Food Chemistry 115 (2009) 456–461

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Synergistic antioxidant and antibacterial activity of rosemary plus butylated derivatives

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ARTICLE INFO

Article history:
Received 21 August 2008
Received in revised form 5 December 2008
Accepted 9 December 2008

Keywords:
Antibacterial
Antioxidant
Carnosic acid
Polyphenols
Rosemary
Rosmarinic acid
Synergism

ABSTRACT

Antioxidant and antibacterial activity of a methanol rosemary extract (RE) containing 30% carnosic acid (CA), 16% carnosol (COH) and 5% rosmarinic acid (RA) was studied *in vitro* alone and in combination with the antioxidant food additives butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA). The antioxidant efficiency of the extract, CA, and RA, was determined by a kinetic analysis of the 2,2-diphenyl-2-picrylhydrazyl hydrate radical (DPPH·) scavenging activity. RE showed two different rate slopes in the reduction of DPPH· vs. time curve, which correlated with the distinct behaviours of RA and CA; pure RA reached the plateau more rapidly than CA. A synergistic antioxidant effect between RE and BHT was demonstrated by isobolographic analysis and a synergistic interaction of RE with BHA to inhibit *Escherichia coli* and *Staphylococcus aureus* growth was observed. Therefore, rosemary not only enhances the antioxidant efficiency of BHA and BHT, but also the antibacterial effect of BHA; allowing a decrease from 4.4 to17 folds in the amounts of the synthetic compounds used.

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1. Introduction

Today there is an increasing interest in the use of natural antioxidants, such as tocopherols, flavonoids and rosemary (*Rosmarinus officinalis* L.) extracts for food preservation (Hras, Hadolin, Knez, & Bauman, 2000; Williams, Spencer, & Rice-Evans, 2004), because these natural antioxidants avoid undesired health problems that may arise from the use of synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) which may have toxic effects (Aruoma, Halliwell, Aeschbach, & Löligers, 1992). In fact, natural polyphenols isolated from aromatic plants are a promising source of compounds scrutinized to reduce or substitute butylated derivatives used as antioxidant in food, animal feed, pharmaceutical preparations and cosmetic formulations (Aherne, Kerry, & O'Brien, 2007; Guo, Wise, Collins, & Meydani, 2008).

Antioxidants (AOXs) have also a wide range of biological and pharmacological activities and are considered to be of great benefit in nutrition and health, as oxidative stress is an important factor in cell damage and it has been implicated in the development of certain cancers and neurodegenerative diseases (Newman, Cragg, & Snader, 2000). Phenolic compounds derived from herbs and spices are an interesting target in the search for health-beneficial phytochemicals (Liu, 2003).

Rosemary (*R. officinalis* L.) belonging to the *Lamiaceae* family, is well known for its antioxidative properties and it is used for flavoring food, beverages, as well as in several pharmaceutical applications (Shylaja & Peter, 2004). The main compounds responsible for its antioxidative activity in non-volatile fractions are carnosic acid (CA), carnosol (COH) and rosmarinic acid (RA) (Cuvelier, Berset, & Richard, 1994). These polyphenols have also important biological activities *in vitro* as anti-tumor, chemopreventive and anti-inflammatory agents (Al Sereiti, Abu Amer, & Sen, 1999; Cheung & Tai, 2007; Danilenko et al., 2003; Shuang-sheng & Rong-liang, 2006). It has been proposed that polyphenols of rosemary may greatly increase functionality of food for health and wellness (Shahidi & Naczk, 2004).

Plant extract may provide a potential additional barrier to inhibit the growth of food-borne pathogens in food products. The use of combined AOXs, also called hurdle technology, has gained acceptance in industry and has been applied to different aspects of food preservation (Davidson, Sofos, & Branen, 2005). In this sense, a promising strategy to enhance and/or broaden the biological antioxidant and antimicrobial activities seems to be the combination of two or more compounds; although up to date a rational basis for the use of phytochemicals against food-borne pathogens is still poorly explored (Alzamora, Tapia, & Welti-Chanes, 1998; Wei & Shibamoto, 2007).

