extract after 24 h at 30°C were, respectively, 7.70 ± 0.70 log CFU/ml and 8.90 ± 0.38 log CFU/ml. NT indicates not tested.

[b] 1.31 = detection limit.

[c] Numbers of bacteria in broth containing MIC after 24 h at 30°C ± SD calculated from two replicates.

ml. Collins and Charles *(4)* showed that a phenolic diterpenoid, carnosol, caused a significant decrease of *S. aureus* at 50 μg/ml, whereas 150 μg/ml was necessary to inhibit *E. coli*. Flavonoidic structures were inactive against the gram-negative bacteria *Enterobacter agglomerans, E. coli, Klebsiella pneumoniae, Salmonella* type B, *Proteus vulgaris*, and *P. aeruginosa (14)*.

One percent rosemary extracts had a moderate antifungal activity and no effect on the yeasts tested. Beuchat and Golden *(2)* reported the reduction of molds and yeasts in the presence of many herbs and spices, but according to Davidson *(6)*, studies on the antifungal properties of hydroxycinnamic acids gave conflicting results. Parmar et al. *(14)* found no activity among 102 flavonoids on the yeast *Candida albicans* and on the fungi *Aspergillus fumigatus, Microsporum canis*, and *Trichophyton rubrum* at 100 μg/ml. Among 21 abietane diterpenes tested, Moujir et al. *(13)* found that only one could inhibit *Candida utilis* at 20 μg/ml.

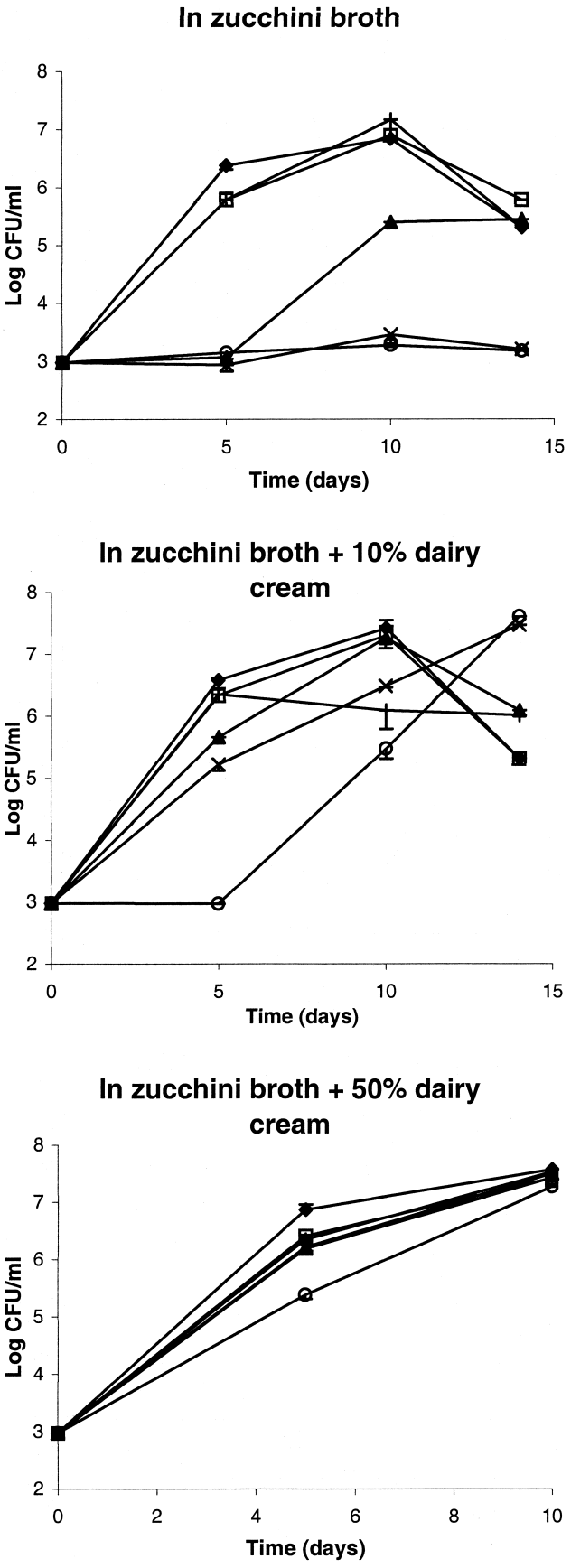

FIGURE 5. *Effect of the rosemary extract (♦: 0%; □: 0.13%; ▲: 0.25%, ×: 0.5%; ○: 1%) on the growth of aerobic mesophilic bacteria (log CFU/ml) in zucchini broth with sterilized dairy cream after 5, 10, and 14 days at 10°C. Bars indicate standard deviation calculated from two replicates. Standard deviations lower than 0.15 log CFU/ml were smaller than the symbols.*

