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Antioxidant and antimicrobial activity of extracts obtained from rosemary (*Rosmarinus officinalis*) and vine (*Vitis vinifera*) leaves

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Summary

The antioxidant properties and antimicrobial activity of rosemary extracts were compared to those of vine leaf extracts. The phenolics were quantified by HPLC. Rosemary extracts were stronger in reducing power than vine leaf extracts but possessed weaker superoxide anion radical (O_2^{-}) scavenging capability. The antimicrobial activity was confirmed by the broth microdilution test using minimal inhibitory (MIC) concentrations against gram-positive (*Staphylococcus aureus, Bacillus cereus, Listeria monocytogenes)* and gram-negative bacteria (*Campylobacter jejuni, Salmonella* Infantis*, Escherichia coli* O157:H7). The kinetics of survival was assessed using the broth macrodilution method. The study confirmed the stronger antibacterial activity of rosemary extracts, especially against gram-positive bacteria and *C. jejuni.* The antioxidant and antimicrobial activity of vine leaf extracts did not correlate with their contents of flavan-3-ols and flavonols. For rosemary extracts the absence of a correlation between their activities and the content of carnosic acid suggests that protective efficiency is achieved through interactions among phenolic constituents.

Keywords: rosemary extracts, vine leaf extracts, antioxidant activity, antimicrobial activity

Introduction

Oxidative degradation of lipids and microbial spoilage in foods leads to deterioration of taste and nutritional quality and could have harmful effect on consumers' health. Many studies have confirmed that food phenolics are a promising way to overcome these phenomena. Among the phenolic compounds in extracts from rosemary, the main constituents determined are phenolic diterpenes, such as carnosol, carnosic acid, methyl carnosate and phenolic acids such as rosmarinic and caffeic acid (Cuvelier et al., 1996). Beside its antioxidant activity, the antimicrobial activity of rosemary plants against both gram-positive and gram-negative bacteria was also confirmed (Moreno et al., 2006; Campo et al., 2000; Cushnie and Lamb, 2005). In the recent literature attention has also been focused on the antioxidant activity of vine leaf extracts (Balik et al., 2008; Doshi et al., 2006; Stopka et al., 2008). Extracts from vine leaves are composed of anthocyanins (-3-glucosides and -3-(6-p-coumaroyl)glucosides of delphinidin, cyanidin, petunidin, peonidin and malvinidin), flavonols (3-O-glycosides of quercetin and kaempherol), a hydroxycinnamic acid derivative (trans-caftaric acid) (Monagas et al., 2006), *trans*-resveratrol and its glucoside (trans-piceid) (Balik et al., 2008). Investigations that evaluated antioxidant activity by a combination of different methods and correlated these properties to the antimicrobial activity of rosemary leaf extracts (Moreno et al., 2006; Božin et al., 2007) or vine leaf extracts (Yigit et al., 2009) are scarce. Extracts with similar concentrations of total phenolics may vary remarkably in their antioxidant and antimicrobial activity (Brul and Coote, 1999; Carneiro et al., 2008; Mimica-Dukić and Božin, 2007; Tripoli et al., 2007).

The aim of this study was to provide basic data on the antioxidant and antimicrobial activities of phenolics in both types of extracts (rosemary leaf extracts with phenolic diterpenes as the main constituents and vine leaf extracts containing predominately flavonoid compounds), and to interpret them in terms of extract composition and to predict their usefulness as functional food ingredients.

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Materials and methods

Plant materials

The present study included four *Rosmarinus officinalis* leaf extracts supplied by Vitiva (Markovci, Slovenia) with carnosic acid as the main active phenolic compound (its content was determined as described recently (Terpinc et al., 2009) and expressed as w/w: ROSM 1 (19.7 %), ROSM 2 (70.0 %), ROSM 3 (13.4 %), ROSM 4 (18.8 %)), and five native *Vitis vinifera* L. leaf extracts obtained from the vine varieties Lasin, Maraština, Merlot, Syrah, and Vranac. Fully expanded, green, healthy vine leaves with petioles were collected from the Teskera vineyards, Kijevo, Dalmatia, Croatia at the end of grape ripening (in September). The plant material was air dried in shade at room temperature. The leaf petioles were carefully manually separated and the dry leaves pulverized (3 × 1 min in a high speed grinder) into a powder.