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doi:10.1016/j.foodchem.2008.12.029

It has been extensively reported that rosemary essential oils have antimicrobial properties against a wide range of microorganisms, although little information exists regarding the specificity and efficacy of non-volatile phenolic compounds as microbicides (Santoyo et al., 2005). We had previously reported the performance as antioxidant and antimicrobial of rosemary extracts without essential oils (Moreno, Scheyer, Romano, & Vojnov, 2006). Here, we further analyzed the free radical scavenging kinetic behavior of a methanol rosemary extract (RE), and investigate the antioxidant performance and the antibacterial activity of binary mixtures with BHA and BHT in order to determine the type of interaction between them.

2. Materials and methods

2.1. Materials

All solvents used in the experiments were HPLC grade and purchased from Merck (USA). 2,2-Diphenyl-2-picrylhydrazyl hydrate (DPPH), α-tocopherol, gallic acid (GA), 3,5-di-tert-butyl-4-hydroxytoluene (BHT), 3-tert-butyl-4-hydroxyanisole (BHA), were purchased from Sigma Aldrich (Milwaukee, WI, USA). Rosmarinic acid (RA), carnosic acid (CA) and carnosol (COH) were obtained from Alexis Co. (USA). All other reagents were of analytical grade. Rosemary leaves were collected in November from flowering plants grown in the north west of Argentina. Dried leaves were separated from the branches and stored at −20 °C until used.

2.2. Preparation and analysis of rosemary extract

The methanol RE used in this study was prepared as previously reported (Moreno et al., 2006). Briefly, dried rosemary leaves (20–200 g) were chopped into small parts with a blender and placed in a 3–l round-bottom flask with 1l of deionized water. The solution was steam-distilled for 60 min in a Clevenger-type apparatus for oil isolation. The residue was extracted using methanol as solvent by a Soxhlet apparatus. The solvent was vacuum-distilled at 37 °C in a rotary evaporator. The final extract was a dark green powder, and it was kept in a freezer at −20 °C until use. The content of CA, COH and RA of the extract was analyzed by high-performance liquid chromatography (HPLC). The extract was resuspended in pure methanol and centrifuged using a 5804 Eppendorf centrifuge at 5000 rpm for 15 min at room temperature before analysis. HPLC was performed with an LKB Bromma instrument equipped with a diode array detector, using a 250 mm × 4 mm C18 Luna analytical column (Phenomenex, USA). The separation was undertaken with a mobile phase consisting of a gradient of 5–100% acetonitrile in water containing 3% (v/v) acetic acid at a flow rate of 1 ml/min and the injection volume was 20 μl. Different criteria were developed for compound identification such as comparison of the retention time (R_{time}) using commercial standards, determination of maximum absorbance at different wavelengths for compounds, UV spectra using a photo-diode array detector and by adding pure standards to the samples prior to HPLC analysis. Stock solutions of pure CA, COH and RA (1 mg/ml) were prepared in ethanol.

2.3. DPPH radical scavenging assay

This assay was carried out as described by Brand-Williams, Cuvelier, and Berset (1995) with some modifications (Fukumoto & Mazza, 2000). Briefly, instead of reading samples spectrophotometrically directly at 515 nm, the assay was performed in a 96-well flat-bottom microplate with 200 μl of DPPH· solution (120 μM) and 22 μl of sample in triplicate testing at least five different concentrations: RE (0.04–1 mg/ml), BHT and α-tocopherol (0.01–0.15 mg/ml), CA, RA and GA (1–30 μg/ml). The DPPH· solution was prepared in 80% methanol instead of 100% methanol in order to decrease evaporation losses. The plate was then covered and left in the dark at room temperature. Absorbance at 492 nm was read in a microplate reader (SLT Lab Instruments 340 ATTC). Standard curves for DPPH· at 492 nm and 515 nm were developed in order to convert the values at 492 nm to the corresponding ones at 515 nm and then to micromolar of DPPH· by the Brand-Williams' equation. The free radical scavenging activity of each solution was calculated as percent DPPH· quenched or percent remnant DPPH· according to the following equations:

