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Antioxidant and antimicrobial activities of rosemary extracts linked to their polyphenol composition

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Abstract

Rosmarinus officinalis extracts were investigated by a combination of bioassays and biochemical analysis to identify bioactive compounds. The 2,2-diphenyl-2-picrylhydracyl hydrate (DPPH) radical scavenging method, Folin–Ciocaulteau method and HPLC chromatography were used to study the distribution and levels of antioxidants (AOXs). Antimicrobial activity analysis was carried out using the disk diffusion and broth dilution techniques. A good correlation between the AOX activities and total phenol content in the extracts was found. Although all rosemary extracts showed a high radical scavenging activity, a different efficacy as antimicrobial agent was observed. Methanol extract containing 30% of carnosic acid, 16% of carnosol and 5% of rosmarinic acid was the most effective antimicrobial against Gram positive bacteria (minimal inhibition concentration, MIC, between 2 and 15 μg/ml), Gram negative bacteria (MIC between 2 and 60 μg/ml) and yeast (MIC of 4 μg/ml). By contrast, water extract containing only 15% of rosmarinic acid showed a narrow activity. MIC value of the methanol and water extracts is in a good correlation with the values obtained with pure carnosic acid and rosmarinic acid, respectively. Therefore, our results suggested that the antimicrobial rosemary extracts efficacy was associated with their specific phenolic composition. Carnosic acid and rosmarinic acid may be the main bioactive antimicrobial compounds present in rosemary extracts. From a practical point of view, rosemary extract may be a good candidate for functional foods as well as for pharmaceutical plant-based products.

Keywords: *Antioxidants, antimicrobial activity, carnosic acid, rosmarinic acid, Rosmarinus officinalis*

Introduction

Antioxidants (AOXs) have been widely used to avoid degradation of foods. They also have an important role in preventing a variety of diseases and aging because they inhibit or delay the oxidation process by blocking the initiation or propagation of oxidizing chain reactions [1,2].

Plant tissues contain a network of compounds that control the level of reactive oxygen species, including phenolic compounds, vitamins C and E, glutathione and several enzymes. Phenolic compounds widely distributed in the natural plants tissues include flavonoids, tannins, hydroxycinnamate esters and lignin [3]. The Lamiaceae family seems to be a rich source of plant species containing large amounts of phenolic acids, so it is considered to be a promising source of natural AOXs [4]. In addition, each plant sample could be specific enough for the presence of different phenolic acids and their derivates [5].

Rosmarinus officinalis L. is a household plant grown in many parts of the world and has many compounds with AOX activity, mostly polyphenols. The most important AOXs constituents of this plant species are carnosic acid, caffeic acid and its derivatives such as

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rosmarinic acid, which have powerful AOX activity. Rosemary leaf extracts were proposed as important human dietary factors, and investigated as therapeutic potential agents against several diseases [6–8]. However, at this time, very little is known regarding their clinical application in human health. Multiple biological activities have been described for rosmarinic acid like antioxidative, antiviral, antibacterial and antimutagen [9,10]. Recently, it was reported that this compound could be a therapeutic agent in Alzheimer's disease treatment [11]. Studies on carnosic acid and its oxidative hydroxylated derivative carnosol, showed that they have anti-inflammatory and anti-tumor effects [12,13].

The antimicrobial effect of essential oil in *R. officinalis* L. plants was reported extensively, although information on non-volatile extract is scarce [14]. It is clear that rosemary extracts have bioactive properties according to traditional use and scientific evidence, but their antimicrobial activities have not been deeply characterized. The aim of this study is to characterize the AOX activity of several *R. officinalis* extracts and to evaluate their potential antimicrobial action. Here, we reported that rosemary plants are rich sources of phenolic compounds with high AOX and antimicrobial properties. Methanol and acetone extracts showed a high antimicrobial activity against both Gram positive and Gram negative bacteria and yeast in correlation to its carnosic acid/carnosol content. On the other hand, water extract, containing exclusively rosmarinic acid, showed a low efficacy.

Materials and methods

Reagents

Rosmarinic acid, carnosic acid and carnosol were obtained from Alexis Co., USA. HPLC grade methanol, acetic acid and acetonitrile were purchased from Merck, USA.

α-tocopherol, Gallic acid, 2,2-diphenyl-2-picrylhydracyl hydrate (DPPH), 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and Butyl hydroxytoluene (BHT) were purchased from Sigma Aldrich, Milwaukee, WI, USA.

