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Rosemary Compounds as Nutraceutical Health Products

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1. Introduction

The oxidation of lipids in food, cosmetic and pharmaceutical products, together with the growth of undesirable microorganisms results in the development of spoilage, off-flavor, rancidity and deterioration of such products turning them unacceptable for human consumption. In consequence, the addition of exogenous antioxidants such as butylated hydroxytoluene and butylated hydroxyanisole is frequently required to improve the stability of these products. As synthetic butylated derivatives may have toxic effects there is an increasing interest in the use of natural antioxidants, such as phenols isolated from plants to avoid undesired food borne diseases (Shylaja & Peter, 2004).

In recent years, a greater emphasis has been placed on the link between the prevention of chronic diseases and the human diet. The present popularity of natural antioxidants, as the dietary polyphenols, on human health has promoted the surge of *in vitro* studies examining the effects of these physiological active components (Williams et al., 2004).

During the past decade, consumers began to view food in a new way. The antioxidant properties of polyphenols have been widely studied, but it has become clear that the mechanisms of action of these compounds go beyond the modulation of oxidative stress (Scalbert et al., 2005). These compounds have great potential in the emerging nutritional industry, because they are often considered as food and medicines as well, therefore they may be used in the prevention and curative treatments (Sies, 2010). This issue has been of interest from ancient times, Hippocrates, 400 B.C. said, "Let food be your medicine and medicine your food".

Different foods have been identified as containing health-promoting properties beyond their basic nutritional value, and stimulating innovation in the field of nutrition and health is the search for nutraceuticals. A nutraceutical is defined as a food or part of a food that provides medicinal benefits or health, including prevention and/or treatment of diseases.

In order to use nutraceuticals in the prevention and treatment of human pathologies, many questions still remain unanswered:

Which natural source is to be used? Which is a good candidate to be used as nutraceutical?.

Rosemary (*Rosmarinus officinalis* L., Lamiaceae) is considered one of the most important sources for the extraction of phenolic compounds with strong antioxidant activity. This specie grows worldwide and has been cultivated since long ago, in ancient Egypt, Mesopotamia, China and India (Bradley, 2006). Rosemary extracts, enriched in phenolic compounds are effective antioxidants due to their phenolic hydroxyl groups but they also possess plenty of other beneficial effects like antimicrobial, antiviral, anti-inflammatory, anticarcinogenic activities and is also known to be an effective chemopreventive agent (al-Sereiti et al., 1999; Aherne et al., 2007).

Therefore, this specie contains bioactive ingredients which provide a complementary value other than the nutritional one, to be applied in the food industry. However, particular bioactives of rosemary responsible for some biological activities, as antimicrobial, have not been deeply characterized. A lesser amount of information exists about their mechanism of action. The present chapter focuses on the most significant rosemary biological properties, reviewing the free radical scavenging and antibacterial actions of non-volatile constituents and essential oils, the antibacterial activity of main rosemary bioactives in combination with antibiotics, as well as possible antibacterial mechanism of action is proposed, among other topics. In addition, a toxicity assay using the nematode *Caenorhabditis elegans* is covered.

2. Rosemary the best natural antioxidant

2.1 Antioxidant action of different fraction of rosemary

Rosemary plants have many phytochemicals which constitute potential sources of natural compounds as phenolic diterpenes, flavonoids phenolic acids and essential oils. About 90% of the antioxidant activity is attributed mainly to a high content of non-volatile components as carnosic acid and carnosol (phenolic diterpenes) and rosmarinic acid (Bradley, 2006). It is clear that *R. officinalis* constituents have antioxidant activity according to traditional use and scientific evidence, although little information is available on the relationship between chemical composition and antioxidant activity of the essential oils and non-volatile extracts.

We investigated the antioxidant activity of volatile and non-volatile fractions isolated from two leaf phenotypes of rosemary plants growing in the same farm in Argentina. Plants showing a wide (W) and a narrow (N) leaves phenotypes were collected from Jardín Botánico Arturo E. Ragonese from National Institute of Agricultural Technology-INTA Castelar, Argentina, January 2008. The essential oils were obtained by hydrodistillation of dried leaves using a Clevenger-type apparatus and samples were analyzed by high resolution gas chromatography coupled with mass spectrometry. Ethanol extracts were prepared according to the method previously reported (Moreno et al., 2006) and 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. Quantification of phenolic compounds and identification was performed on an HPLC (LKB Bromma) equipped with a diode array detector, using a 250 mm × 4 mm C18 Luna analytical column (Phenomenex, USA), as previously described (Moreno et al., 2006).

The antioxidant activity was tested using a stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) as described by Brand-Williams et al., 1995. The percentage of DPPH radical was calculated measuring the change in absorbance at 517 nm and EC_{50} (extract concentration necessary to decolorate DPPH radical in a 50%) was determined for each fraction.

Results showed that different amounts of key bioactive compounds were present in the essential oils of both phenotypes. The main constituents of W phenotype essential oil were α-pinene and 1,8-cineole, while the N phenotype contained a comparable amount of 1,8 cineole and high contents of myrcene (Table 1). Therefore, the first is referred as an α-pinene chemotype and the other one as a myrcene chemotype. The essential oil of the myrcene chemotype exhibited approximately a double fold antioxidant activity than the α-pinene phenotype. Recently, other authors reported that pure myrcene showed antioxidant activity and eliminated oxidative stress in rats in a time-dependent manner (Ciftci et al., 2011).

Essential oil	EC_{50} (µl/ml)	Main constituents	(%)
W Phenotype	25.0 ± 1.0	α-Pinene	31.2 ± 2.5
		1,8 -Cineole	21.6 ± 1.6
N Phenotype	10.0 ± 0.4	Myrcene	31.1 ± 2.3
		1,8-Cineole	18.7 ± 1.4

Table 1. Antioxidant activity and main constituents of essential oils isolated from two phenotypes of rosemary plants. Values ± SD

Chemical analysis of ethanol rosemary extracts indicated that W phenotype contained about 36.8% of diterpenes (carnosic acid plus carnosol) and 8.4% of rosmarinic acid, while the other phenotype contained a higher amount of diterpenes and lesser amounts of rosmarinic acid (Table 2). The ethanol extract isolated from the N phenotype, containing a high amount of diterpenes, exhibited approximately double fold antioxidant activity than plants of W phenotype.

Our results showed that the volatile and non-volatile fractions isolated from the same phenotype had similar antioxidant activity.

