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New insights into antibacterial and antioxidant activities of rosemary essential oils and their main components

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ABSTRACT

Rosemary plants growing in Argentina were studied to evaluate any relationship between the chemical composition of their essential oils and the free radical scavenging capacity. The antibacterial activity against human pathogenic and food decay bacteria was also assessed. The chemotype of rosemary essential oil rich in myrcene had greater free radical scavenging capacity, probably due to the high content of myrcene, which demonstrated the highest activity in the DPPH assay. The results obtained suggested a relationship between the antibacterial activity of rosemary essential oil, against Gram-positive and Gram-negative bacteria and the content of α-pinene. In this work, for the first time, changes in the membrane permeability of *Escherichia coli* and *Enterococcus faecalis* after treatment with α-pinene or 1,8-cineole were assessed by the SYTOX Green assay. 1,8-cineole, the other main compound present in both essential oils, was found to disrupt the cell membrane of *E. coli* at ½ × MIC (4 μL/mL).

1. Introduction

A great deal of essential oils is used in food industry as flavoring agents and as pharmaceuticals due to their functional properties, as well as biopreservatives to prolong the shelf life of foods, by reducing or eliminating pathogenic bacteria and increasing the overall quality of food products (Ponce, Roura, & Moreira, 2011). It is well known that some essential oils exert antimicrobial and antioxidant properties (Burt, 2004; Oussalah, Caillet, Saucier, & Lacroix, 2007; Tajkarimi, Ibrahim, & Cliverb, 2010). Few preservatives containing essential oils are commercially available in some countries and are classed as generally recognized as safe (GRAS) food additives (Burt, 2004). Even though manufactures claim such preservatives to contain 50% of essential oils from rosemary, sage and citrus, the precise amounts are not informed.

Microbial activity and oxidation reactions are considered to be the major causes of food decay. In addition, the presence of pathogenic bacteria in foods has prompted researchers to search for new alternatives to reduce the incidence of food borne diseases by controlling the microbiological quality of food products (Rahman & Kang, 2009) and by controlling consumer behaviors (Losasso et al., 2012).

Rosemary (*Rosmarinus officinalis* L.) plants grow worldwide and have been cultivated since long ago for its strong antioxidant and antimicrobial activities. This plant species also has many other beneficial activities such as antiviral, anti-inflammatory and anti-carcinogenic (Aherne, Kerry, & O'Brien, 2007; Barni, Carlini, Cafferata, Puricelli, & Moreno, 2012; Barni, Fontanals, & Moreno, 2009; Mengoni et al., 2011; al-Sereiti, Abu-Amer, & Sen, 1999) activity. This species is considered to be one of the most important sources of both volatile and non-volatile bioactive compounds (Bradley, 2006; Moreno, Scheyer, Romano, & Vojnov, 2006).

Significant variations in the chemical composition of rosemary essential oils have been reported in relation to the geographic origin (Bradley, 2006). Moreover, variations in the antioxidant and antimicrobial properties of rosemary oils from natural populations were also detected. The latter variations were found to be due to regional, environmental and agronomic conditions, the time of harvest, the stage of development of plants, the method of extraction and methodologies used to evaluate their biological activities (Burt, 2004; Celiktas et al., 2007; Jamshidi, Afzali, & Afzali, 2009; Okoh, Sadimenko, & Afolayan, 2010; Zaouali, Bouzaine, & Boussaid, 2010). Although many works have dealt with the

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antimicrobial and antioxidant activities of the essential oils, the correlation between the presence and content of specific compounds and their activity and mechanisms of action has not been investigated (Baratta, Dormans, Deans, Biondi, & Ruperto, 1998; Ivanovic, Misic, Zizovic, & Ristic, 2012; Santoyo et al., 2005).

The present work was undertaken to determine the compounds responsible for the antioxidant and antibacterial activities of essential oils from two phenotypes of rosemary growing in the same location of Argentina. Additionally, the SYTOX Green assay was used to test the mechanism of antibacterial action of the main components of the oil, α-pinene and 1,8-cineole in *Escherichia coli* and *Enterococcus faecalis*.

2. Materials and methods

2.1. Plant material and reagents

Rosemary plants were cultivated in the "Arturo E. Ragonese" Botanical Garden of the INTA-Castelar, Argentina. Leaves of a wide phenotype (WP) and a narrow phenotype (NP) were collected at flowering stage in January 2008. Voucher specimens have been deposited in the Herbarium of the INTA as *R. officinalis* WP Juárez N° 559 and *R. officinalis* NP Juárez N° 560.