R. officinalis extracts

R. officinalis grown in San Luis Province of Argentina were collected between October and November 2003. The fresh aerial parts (20–200 g) of leaves, flowers, branches and flowering plants were chopped into small parts with a blender and placed in a 3-litre round-bottom flask with 1 l of deionized water. The solution was steam-distilled for 1 h in a Clevenger-type apparatus to oil isolation. The residue was extracted using methanol and acetone by a Soxhlet apparatus, as previously described [15]. Water-soluble AOX material was obtained as described [16]. The extracts were stored at −20°C. To determine the dry weight of each extract, 1 ml of the sample was dried in an oven to constant weight. The extracts were centrifuged using a 5804 Eppendorf centrifuge at 5000 rpm for 15 min at room temperature before HPLC analysis.

HLPC analysis

Quantification of phenolic compounds was conducted with an HPLC LKB Bromma equipped with a diode array detector working in the range of 200–400 nm. A 20–100 μl amount of each extract (5–20 mg/ml) was subjected to HPLC. A 250 mm × 4 mm C18 Luna analytical column (Phenomenex, USA) was used and temperature was maintained at 30°C. A continuous gradient of 5–100% acetonitrile in water containing 2.5% acetic acid was applied at a flow rate of 1 ml/min. Chromatograms were acquired at wavelengths 280 and 330 nm and the quantification of each compound was performed by the calculation of the peak areas after HPLC separation. Mean total content was expressed in % (g/100 g dry weight extract). HPLC fractions were concentrated by Speed Vac (Heto Lab equipment VR-1). Stock solutions of pure carnosic acid, rosmarinic acid and carnosol (8 mg/ml) were prepared in methanol and a working solution was made by diluting 40 μl in mobile phase to a final volume of 300 μl.

AOX activity assay

For antiradical assays, DPPH was used as the free radical source [17] and the free radical scavenging activities of the extracts were measured using the original method of Brand-Williams et al. [18] with modifications [15]. Briefly, 1 ml of 90 μM DPPH in methanol was added to 100 μl of the rosemary extract at different concentrations (0.1–1 mg/ml). After 30 min in the dark at room temperature, the absorbance was measured at 515 nm. In other experiments, the assay was performed in a microplate. Twenty two microlitre of sample (in triplicate and five different concentrations, 0.1–1 mg/ml) and 200 μl of DPPH solution (120 μM) in 80% methanol, were added to a well in a 96-well flat-bottom microtitration plate (ICN Biomedical Inc.). The plate was then covered and left in the dark at room temperature and read in a plate reader (SLT Lab Instruments 340 ATTC) using a 492 nm filter at different times between 30 min and 2 h. A standard curve for DPPH at 492 nm was developed in order to convert the values to the corresponding ones at 515 nm and then to micromolar of DPPH by Brand Williams' equation [18]. The AOX activity was expressed as % inhibition, which was calculated