Ethanol extract	EC_{50} (µg/ml)	Main constituents	(%)
W Phenotype	20.0 ± 0.8	Carnosic acid + carnosol	36.8 ± 2.7
		Rosmarinic acid	8.4 ± 0.5
N Phenotype	10.8 ± 0.4	Carnosic acid + carnosol	50.0 ± 4.5
		Rosmarinic acid	2.9 ± 2.5

Table 2. Antioxidant activity and main constituents of ethanol extracts isolated from two phenotypes of rosemary plants. Values ± SD

2.2 Rosemary as protective agent against oxidative protein damage

A large number of reports have shown rosemary constituents to be an efficient antioxidant against lipid peroxidation and DNA damage induced by radical oxygen species in rat liver mitochondria and microsomes at concentrations of 3 - 30 µM, demonstrating their ability to protect tissues and cells against oxidative stresses (Bradley, 2006). On the other hand, it is

well-known that several antioxidants exhibited pro-oxidant effect producing protein damage under certain conditions as in the presence of transition metals such as Fe and Cu. In order to study the protection of rosemary compounds against protein damage in comparison with ascorbate and 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), hydroxyl radical-mediated oxidation experiments, were carried out using a metal-catalyzed reaction. Bovine serum albumin (4 µg) was incubated with or without Cu^+ (100 µM) and H_2O_2 (1 mM) in the absence or presence of ascorbate, Trolox or the methanol rosemary extract (obtained as described in Moreno et al., 2006). Reactions were performed in opened tubes at 37°C, mixed with loading buffer and loaded in 12.5% dodecyl sulfate-polyacrylamide gel electrophoresis as reported by Mayo et al., 2003. Figure 1 shows that 20 µg of the plant extract used containing a concentration of 18 µM diterpenes, reduced significantly protein damage compared with 20 µM of ascorbate (compare the intensity of the protein's monomer in lane 8 vs. lane 6). Figure 1 also shows that Trolox and ascorbate only protected protein modifications when they were present at lower concentrations, and no protection of the protein was observed at higher concentrations. Ascorbate reveals a higher pro-oxidant action than Trolox.
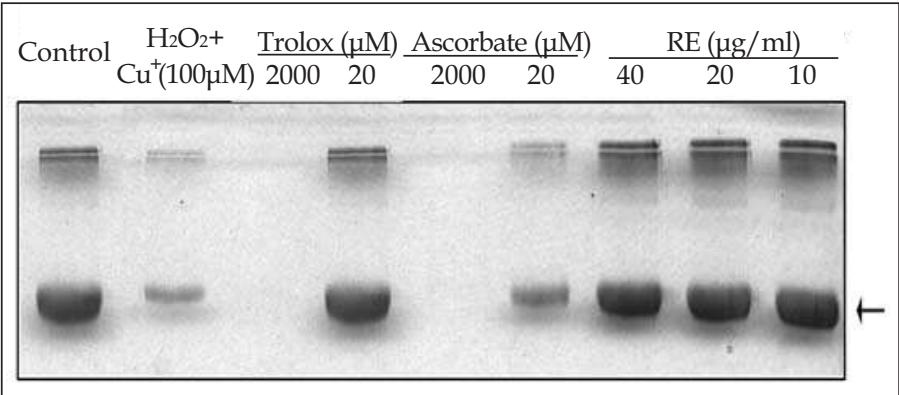

Fig. 1. Gel electrophoresis of bovine serum albumin protein after treatment with H_2O_2 + $CuSO_4$ in the presence of Trolox, ascorbate and methanol rosemary extract (RE) at the concentrations indicated. Control was performed with the addition of ethanol 0.2%. Arrow indicates the bovine serum albumin monomer

3. Antibiotics and antioxidants of rosemary working together

Due to the recent trend in green consumers, there is an increasing interest in the antimicrobial properties of rosemary compounds. Essential oils and organic or aqueous extracts of isolated from this specie not only have antioxidant activity but also present antibiotic effects, therefore they have gained acceptance in industry to replace existing synthetic preservatives in foods (Davidson, Sofos, & Branen, 2005).

In the area of health, the near-term interest of plant products as antimicrobial agents is related to the development of new strategies/therapies for infections caused by bacterial species (Cowan, 1999; Lewis and Ausubel, 2006). Recently, it was reported that natural plant products can potentiate the activity of antibiotics in combination (Coutinho et al., 2009). Moreover, the use of bacterial resistance modifiers derived from natural sources, mainly from plants, such as efflux pump inhibitors, was suggested to be useful to suppress the emergence of multidrug resistant strains (Stavri et al., 2007).

To determine the validity of rosemary compounds as nutraceuticals, a rigorous analysis of the biological activities of their bioactives is required as well as the further study of their antibacterial mechanism of action.

3.1 Antibiotic action of different fractions of rosemary

We previously reported the effective antimicrobial action of 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* (Moreno et al., 2006). These microorganisms cause severe problems in human health (NNIS, 2004). According to a 2011 research study, *S. aureus* was found in ~50% of beef, pork, and poultry products throughout the United States, 96% of these isolates were resistant to at least 1 antibacterial agent relevant in human medicine and 52% were resistant to three or more types (Waters et al., 2011).

Here, we show the antibacterial performance of a methanol rosemary extract (obtained as described in Moreno et al., 2006) in comparison with common food preservatives. The results are expressed in percent of inhibition of bacterial growth (see Equation 1)

$$\% \text{ Inhibition of bacterial growth} = \frac{(A_{595}\text{Control} - A_{595}\text{Sample})}{A_{595}\text{Sample}} \times 100 \qquad (1)$$

Where, A_{595} Control is the absorbance of the bacterial culture without compounds.

Rosemary extracts presented a higher antimicrobial efficacy than benzoic acid, butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) to inhibit *S. aureus* growth (Fig. 2A), while a similar action than BHT and benzoic acid and a minor antimicrobial activity in relation to BHA was seen against *E. coli* (Fig. 2B).
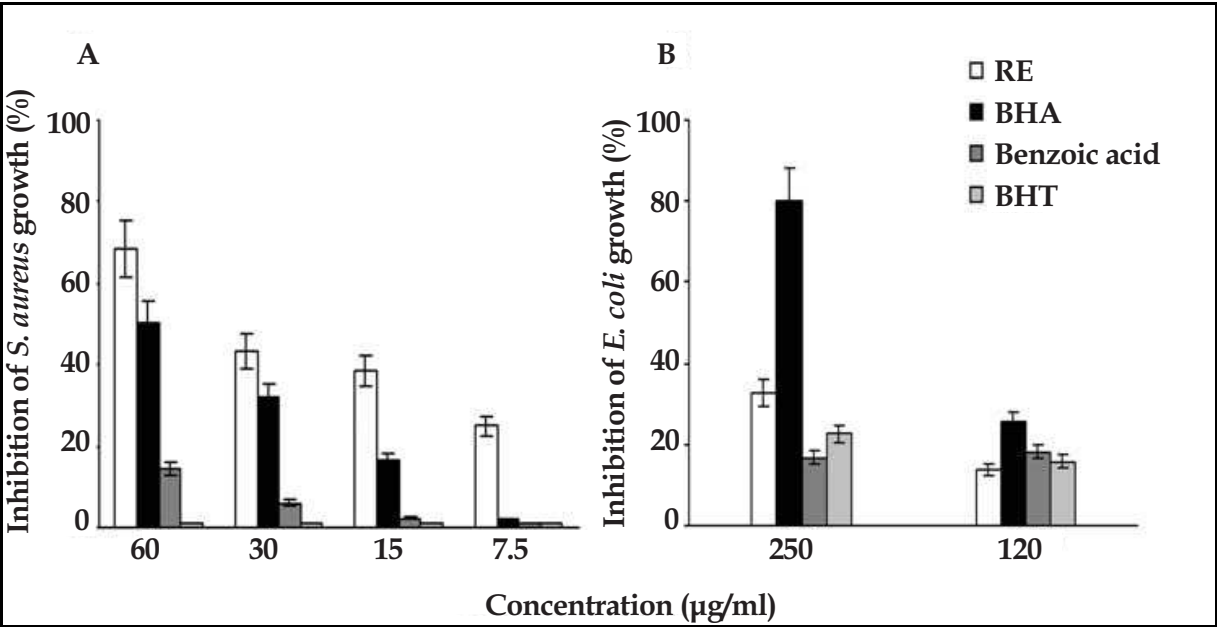

