

Antibacterial potential of non-volatile constituents of *Rosmarinus officinalis* against 37 clinical isolates of multidrug-resistant bacteria

[Actividad antibacteriana de constituyentes no volátiles de *Rosmarinus officinalis* contra 37 aislamientos clínicos de bacterias multirresistentes]

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Abstract

In this paper we investigated the antibacterial activity of a methanolic extract of *Rosmarinus officinalis* L. and their main constituents, carnosic acid and rosmarinic acid, against 37 nosocomial strains of multidrug-resistant bacteria. Results obtained showed that both the rosemary extract and carnosic acid inhibited all clinical isolates of *Staphylococcus aureus* methicillin-resistant and *Enterococcus faecalis* gentamicin and streptomycin-resistant bacteria examined (MICs 60 µg/mL vs. 200 µg/mL, respectively). Rosemary extract showed MIC values between 400 and 1600 µg/ml against the Gram-negative multidrug-resistant bacteria: *Escherichia coli*, *Proteus mirabilis*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, *Morganella morganii* and *Providencia stuartii*, while carnosic acid showed MIC of 120 to 240 µg/mL. Bactericidal effect of carnosic acid against *S. aureus* and *E. faecalis* was observed at their MIC value, while 2 x MIC to 4 x MIC were needed to kill Gram-negative bacteria. Rosmarinic acid showed a narrow spectrum of action against a few Gram-negative clinical isolates. Our findings suggest that carnosic acid would be a good lead candidate useful in counteracting drug-resistant infections.

Keywords: Antibacterial activity; Carnosic acid; Multidrug-resistant; *Rosmarinus officinalis*; Rosmarinic acid

Resumen

En este trabajo evaluamos la actividad antibacteriana de un extracto metanólico de *Rosmarinus officinalis* L. y sus principales componentes el ácido carnósico y ácido rosmarínico, contra 37 cepas de bacterias multirresistentes nosocomiales. Los resultados muestran que el extracto de romero y el ácido carnósico, inhibieron las bacterias Gram-positivas *Staphylococcus aureus* resistentes a metilicina y *Enterococcus faecalis* resistentes a gentamicina y estreptomina (CIM 200 µg/mL y 60 µg/mL, respectivamente). El extracto de romero inhibió los Gram negativos multirresistentes: *Escherichia coli*, *Proteus mirabilis*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, *Morganella morganii* y *Providencia stuartii* (CIM 400 a 1600 µg/mL), mientras que el ácido carnósico mostró valores de CIM entre 120 a 240 µg/mL. El ácido carnósico mostró actividad bactericida contra *S. aureus* y *E. faecalis* a su CIM, mientras que 2 a 4 X CIM se requirieron para matar las bacterias Gram-negativas. El ácido rosmarínico mostró inhibió unos pocos aislados clínicos Gram-negativos. Estos hallazgos sugieren que el ácido carnósico puede ser de utilidad contra infecciones bacterianas multirresistentes a antibióticos.

Palabras Clave: Actividad antibacteriana; Ácido rosmarínico; Ácido carnósico; Bacterias multirresistentes; *Rosmarinus officinalis*.

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INTRODUCTION

Rosemary (*Rosmarinus officinalis* L.) plants grow worldwide and have been cultivated since long ago for its strong antioxidant and antimicrobial activities. Nowadays, a long list of claims including antimicrobial, antioxidant, chemopreventive, and anticancer properties were described (Shahidi and Naczki, 2004; Barni et al., 2012). This species is considered to be one of the most important sources of both volatile and non-volatile bioactive compounds (Bradley, 2006).

The antimicrobial effect of rosemary extracts have been attributed to the high content of phenolic compounds including non-volatile components (Del Campo, 2000). We previously reported the effective antimicrobial action of several non-volatile rosemary extracts containing 33 - 46% of diterpenes (carnosic acid plus carnosol) against common food pathogenic Gram positive bacteria as *Staphylococcus aureus* and *Enterococcus faecalis* as well as the Gram negative bacteria *Escherichia coli*, which were all sensitive to antibiotics (Moreno et al., 2006). The 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, a water extract containing only 15% of rosmarinic acid showed a narrow activity. MIC values of the methanol and water extracts were in a good correlation with the values obtained using the commercial pure compounds, carnosic acid and rosmarinic acid, respectively. Therefore, carnosic acid and rosmarinic acid may be the main bioactive antimicrobial compounds present in these rosemary extracts (Moreno et al., 2006). Accordingly, a bioassay-guided fractionation of the leaf extract of *R. officinalis* led to the identification of carnosic acid and carnosol as the major compounds displaying the highest activity against oral pathogens as *Streptococcus mutans*, *S. salivarius*, *S. sobrinus*, *S. mitis*, *S. sanguinis*, and *Enterococcus faecalis* (Bernardes et al., 2010). In addition, carnosic acid was the major bioactive of commercial rosemary extract formulations having antimicrobial and antioxidant activity *in vitro* against gram-positive (*Bacillus* and *Staphylococcus*) and gram-negative (*Campylobacter* and *Salmonella*) bacteria (Klančnik et al., 2009).