$$\%\ \text{DPPH.quenched} = 100(A_{blank} - A_{sample})/A_{blank}$$

$$\%\ \text{remnant DPPH.} = 100 - \%\ \text{DPPH.quenched}$$

The AOXs were also characterized by their EC_{50} value, the concentration necessary to quench 50% of initial DPPH·. Kinetic studies were conducted at room temperature by measuring the time course disappearance of DPPH· absorbance at 492 nm following the addition of the antioxidant in 1–2 orders of magnitude higher than the radical compound, thus forcing the reaction to behave as pseudo-first-order, as described (Sanchez-Moreno, Larrauri, & Saura-Calixto, 1998). The initial slope of the curve ($k1$) is linearly dependent on the concentration of the antiradical so determinations of $k1$ were conducted in triplicate using at least five different extract concentrations per sample and plots of remnant DPPH· vs. concentration of the AOX were made using the results from the time interval with the steepest slope. The slope of these curves ($k2$) was calculated by linear regression ($r^2 > 0.800$), and its absolute value is defined as the AOX power. DPPH solution in methanol was freshly prepared for each experiment.

2.4. Antibacterial activity

To test the antibacterial activity the strains *Escherichia coli* XL1Blue and *Staphylococcus aureus* ATCC 25923 were cultured in Mueller Hinton Broth purchased from Difco (MD, USA). The minimal inhibitory concentration (MIC) was determined by a broth microdilution method in MuellerHinton media following the recommended procedures of the Clinical and Laboratory Standards Institute (1999). The MIC was defined as the lowest concentration at which the substance inhibited the bacterial growth in a 50% ± 5% for *S. aureus* and a 15% ± 4% for *E. coli*. The strains were cultured for 24 h at 37 °C with agitation at 100 rpm. Rosemary compounds (1–1000 μg/ml) and commercial AOX compounds (15–250 μg/ml) were dissolved in methanol or ethanol. Twenty microlitres of each antibacterial compound were dispensed into wells in a 96-well flat-bottom sterile microplate with 240 μl of the inoculum. Plates were incubated 24 h with the lids on, at 37 °C with agitation at 100 rpm. After incubation the microorganism growth inhibition was evaluated by measuring absorbance at 590 nm using the microplate reader mentioned above. Controls were set up with ethanol or methanol alone at a final concentration of 1–5%. All experiments were performed three independent times and each sample assayed in duplicate. The antibacterial activity was expressed as percentage of bacterial growth inhibition, as follows:

$$\%\ \text{inhibition} = 100(A_{Control} - A_{Sample})/A_{Control}$$

Where $A_{Control}$ is the absorbance of bacteria cultured alone.

2.5. Combination system

Isobolograph analysis was performed according to Tallarida (2001), to evaluate the presence of synergism or antagonism. It requires experimental data for agents used alone and in different dose combinations at equieffective levels. The data is plotted on

graphs with the axes representing the doses of each agent. If two agents do not interact, the line that forms with the points corresponding to the different combination of doses representing the sum of the effects will be a straight line. When agents in combination are more effective than what might be expected from their dose-response curves (synergy), smaller amounts will be needed to produce the effect under consideration, and a concave-up isobole results. On the other hand, when agents in combination are less effective than expected (antagonism), greater doses than expected will be needed to produce the same effect, and a concave-down isobole is generated. Different doses of the compounds were selected using the checkerboard method (Davidson & Parish, 1989). Compounds were placed into 96-wells tissue culture plate (Greiner Bio-one, USA) to obtain mixtures covering a broad range of suboptimal concentrations of both compounds, so the concentrations chosen were lower than the EC_{50} or MIC values of the compounds alone. Later, isobolograms were carried out, in which data for individual compounds defined a straight line called the additivity line. The EC_{50} and MIC values, normalized to the unit, are represented by the points in abscise and *Y*-axis. For the binary mixtures (A + B) experimental data were transformed to fractional inhibitory concentration (FIC) as:

$$FIC_A = \frac{\text{Activity of compound A in the presence of B}}{\text{Activity of compound A individually}}$$

$$FIC_B = \frac{\text{Activity of compound B in the presence of A}}{\text{Activity of compound B individually}}$$