Reagents and solvents

The following reagents were obtained from Merck (Darmstadt, Germany): ethanol (96 %), sodium carbonate, trichloroacetic acid (99.5 %), acetic acid and acetonitrile. Nitroblue tetrazolium (NBT), β-nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), 2-p-iodophenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride (INT) and Folin–Ciocalteu reagent were purchased from Sigma (Sigma–Aldrich GmbH, Steinheim, Germany). Potassium ferricyanide and potassium hydrogen phosphate were obtained from Kemika (Zagreb, Croatia). Ferric trichloride was obtained from Carlo Erba Reagenti (Rodano, Italy). The phosphate buffer was made from disodium hydrogen phosphate purchased from Zorka (Šabac, Croatia) and HPLC standards were purchased from Extrasynthese (Genay, France), Sigma (Milwaukee, USA) and Polyphenols Laboratories (Sandnes, Norway). BacTiter-Glo reagent from Promega Corp. (Madison, WI) was also used. Water was prepared by purification with a Millli-Q-water purification system (Millipore, Bedford, Massachusetts).

Extraction of phenolic compounds from vine leaves

The polyphenolic constituents of vine leaves were extracted using a conventional solvent extraction procedure. Homogenized dry plant material (20 g) was extracted with alcoholic solvent (100 mL) (ethanol/water 80:20, v/v) at 60 °C. The contact time was 60 min. After extraction, samples were filtered with Whatman No. 1 filter paper and the residual tissue washed with solvent (2 × 25 mL). The filtrates were combined into a total extract and dried in a vacuum rotary evaporator at 50 °C. The dry residues were redissolved in 50 % methanol (10 mL) and centrifuged at 5000 rpm for 10 min. The vine leaf extracts obtained were used for spectrophotometric and HPLC measurements.

Determination of total phenolic content

The content of total phenolic compounds in rosemary leaf extracts solubilized in 96 % ethanol and in the solution of vine leaf extracts prepared as described above was determined using a slightly modified method by Gutfinger (1981). A reaction mixture containing extract solution, freshly prepared Folin-Ciocalteu reagent and sodium carbonate solution (20 %) was prepared. After 40 min of incubation the absorbance at 765 nm was measured on a model 8453 Hewlett Packard UV-Visible spectrophotometer (Hewlett Packard, Waldbronn, Germany) with a 1 cm cell. Results are expressed as gallic acid equivalents. The reaction was conducted in triplicate and results were averaged.

HPLC analysis

The polyphenolic compounds in vine leaf extracts were separated on an octadecyl column (Zorbax Eclipse XDB-C18; 4.6x250, 5µ, Agilent) maintained at 25 °C. The vine leaf extract was filtered through 0.45-µm syringe filter and directly injected through a 20 µL fixed loop into a guard C_{18} column. Separation of polyphenols was carried out as follows: a gradient elution consisting of solvent A (water/acetic acid, 98:2, v/v) and solvent B (acetonitrile/acetic acid, 98:2, v/v) was applied at a flow rate of 1.0 mL/min in the sequence: 0 min 9 % A and 8 % B; 18 min 80 % A and 20 % B; 25 min 60 % A and 40 % B; 30 min 55 % A and 45 % B; 40 min 35 % A and 65 % B; 50 min 20 % A and 80 % B; 54 min 20 % A and 80 % B; 57 min 90 % A and 10 % B; 60 min 90 % A and 10 % B. The signal was monitored at 280 nm wavelength. Each sample was injected twice into the chromatographic system. The phenolics were quantified from the areas of their peaks at 280 nm using external standard calibration curves. Flavan-3-ols were calculated as the sum of

(+)-catechin and (-)-epicatechin monomers. Flavonols were calculated as the sum of free quercetin and quercetin derivatives (quercetin-4-glucoside and rutin).