In another work, we studied the antibacterial efficacy of an rosemary ethanolic extract containing a high amount of carnosic acid in two skin infection models in mice infected with an antibiotic sensitive strain of *S. aureus* (Barni et al., 2009). Results showed that this rosemary extract is able to carry out bactericide as well as bacteriostatic action comparable with the antibiotic action of the well-recognized antibiotic fusidic acid. Moreover, we found that carnosic acid showed a broad spectrum of pharmacological action as anti-inflammatory and anticarcinogenic (Mengoni et al., 2011; Barni et al., 2012). Other researchers found that carnosic acid was active against a strain of *S. aureus* exhibiting multidrug-resistant efflux proteins with MIC values of 16 to 60 µg/mL (Oluwatuyi et al., 2004) and in vancomycin-resistant enterococci (Horiuchi et al., 2007). In this sense, recently we reported that carnosic acid showed a selective synergistic interaction with tetracycline and aminoglycosides in *S. aureus* ATCC 25923 (Moreno et al., 2012).

The emergence of antibiotic resistance among both pathogenic and opportunistic microbes (*S. aureus* and *E. faecalis*) resident in hospitals represents a serious and recurrent problem for the treatment of infections (Hancock, 2005; Shepard and Gilmore, 2002). Methicillin-resistant *S. aureus* (MRSA) can cause a multitude of diseases as a result of infection of various tissues of the body, its distribution is worldwide: as many as 11% - 40% of the population is estimated to be colonized and usually it shows resistance to many antibiotics. Bacterial resistance to β-lactam antibiotics has also risen dramatically (Paterson and Bonomo, 2005), contributing to this increase has been the spread of extended-spectrum β-lactamase-producing bacteria that have spread worldwide by nosocomial routes. Treatment of the infections caused by these organisms is difficult, not only because of the resistance to the extended-spectrum cephalosporin themselves, but also because they are often associated with resistance to other antimicrobial agents coded either by the same or different plasmids. The progress of resistance to the last line of antibiotic defense has led to the search of new antibiotics and nowadays natural drugs could represent a remarkable approach to limit the emergence and spread of multi-drug resistant organisms (Lewis and Ausubel, 2006; Smith et al., 2007).

Therefore, although it has been reported that non-volatile rosemary compounds have antimicrobial properties against antibiotic sensitive microorganisms, little information exists regarding their efficacy as microbicide against multidrug-resistant bacteria. This contribution reports the efficacy and potency of non-volatile rosemary constituents, carnosic and rosmarinic acid, against several multi-drug resistance Gram-positive and Gram-negative strains isolated from a Hospital in Argentina.

MATERIALS AND METHODS

Materials

All solvents used in the experiments were purchased from Merck (USA). Carnosic acid (Sigma, Aldrich Lot #061M1862v) and rosmarinic acid (Extrasynthase Lot #08041513, Genay, France). All other reagents were of analytical grade.

Preparation and analysis of rosemary extract

The methanol rosemary extract 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 3L 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 carnosic acid, and rosmarinic acid 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 x 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 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.

The methanol rosemary extract contained 33 - 46% of diterpens (carnosic acid plus carnosol) and 5% of rosmarinic acid determined by high-performance liquid chromatography according to Moreno *et al.*, 2006. The extract was kept in a freezer at -20° C until use. Stock solutions of pure carnosic acid, and rosmarinic acid (1 mg/mL) were prepared in ethanol.

Bacterial strains

The microorganisms used in this study consisted of bacterial isolates collected from clinical samples obtained from the Hospital Nicolás Avellaneda, Tucumán, Argentina: *S. aureus* (n = 7), *E. faecalis* (n = 13), *E. coli* (n = 7), *E. cloacae* (n = 4), *P. mirabilis* (n = 3), *P. aeruginosa* (n = 1), *Morganella morganii* (n = 1) and *Providencia stuartii* (n = 1). *S. aureus* ATCC 25923 and *E. faecalis* ATCC 29212 were also included in the investigation. All samples were identified by standard methods according to the recommendations of the Manual of Clinical Microbiology (Murray *et al.*, 1999) and the CLSI guidelines, 2007. Resistance to antibiotics was determined as previously described (Arias *et al.*, 2004; Zampini *et al.*, 2005). The strains were maintained in brain-heart infusion broth containing 30% (v/v) glycerol at -20° C. Before testing, the suspensions were transferred to trypticase soy agar supplemented with 5% of sheep blood (Difco) and aerobically grown overnight at 35° C. Individual colonies were isolated and suspended in 5 mL of 0.9% NaCl solution. The inoculums were prepared by adjusting the turbidity of the suspension to match the 0.5 McFarland and diluted in cation-adjusted Müller Hinton broth (Difco, MD, USA).