Myrcene, camphor, borneol, thymol and 1,8-cineole standards were purchased from Treatt (Lakeland, Florida, USA), α-pinene from Moellhausen (Italy), geraniol from Extrasynthése (Genay, France).

2.2. Essential oils isolation and analysis

Essential oils were isolated by hydrodistillation for 4 h from 810 to 968 g of air-dried rosemary leaves of WP and NP using a Clevenger-type apparatus. After cooling, settling and drying over anhydrous sodium sulfate, oils were recovered and stored at 4 °C until analysis. Quantitative and qualitative analysis were performed by GC–FID–MS using a Perkin–Elmer Clarus 500 GC–FID–MS system with a special configuration (Gil, van Baren, Di Leo Lira, & Bandoni, 2007), equipped with a single split-splitless injector connected by a flow splitter to two capillary columns: a polyethylene glycol MW *ca.* 20,000 column and a 5% phenyl-95%-methyl silicone column, both 60 m × 0.25 mm with 25 μm of fixed phase (J&W Scientific). The polar column was connected to an FID, whereas the non-polar column was connected to an FID and a quadrupole mass detector (70 eV) by a vent system. Helium was used as gas carrier (flow rate: 1.87 mL/min). The column temperature was programmed according to the following gradient: 90 °C during 5 min, increasing at a rate of 3 °C/min–230 °C and maintained for 13 min. The injector temperature was set at 255 °C. Both FID temperatures were 240 °C, and temperatures for the transference line and the ionic source were set at 180 °C and 150 °C, respectively. Mass range (m/z) and scan time were 40–350 Da and 1 s, respectively. The autosampler injection volume was 0.5 μL of a 10% solution of the oil in hexane, the split ratio was 80:1. A mixture of aliphatic hydrocarbons (C_6–C_{24}, Sigma–Aldrich) in hexane was co-injected at the temperature program mentioned above to calculate the retention index (RI) using a generalized equation. This system configuration allowed achieving three identification parameters in a single run: RI in both polar and non-polar columns as well as the mass spectra of each compound.

Identification of compounds was then performed by comparison of mass spectra and retention indexes obtained in both columns with those of reference compounds or those reported in the literature or with those of mass spectra libraries (Adams, 2007; Wiley, 2008). For the comparison of the two oils composition, percentage composition of the essential oils components was calculated by peak area normalization (FID responses) without considering corrections for response factors. The lowest response obtained from both columns for each component was considered. In order to correlate absolute content with minimal inhibitory concentration (MIC) values of the major compounds (α-pinene, myrcene, camphor, borneol and 1,8-cineole), quantification of those compounds in the essential oil was performed using geraniol as internal standard and employing the same operating conditions as described above.

2.3. Antioxidant activity

The antioxidant activity was evaluated by the DPPH (1,1-diphenyl-2-picrylhydrazyl) radical method. The ability of the essential oils and main compounds to donate an electron and scavenge DPPH radical was determined by the method of Brand-Williams, Cuvelier, and Berset (1995). Briefly, 20 μL of each sample in triplicate and five different concentrations (3–50 μL/mL) and 180 μL of DPPH solution (160 μM) in 80% methanol, were added to a well in a 96-well flat-bottom microtitration plate. A DPPH$^{\bullet}$ solution was used as blank sample, and thymol at concentrations of 0.45–3 mg/mL in ethanol was used as positive control. The plate was covered and incubated on a shaker for 1 h at 50 rpm at room temperature. Samples were read in a plate reader (DTX 880 Multimode detector, Beckman Coulter) using a 515 nm filter. The DPPH$^{\bullet}$ solution employed was freshly prepared, and stored protected from light. The antioxidant activity of the tested samples, expressed as percentage inhibition of DPPH, was calculated according to the formula $IC\,(\%) = [(A_0 - A)/A_0] \times 100$, where A_0 and A are the absorbance values of the blank sample and the test, respectively. Percent inhibition after 30 min was plotted against concentration, and a linear regression was applied to obtain the IC_{50} value. IC_{50} values were inversely proportional to the antioxidant activity.

2.4. Antibacterial activity

American Type Culture Collection of *Staphylococcus aureus* ATCC 25923, *E. faecalis* ATCC 29212, *E. coli* ATCC 35218, and *Klebsiella pneumoniae* (from the Food Microbiology Laboratory, Faculty of Natural Sciences, Buenos Aires University) were used in this study.