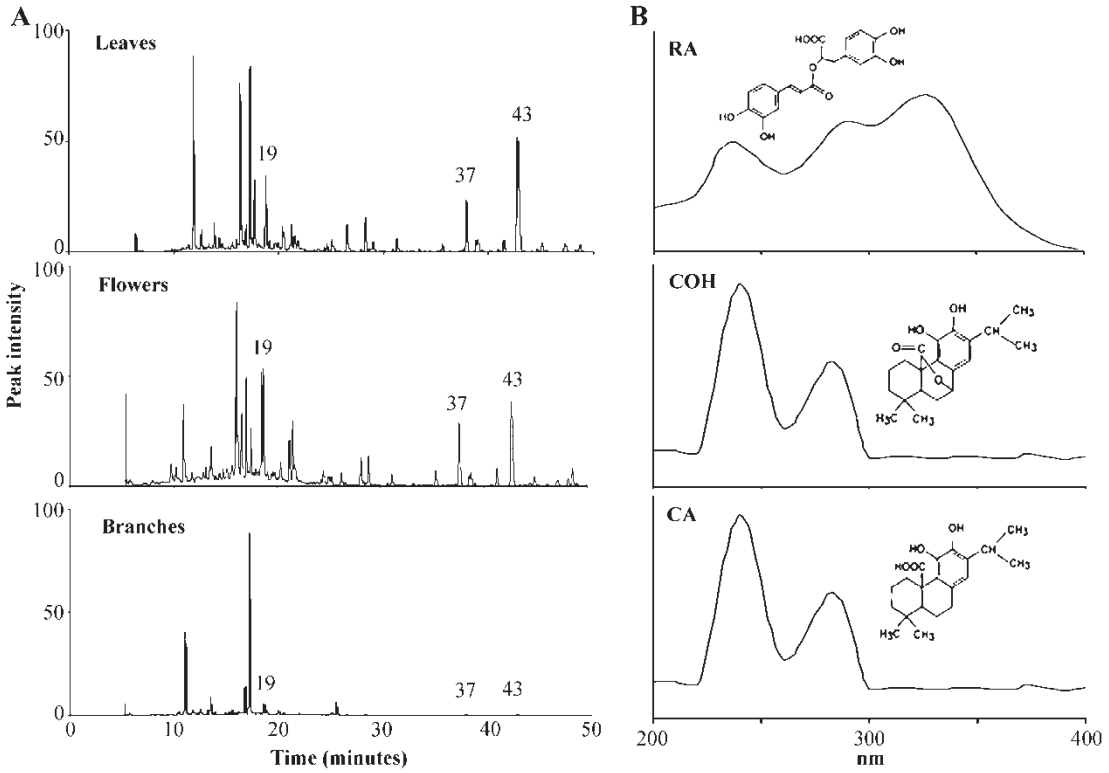

Figure 1. Characteristic chromatogram of leaves, flowers and branches extracts obtained from rosemary plants (A). Peaks with an R_{time} of 19, 37 and of 43 min corresponding to rosmarinic acid (RA), carnosol (COH) and carnosic acid (CA), respectively. Spectrum of rosmarinic acid, carnosol and carnosic acid (B).

according to the formula of Yen and Duh [19].

$$\%\,\text{Inhibition} = \frac{(AC_0 - AC)}{AC_0} \times 100$$

where AC_0 and AC is the absorbance of the initial DPPH and the test sample, respectively. In other cases AOX activity was expressed as the EC_{50} value corresponding to the concentration of sample necessary to decrease by 50% the initial DPPH absorbance.

α-tocopherol and the synthetic AOXs BHT and Trolox, were used as reference materials. The latter was used to obtain the Trolox equivalent AOX capacity (TEAC), which indicates the AOX activity of the sample as compared to Trolox (EC_{50} of 9.7 μg/ml).

Total phenolic assay

Total phenolics were assayed using a method based on the Folin–Ciocaulteau reaction [20] and expressed as grams of Gallic acid equivalents (GAE) per 100 g of dry extract.

Antimicrobial activity

The methanol, acetone and water rosemary extracts were individually tested against Gram negative bacteria *Escherichia coli* XL1Blue, *Xanthomonas campestris* pv *campestris*, *Klebsiella pneumoniae* and *Proteus mirabilis* (from the Food Microbiology Laboratory, Organic department, Faculty of Science, Buenos Aires University); Gram positive bacteria (*Staphylococcus aureus* ATCC 25922, *Bacillus megaterium* PV447, *Bacillus subtilis* GSY1604, *Enterococcus faecalis* ATCC 29212) and yeast (*Saccharomyces cerevisiae* PRY225, *Candida albicans*, *Picchia pastoris* X33). In order to reach stationary growth phase, bacteria were cultured at 37°C for 24 h in Mueller Hinton Broth (MHB, Difco, MD, USA) and yeasts were cultured at 30°C in Sabouraud dextrose. YPM medium (0.3% yeast extract 0.5% peptone, 0.3% malt extract) with 1% glucose was used to culture *X. campestris* pv *campestris*.

The rosemary extracts' antimicrobial activity was estimated qualitatively by using the disk diffusion technique [21]. The surface of agar medium plates was inoculated with mother cultures adjusted to a microorganism concentration of 1×10^6 colony forming units (CFU)/ml. Filter paper disks (6 mm in diameter; paper chromatography Whatman No. 1, USA) were impregnated with 40 μl of extracts (concentration 6–18 mg/ml). The disks were allowed to dry at room temperature. Petri dishes were then incubated at 37°C for 24 h. Antibacterial activity was determined through the zone of bacterial growth inhibition around the disk.

Quantitative evaluation of the antimicrobial activity was made through the broth dilution technique. Geometric dilutions, ranging from 1 to 1000 μg/ml of rosemary extracts were added in tubes with nutrient broth (5 ml MHB). Replicas at each concentration were performed. The inoculum was $\sim 10^2$–10^3 CFU/ml. Inhibition of microorganism growth was determined by measuring the absorbance at 625 nm.

The minimal inhibitory concentration (MIC) was the lowest concentration of extract or substance at which bacterial growth was inhibited after 24 h. The minimum bactericide concentration (MBC) was the lowest concentration of the substance at which survival of any bacterial cell was not possible after incubation for 48 h and was determined by inoculating on agar plates a portion of the broth culture, where MIC values were previously defined. In addition, the cultures showing no growth were sub-cultured in a fresh medium to test for bacterial survival.

The antimicrobial activity of some pure compounds of the rosemary extract under study was also tested by the broth dilution technique. Those compounds were pure carnosic acid (1–50 μg/ml) and rosmarinic acid (5–250 μg/ml). Experimental conditions were the same as for the rosemary extracts. Controls were set up with solvents alone in amounts corresponding to the highest quantity present in the test. All experiments were performed at least three times.

Results

Identification and quantification of the main polyphenols in rosemary plant

In order to identify bioactive compounds in rosemary plants responsible for the AOX biological activity, it is important to know their distribution within the plant tissues for maximizing their extraction. Here, we investigated the polyphenol composition of fresh leaves, flowers and branches, from the plant *R. officinalis*. 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 finally by adding pure standards to the samples prior to HPLC analysis.