Fig. 2. Effect of the methanol rosemary extract (RE) on the *S. aureus* growth (A) or on the growth of *E. coli* (B), in comparison with BHA, BHT and benzoic acid. Values are shown as the mean of three independent experiments ± SD

The performance of rosemary oils and ethanol extracts isolated from the W phenotype and N phenotype plants against the human pathogen *E. faecalis,* was also compared (Table 3). Results showed that the essential oil from the α-pinene chemotype (W phenotype) exhibited a higher antibacterial activity than the essential oil isolated from N phenotype. By contrast, the ethanol extract isolated from the N phenotype containing higher amounts of carnosic acid and carnosol exhibited the highest antimicrobial action (Table 4).

Phenotype	Essential oil	Inhibition of *E. faecalis* growth (%)
W	26 μl/ml	100
N	26 μl/ml	45.5 ± 3

Table 3. Antibiotic activity of essentials oils isolated from two phenotypes of rosemary plants. Values are shown as the mean of three independent experiments ± SD

Phenotype	Ethanol extract	Inhibition of *E. faecalis* growth (%)
W	0.25 mg/ml	50.0 ± 6
N	0.25 mg/ml	100

Table 4. Antibiotic activity of ethanol extracts isolated from two phenotypes of rosemary plants. Values are shown as the mean of three independent experiments ± SD

All findings suggested that carnosic acid and α-pinene are key ingredients responsible that confer the antibacterial properties in the non-volatile and volatile fractions isolated from rosemary plants, respectively.

The essential oils isolated from N phenotype exhibited minor antibacterial activity than the other one, although it presented a higher performance as antioxidant.

We are investigating the antibacterial action against several Gram positive and negative microorganisms. Others authors demonstrated antibacterial activity of volatile compounds against *Listeria monocytogenes*, *Salmonella typhimurium*, *Escherichia coli*, *Shigella dysenteria*, *Bacillus cereus* (For review see Burt, 2004).

3.2 Antibiotic potentiation by rosemary bioactives

The use of combined antioxidants has gained acceptance in industry and has been applied to different aspects of food preservation (Davidson, Sofos & Branen, 2005). Although, up to date, a rational basis for the use of phytochemicals enhancing and/or broadening the biological antioxidant and antimicrobial activities against food-borne pathogens, is still poorly explored (Wei & Shibamoto, 2007). In the health's area, natural compounds are usefull strategies for the development of therapies against infections caused by bacterial species, and they are used in combination with common antibiotics potentiating their activity (Coutinho et al., 2009).

We previously reported a synergistic antioxidant effect between the methanol rosemary extract and BHT and a synergistic interaction with BHA to inhibit *E. coli* and *S. aureus* growth (Romano et al., 2009). Here, we reported the *in vitro* antibiotic interaction of rosemary extracts with common antibiotics using the broth microdilution method against *S. aureus* and *E. faecalis.*

Figure 3A shows the dose-response curve, in which the addition of 6.25 µg/ml of the plant extract clearly displaced the curve to the left, meaning that an increment in the antimicrobial action against *S. aureus* took place in the binary mixture at all gentamicin concentrations tested. It can be extrapolated from the curve that 0.035 µg/ml of pure gentamicin is needed to achieve 50% of inhibition, while the same effect can be achieved with 0.015 µg/ml of the aminoglycoside in the presence of rosemary extract.

Then, to study the type of interactions binary mixtures with different concentrations of rosemary extract and pure carnosic acid with gentamicin were analyzed by isobolograms (Fig. 3B).
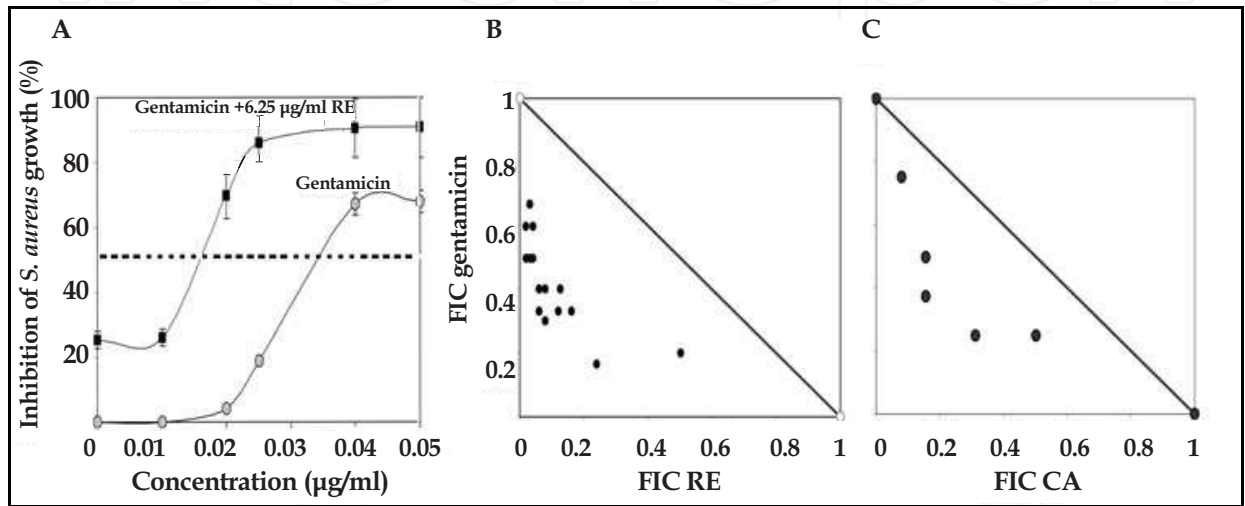
Fig. 3. Rosemary extract (RE) and carnosic acid (CA) acts synergistically with gentamicin against *S. aureus*. Dose-response curve (A), isobologram of gentamicin in combination with RE (B) or pure carnosic acid (C). Inhibition of *S. aureus* 25923 growth was determined after 24 h of incubation. FIC, fractional inhibitory concentration, normalized to the unit of RE, gentamicin or pure carnosic acid, corresponds to the $MIC_{50} = 12$ µg/ml, 0.035 µg/ml and 8 µg/ml, respectively. Values are shown as the mean of three independent experiments ± SD

Compounds concentrations lower than their MIC_{50} values were prepared for every combination tested as described previously (Romano et al., 2009) and isobolograms were performed. When compounds 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 (Tallarida, 2001).