The antibacterial activity of the essential oils and pure compounds was estimated by the microplate bioassay with slight modifications (CLSI, 2006). Dilutions in Mueller Hinton broth (MH) (Difco, MD, USA), containing 0.5% Tween 80 were prepared from an 80% (v/v) essential oil or pure compound solution in ethanol. Each compound (0.4–40 μL/mL, 200 μL per well) was incubated in 96-well flat bottom microplates together with each bacterial strain at a final bacterial density of 5×10^5 CFU/mL (which was achieved by adding 10 μL/well of a 1×10^8 CFU/mL suspension of the investigated strain in MH). The microplate was aseptically sealed, and incubated at 37 °C for 16–24 h under constant shaking (100 rpm). All experiments were performed in triplicate. The MIC was defined as the lowest plant compound concentration that was able to inhibit the bacterial growth after 24 h incubation at 37 °C determined by measuring the absorbance at 625 nm. The bactericide concentration was determined by time-kill studies. Five milliliters of MH broth was inoculated with 1×10^6 CFU/mL of *E. coli* ATCC 35218 (Aeschlimann & Rybak, 1998). The concentrations used for the test were: ½ × MIC, MIC and 2 × MIC. CFU/mL was estimated at 1, 2, 4, 8, 12 and 24 h of exposure to the compounds and employing Trypticase soy broth (TSB) (Merck, Darmstadt, Germany) agar plates incubated at 37 °C. To avoid an antibiotic carryover effect samples were centrifuged; and resuspended in fresh MH medium and serially diluted 10 fold in 0.9% saline before plating.

2.4.1. Evaluation of bacterial membrane permeability

It is known that the SYTOX Green dye is always excluded from the cell, however, when the integrity of the plasma cell membrane is affected, this fluorophore enters the cells freely giving rise to intense green fluorescence (Roth, Poot, Yue, & Millard, 1997). Bacterial cultures in logarithmic phase growth were harvested, incubated in fresh MH medium at 26 °C for 1 h in the presence of each plant compound, pure dissolvent (negative control) or 70% isopropyl alcohol (positive control). Cells were then washed three times with ice cold sterile 0.9% NaCl solution. Bacterial suspensions containing 1×10^8 CFU/mL were stained with 5 μM SYTOX Green (Invitrogen-Molecular Probes, Carlsbad CA, USA) in a black microplate for 10 min in the dark. The fluorescence of the DNA-bound dye was monitored on a fluorescence multiwell plate reader (DTX 880 Multimode detector, Beckman Coulter) with excitation and emission wavelengths of 535/595 nm, respectively. The membrane-permeable activity was expressed as the percentage of permeability in cell membrane bacteria in relation to the maximum permeability (100% value) obtained after incubation with 70% isopropyl alcohol for 10 min. Values were expressed as the mean of at least three independent experiments, each performed in triplicate. Stock solutions of SYTOX Green stain were prepared in dimethyl sulfoxide to a final concentration of 5 mM.

2.5. Statistical analysis

Results were expressed as means ± standard deviation of at least three independent experiments using the software InfoStat (2008). All data were analyzed by ANOVA followed by Tukey test. Differences were considered significant when $p < 0.05$.

3. Results and discussion

3.1. Essential oils analysis

Essential oils isolated from leaves of WP and NP (yields of 2.28% and 2.58% v/w, respectively) cultivated in the same farmhouse were analyzed by GC–FID–MS to determine the chemical composition.

Table 1 shows the list of chemical components of both essential oils expressed as percentages. Forty five compounds representing 93.7% and 94.2% of the total WP and NP oils, respectively, were determined. Two chemotypes of rosemary essential oils were recognized: a WP, rich in α-pinene (31.2%) and the other one, NP, rich in myrcene (31.1%). Other major components were: 1,8-cineole (18.7%–21.6%), borneol (0.4%–15.4%) and camphor (7.0%–15.4%).

Typical rosemary essential oils are rich in α-pinene, 1,8-cineole and camphor, associated with variable amounts of other compounds such as borneol and verbenone (Bradley, 2006). The main component of the Tunisian, Turkish, Moroccan and Italian oils is 1,8-cineole with over 40% (Boutekedjiret, Bentahar, Belabbes, & Bessiere, 1999; Bradley, 2006) whereas French, Spanish and Greek oils have 1,8-cineole, α-pinene and camphor with approximately equal ratios (20–30%) (Lawrence, 1995). The myrcene-rich oil has only been reported in South America, in Argentina (Mizrahi, Juarez, & Bandoni, 1991), Uruguay and Brazil (Dellacassa et al., 1999; Larrán et al., 2001; Porte et al., 2000).

The differences in the chemical composition detected in the two Argentinean rosemary phenotypes grown under the same conditions for over 7 years are mainly determined by the genotype of the plants. Our results are in accordance with those reported by Zaouali et al. (2010), these authors analyzed the chemical composition of essential oils of two varieties of Tunisian rosemary and found a high variation in the chemical composition, suggesting that these differences could be genetically determined.