Figure 1A shows the HPLC chromatographic profiles of leaves, flowers and branches after a methanol extraction. Leaf extract of the analyzed chemotype showed the presence of rosmarinic acid, carnosol and carnosic acid, identified on the basis of retention time. To confirm the identity of each compound, their UV spectra were obtained (Figure 1B). A spectrum typical of rosmarinic acid was observed for the compound eluting at 19 min, while peaks eluting at 37 and 43 corresponded to the spectra of carnosol and carnosic acid, respectively, [22]. Finally, the addition of the respective pure compounds to the samples increased the concentration of each peak (data not shown).

To quantify the rosmarinic acid, carnosic acid and carnosol present in each part of the plant, the individual peak areas obtained from the HPLC chromatograms, at 330 nm for rosmarinic acid and at 280 nm for carnosic acid and carnosol, were compared with areas from standards of known concentrations. Then, the amount of each compound was expressed as grams of the compound per 100 g of dry weight of rosemary extract (Table I). A higher yield of rosmarinic acid and carnosic acid was found in leaves in comparison to flowers, whilst branches did not show significant presence of these polyphenol

Table I. Quantification of rosmarinic acid (RA), carnosol (COH) and carnosic acid (CA) in leaves, flowers and branches extracts after HPLC chromatography.

Compound	R_{time} (min)	Yield (g/100 g extract) in		
		Leaves	Flowers	Branches
RA	19	7.9 ± 0.8	4.6 ± 0.5	0.1 ± 0.1
COH	37	8.5 ± 0.8	8.7 ± 0.9	NF
CA	43	29.3 ± 2.9	13.6 ± 1.3	NF

NF = Not found.

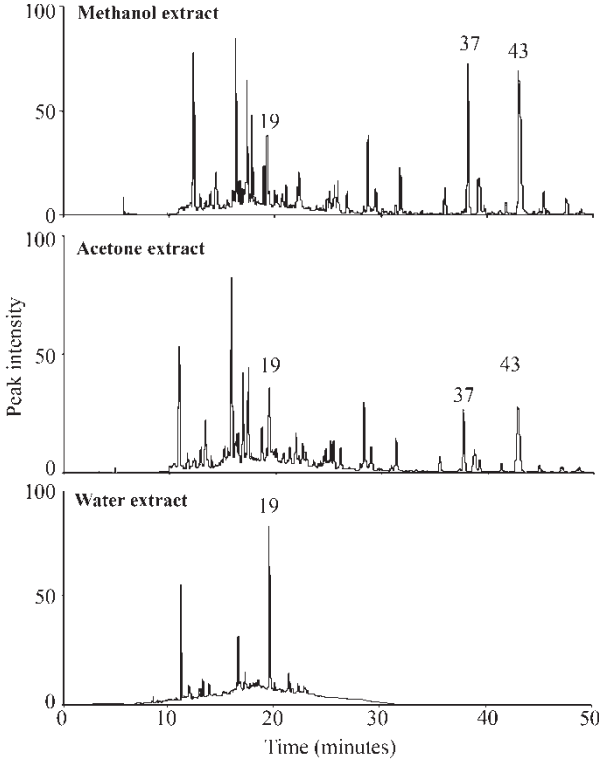

Figure 2. HPLC chromatogram of methanol (A), acetone (B) and water (C) extracts from *R. officinalis*. Peaks with an R_{time} of 19, 37 and of 43 min corresponding to rosmarinic acid, carnosol and carnosic acid, respectively.

Table II. Quantification of rosmarinic acid (RA), carnosic acid (CA) and carnosol (COH) and total phenolic content in different extracts of flowering plants.

Extracts	Yield (g/100 g extract) of			Phenol content (g GAE/100 g extract)
	RA	CA	COH	
Acetone	4.0 ± 0.4	21.5 ± 2.1	11.0 ± 1.1	19 ± 8
Methanol	5.5 ± 0.5	30.5 ± 3.0	16.2 ± 1.6	12 ± 5
Water	14.5 ± 1.4	NF	NF	3 ± 2

NF = Not found; GAE = Gallic acid equivalents.

compounds. In leaf extract the amount of the three polyphenols studied corresponded to 45.7% of the extract, while in flower extract 26.9% of the dry weight corresponded to these compounds.

Leaves and flowers showed the highest amount of the main active polyphenols. Different extraction procedures using water or organic solvents were carried out (see "Materials and Methods" section) and their polyphenol compositions were analyzed by HPLC chromatography. Figure 2 shows that methanol and acetone extracts contained main peaks corresponding to rosmarinic acid, carnosol and carnosic acid with a total yield for these polyphenols of 52.2 and 36.5% of extracts, respectively (Table II). On the other hand, water extracts contained only rosmarinic acid with a yield of this compound of 14.5% of extract (Table II).