Antibacterial activity

Serial agar macrodilution method was used to measure the antibacterial activity against clinical isolate strains as previously described (Zampini *et al.*, 2005). Müller Hinton Agar (MHA) (9 mL) was mixed with 1 mL of serial dilutions of carnosic acid and rosmarinic acid performed with ethanol at a final concentration of 15 - 480 µg/mL or with the methanol rosemary extract at a final concentration of 15 - 1600 µg/mL. Then, plates were inoculated with 2 µL of each bacterial cell suspension (5×10^4 CFU/mL) and aerobically incubated for 18 h at 35° C. A growth control of each tested strain was included. MIC values were defined as the lowest concentration of compounds or antibiotics where no colony was observed after incubation.

The broth microdilution method has been used in a microtiter plate-based assay according to the National Clinical Laboratory Standard (CLSI, 2007) recommendations. The Müller-Hinton medium containing carnosic acid and rosmarinic acid (15 - 800 µg/mL) was dispensed at 100 µL/well in 96-well microtiter sterile plate. Each well was inoculated with 10 µL of bacterial culture (1×10^5 CFU/mL). The plates were incubated at 37° C for 20 h with agitation at 200 rpm and the bacterial growth was monitored by absorbance at 595 nm in a microtiter plate reader (Beckman Coulter DTX880 Multimode Detector). All experiments were performed in three independent times and each sample assayed in triplicate. The antibacterial activity was expressed as the minimal inhibitory concentration (MIC) of compounds that will inhibit the visible growth of bacteria alone. 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.

RESULTS

We used the methanol rosemary extract to perform this study since it was the more efficient as antibacterial against several Gram-positive and Gram-negative bacteria and yeast all sensible to antibiotics (Moreno *et al.*, 2006). Here, this rosemary extract rich in carnosic acid and the main pure constituents, carnosic acid and rosmarinic acid were evaluated against MRSA and *E. faecalis* multi drug-resistant strains isolated from a Hospital in Argentina (**Table 1**). The rosemary extract inhibited the growth of MRSA with a MIC value of 200 µg/mL. Similar result was obtained for the ATCC strain. Carnosic acid was active against MRSA with a MIC value of 60 µg/mL. Rosmarinic acid was ineffective even at high concentration as 480 µg/mL. **Table 1** also presented the antibacterial activity of the rosemary extract as well as carnosic acid and rosmarinic acid against thirteen strains of *E. faecalis* resistant to gentamicin, streptomycin or to strains exhibiting dual resistance isolated from the Hospital. The plant extract showed a MIC value of 200 to 400 µg/mL against multidrug-resistant as well as sensitive bacteria. Carnosic acid was also able to inhibit the growth of these pathogenic strains at a concentration of 60 µg/mL. Rosmarinic acid was inactive against these bacterial strains even at high concentration as 480 µg/mL.

Table 2 shows the minimal bactericide concentration of the rosemary extract at $2 \times \text{MIC}$ to $4 \times \text{MIC}$ and carnosic acid at the MIC value against *S. aureus* and *E. faecalis* strains. Notably, carnosic acid inhibited both antibiotic-resistant as well as sensitive bacteria at the same concentration.

Carnosic acid was also active against all the multidrug-resistant Gram-negative bacteria tested **Table 3**. This compound was particularly active towards *E. cloacae* 302 strain expressing extended-spectrum β-lactamases resistant to levofloxacin, piperazilina/tazobactam, ceftriaxone, cefotaxime, cefuroxime, ampicillin/sulbactam and meropenem as well as against *P. mirabilis* 304 resistant to cefuroxime, ampicillin/sulbactam and meropenem with a MIC of 30 µg/mL and 60 µg/mL, respectively. However, a lower efficacy of carnosic acid against *E. coli*, *E. cloacae* and *P. mirabilis* strains was observed with MIC values of 120 µg/mL to 240 µg/mL.