Table 1
Chemical composition of rosemary essential oils (EO) expressed as percentages obtained by hydrodistillation of leaves of *Rosmarinus officinalis* from a wide phenotype (WP) and a narrow phenotype (NP).

Compound	RI	RI	WP EO	NP EO
trans-2-Hexenal	850[a]	1225[b]	t	t
α-Thujene	930	1034	0.1	0.2
Tricyclene	932	1014	0.2	0.1
α-Pinene	939	1032	31.2	4.9
Camphene	970	1076	5.0	1.8
3-Octenol	977	–	t	t
3-Octanone	980	–	0.2	t
Myrcene	982	1174	1.1	31.1
β-Pinene	983	1118	1.7	t
α-Phellandrene	1005	1176	0.2	0.5
α-Terpinene	1018	1188	0.6	1.1
p-Cymene	1025	1280	0.5	1.5
Limonene	1026	1203	3.3	2.4
1,8-Cineole	1030	1213	21.6	18.7
γ-Terpinene	1049	1255	1.0	1.9
Terpinolene	1090	1290	0.8	0.5
Linalool	1099	1553	2.8	1.9
Crysanthenone	1130	–	0.3	t
Camphor	1145	1532	7.0	15.4
t-Pinocamphone	1100	–	0.3	0.2
Pinocarvone	1160	1586	0.2	0.1
δ-Terpineol	1166	1682	0.4	0.4
Borneol	1170	1719	4.2	0.4
Terpinen-4-ol	1175	1611	1.1	1.5
α-Terpineol	1190	1706	2.4	2.7
Verbenone	1210	1725	2.1	3.0
Piperitone	1250	1757	0.4	0.1
Bornyl acetate	1290	1590	1.6	0.2
Piperitenone	1340	–	0.1	t
α-Terpenyl acetate	1348	–	t	t
Linalyl isobutanoate	1370	–	t	t
α-Ylangene	1372	1493	0.1	t
α-Copaene	1374	1497	0.1	t
Methyl eugenol	1405	1830	0.1	0.4
β-Caryophyllene	1420	1612	2.2	1.7
α-Humulene	1455	1687	0.4	0.7
allo-Aromadendrene	1460	1661	t	t
α-Amorphene	1488	–	t	0.1
β-Bisabolene	1505	1741	0.1	t
δ-Amorphene	1510	–	0.1	0.1
α-Calacorene	1545	1941	0.1	t
E-Nerolidol	1555	2050	t	t
Caryophyllene oxide	1584	2001	0.1	0.1
Humulene epoxide II	1610	2071	t	0.1
epi-α-Bisabolol	1680	–	0.1	0.3
Total			**93.7**	**94.2**

Compounds are listed in order of elution.
t < 0.05%.
[a] RI: Retention index in the apolar column.
[b] RI: Retention index in the polar column.

3.2. Antioxidant activity

In the DPPH assay, the ability of both essential oils and their main components to act as donors of hydrogen atoms or electrons for the transformation of DPPH into its reduced form DPPH• was investigated. Both essential oils studied were able to change the stable violet DPPH radical into yellow-colored DPPH•, reaching 50% of reduction with IC_{50} values ranging from 11 μL/mL and 25 μL/mL for the NP and WP essential oils, respectively (Table 2). Our results are in agreement with previous data reported on the antioxidant activity of essential oils of rosemary (Bozin, Mimica-Dukic, Samojlik, & Jovin, 2007; Gachkar et al., 2007).

Results also showed that the chemotype of rosemary myrcene-rich essential oils showed twice the activity than the other and as there were no reports on the antioxidant activity of this chemotype, antioxidant activity of the essential oils and the three major compounds (α-pinene, myrcene and 1,8-cineole) was also

Table 2
Neutralization of DPPH• by rosemary essential oils (EO) and their main components.

Samples	IC_{50} (μL/mL)
WP EO	25 ± 0.7
NP EO	11 ± 0.5
Myrcene	4.5 ± 0,9
1,8-Cineole	n.a.
α-Pinene	18 ± 0.5
Thymol	0.4[a] ± 0.03

n.a.: not active.
[a] mg/mL.

evaluated. The results showed a significant antioxidant activity of myrcene with an IC_{50} of 4.5 μL/mL, followed by the α-pinene with an IC_{50} of 18 μL/mL.