The Fractional inhibitory concentration (FIC index) was determined (see Equation 2).

$$\text{FIC index} = FIC_A + FIC_B = [A]/MIC_A + [B]/MIC_B \qquad (2)$$

FIC_A, FIC_B: Fractional inhibitory concentration of drug A & B respectively. MIC_A, MIC_B: Minimum inhibitory concentration of drug A & B respectively. [A], [B]: Concentration of drug A & B respectively.

FIC index obtained by checkerboard method is interpreted as follows: ≤ 0.5- synergy; > 0.5 and ≤ 4- additivity; and > 4– antagonism.

Figure 3B shows that different combinations of rosemary extracts corresponding to FIC 0.01 to 0.25 with gentamicin (FIC of 0.2 to 0.4) resulted in fixed-ratio points below the additive line and exhibited values of FIC index ≤ 0.5, verifying synergistic antimicrobial effects of the plant extract with gentamicin.

As carnosic acid was shown to be the main antimicrobial compound of non-volatile rosemary extracts against *S. aureus* (Moreno et al., 2006), this compound was assayed in combination with gentamicin at concentrations equivalent to those found in the extract (1 to 10 µg/ml). The minimal inhibition concentration (MIC) of carnosic acid against the bacteria was 16 µg/ml and was referred as a FIC of 1. Values of FIC index ≤ 0.5 (FIC 0.3 of carnosic acid in combination with FIC 0.2 to 0.3 of gentamicin) were observed (Fig. 3C).

On the other hand, rosmarinic acid exhibited a minor antibacterial effectiveness than carnosic acid and exhibited additive antibacterial actions with gentamicin (Data not shown). Therefore, data showed that the addition of both the extract and carnosic acid allows a reduction of the gentamicin amount in approximately 3 - 4 folds to obtain the same antibiotic action. The bactericidal action of rosemary extracts and pure carnosic acid were confirmed by time-kill curves.

Table 5 shows that carnosic acid plus tetracycline, tobramycin, kanamycin, ciprofloxacin and gentamicin exhibits significant synergistic antibiotic activity against *S. aureus.*

Antibiotics	MIC values of antibiotic in combination with MIC of carnosic acid[a] (µg/ml)				FIC Index
	0	1/8	1/4	1/2	
Tetracycline	0.50	<u>0.06</u>	0.06	0.06	0.25
Tobramycin	0.50	<u>0.12</u>	0.12	0.06	0.37
Kanamycin	2.00	1.00	<u>0.50</u>	0.25	0.50
Vancomycin	2.00	<u>1.00</u>	1.00	0.50	0.62
Ciprofloxacin	0.50	<u>0.06</u>	0.12	0.12	0.25
Penicillin	0.06	-	<u>0.03</u>	0.03	0.75
Gentamicin	0.50	<u>0.06</u>	0.06	0.06	0.25

Table 5. Antibiotic activity of carnosic acid in combination with several antibiotics against *S. aureus* ATCC 25923. [a]Underlined values: combinations of minimum FIC used to calculate the FIC index. Values are the mean of three independent experiments

In order to confirm the role of carnosic acid as the main bioactive compound of rosemary extracts involved in the synergistic effect with gentamicin, additional experiments were carried out, examining carnosic acid in combination with gentamicin against *E. faecalis* (Fig. 4). Results showed that 30 µg/ml of the diterpene in combination with 0.25 µg/ml of gentamicin resulted in an strong antibiotic synergistic effect as the expected value for the sum corresponding to individual effects was 42% of inhibition, whereas experimental data showed a100 % of inhibition on bacteria growth for the combination mixture.
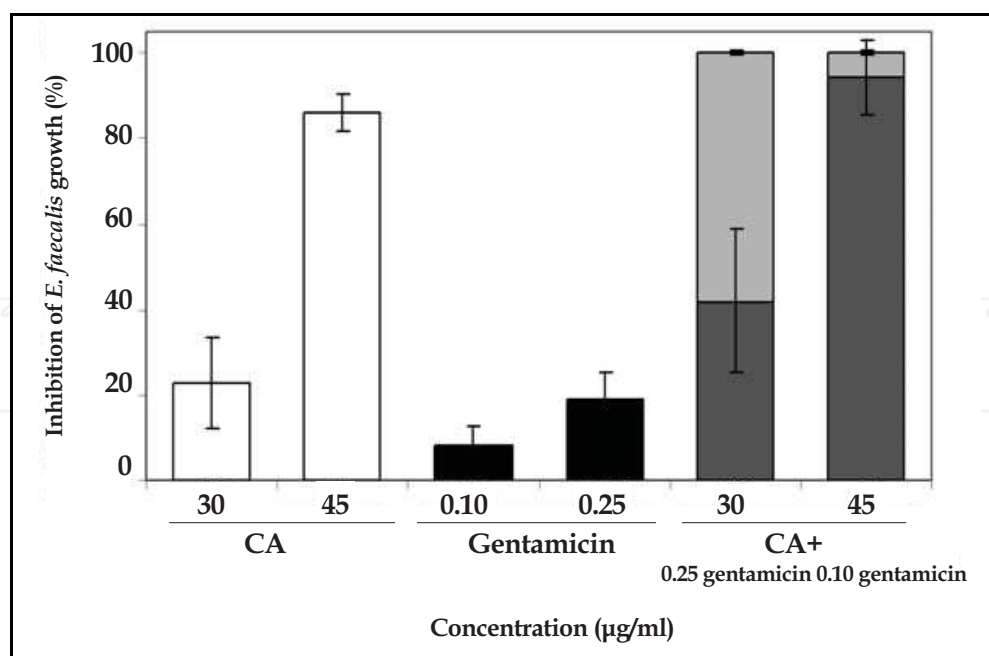
Fig. 4. Antibiotic activity of carnosic acid in combination with gentamicin against *E. faecalis* ATCC 29212. Dark grey bars represent the expected results if an additive interaction took place and light grey bars show the experimental data