Total phenolics were estimated by the Folin–Ciocaulteau method (Table II). Water extract presented 3 g of GAE per 100 g of extract, while organic extracts had considerably higher phenolic content.

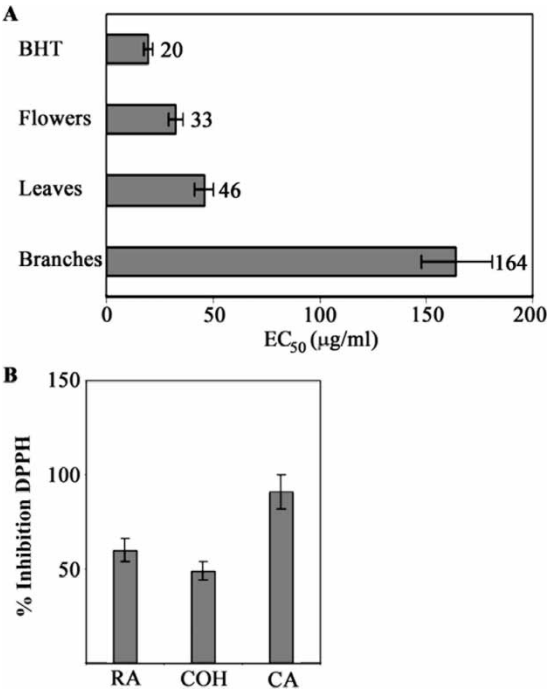

Figure 3. AOX activity in leaves, flowers and branches extracts (A) and in the fractions corresponding to the elution of rosmarinic acid, carnosol and carnosic acid after separation of the extract leaves by HPLC chromatography (B).

Radical scavenging activity

The AOX activity measured by the DPPH method was assayed in aliquots of extracts from leaves, flowers and branches. Figure 3A shows that leaves and flowers exhibited lower EC_{50} values indicative of a high AOX activity, comparable to the AOX activity of commercial BHT, whereas branches presented higher EC_{50} values indicative of a low activity.

When AOX activity was measured in the HPLC chromatography fractions after separation of the methanol extract of leaves, a high activity was associated with fractions containing rosmarinic acid, carnosol and carnosic acid (Figure 3B). The peak corresponding to carnosic acid, the main compound found in the methanol extract from leaves (Table I), showed the higher activity. Other fractions, eluting at R_{time} 14 min and at R_{time} 24, showed minor AOX activity. The first peak may correspond to caffeic acid, the rosmarinic acid precursor, since pure caffeic eluted with a similar R_{time} under the HPLC conditions used (data not shown). The other minor peak with an R_{time} 24 is under investigation.

The screening of AOX activity in methanol, acetone and water rosemary extracts was performed by a micro titer model system. The AOX property of these methanol extracts in comparison with those of known natural and synthetic AOX is shown in Figure 4. Higher radical scavenging activities were observed in methanol and acetone extracts with EC_{50} of 18 and 25.6 μg/ml. These AOX activity values were comparable to α-tocopherol and BHT. By contrast, water extract showed lower AOX activity with an EC_{50} of

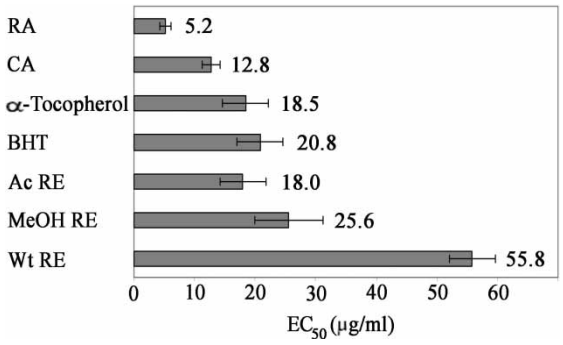

Figure 4. AOX activities of several rosemary extract in comparison with commercial AOX standards.

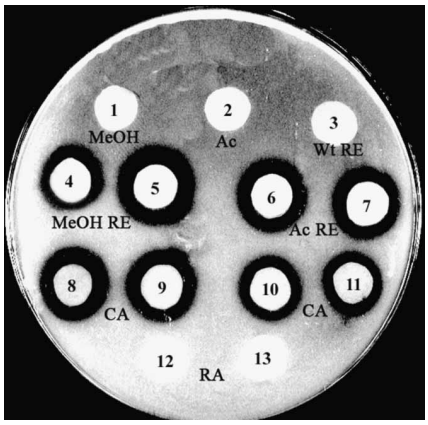

Figure 5. Evaluation of the antimicrobial activity of rosemary extract by means of *in vitro* disk diffusion test. A measure of 250 and 500 μg of methanol (spot 4 and 5), 500 and 750 μg of acetone (spot 6 and 7) and 500 μg of water (spot 3) extracts were assayed. A measure of 250–25 μg of pure carnosic acid (spot 8–11) and 250 and 500 μg of pure rosmarinic acid (spot 12 and 13). Methanol (MeOH) alone or acetone (Ac) alone (spot 1 and 2), as control.