Antioxidants minimize oxidation of the lipid components in foods and there is an increasing interest in the use of natural antioxidants in food preservation. Thymol was used as reference since it possesses well-known antioxidant properties (Yanishlieva, Marinova, Gordon, & Raneva, 1999). The antioxidant *in vivo* effects of myrcene determined by peroxidation assay have previously been reported (Ciftci, Ozdemir, Tanyildizi, Yildiz, & Oguzturk, 2011; Mitic-Culafic et al., 2009). In mammalian cells, myrcene not only has not proved to be mutagenic, but also it was shown to have antimutagenic properties (Kauderer, Zamith, Paumgartten, & Speit, 1991). Therefore, our results suggest that the rosemary myrcene-rich oil can be a suitable target in the search for 'natural' replacements for 'synthetic' antioxidant food additives.

3.3. Antibacterial activity

The chemical composition of essential oils is critical for their antibacterial activities (Zaouali et al., 2010). For these reason a quantitative evaluation of the antibacterial activity of the two rosemary essential oils was determined by the microplate bioassay against the Gram-positive bacteria, *S. aureus* and *E. faecalis*, and against the Gram-negative bacteria, *E. coli* and *K. pneumoniae*. Both essential oils exerted a moderate antibacterial activity, although the dose–response curves showed that the α-pinene-rich oil had a higher antibacterial activity than the myrcene-rich one obtained from the NP against *S. aureus*, *E. faecalis* and *K. pneumoniae*; meanwhile both oils were equally active against *E. coli* (Fig. 1). The 100% of growth inhibition (MICs values) of *S. aureus* and *E. faecalis* of the α-pinene-rich oil were obtained using 10 μL/mL and 26 μL/mL, respectively; while this oil was active against *E. coli* and *K. pneumoniae* at 14 μL/mL and 20 μL/mL, respectively (Table 5). The myrcene-rich oil obtained from NP showed a lower efficacy as antibacterial and it was unable to induce a total inhibition of the growth of *S. aureus*, *E. faecalis* and *K. pneumoniae* (Fig. 1).

Given the observed difference in the antibacterial efficacy of the two oils studied, we hypothesized that the antibacterial activity of these essential oils could be related to one of their main compounds, α-pinene, myrcene, 1,8-cineole, camphor and borneol. The antibacterial activity of these compounds was then evaluated under the same experimental conditions. Table 3 shows that α-pinene was the only compound that was able to inhibit all the microorganisms tested with MIC values ranging from 0.8 to 8 μL/mL. On the other hand, camphor and borneol inhibited only Gram-positive bacteria, while 1,8-cineole was active against Gram-negative bacteria with MIC values from 8 to 20 μL/mL. Myrcene, the major constituent of essential oil obtained from NP, did not show any activity against the tested bacteria.

Our results differ from those reported by Santoyo et al. (2005), regarding the effectiveness of the pure compounds tested, in particular that of α-pinene against *S. aureus* ATCC 25923. These authors have found that the most active compound was borneol, followed by camphor, 1,8-cienole and α-pinene. Other authors have also reported different efficiencies for these compounds (Leite et al., 2007). Burt (2004) reviewed the antimicrobial activity of essential oils, reporting a multiplicity of factors ranging from the culture of the plant to variations in the experimental conditions for the measurement of the antibacterial activity, as well as differences in the methodology as the type of Tween used to dissolve the hydrophobic components.

The hydrophilic cell wall structure of Gram-negative bacteria, constituted essentially by a lipo-polysaccharide, blocks the penetration of hydrophobic components of oils and for this reason, Gram-positive bacteria are found to be more sensitive to the essential oils effects (Burt, 2004). Surprisingly, we found a marked antibacterial activity for α-pinene and 1,8-cineole against the Gram-negative bacterium *E. coli* (Fig. 1). Therefore, the time-kill dynamic process of these two main bioactive components of rosemary toward *E. coli* is presented in Fig. 2. At the MIC values, both α-pinene and 1,8-cineole had bactericidal effects on *E. coli* within the first 12 h (Fig. 2); while at ½ × MIC 1,8-cineol was four times more potent than α-pinene. In addition, at 2 × MIC 1,8-cineole triggered a reduction of ~2 log10 in bacterial growth in 1 h (Fig. 2b), whereas this effect was observed 2 h later for α-pinene using 2 × MIC (Fig. 2a). Recently, Jiang et al. (2011) have reported a bactericidal effect of α-pinene and 1,8-cineole using 1 × MIC against *S. aureus* after 8 h and 12 h, respectively. In the present work, the lethal effect of α-pinene and 1,8-cineole on *E. coli* is reported for the first time.