Superoxide anion radical scavenging effectiveness

Measurement of $O_2^{\cdot-}$ scavenging activity of the extracts was based on a method described by Roback and Grygrewski (1988). All reagents were prepared in phosphate buffer (pH 7.4). To a mixture consisting of extract (or ethanol for control), NBT solution and NADH solution, a PMS solution was added. The extracts were added to the reaction mixture in amounts such that a concentration of 5.0 mg/L total phenolics was achieved. After 5 min the absorbance at 560 nm was measured against blank samples (without PMS). All tests were done in duplicate.

Reducing power

The reducing power of extracts was determined according to Juntachote et al. (2006). A solution of extract at different concentrations was mixed with phosphate buffer (pH 6.8) and potassium ferricyanide. Then trichloroacetic acid solution was added. After centrifugation the supernatant was mixed with water and ferric trichloride solution. The absorbance was measured at 740 nm against a blank containing the corresponding solvent instead of extract. Each determination was repeated twice.

Bacterial strains and cultivation conditions

Six bacterial strains: *Bacillus cereus* WSBC-10530 (clinical isolate), *Staphylococcus aureus* ATCC 25923 (clinical isolate), *Escherichia coli* O157:H7 ZMJ 129 (clinical isolate), *Listeria monocytogenes* ZM58 (IHM, Würzburg, Germany), *Campylobacter jejuni* ATCC 33560 (bovine faeces isolate) and *Salmonella* Infantis ZM9 (poultry meat isolate) were used for antibacterial testing. The cultivation/assay media were as described previously (Klančnik et al., 2009). Bacterial cultures for antimicrobial testing were prepared by picking a colony from 24-h-old plates, suspended in an appropriate medium (5 mL) and grown aerobically for 20 h at 37 °C, while *Campylobacter* was grown microaerobically at 42 °C. For antibacterial activity assays each culture (1 mL) was diluted with Tryptone soya broth (TSB) or Müeller-Hinton broth (MHB) medium to 10^5 - 10^6 CFU/mL.

Broth microdilution method

The extracts were diluted to 10 and 15 % (v/v) stock solutions in MHB (Oxoid, Basingstoke, UK) or TSB (Oxoid, Hampshire, UK) when *E. coli* was tested. The bacterial culture (50 µL) in the early stationary phase (ca. 10^6 CFU/mL) was added to the wells of a sterile 96-well microtitre plate containing two-fold serially diluted plant extract stock solutions (50 µL). The concentrations of total phenols ranged from 4.0 - 0.01 mg/mL of growth medium. To indicate respiratory activity the presence of colour was determined after adding INT (10 µL/well, 2 mg/mL, dissolved in water) and incubated for 30 min in the dark (Klančnik et al., 2010). To determine adenosine triphosphate (ATP) activity, the bioluminescence signal was measured by a Microplate Reader (Tecan, Mannedorf/Zurich, Switzerland) after adding BacTiter-Glo™ reagent (100 µL/well) and 5 min incubation in the dark (Klančnik et al., 2009). The minimum inhibitory concentrations (MICs) were tested in triplicate and calculated from the lowest concentration where no metabolic activity was observed (Katalinić et al., 2010).

Broth macrodilution method

The broth macrodilution method was used to follow the kinetics of inactivation. The extracts were added to growth medium (5 mL) to give final concentrations in accordance with the results obtained by the microdilution method. Bacterial growth was followed by taking samples at 0, 3, 6, 9 and 24 h and plating on cultivation media after serial sample dilutions. Control samples were performed without adding the plant extract. The MIC was defined as the lowest concentration of plant extract resulting in a significant decrease (> 90 %) in inoculum viability after 24 h of incubation (Klančnik et al., 2010). All experiments were independently repeated three or more times and the mean log CFU/mL as well as the standard deviations were calculated.