3.3 Mechanism of antibacterial action of rosemary bioactives

R. officinalis plants exhibited antibiotic activity although less is known regarding the mechanisms of action of the particular bioactives. Polyphenols still constitute a promising source of new drugs and there is a high interest in understanding their mechanisms of action (Scalbert, 2005). We carried out studies to determine the antibacterial effect of the carnosic acid. This liphophilic diterpene has been found associated with chloroplasts membranes (Perez-Fons et al., 2006). Herein the cell membrane permeabilization effect by carnosic acid using the membrane potential sensitive cyanine dye DiS-C3-(5). This fluorescent probe is a caged cation, which distributes between cells and medium depending on the cytoplasmic membrane potential. Once it is inside the cells, it becomes concentrated and self-quenches its own fluorescence. The fluorescence monitored with an excitation wavelength of 610 nm and an emission wavelength of 670 nm. A blank with only cells and the dye was used to subtract the background and compounds as the proton motive force inhibitor carbonyl cyanide-m-chlorophenylhydrazone (CCCP) and Polimycyn B were used as controls for their ability to decreased or increase the membrane potential, respectively. *S aureus* suspension was incubated with 1.6 µM DiS-C3-(5) until a stable reduction in fluorescence and 100 mM KCl was added to equilibrate the cytoplasmic and external K^+ concentration. Figure 5 shows time decreases of the fluorescence intensities upon addition of 32 µg/ml of carnosic acid to *S. aureus* cells in a similar way than the inhibitor CCCP, while the cationic antimicrobial peptides Polimyxyn B produced an increment on the fluorescence. CCCP is a small amphipathic molecule which dissolves in phospholipid bilayers, providing a polar environment for the ion and an hydrophobic face to the outside world, it is an uncoupling agent that specifically increases the proton permeability, and disconnects the electron transport chain from the formation of ATP, discharging the pH gradient, and destroying the membrane potential.
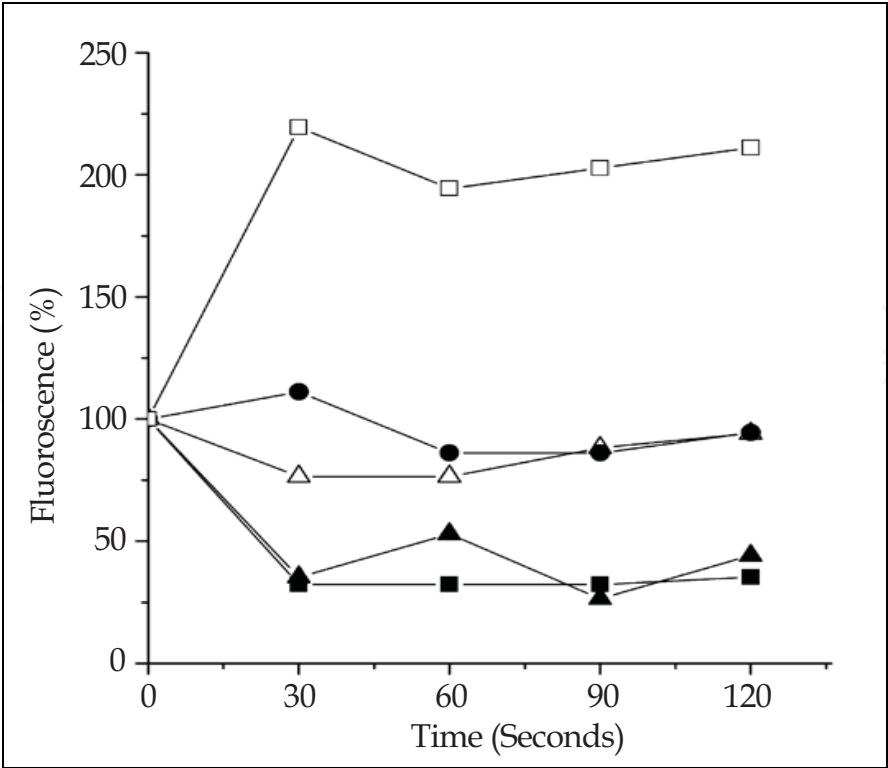
Fig. 5. Time changes of the fluorescence intensities of DiS-C$_3$-(5) upon addition of carnosic acid to *S. aureus* cells: 32 µg/ml (▲), 16 µg/ml (△), cells treated with CCCP (■), cells treated with Polimyxyn B (□) and untreated cells (●). Representative records from two similar independent experiments are shown
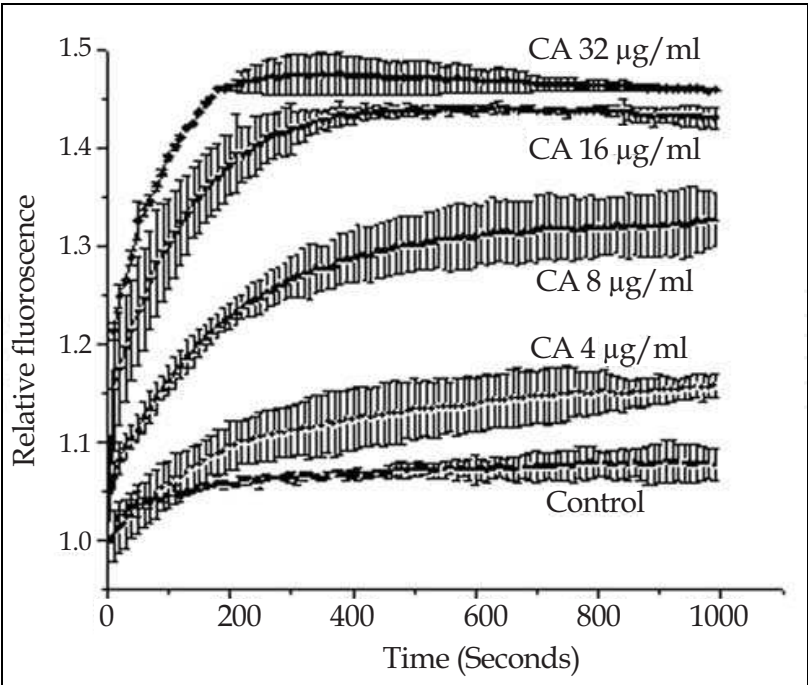

Fig. 6. Effect of carnosic acid on EtBr efflux in *S. aureus* cells. Control corresponds to the initial intracellular fluorescence of EtBr at time 0. Representative efflux records from two similar independent experiments are shown

As it can be seen above, carnosic acid at 1/4 - 1/8 MIC value (2 to 4 μg/ml) showed a selective synergistic interaction with tetracycline and aminoglycosides (Table 5) that may be explained by selective modulation of the antibiotics accumulation possibly by inhibition of their efflux in *S. aureus*. To assess whether this compound may have an efflux pump inhibitor activity in *S. aureus*, the efflux substrate ethidium bromide (EtBr) was used. EtBr shows a characteristic fluorescence when it enters the cell and binds to DNA and its uptake was determined by monitoring the fluorescence using a multiwell plate reader (DTX 880 Multimode detector, Beckman Coulter) at 29ºC every 10 sec intervals for 3 min with excitation and emission wavelengths of 535 nm and 485 nm, respectively. Figure 6 shows that carnosic acid rapidly increased EtBr uptake and this effect was dose dependent indicating a positive effect on the intracellular accumulation of the fluorescent molecule.