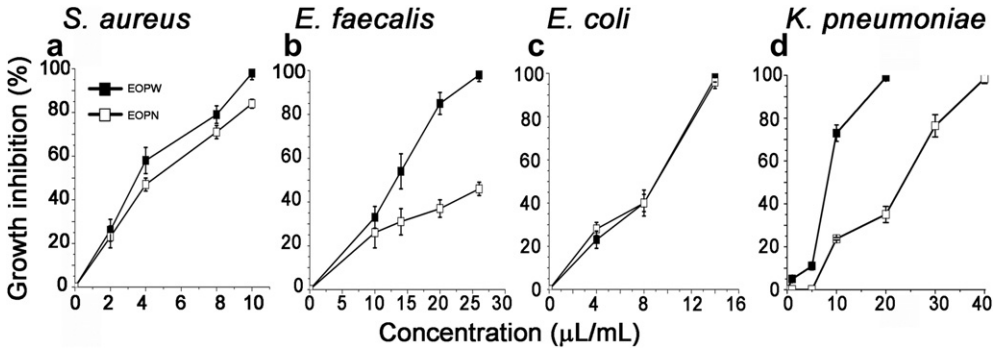

Fig. 1. Antibacterial activity of wide phenotype (WP) and narrow phenotype (NP) rosemary essential oils against *S. aureus* (a), *E. faecalis* (b), *E. coli* (c), and *K. pneumoniae* (d), evaluated by the microplate bioassay.

Table 3
MIC of pure main compounds of rosemary essential oils.

Compounds	MIC (μL/mL)			
	S. aureus	*E. faecalis*	*E. coli*	*K. pneumoniae*
α-Pinene	0.8	8.0	0.8	5.0
Camphor	0.4	8.8	n.t.	n.a.
Borneol	2.0	4.0	n.t.	n.a.
1,8-Cineole	n.a.	n.a.	8.0	20
Myrcene	n.a.	n.a.	n.a.	n.a.

n.a.: not active, n.t.: not tested.

Table 4
Content of the main compounds of rosemary essential oils (EO).

Compounds	Content (mg/mL)	
	WP EO	NP EO
α-Pinene	349 ± 8.9	61 ± 0.1
Myrcene	21 ± 0.5	466 ± 1.9
1,8-Cineole	226 ± 5.7	198 ± 1.3
Borneol	13 ± 0.4	6 ± 0.1
Camphor	64 ± 1.0	143 ± 0.4

3.4. Relationship between the biological activities and chemical composition of essential oils

The biological activities of the essential oils are often attributed to their major compounds (Burt, 2004). Herein, we determined the essential oils composition to establish a relationship with the antioxidant and antibacterial activities of the oils. Table 4 shows the quantification of the main bioactive compounds of both rosemary essential oils performed by GC–FID–MS and expressed in mg/mL.

Considering these values, we calculated the content of each bioactive compound in the rosemary essential oils volume determined as IC_{50} for the antioxidant activity and the MIC values for each tested bacterium. Thus, the compounds were referred to as mass units considering the density of the compounds (density of α-pinene: 0.858 g/mL, myrcene: 0.794 g/mL and 1,8-cineole: 0.9225 g/mL Table 5).

The myrcene-rich oil at its IC_{50} value (11 μL/mL) determined by the DPPH assay (Table 1), contained 4.15 mg of myrcene, amount that is nearly equivalent to the antioxidant activity exerted by the pure compound (IC_{50} 4.5 ± 0.9) (Table 2). Therefore, it is possible to infer that this component may be responsible for the overall antioxidant activity observed for the rosemary myrcene-rich essential oil. In line with the findings of other authors, no significant antioxidant activity was observed for the other evaluated components (Park et al., 2008; Slamenová, Horváthová, Wsólová, Sramková, & Navarová, 2009).

The α-pinene-rich oil that had an IC_{50} = 25 μL/mL did not show an antioxidant activity associated with any particular compound studied here, since α-pinene, 1,8-cineole and myrcene were all found to be in lower amounts (8.73 mg, 5.65 mg and 0.525 mg, respectively) to the active concentrations exhibiting antioxidant activity (Table 2). Therefore, the antioxidant activity exhibited by this essential oil can be the result of the contribution of different compounds, as suggested by Wang, Wu, Zu, and Fu (2008). In this sense, although we were not able to detect antioxidant activity for 1,8-cineole, this compound could exert a synergistic activity with other antioxidant compounds. Further studies are needed to address this issue.