Rosmarinic acid exhibited a narrow spectrum of antibacterial action inhibiting the β-lactamase-producing *P. mirabilis* 304 and 359 strains, as well the multidrug-resistant *P. aeruginosa* strain with MIC values of 120 µg/mL, while it was ineffective against the other bacteria at high concentration as 480 µg/mL (**Table 3**).

DISCUSSION

The activity of the rosemary extract against the human Gram-positive bacteria, which exhibited bactericidal activity at 60 µg/mL, seems to be related to the presence of carnosic acid, while the other constituents of the extract, rosmarinic acid, was inactive. Carnosic acid showed the broadest spectrum of action inhibiting all the Gram-positive and Gram-negative clinical strains tested. These results are in accord with previous suggestion that carnosic acid was the major bioactive compounds of the rosemary extract exhibiting antimicrobial activity (Moreno *et al.*, 2006).

It is important to note that carnosic acid is active toward extended-spectrum β-lactamases strains, which are capable of hydrolyzing penicillins, broad-spectrum cephalosporins and monobactams (Morosini *et al.*, 2006; Paterson and Bonomo, 2005). Therefore, carnosic acid may be a valuable alternative to imipenem and meropenem to treat infections with these organisms. The antibacterial potency of carnosic acid observed in this study against clinical isolates of multidrug-resistant bacteria is comparable with the activity reported for other phenolic diterpene isolated

from plants as the terpene totarol and the alkaloid reserpine (Gibbons and Udo, 2000; Otsuka *et al.*,

2008).

Table 1
Antibacterial activity of the rosemary extract (RE) and carnosic acid (CA) against multidrug resistant Gram-positive bacteria *Staphylococcus aureus* and *Enterococcus faecalis*

Bacterial strains	Fenotype of clinical isolates	MIC ($\mu\text{g/mL}$)		
		RE	CA	RA
<i>S. aureus</i>				
F 01	MET ^R OXA ^R GEN ^R	200	60	n.a.
F 02	MET ^R OXA ^R GEN ^R	200	60	n.a.
F 03	MET ^R OXA ^R GEN ^R	200	60	n.a.
F 05	MET ^R OXA ^R GEN ^R	200	60	n.a.
F 07	MET ^R OXA ^R GEN ^R	200	60	n.a.
F 08	MET ^R OXA ^R GEN ^R	200	60	n.a.
F 23	MET ^R OXA ^R GEN ^R	200	60	n.a.
ATCC 25923	MET ^S OXA ^S GEN ^S	200	60	n.a.
<i>E. faecalis</i>				
F 202	GEN ^S STR ^S	200	60	n.a.
F 203	GEN ^R STR ^R	200	60	n.a.
F 204	GEN ^R STR ^S	200	60	n.a.
F 205	GEN ^R STR ^R	200	60	n.a.
F 210	GEN ^S STR ^R	200	60	n.a.
F 217	GEN ^S STR ^R	200	60	n.a.
F 218	GEN ^R STR ^R	200	60	n.a.
F 222	GEN ^R STR ^S	200	60	n.a.
F 228	GEN ^S STR ^R	200	60	n.a.
F 231	GEN ^S STR ^S	200	60	n.a.
F 242	GEN ^S STR ^R	200	60	n.a.
F 253	GEN ^S STR ^S	200	60	n.a.
F 256	GEN ^S STR ^R	200	60	n.a.
ATCC 29212	GEN ^S STR ^S	200	60	n.a.

MET, methicillin; GEN, gentamicin; OXA, oxacillin, STR, streptomycin. R: Resistance. S: Sensitive. n.a.: no active until 480 $\mu\text{g/mL}$.

Regarding the antibacterial activity of rosmarinic acid, earlier we reported that this ester of caffeic acid is active only against *S. aureus* sensitive to antibiotics (Moreno *et al.*, 2006). In this work, this compound is inactive against all the MRSA tested. However, the Gram-negative bacteria *P. mirabilis*, *P. aeruginosa*, *P. stuartii* and *M. morgani* strains showed some sensitivity to the rosmarinic acid. According to these results, rosmarinic acid inhibited the growth of planktonic cells of *P. aeruginosa* (Walker *et al.*, 2004).

In general Gram-positive bacteria were found to be more sensitive to the hydrophobic plant

compounds than the Gram-negative bacteria (Burt, 2004). Nevertheless, our results showed that the lipophilic carnosic acid is able to inhibit the growth of both Gram-negative and Gram-positive clinical strains with a comparable potency. Although the exact antibacterial mechanism of carnosic acid is not fully known, we reported recently that this compound can function as an efflux pump modulator by dissipation of the membrane potential (Moreno *et al.*, 2012). Inside the rosemary plants this diterpene has been found associated with chloroplasts membranes (Perez-Fons *et al.*, 2010).