Subsequently, to establish if the binary mixtures tested are synergistic, antagonistic or additive, the fractional inhibitory concentration index (FIC_{index}) was calculated as: $FIC_{index} = FIC_A + FIC_B$

Data for doses points appearing below the additivity line are considered as synergic effects in a range of $FIC_{index} < 0.9$, additive effects in a range $0.9 < FIC_{index} < 1.1$ and antagonic effects for $FIC_{index} > 1.1$, according to Santiesteban-López, Palou, and López-Malo (2007).

3. Results and discussion

3.1. Analysis of rosemary extract

We had previously reported the preparation and characterization of different rosemary extracts having a high antioxidant and antimicrobial activity (Moreno et al., 2006). Among them, the methanol extract was the more efficient as an antioxidant and antibacterial. Thus, in this study a similar methanol extract showing a content of 30% CA + 16% COH + 5% RA by HPLC analysis was selected to perform the experiments.

3.2. Antiradical efficiency of rosemary

Many in vitro studies have addressed the antioxidant properties of rosemary polyphenols (Erkan, Ayranci, & Ayranci, 2008; Hras et al., 2000) and reasonably consistent structure activity relationships have been published concerning the trapping of reactive oxygen species and coloured radicals. In particular, DPPH· is widely used for quickly assessing the ability of antioxidants to transfer labile H atoms to radicals (Brand-Williams et al., 1995). In the DPPH test, antioxidants are typically characterized by their EC_{50} value, concentration necessary to reduce 50% of DPPH·. As an extension of previous investigations by our group (Moreno et al., 2006), in the present study a kinetic analysis of the decay in the DPPH· absorption band at 492 nm that follows the addition of the antioxidants was performed in order to obtain additional interesting information about the AOX power of rosemary methanol extract in relationship to the relative reactivity of its distinct phenolic main components, RA and CA. We found that RA reacts rapidly with DPPH· since it reaches a steady state at about 10 min (Fig. 1). By contrast, CA reacts more slowly reaching the plateau at about 30 min. The butylated derivative BHT, as a reference antioxidant compound that shows a characteristic slow reaction, reached the plateau after 100 min. RE showed first a fast reduction of DPPH· with a short plateau that was immediately followed by another slow DPPH· reduction starting after 8 min. These results indicate that the antioxidant efficiency of RE involved the combinatory effect of two or more individual constituents, and that some constituents react more rapidly than others. This observation correlates with the distinct behaviour of pure RA and CA and suggest that the kinetic behaviour of RE might by due to the combinatory effect of these polyphenols.

Taking into account that both concentration and time characterize the antioxidant activity we went on studying these two parameters in the antioxidant behaviour of RE by analyzing the decay in the DPPH· absorption at 492 nm against different concentrations of AOX and the slope of this curve was calculated. The absolute value of the slope is used to measure the AOX power as a compound with high activity would have a steep slope. The activity of RE was compared with the pure polyphenols RA and CA; with GA, as a representative natural polyphenol which shows a strong antioxidant activity (Schlesier, Harwat, Böhm, & Bitsch, 2002); and with other synthetic AOXs like α-tocopherol and BHT (Table 1). The results indicate a comparable antioxidant activity of rosemary, BHT and α-tocopherol, and the activity of RA and CA was considerably higher than BHT. The AOX power in decreasing order was: GA > RA > CA > α-tocopherol > RE > BHT. The AOX power of RE was more similar to the power of CA than to the power of RA; this may be due to a higher content of CA (30%) than the content of RA (5%) in the methanolic extract. This observation is consistent with the proposal that the antioxidant activity of the extract is determined by the percentage content of its main polyphenols CA and RA.