55 μg/ml. Pure rosmarinic acid and carnosic acid presented high AOX activity.

In addition, a comparison of AOX activity of the extracts was performed by the TEAC index. TEAC values of 2.64, 1.85 and 5.75 for the methanol, acetone and water extracts, respectively, were found, considering the Trolox EC_{50} value as one.

Antimicrobial activity

Disk diffusion test was used as a preliminary screen of rosemary extracts antibacterial activity since it is a qualitative technique. Figure 5 shows that methanol extract (spots 5) had the greatest growth inhibition zone against *E. coli* followed by the acetone extract (spots 6). By contrast, water extract did not show antibacterial activity (spot 3). Pure carnosic acid and rosmarinic acid were also assayed and it was found that although 25–200 μg of carnosic acid were effective in inhibiting bacterial growth (spot 8–11), 250 and 500 μg of rosmarinic acid did not (spots 12

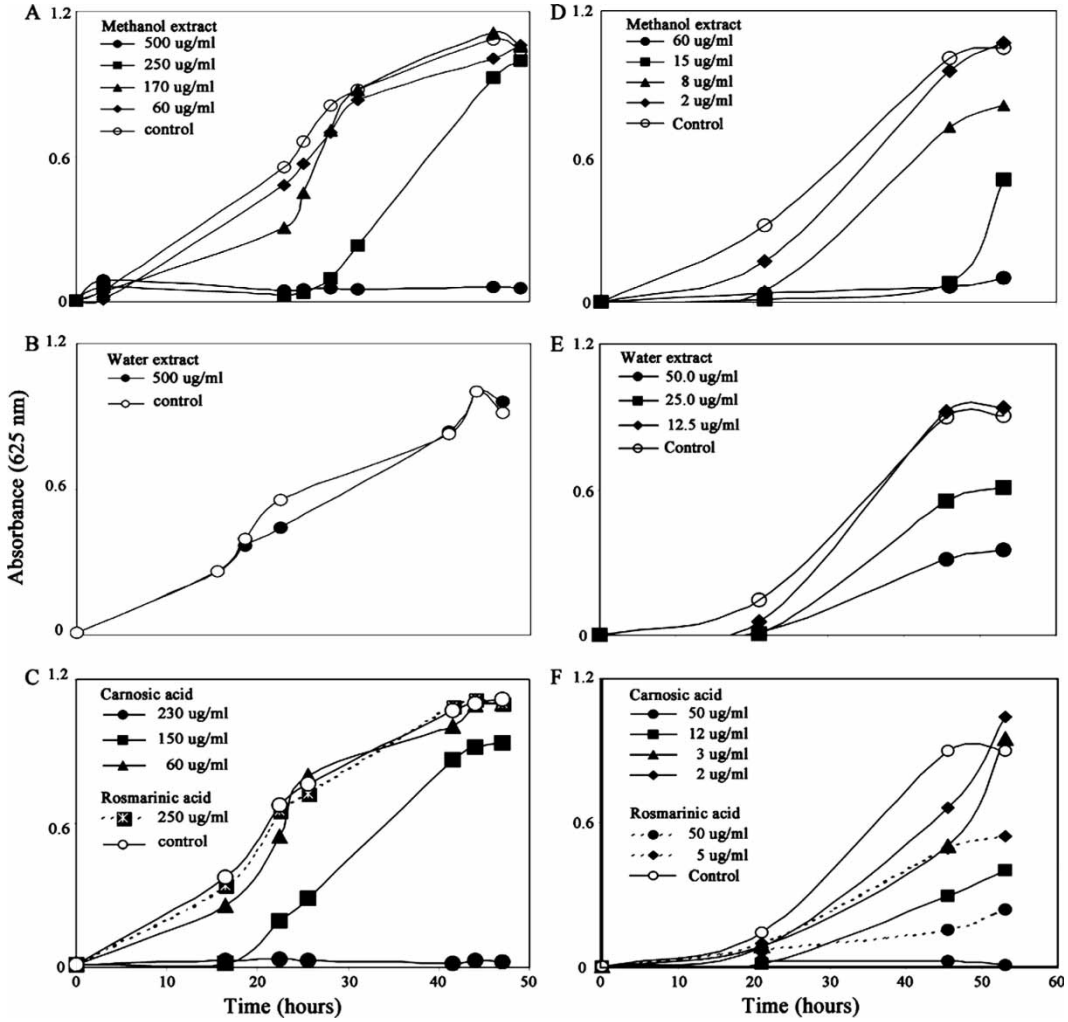

Figure 6. Effect of rosemary extract on the growth of *E. coli* (A–C) and *S. aureus* (D–F) in liquid medium. Methanol (A and D), water (B and E) and carnosic acid and rosmarinic acid (C and F) were assayed. 5% methanol alone was included as control. The experiments were repeated at least three times.

and 13). The solvents alone did not affect the bacterial growth (1 and 2 spots).