Whether carnosic acid is a general disruptor of the transmembrane electrochemical potential, it should display a synergistic effect with chloramphenicol which is effluxed out of the cell by secondary transport relying in the membrane potential. Additionally, an antagonist effect with ampicillin and penicillin G, which enter inside cells by a proton symport mechanism, should be displayed however, none of these effects were observed (Table 5).

Other studies from our laboratory on the antibiotic action of carnosic acid against *E. faecalis* showed similar results. In time-kill studied, carnosic acid displayed a bacteriostatic effect at its MIC value (64 μg/ml), whereas a bactericidal effect was achieved at 2 x MIC (128 μg/ml) increasing the permeability of cell membrane effects. At sub MIC values, the diterpene inhibited the drug efflux pumps of secondary transporters (Repetto, 2009). Other authors reported the antibacterial and resistance modifying activity of *R. officinalis* and suggested that the carnosic acid has the capacity to modify the resistance pattern of strains of *S. aureus* expressing multidrug efflux pumps (Oluwatuyi et al., 2004). Regarding this feature, we decided to investigate several bioactive compounds of *R. officinalis* with antimicrobial activity per se, or as modulators of bacterial resistance, against multidrug-resistant clinical strains of *S. aureus* isolated from pediatric patients (Ojeda Sana et al., 2011).

Our findings, together with results from other authors, suggest that this diterpene is a potential antibacterial agent to be used in combinational therapy (at least with aminoglycosides, tetracyclin and fluoroquinolones) against sensitive as well as multidrug resistant, vancomicyn and/or methicillin resistant Gram-positive cocci. Moreover, as an antibacterial compound, carnosic acid can not only target membrane permeability and enhance drug uptake, but also this compound, at sub MIC values inhibited the drug efflux transport probable by altering the cell membrane potential. We proposed a model for the antibacterial action of carnosic acid (Fig. 7).

3.4 Rosemary bioactives kill inthaphagocytic *S. aureus* cells

We examined the intracellular antibacterial activity of carnosic acid against *S. aureus*. A model of *S. aureus* infected RAW 654.7 mouse macrophages has been monitored for long-term (24 h) experiments. This bacterium adheres to phagocytes and easily invades them and tends to restrict the phagolysosomal compartment, where it largely escapes destruction and survives in a semiquiescent state for prolonged periods (Maurin et al., 2001). These intraphagocytic forms are considered responsible for the well-known recurrent character of

staphylococcal infections as well as for the many failures of apparently appropriate antibiotic treatments.
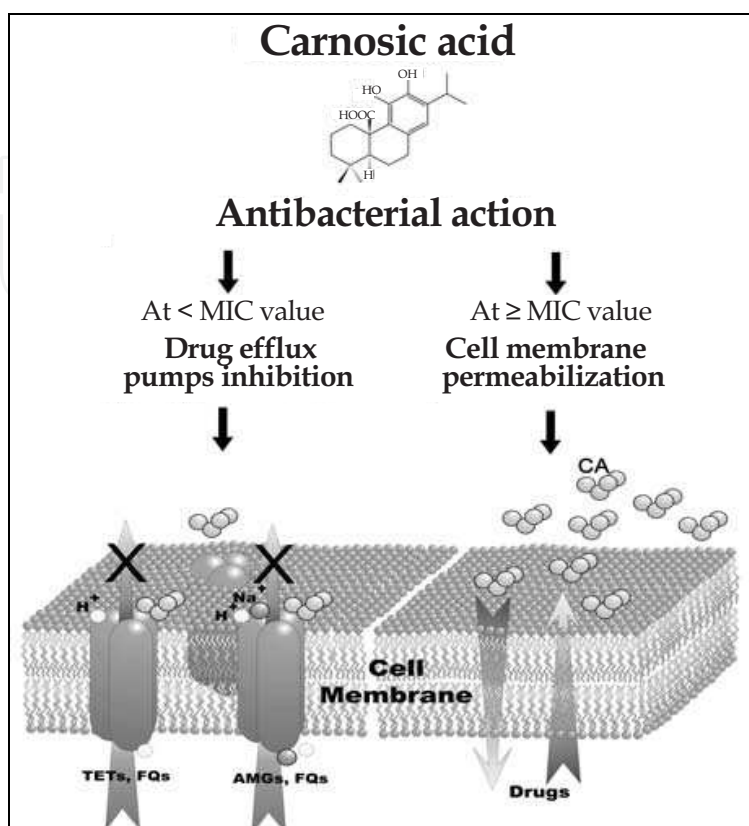

Fig. 7. Proposed model for the antibacterial action of carnosic acid. NMGs, aminoglycosides; TETs, Tetracyclins and FGs, fluoroquinolones

Confocal microscopy was used to ascertain the localization of the bacteria inside RAW 264.7 macrophages observed 24 h after phagocytosis of opsonized *S. aureus.* As shown in Fig. 8 A and B *S. aureus* clearly appeared intracellular. Pericellular membrane, located mostly on the inner face was labeled with FITC- phalloidin (green signal), and bacteria were labeled with DAPI (Blue signal). Later, macrophages were incubated for 24 h with carnosic acid from the MIC (16 μg/ml, determined in broth at pH 7.3). Carnosic acid caused a marked reduction in CFU, diminishing 3-4 log the bacterial growth at a concentration of 1 ½ MIC (24 μg/ml) (Fig. 8C). This effect was comparable to the control cells incubated with 0.5 μg/ml ciprofloxacin, a fluoroquinolone antibiotic that has excellent antibacterial activity against gram positive bacteria and intracellular penetration.

Earlier we demonstrated the *in vivo* antibacterial efficacy of a rosemary extract containing high amounts of carnosic acid against *S. aureus* in two skin infection models in mice (Barni et al, 2009).