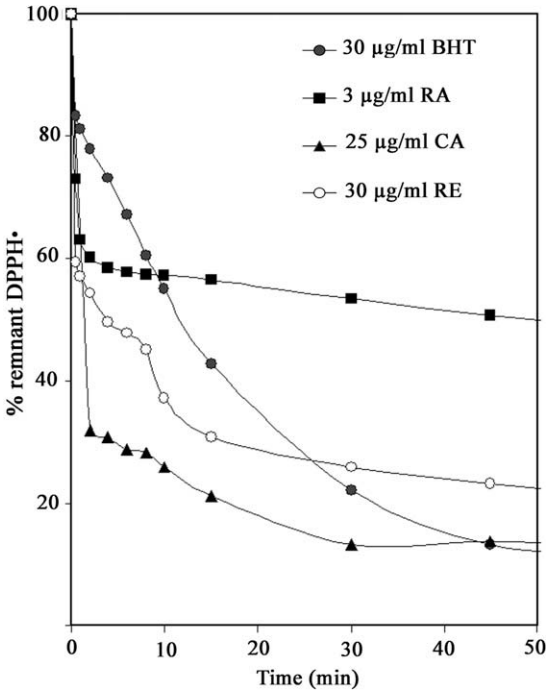

Fig. 1. Free radical scavenging activity, determined by the DPPH· assay, of rosemary extract (RE), CA, RA and BHT vs. time plots. The concentration of each compound was chosen so they reached to the steady state between 5–100 min.

Table 1
AOX efficiency of rosemary compounds vs. α-tocopherol, gallic acid and BHT by the DPPH· scavenging assay.

Sample	AOX power[*,a]
Rosemary extract	1.78 ± 0.12
Carnosic acid	3.05 ± 0.15
Rosmarinic acid	7.59 ± 0.25
α-tocopherol	1.88 ± 0.13
Gallic acid	8.96 ± 0.29
BHT	1.67 ± 0.08

[*] Defined as the slope of the curve of remnant DPPH· (μM) vs. concentration of AOX (μg/ml), express in absolute values.
[a] Data is given as means ± SD.

3.3. Performance of rosemary as an AOX in combination with BHT and BHA

In concert with the antioxidant activity of rosemary, this aromatic plant has gained increasing interest in the food industry as a natural AOX since it has an activity comparable to the synthetic AOXs BHA and BHT (Shylaja & Peter, 2004), and although both types of AOXs may be used as food additives, little is known about the interaction of rosemary with butylated derivatives. Therefore, we investigated the antioxidant properties of mixtures of methanolic RE with BHA and BHT in order to determine the type of interaction between them. Previously, we reported the antioxidant activity of RE and BHT alone assayed by the DPPH method (Moreno et al., 2006) and we had observed that RE and BHA may have a positive interaction (Romano et al., 2006). Here, we further analyzed this possibility including BHT in our study (Fig. 2). Similar results than those obtained with BHA were obtained with BHT, showing that a similar performance in the DPPH· scavenging assay than the one of the AOXs alone could be achieved with a mix of RE plus BHT or BHA with only half the initial dose of both compounds. These data indicate that a positive antioxidant interaction between rosemary methanolic extract and both butylated derivatives might take place.

To confirm this observation, a subsequent study by isobologram, where a diverse range of concentrations of both components of the binary mixture that reached the EC_{50} value of 50% initial DPPH· quenching are evaluated, was done (Fig. 3). This study revealed that when RE is mixed with BHT lower doses than those expected if an additive interaction took place are needed to achieve the same antioxidant effect (Fig. 3A). Furthermore, data corresponds with a synergistic interaction since a minimum amount of 10 μg/ml of RE and 4.5 μg/ml of BHT is needed to achieve the EC_{50} value, combination showing a $FIC_{index} = 0.55$. This combination also allows a decrease of 4.4 folds in the amount of the synthetic AOX and a decrease of 3 folds in the amount of RE. When the antioxidant effect of the mix of RE plus BHA was evaluated, all the dose pairs showed an activity similar to that expected for an additive interaction (Fig. 3B) and the range of FIC_{index} values was 0.85–1.15; which clearly corresponds to an additive type of interaction.