To use rosemary extracts as antimicrobials, it must be active in specified physicochemical conditions of foods. At low temperatures (4 and 10°C compared with 30°C), the inhibitory effect of the rosemary extract was enhanced, but the bactericidal effect disappeared. Ting and Deibel *(22)* showed that refrigeration temperature could enhance the inhibitory effect of sage but not that of clove or oregano against *L. monocytogenes.* Ultee et al. *(23)* observed that the inhibitory effect of 1 and 1.5 mmol/liter of carvacrol, a phenolic compound found in essential oils of thyme and oregano, was respectively two- and 10-fold more pronounced at 30 than at 8°C. The inhibitory effect of the rosemary extract increased with low pH and high NaCl concentrations. Bacterial cells stressed by the low pH or the high NaCl concentrations were presumably more sensitive to the effect of the rosemary extract. Stern et al. *(19)* found that increasing NaCl from 3 to 7% gave a twofold reduction of the amount of butylated hydroxyanisole (BHA) necessary to inhibit *S. aureus.* In the case of phenolics from olives, Fleming et al. *(9)* showed that 5% salt accentuated the antibacterial effect of oleuropein. Davidson *(6)* noted that the effect of pH on the antimicrobial activity of BHA depended on the microorganism tested: *Clostridium perfringens* was more susceptible at pH 5.5 and 8.5, whereas *S. aureus* was most susceptible at neutral pH. At 4 and 10°C, Tassou et al. *(20)* showed a bactericidal effect on *Salmonella* Enteritidis and on *L. monocytogenes* with, respectively, 0.5 and 2% essential oil from mint at pH 4.5, whereas it was inactive at neutral pH. At 30°C, we found that the MIC of the rosemary extract was eightfold lower at pH 6 and 2% sodium chloride than in the normal broth. Similarly, Lachowicz et al. *(12)* observed a synergistic effect with low pH and high NaCl concentration on the antimicrobial activity of 0.1% anise oil on *Lactobacillus curvatus* at 30°C.

Among food ingredients, pectin did not affect the inhibitory activity of the rosemary extract, but surface-active agents, bovine serum albumin and dairy cream, reduced the inhibitory effect. Juven et al. *(11)* showed that a concentration of 140 μg/ml of thymol in the presence of 125 μl/liter of Tween 80 caused a 4-log CFU/ml reduction (a growth of 9 log CFU/ml was observed without thymol) of *Salmonella* Typhimurium, whereas in the presence of 1,000 μl/liter of Tween 80, the inhibitory effect disappeared. They observed that 9 mg/ml of bovine serum albumin eliminated the antibacterial action of 175 μg/ml of thymol on *Salmonella* Typhimurium. Davidson *(6)* reported that bovine serum albumin and casein decreased the antimicrobial activity of BHA. According to Davidson *(6)*, the presence of lipids dramatically decreases the effectiveness of BHA on bacteria, molds, and yeasts. In our study, the rosemary extract totally lost its antibacterial activity in skim milk. Tassou and Nychas *(21)* noted that 2% olive phenolic did not inhibit significantly the growth of *S. aureus* in nonfat milk. This effect of food components reduces the use of the rosemary extract in foods.