Clinically effective antimicrobial agents exhibit selective toxicity towards the bacterium rather than the host. It is this characteristic that distinguishes antibiotics from disinfectants. The basis for selectivity will vary depending on the particular antibiotic. Carnosic acid at the concentrations that kill *S. aureus* has a high selectivity to bacteria and is not toxic to macrophages.
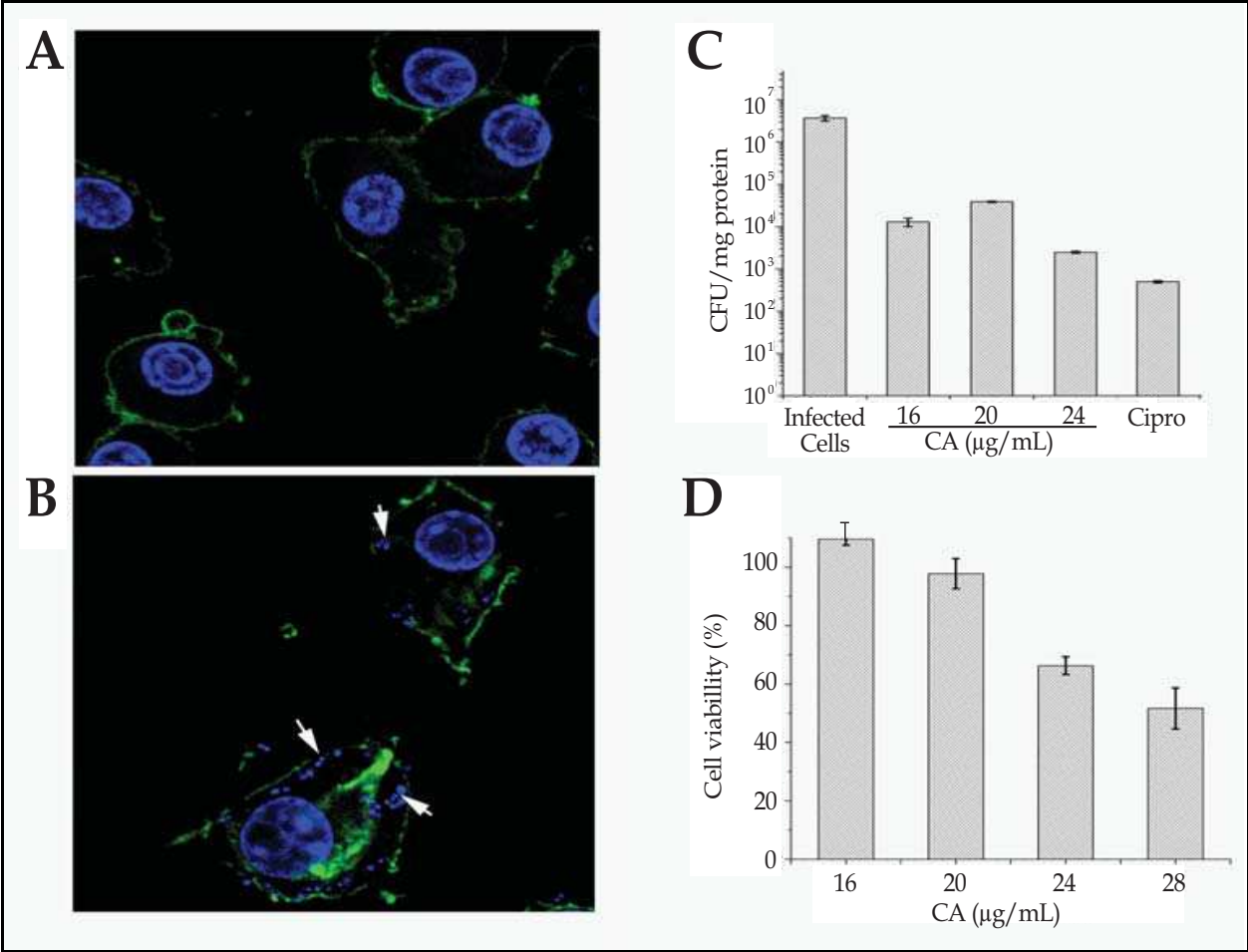
Fig. 8. Intraphagocitic killing of *S. aureus* by carnosic acid. Confocal microscopy of uninfected RAW 264.7 macrophages (A) or after phagocytosis of *S. aureus* (B). Arrowheads indicate intraphagocytic bacteria. (C) variations in the number of CFU per mg of protein (± SD; n= 2) after 24 h of incubation with carnosic acid or with 0.5 µg/ml of ciprofloxacin (Cipro). (D) Effect of carnosic acid following a 24-h exposure using a standard MTS cell viability assay

4. Safety of rosemary: Toxicity evaluation

Due to the potential use in human nutrition and health in foods of carnosic acid, it is important to determine its toxicological effects in a living organism.

In this study, we used *Caenorhabditis elegans* as a model system to examine the toxic effects of rosemary bioactives. This nematode, present in soil and found in temperate regions of the world, has emerged as an important animal model in various fields including toxicological research and rapid toxicity assessment for new chemicals (Moy T et al., 2006). *C. elegans* is easily cultured on agar medium plates with *Escherichia coli* OP50 as a food source. The ease of laboratory cultivation, its small size, large brood size, short development time (the entire complete life cycle from egg to egg producing hermaphrodite occurs over 3 days at 20°C under normal laboratory conditions) and the well-studied biology, makes the *C.elegans* an ideal model organism for biological studies. The transparency of this nematode allows for high quality microscopic images to be taken. Toxicity assays will be used in this study to test

how carnosic acid affects *C. elegans* survival by exposing them to various concentrations of this compound. The *C. elegans* life cycle is comprised of an embryonic stage, four larval stages designated L1-L4 and adulthood. As it had been previously described that some nematode killing observed in this assays was a consequence of eggs being retained in the uterus and hatching internally, we substituted wild type worms by the *glp-4* temperature-sensitive sterile mutants to prevent internal hatching of progeny. *glp-4* does not make a germline at the restrictive temperature and survive without a bacterial food source, whereas WT *C. elegans* die by internal hatching of progeny when transferred to bacteria-free media. Worms were synchronized by isolating eggs from gravid adults, hatching the eggs overnight in M9 buffer, and plating L1-stage worms onto lawns of OP50 *E. coli* on nematode growth medium (NGM) agar media. Worms were grown to sterile, young adults by incubation at 25°C for 48 – 52 h, washed off the plates with M9 buffer. Approximately 40 worms were transferred to 24 well plates containing 500 microliters of M9 medium and the compounds to be tested. Each compound was tested in individual wells, and the screen was performed by using triplicate 24-well plates. To score for survival, the plates were shaken by hand, worms were considered to be dead if they did not move or exhibit muscle tone when viewed using a stereo microscope at 20X magnification. Worms were observed every two days and scored for survival analysis at day 8 after transfer. As carnosic acid was dissolved in ethanol, we first tested if the 1.6% final ethanol concentration found in the vehicle of this assay had any effect over the survival of worms. We didn't find significative differences between the survival of ethanol treated and untreated worms. Our results demonstrated that up to 250 µg/ml of carnosic acid tested the compound does not adversely affect the normal physiology of the nematodes, while the highest concentration tested (500 µg/ml) of carnosic acid had moderate worm mortality (30%) (Fig. 9).
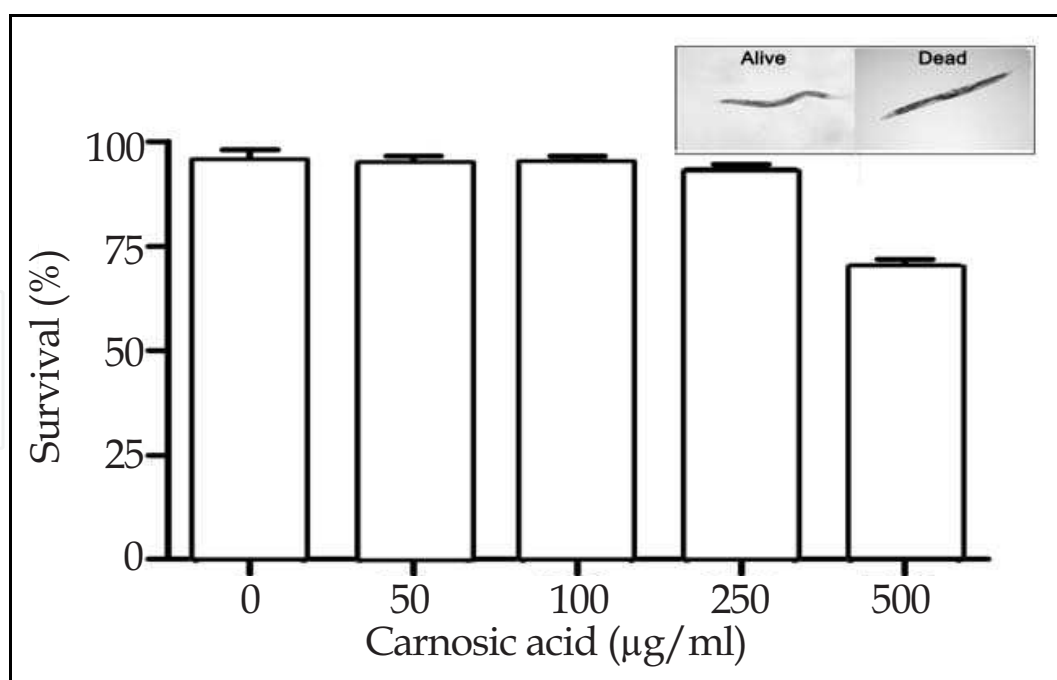