This research indicates that the interaction of rosemary components with BHT appears to increase the reactivity of the AOXs involved in the mixture. The reason for this is unclear, but one possible explanation would be that an heterologous activation of an oxydryle group in an AOX molecule by another AOX takes place to enhance the formation of an hydrogen radical which rapidly reacts with DPPH· to quench it, although there is still much to be understood about the exact mechanism of action of rosemary polyphenols such as RA and CA to get a deeper understanding of their interaction with BHT.

Previously, we reported positive interactions of the rosemary methanolic extract with ascorbic acid and α-tocopherol (Romano et al., 2006). These studies indicate that the ability of RE to enhance the antioxidant efficiency of synthetic AOXs might present a good reason for the food industry to use a combination of natural antioxidants with synthetic ones to improve storage stability for processed food items, and avoid the toxicity effects that may arise from the use of high doses of AOXs.

3.4. Antibacterial action of rosemary in combination with BHA and BHT

AOXs food additives may also play a role in the growth inhibition of food-borne pathogens (Alzamora et al., 1998). In addition, we had previously reported that a rosemary methanolic extract exhibited not only a high antioxidant activity but also a high anti-

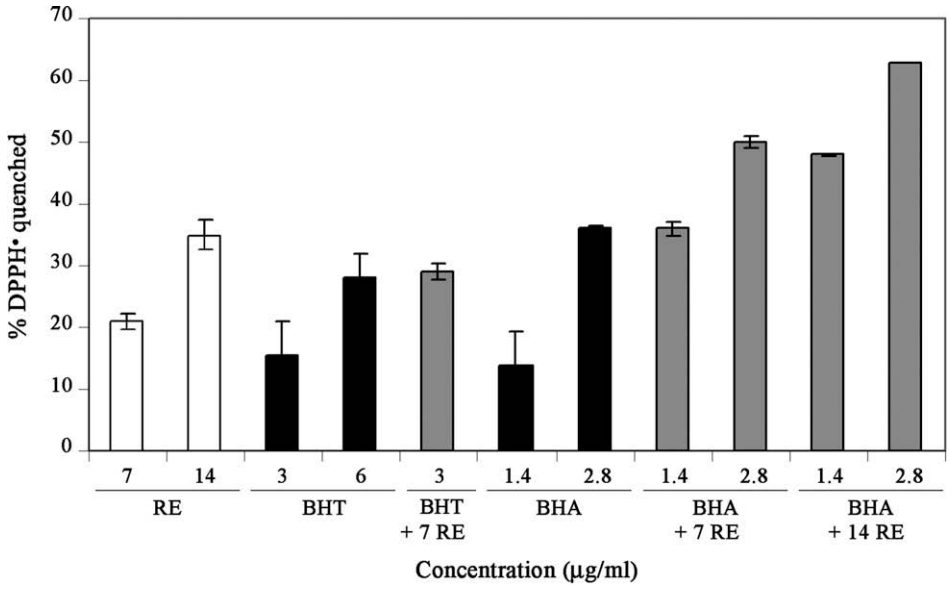

Fig. 2. Antioxidant activity of rosemary extract (RE), BHA and BHT alone and in combination assayed by the DPPH method. The AOXs were allowed to react for 30 min before the determination of % DPPH· quenched. Values are given as the means of three independent experiments and error bars represent the SD.