The absence of inhibition zones does not necessarily mean that compounds are inactive. For example, non-polar compounds may not diffuse into the culture medium.

Later, a quantitative evaluation of the rosemary extracts antimicrobial activity was made through the broth dilution technique. Methanol, acetone and water rosemary extracts were individually assayed against Gram negative bacteria (*E. coli*, *K. pneumoniae*, *P. mirabilis*, *X. campestris* pv *campestris*); Gram positive bacteria (*S. aureus*, *B. megaterium*, *B. subtilis, E. faecalis*) and yeast (*S. cerevisiae*, *C. albicans*, *P. pastoris*) (Figure 6 and Table III).

Methanol extract was effective against *E. coli* (Figure 6A) within the range of 60–500 μg/ml. Compared to the control, the rate of growth was reduced during the 24 h incubation period with a significant effect ($p < 0.05$). When 250 μg/ml of extract was used, a 100% inhibition was observed after 24 h of incubation. However, after 48 h, bacterial growth was recovered. When 500 μg/ml of the same extract was assayed a total inhibition effect was observed for all incubation times assayed, suggesting a bactericide effect of the extract at this concentration.

A minor inhibitory effect was found testing the acetone extract since 500 μg/ml are necessary to obtain no growth after 24 h incubation and the addition of 1 mg/ml of this extract is necessary to cause a total growth inhibition at all tested times (data not shown).

A different behavior was observed when the water extract was tested against *E. coli*, because it was not able to inhibit the bacterial growth even when assayed at high concentration (Figure 6B).

In order to identify the bioactive compounds exhibiting antimicrobial activity in the rosemary extract, the effect of pure rosmarinic acid and carnosic acid on the bacterial growth was studied (Figure 6C). A bacteriostatic effect was observed after 16 h incubation testing carnosic acid at concentrations >150 μg/ml, but bacterial growth recovered later at concentrations <150 μg/ml. This compound assayed at 60 μg/ml presented a moderate growth inhibition of approximately 40% after 16 h incubation. While carnosic acid showed a high antibacterial effect, rosmarinic acid did not, even if it was assessed at a high concentration of 250 μg/ml (Figure 6C).

A different antimicrobial property of the rosemary extracts was observed testing *S. aureus*. Methanol extract and carnosic acid presented a higher antibacterial activity on *S. aureus* than on *E. coli* (Figure 6D and F). Moreover, the water extract, containing approximately 15% of rosmarinic acid as well as the pure rosmarinic acid, presented activity only against *S. aureus* (Figure 6E and F). Pure carnosol showed similar MIC values as carnosic acid against *S. aureus* (data not shown).

Later, to confirm that carnosic acid and carnosol are the main bioactive compounds in the rosemary extracts responsible for the inhibition of bacterial growth, those compounds were isolated by HPLC from the extract and their antimicrobial activity was assayed against *S. aureus*. The isolated compounds showed similar MIC values to the commercial ones. These results are in accord with the proposal that carnosic acid and its derivative were the major bioactive compounds of the rosemary extract exhibiting antimicrobial activity. The presence of minor unidentified compounds with antimicrobial activity in the extracts cannot be discounted.

Tests of the effect of rosemary extract on the growth of the other microorganisms including bacteria and yeasts were carried out (Table III). Both methanol and acetone extracts presented a moderate inhibitory effect against *E. coli*, *K. pneumoniae* and *P. mirabilis* with similar MIC values within the range of 60–125 μg/ml. Pure carnosic acid showed, in the same way as methanol and acetone extracts, a moderate

Table III. Antimicrobial activity of rosemary extracts and pure carnosic acid (CA) and rosmarinic acid (RA).

Microorganism	Methanol		Acetone		Water		CA		RA	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
E. coli	60	500	125	1000	NA	–	30	230	NA	–
K. pneumoniae	60	500	60	NA	NA	–	30	NA	NA	–
P. mirabilis	60	NA	125	NA	NA	–	30	NA	NA	–
X. campestris pv *campestris*	1	60	NT	NT	NA	–	3	50		
S. aureus	2	60	4	120	25	NA	2	50	5	NA
B. megaterium	8	15	8	15	NA	–	15	60	NA	–
B. subtilis	8	15	4	30	NA	–	4	60	NA	–
E. faecalis	15				NA	–	–	–	NA	–
S. cerevisiae	4	NA	8	NA	NA	–	2	NA	NA	–
C. albicans	4	NA	4	NA	NA	–	2	NA	NA	–
P. pastoris	4	NA	4	NA	NA	–	2	–	NA	–

Minimal inhibitory concentration (MIC) and minimal bactericide concentrations (MBC) values are expressed as μg of rosemary extract per ml of culture medium; NA = Not active.

antimicrobial activity against *E. coli*, *K. pneumoniae* and *P. mirabilis* with a MIC value of 30 μg/ml.