Table 2
Minimal bactericide concentration (MBC) of the rosemary extract (RE) and carnosic acid (CA) against *S. aureus* and *E. faecalis* strains

Bacterial strains	MBC ($\mu\text{g/mL}$)	
	RE	CA
<i>S. aureus</i>		
F 05	800	60
F 07	800	60
ATCC 25923	200	60
<i>E. faecalis</i>		
F 202	200	60
F 204	400	60
F 205	800	60
F 210	800	60
F 218	400	60
F 222	400	60
F 231	400	60
ATCC 29212	400	60

All together, the available evidence indicates that rosemary compounds might be of therapeutic value against multidrug-resistant bacterial infections. Carnosic acid is a potential antibacterial agent which exhibited a moderate activity against several multidrug-resistant Gram-positive and Gram-negative bacteria. A future therapeutic challenge is needed to demonstrate its in vivo efficacy and the possibility to use this compound in combinational therapies.

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Table 3
Antibacterial activity of rosemary extract (RE), carnosic acid (CA) and rosmarinic acid (RA) against Gram-negative multidrug-resistant strains

Bacterial strains	Phenotype of clinical isolates	MIC ($\mu\text{g/mL}$)				
		RE	CA	RA		
<i>Escherichia coli</i>						
F 301	LEV ^R CRO ^R CTX ^R CXM ^R SAM ^R MEM ^R TZP ^S CAZ ^S FEP ^S	400	120	n.a.		
F 306	LEV ^S CRO ^R CTX ^R CXM ^R SAM ^R MEM ^R TZP ^S CAZ ^S FEP ^R	1600	240	n.a.		
F 330	LEV ^R CRO ^R CTX ^R CXM ^R SAM ^R MEM ^R TZP ^S CAZ ^S FEP ^S	1600	240	n.a.		
F 331	LEV ^R CRO ^R CTX ^R CXM ^R SAM ^R MEM ^R TZP ^R CAZ ^R FEP ^R	1600	120	n.a.		
F 345	LEV ^R CRO ^R CTX ^R CXM ^R SAM ^R MEM ^R TZP ^S CAZ ^S FEP ^S	1600	240	n.a.		
F 348	LEV ^R CRO ^R CTX ^R CXM ^R SAM ^R MEM ^R TZP ^S CAZ ^S FEP ^S	1600	240	n.a.		
F 350	LEV ^R CRO ^R CTX ^R CXM ^R SAM ^R MEM ^R TZP ^R CAZ ^S FEP ^R	1600	240	n.a.		
<i>Enterobacter cloacae</i>						
F 302	LEV ^R CRO ^R CTX ^R CXM ^R SAM ^R MEM ^R TZP ^R CAZ ^S FEP ^R	400	30	n.a.		
F 308	LEV ^S CRO ^S CTX ^R CXM ^R SAM ^R MEM ^R TZP ^S CAZ ^R FEP ^R	800	120	n.a.		
F 326	LEV ^S CRO ^S CTX ^S CXM ^R SAM ^S MEM ^R TZP ^S CAZ ^S FEP ^S	1600	240	n.a.		
F 349	LEV ^R CRO ^R CTX ^R CXM ^S SAM ^R MEM ^R TZP ^R CAZ ^R FEP ^S	400	240	n.a.		
<i>Proteus mirabilis</i>						
F 304	LEV ^S CRO ^S CTX ^S CXM ^R SAM ^R MEM ^R TZP ^S CAZ ^S FEP ^S	400	60	120		
F 359	LEV ^R CRO ^S CTX ^S CXM ^R SAM ^R MEM ^S TZP ^S CAZ ^S FEP ^S	400	120	120		
F 361	Clinical isolate ^S	400	120	120		
<i>P. aeruginosa</i>						
F 305	LEV ^R CRO ^R CTX ^R CXM ^R SAM ^R MEM ^R TZP ^R CAZ ^R FEP ^R	400	120	120		
<i>Morganella morganii</i>						
F 339	LEV ^R CRO ^S CTX ^R	MEM ^R TZP ^R CAZ ^R	AMK ^R IPM ^R	400	120	480
<i>Providencia stuartii</i>						
F 343	Clinical isolate ^S	400	120	120		

LEV, levofloxacin; CRO, ceftriaxone; CTX, cefotaxime; CXM, cefuroxime; SAM, ampicillin/sulbactam; MEM, meropenem; TZP, piperacillin/tazobactam; CAZ, ceftazidime; FEP, cefepime; AMK, amikacin; IPM, imipenem. R: Resistance. S: Sensitive. n.a: no active until 480 $\mu\text{g/mL}$.

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