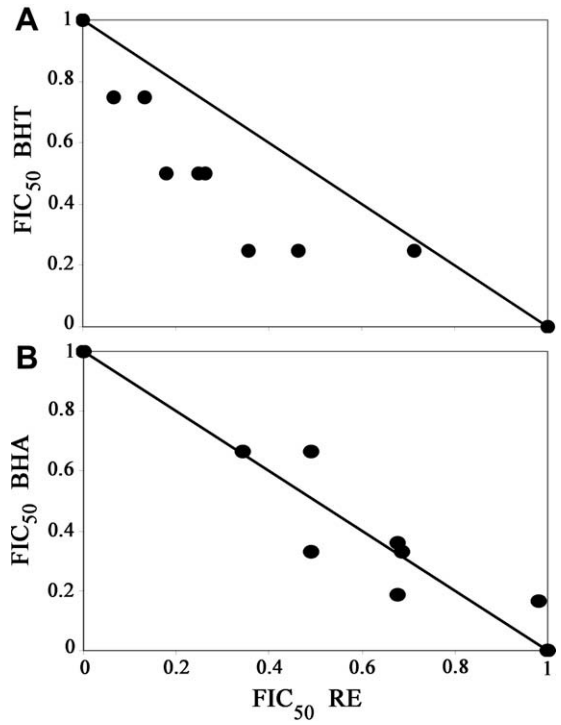

Fig. 3. Antioxidant performances of rosemary extract (RE) in binary mixtures with BHT (A) or with BHA (B) studied by isobolograph. The fractional inhibitory concentrations needed to achieved a 50% of initial DPPH· quenching (FIC_{50}) were normalized to the unit, so 1 represent the FIC_{50} of the compound alone: 20 μg/ml for BHT, 4.25 μg/ml for BHA and 30 μg/ml for RE.

Table 2
The minimal inhibitory concentration (MIC) of rosemary extract in comparison with phenolic compounds.

Sample	MIC (μg/ml)	
	S. aureus	*E. coli*
Rosemary extract	50	105
BHT	250	250
BHA	35	60
Benzoic acid	120	250

microbial action against several gram-positive and gram-negative bacteria (Moreno et al., 2006). But the question in the present study remains whether RE may interact with synthetic AOXs. In order to address an answer, the antibacterial action of RE and butylated derivatives was determined against two model food-borne bacteria: *E. coli* and *S. aureus* as a gram-negative and a gram-positive model respectively.

The MIC values of the RE in comparison with BHT, BHA and benzoic acid, one of the oldest phenolic chemical preservatives used in the cosmetic, drug and food industries (Davidson et al., 2005), were determined by the broth microdilution technique (Table 2). Results indicate that RE exhibited a higher antibacterial activity than BHT and benzoic acid, while a similar antibacterial effect than BHA was observed against both bacteria. The MIC for *S. aureus* in increasing order was: BHA < RE < benzoic acid < BHT, and for *E. coli*: BHA < RE < benzoic acid = BHT. In both cases BHT was the less effective compound to inhibit the bacterial growth, as was expected from early reports (Davidson et al., 2005). It is important to note that not only rosemary shows a significant higher bacterial growth inhibition in comparison to benzoic acid and BHT, but it also exhibited a comparable antibacterial activity to BHA. Yet another observation is that rosemary exhibited a higher antibacterial activity against the gram-positive bacteria *S. aureus* than against the gram-negative bacteria *E. coli;* this is consistent with previous studies which indicate that in general the later type of bacteria is more resistant to most of the known antimicrobial agents (Cowan, 1999).

The above results prompted us to look for possible interactions between RE and BHA since they had a similar activity, while BHT was not included in this study since it had only a very low antibacterial activity. Isobolograms where perform to study the type of interaction between RE and BHA in the growth inhibition of *S. aureus* (Fig. 4A) and *E. coli* (Fig. 4B). Results show that a numerous and diverse range of dose pairs, with the same biological action, are lower than those values expected if an additive interaction took place. Moreover, a minimum amount of 18.5 μg/ml of RE and 5 μg/ml of BHA is needed to achieve the MIC of *S. aureus*, a combination showing a FIC_{index} = 0.51; and a minimum amount of 43 μg/ml of RE and 5 μg/ml of BHA is needed to achieve the MIC of *E. coli*, a mix with a FIC_{index} = 0.49. Data shows that the combination of RE with BHA allows a decrease of 7 or 12 folds in the amount of the synthetic compound used to inhibit the growth of *S. aureus* or *E. coli* respectively, and also a decrease of near 2.5 folds of RE against both bacteria. This study indicates that RE has a synergistic interaction with BHA to inhibit the growth of both types of bacteria. It has been shown by other authors that BHA enhances the antimicrobial effect of other compounds through permeabilization of the cell membrane (Simonetti, Simonetti, & Villa, 2003) and it has also been indicated that rosemary polyphenols may affect membrane fluidity (Pérez-Fons, Aranda, Guillén, Villalaín, & Micol, 2006). Thereby a possible mechanism for BHA enhancing the activ-