According to Naturex, 1,000 μg/ml is the maximal quantity of Oxy'less used in foods. This concentration corresponds to the maximal concentration of rosemary extract

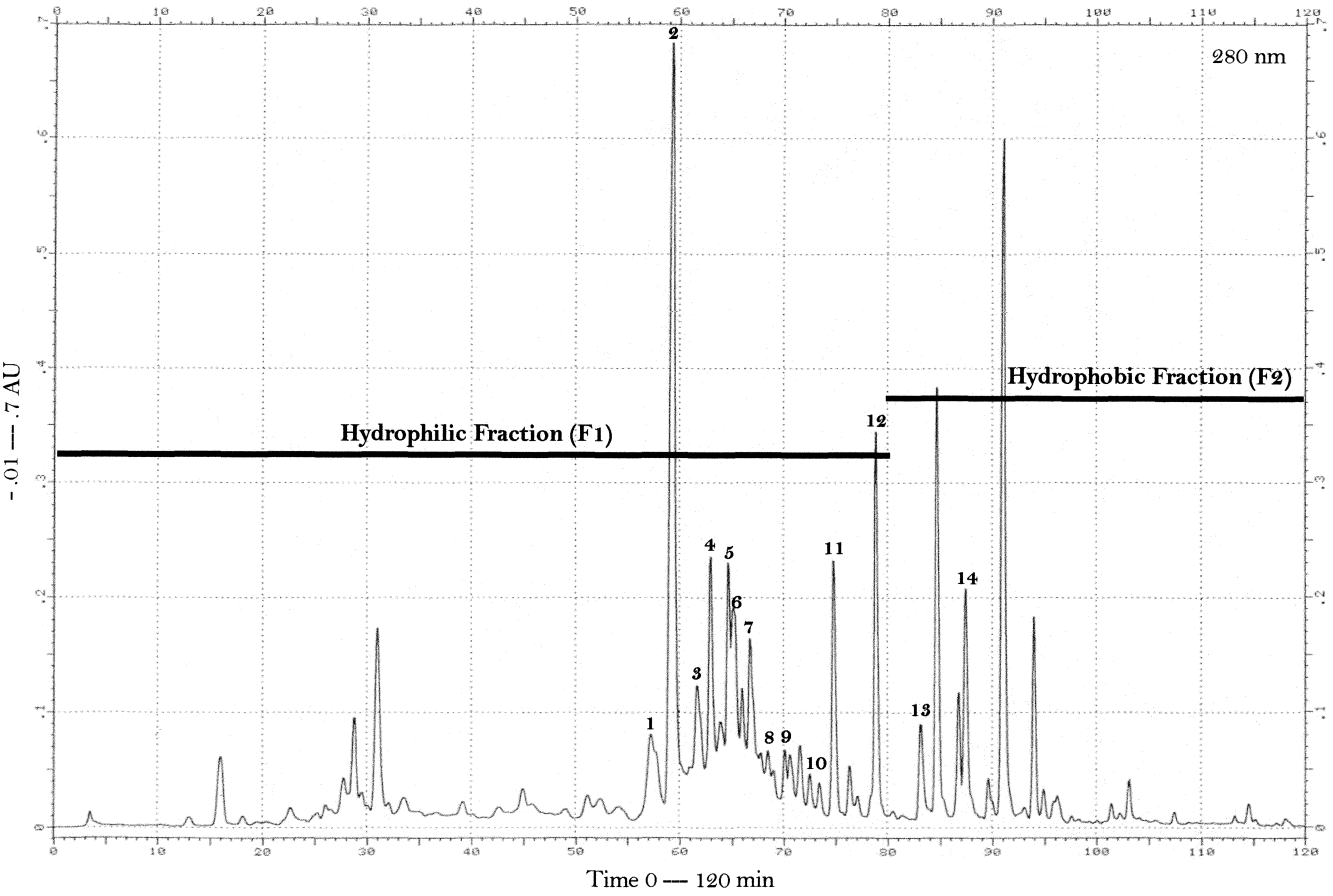

FIGURE 6. *Chromatogram of analytical RP-HPLC of rosemary extract at 280 nm: compounds of the hydrophilic fraction (F_1) and hydrophobic fraction (F_2) are shown. Peaks 1, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, and 14 were characterized as flavones. Peak 2 was identified as rosmarinic acid.*

used in our works (i.e., 1% of an ethanolic solution of 10 g Oxy'less in 100 ml). The rosemary extract should be more appropriate in foods with low fat and protein contents in which gram-positive bacteria cause the major problems. The efficiency of the rosemary extract would be improved in refrigerated foods, and the rosemary extract withstands moderate heat treatment. Therefore, the rosemary extract could be a useful preservative in refrigerated foods with vegetables pasteurized in their final package, which microflora consists of *Bacillus* spp. *(3)*, as shown by its antimicrobial effect on the microflora of pasteurized zucchini broths.

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