Likewise, the antibacterial activity of the α-pinene-rich oil contained an amount of α-pinene greater than the MIC values for all the bacteria tested. Therefore, the inhibitory activity of the oil observed for Gram-positive and Gram-negative bacteria may be attributed to this compound in this rosemary oil chemotype. It can also be speculated that the myrcene-rich oil would have a lower antibacterial activity against *E. faecalis*, possibly due to the fact that it contained an amount of α-pinene far below (1.52 mg/mL) the MIC value of the pure compound (7.56 mg/mL) (Table 5). Camphor and borneol showed activity against *S. aureus* and *E. faecalis*, but as their levels in rosemary oils are low, they probably did not contribute to the antibacterial activity.

The antibacterial activity of the myrcene-rich oil could not be attributed to the presence of a particular bioactive compound present in amounts greater than the MIC values obtained for the bacteria tested. Therefore, it is quite possible that the antibacterial effect of this oil on *E. coli* and *K. pneumoniae* is the result of a combination of α-pinene and 1,8-cineole (40 μL/mL of this oil contained 2.44 mg and 7.92 mg of α-pinene and 1,8-cineole, respectively). On the other hand, myrcene which is the main compound present in the myrcene-rich oil chemotype was not active against all bacteria tested.

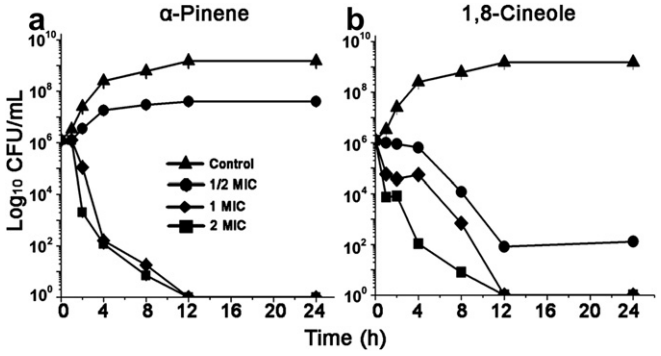

Fig. 2. Time-kill curves of *E. coli* ATCC 35218 in the presence of α-pinene (a) and 1,8-cineole (b). The concentrations used for the test: ½ × MIC, MIC and 2 × MIC, the control did not contain the test sample.

3.5. SYTOX Green assay

Due to the lipophilic nature of α-pinene and 1,8-cineole, it is believed that their antibacterial activity is accomplished by the alteration of the architecture of cell membranes (Sikkema, de Bont, & Poolman, 1995). Here, the effect of these compounds on the cell membrane permeability of *E. coli* and *E. faecalis* was investigated using the SYTOX Green stain, a high-affinity nucleic acid stain that does not cross the membranes of live cells and yet easily penetrates cells with compromised plasma membranes.

We found that α-pinene did not change significantly the cell membrane permeability in *E. coli* (Fig. 3a and c) and *E. faecalis* (Fig. 3b and d) at concentrations around or higher than its MIC

Table 5
MIC of rosemary essential oils (EO) against bacteria and α-pinene content present at the MIC values.

Bacteria	MIC (μL/mL)		
	WP EO	NP EO	α-Pinene
S. aureus	10 (3.40)[a]	10[b] (0.70)	0.8 (0.69)
E. faecalis	25 (8.75)	25[c] (1.52)	8.0 (7.56)
E. coli	14 (4.88)	14 (0.86)	1.0 (0.86)
K. pneumoniae	20 (6.98)	40 (2.44)	5.0 (4.29)

[a] Number in brackets indicate the α-pinene content present at the MIC values of the oils, expressed in mg/mL.
[b] Concentration of essential oil inhibiting the bacterial growth in an 80%.
[c] Concentration of essential oil inhibiting the bacterial growth in a 40%.