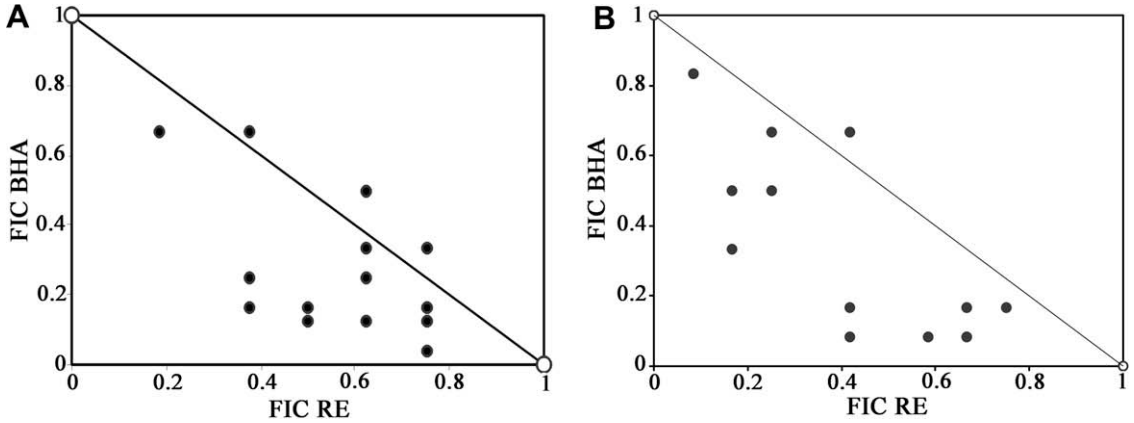

Fig. 4. Antimicrobial action of rosemary extract (RE) in binary mixtures with BHA against *S. aureus* (A) and *E. coli* (B) studied by isobolograph. The fractional inhibitory concentrations of the bacterial growth (FIC) were normalized to the unit. The MIC of the compounds alone is shown in Table 2.

ity of RE may be by a cooperative interaction between both compounds which act together on the bacterial membrane. However, further studies are needed to investigate the action mechanisms of these AOXs against bacterial growth.

4. Conclusion

The present study indicates that the radical scavenging activity of a rosemary methanolic extract can be explained by the combinatory activities of its main polyphenols CA and RA. This observation highlights the importance of choosing the best combination of bioactive polyphenols in rosemary extracts depending on the process to be performed. We think that a specific plant phenolic profile can be designed for different foods, and this is a key factor in the election of rosemary for biotechnological applications.

Another theme arising in the study of rosemary as a natural food additive is the interaction with synthetic AOXs additives like BHA and BHT. This research revealed that rosemary methanolic extract enhances the antiradical efficiency of BHT and the antibacterial activity of BHA through synergistic interactions. These findings provides interesting information for the use of combined AOXs, also called hurdle technology, for food preservation, including the prevention of food oxidation and the inhibition of food-borne pathogens. These *in vitro* interactions are considerably interesting, although it remains to be shown whether rosemary may allowed lowering the dose of synthetic AOXs in foods.

Acknowledgments

We would like to thanks to National Agency of Scientific and Technological Promotion, Argentina for the support of this work through the Grant PICT 2005, N° 35401, and the National Council for Scientific and Technological Research. We also thank Laboratorio Pablo Cassará for its consideration in our work.

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