Surprisingly, methanol extract as well as carnosic acid showed a strongly inhibitory effect against other Gram negative bacteria such as *X. campestris* pv *campestris* with a MIC value of 1 μg/ml and a MBC value of 60 μg/ml. By contrast, water extract and pure rosmarinic acid did not demonstrate any effect.

Methanol and acetone extracts, as well as carnosic acid, were active against Gram positive bacteria at relatively low concentrations.

All the extracts tested presented only fungistatic activity under the assessed conditions (the maximum extract concentration tested was of 1 mg per ml). However, fungicidal effect of the methanol and carnosic acid was observed against *S. cerevisiae* when culture medium contained 2% glucose. When 1% of glucose was used no fungicide effect was observed.

All these results point at methanol and acetone extracts as broad-spectrum antimicrobial agents inhibiting the growth of a number of Gram positive and Gram negative bacteria and yeasts. By contrast, water extract and pure rosmarinic acid seem to have a narrow antimicrobial activity.

Discussion

This paper describes the AOX performance and effectiveness as antimicrobial chemical of the organic and water extracts devoid of essential oils obtained from plants of *R. officinalis*. Our results showed that methanol extracts from leaves and flowers of rosemary plants contained high amounts of polyphenol compounds (Figure 1 and Table I) as well as a high AOX activity (Figure 3). For this reason, water and organic extracts were later prepared from flowering plants.

It was reported that both genetic and environmental elements such as water, light and heat stress affect the carnosic acid concentrations [23,24]. In those reports, 6 percent of carnosic acid was present in Mediterranean rosemary leave extracts, whereas in our trial plants a concentration of around 30 percent of carnosic acid was found in leaf extracts (Table II). These data suggest that the Argentinean climate favors the production of this kind of compound. Similar to our result, a de-odorized aqueous extract from a Finland chemotype of *R. officinalis* L. also presented rosmarinic acid as the main phenolic component [16].

We showed, using DPPH method, a high radical scavenging activity in the methanol and acetone extract similar to the synthetic compound BHT and α-tocopherol. Also, when the values are referred as a TEAC index, acetone and methanol extracts showed again higher performances than water extracts—consistent with their relative phenolic content. When the AOX activity of rosemary extracts was measured by the β-carotene bleaching test similar results were obtained (data not shown).

The antimicrobial activity of essential oils fractions from rosemary has been reported several times, [20,25] although this activity was scarcely investigated in other fractions. Here, we describe the antimicrobial activity of water, methanol and acetone rosemary extracts (Figures 5 and 6; Table III). Methanol and acetone extracts showed a good antimicrobial activity against all microorganisms tested. Water extracts were active against *S. aureus* only. Similar results were obtained assaying pure rosmarinic acid.

Other papers have reported a MIC value for rosemary extract of 16–60 μg/ml for the inhibition of another strains of *S. aureus*, [26] while our results (MIC: 2 μg/ml) indicated a more efficient antibacterial activity for the rosemary extract. In another report an inhibitory activity of a commercial rosemary extract against *S. aureus* was described, but in this case the composition and the active compound was not given [27].

In summary, our results show, for the first time, that both water and organic rosemary extracts linked their antimicrobial properties to their different polyphenol compositions.

It was reported that an antimicrobial action of phenolic compounds was related to the inactivation of cellular enzymes, which depended on the rate of penetration of the substance into the cell or caused by membrane permeability changes [28]. The mechanism of action of terpenes is not fully understood but it is in general speculated to involve membrane disruption by the lipophilic compounds [29]. Thus, the higher activity of carnosic acid may result from its lipophilic character.

All our data suggested that the antimicrobial rosemary extracts efficacy was associated with their specific phenolic composition.

The increased public awareness of the negative effects caused by overexposure to synthetic chemicals led to the search for "green solutions", such as organic and synthetic chemical-free food products. Rosemary is an available herb, inexpensive, and has been shown to be relatively non-toxic in animal models and as antimutagenic [30,31]. From a practical point of view, the rosemary extracts studied here could be considered as good candidates for food preservation [32] or functional foods, as well as for pharmaceutical and natural plant-based products. Up to date information from *in vivo* experiments on the antimicrobial properties of phenolic compounds of rosemary extracts is scarce. Our *in vitro* bioassays results showed that Argentinean methanol rosemary extracts contained an activity higher than previously described, and a study of its *in vivo* effect is in progress.

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