Fig. 9. Effect of carnosic acid over *C.elegans* survival. (Inset) typical morphology of living nematodes maintaining a sinusoidal shape (left) and dead ones appearing as straight, rigid rods (right) (at ×20 magnification). Values are shown as the mean of three independent experiments ± SD

5. Conclusions

The present chapter summarizes the most significant rosemary biological properties, reviewing its free radical scavenging, antibacterial actions of essential oils and non-volatile pure constituents and in combination with antibiotics.

Both volatile and non-volatile secondary metabolites isolated from rosemary plants growing in the same place exhibited comparable antioxidant activities. Non-volatile constituents exhibited at least a minor pro-oxidant property than ascorbic acid and Trolox, at the concentration assayed. The non-volatile fractions containing high content of carnosic acid as well as the volatile oils rich in α-pinene exhibited the highest antibiotic activity.

Rosmarinus officinalis extract as well pure carnosic acid, showed synergistic effect in combination with aminoglycosides and ciprofloxacin against *S. aureus*. We proposed a model for the antibacterial action of carnosic acid which may be useful for future applications.

Unexpectedly, carnosic acid showed a clear antistaphylococcal action towards extracellular and intraphagocytic forms of *Staphylococcus aureus*, pointing out a potential use of this compound in the treatment of *S. aureus* infections, without significant effect on the macrophage viability. Moreover, carnosic acid at bactericide concentrations did not show adverse effects on the viability of a living organism as the nematode *C. elegans.*

6. Rosemary in human nutrition and health: Future and prospect

All together, the available evidence indicates that rosemary compounds might be of therapeutic benefit in bacterial infections and be an ideal candidate for nutraceutical health products. Non-volatile extracts of rosemary containing approximately an amount of 20 μg/ml (18 μM) of carnosic acid as the key compound, killed several bacteria and represent a therapeutic alternative against extracellular-intracellular *S. aureus* infections. This compound did not show pro-oxidant effects and its use is safe at least until a concentration of 250 μg/ml (750 μM). The *in vivo* antibacterial efficacy of an ethanol extract of *Rosmarinus officinalis* L. containing high amounts of carnosic acid against the pathogenic bacteria *S. aureus* has been demonstrated previously in mouse (Barni et al, 2009). Even though, prospective controlled clinical studies are still lacking.

Rosemary is the only spice commercially available for use as an antioxidant in Europe and the United States. This specie has the advantage to contain different antioxidant molecules (lipophilic monoterpenes and diterpenes, as well as hydrophilic derivatives of caffeic acid as rosmarinic acid) that could be effective in both, aqueous fluids as well as in lipophilic parts of the body as a very effective antioxidant to scavenge free radicals. In addition, non-volatile extracts of rosemary can also be used to decrease 4.4 - to 17-fold the amounts of the synthetic butyl derivatives used as food or cosmetic preservatives (Romano et al., 2009).

Although not discussed in this chapter, due to the associated bioactivities of carnosic acid (anti-inflammatory-and anticarcinogenic effects), rosemary polyphenols can be considered as a potential source of promising new nutraceuticals formulations (Mengoni et al., 2011).

Improvements in the processes of regulation of rosemary bioactives are needed, and the general tendency is to perpetuate the German Commission E experience, which combines scientific studies and traditional knowledge (monographs).

There is still work to be done regarding how to use rosemary derivatives inside the human diet. Particular attention needs to be given to stability studies and interactions of rosemary constituents with other food constituents. Further investigations will be directed towards the application of phenolic compounds in various food matrices. The use of functional foods enriched with rosemary compounds need technologies for incorporating health-promoting ingredients into food without reducing their bioavailability or functionality. In this sense, we are also working on the addition of rosemary bioactives into edible films (Proyect CYTED, 309AC0382 action: Getting additive materials from plant by-products of the region and its application in the development of biodegradable packaging food processing and nutraceutical use). Finally, the production and biotechnological studies and genetic improvement of rosemary plants will offer great advantages, since it will be possible to obtain uniform and high quality raw materials which will ensure the efficacy and safety of rosemary products.

7. Acknowledgment

8. References

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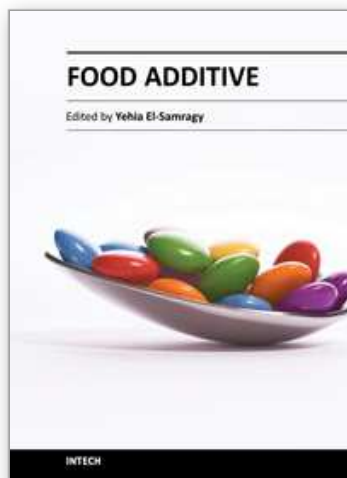
Food Additive

Edited by Prof. Yehia El-Samragy

A food additive is defined as a substance not normally consumed as a food in itself and not normally used as a characteristic ingredient of food whether or not it has nutritive value. Food additives are natural or manufactured substances, which are added to food to restore colors lost during processing. They provide sweetness, prevent deterioration during storage and guard against food poisoning (preservatives). This book provides a review of traditional and non-traditional food preservation approaches and ingredients used as food additives. It also provides detailed knowledge for the evaluation of the agro-industrial wastes based on their great potential for the production of industrially relevant food additives. Furthermore the assessment of potential reproductive and developmental toxicity perspectives of some newly synthesized food additives on market has been covered. Finally, the identification of the areas relevant for future research has been pointed out indicating that there is more and more information needed to explore the possibility of the implementation of some other materials to be used as food additives.

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