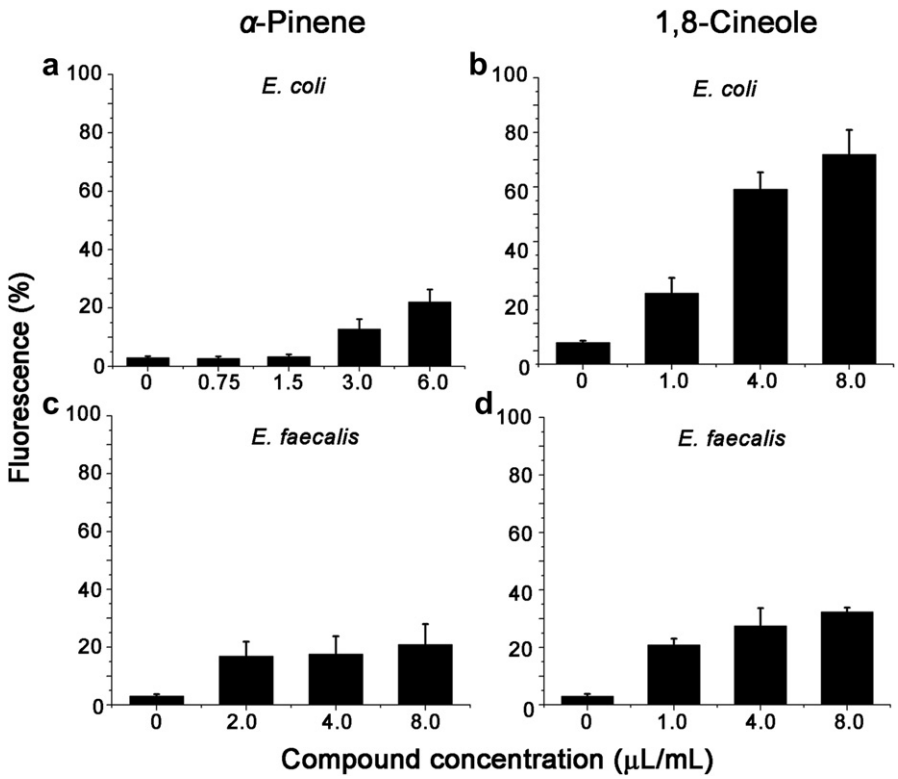

Fig. 3. Effect of α-pinene and 1,8-cineole on the cell membrane permeability of *E. coli* (a, b) and *E. faecalis* (c, d) by the SYTOX Green assay; the 100% of membrane permeability was obtained after incubation with 70% isopropyl alcohol.

value. However, in yeast cells and isolated mitochondria, α-pinene destroys cellular integrity increasing the membrane permeability (Cox et al., 2000). Recently, Matsuo et al. (2011) have reported that this monoterpene induces apoptosis and confers antimetastatic protection in a melanoma model and was speculated that this compound was able to permeabilize only the outer mitochondrial membrane.

At the MIC value (8 μL/mL) 1,8-cineole caused an increase in the fluorescence of near 70% in *E. coli* (Fig. 3b). Therefore, this main bioactive component of the rosemary essential oil is an effective antibacterial agent causing damage to the plasma membrane at least in the Gram-negative bacteria *E. coli*, showing a bactericidal effect at the MIC value as demonstrated by the time-kill curve (Fig. 2). In *E. faecalis*, the 1,8-cineol increased near 30% the fluorescence, but this effect seems not to affect the cell viability (see Table 3).

Many compounds present in essential oils have been proven to have antibacterial activity, e.g. carvacrol, thymol, eugenol, perillaldehyde, cinnamaldehyde and cinnamic acid, although the mechanisms by which these compounds exert their activity are poorly understood. As a rule, Gram-positive bacteria are more sensitive to the essential oils, as it is known that the hydrophilic cell wall structures of Gram-negative bacteria block the penetration of hydrophobic components in the cell membrane (Burt, 2004). In the present study, the bactericidal effect associated with injury to the cell membrane exerted by1,8-cineole on *E. coli* is reported.

4. Conclusion

In the present work, a relationship between the composition of the rosemary essential oils studied and the radical scavenging capacity and antibacterial activity was observed. The rosemary myrcene-rich oil was found to have the highest antioxidant activity, while the rosemary α-pinene-rich oil had the highest antibacterial activity against Gram-positive/Gram-negative bacteria.

It is difficult to attribute the antibacterial effect of an essential oil to one or a few active compounds, because in general they contain a mixture of different chemical compounds. Taking into the account the results presented herein, it could inferred that the effectiveness of rosemary essential oils as antioxidant and antibacterial agents depend on an adequate number and content of key bioactive/s compound/s. Therefore, the relationships between composition and the scavenging capacity and antibacterial activity of rosemary essential oils and the biological activity of each main component is crucial in the search for natural alternatives to synthetic bactericides used for food preservation.

The rosemary oils studied in the present work proved to have α-pinene, which exhibited a broad antibacterial spectrum against Gram negative and Gram positive bacteria, and 1,8-cineole which displayed antibacterial activity against Gram-negative pathogenic bacteria by inducing cell membrane disruption. In addition, essential oils with ability to scavenge the DPPH radical are of main interest in food industry as the antioxidant capacity is nowadays accepted as a criterion of high food quality. The scavenging activity is also employed to monitor the impact of food processing in the nutraceutical value of food products (Cabrera & Prieto, 2010). For this reason, the use of rosemary essential oils in foods, cosmetics and drugs, requires the identification of the bioactive compounds as well as to perform further studies on their mechanism of action.

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