Results and Discussion

As presented in Table 1 the investigated rosemary leaf extracts differed in the content of total phenolics and also in the amount of carnosic acid. The content of total phenolics in rosemary leaf extracts ranged from 99 mg/g to 318 mg/g. Among vine leaves the Merlot and Syrah varieties were the richest in the content of

total phenolics amounting to 23 mg/g (expressed as mg of gallic acid per g of dry grape leaves). Flavan-3-ols were the most abundant in the leaves of the Merlot variety while flavonols were the most abundant in the leaves of the Maraština variety.

Table 1. Contents of the total phenolics, flavan-3-ols and flavonols in rosemary and in vine leaf extracts

Rosemary leaf extract	Total phenolic content (mg/g)[a]	Vine leaf extract	Total phenolic content (mg/g)[b]	Flavan-3-ols (mg/g)[c]	Flavonols (mg/g)[d]
ROSM 1	108 ± 3	Lasin	16.2 ± 0.2	0.05	0.97
ROSM 2	318 ± 1	Maraština	17.3 ± 0.3	0.05	1.53
ROSM 3	92 ± 2	Merlot	22.7 ± 0.3	0.72	0.82
ROSM 4	109 ± 2	Syrah	22.9 ± 0.2	0.01	0.67
		Vranac	18.9 ± 0.4	0.06	0.99

[a]Total phenolic content: determined by the Folin-Ciocalteau assay and expressed as mg of gallic acid per g of ground rosemary extract. [b]Total phenolic content: determined by the Folin-Ciocalteau assay and expressed as mg of gallic acid per g of dry grape leaves. [c]Flavan-3-ols: determined using HPLC and calculated as the sum of (+)-catechin and (-)-epicatechin. [d]Flavonols: determined using HPLC and calculated as the sum of quercetin, quercetin-4-glucoside and rutin.

The effectiveness of the extracts in scavenging $O_2^{\cdot-}$ radical was expressed as the coefficient of superoxide radical scavenging activity (C_{SASA}):

$$C_{SASA} = [1 - A_{s\,560\,nm} / A_{c\,560\,nm}] \times 100\ \% \quad (1)$$

where $A_{s\,560\,nm}$ is the absorbance of the mixture containing the sample, and $A_{c\,560\,nm}$ is the absorbance of the control (without sample). As seen on Fig. 1 vine leaf extracts showed appreciably higher C_{SASA} values than rosemary leaf extracts, indicating that the phenolics in vine leaf extracts have a stronger ability to transfer an electron and/or hydrogen atom to an $O_2^{\cdot-}$ radical than phenolic diterpenes (the main constituents of rosemary leaf extracts). Among the extracts investigated, the extract of the Merlot variety with a C_{SASA} value of (66 ± 2) % exhibited the highest efficiency in scavenging $O_2^{\cdot-}$ radicals.

The reducing power of the extracts was quantitatively expressed as the slope of the lines representing the dependence of A_{740nm} on the concentration of total phenolics in the reaction mixture (not shown) and denoted as C_R. Contrary to the $O_2^{\cdot-}$ scavenging activity assay rosemary leaf extracts showed higher C_R values, ranging to (0.263 ± 0.005) mL/mg for ROSM 1, than vine leaf extracts (Fig. 1). Of the vine leaf extracts the highest efficiency in reducing metal ions with a C_R value amounting to (0.222 ± 0.003) mL/mg was shown by the extract from the Vranac variety.

Although it has been shown by many authors that the antioxidant activity of rosemary extracts is primarily related to the presence of the two phenolic diterpenes carnosic acid and its derivative carnosol (Nogala-Kalucka et al., 2005; Frankel et al., 1996), we can confirm that the extract ROSM 2 with a highly predominant content of carnosic acid had an antioxidant efficiency that was quite comparable or even lower than the effeciencies of rosemary leaf extracts ROSM 1, ROSM 3 and ROSM 4. These extracts, despite their appreciably lower amount of carnosic acid, possessed strong antioxidant activity, probably due to interactions among their various phenolic constituents, thus achieving a synergistic effect. The results reported by Cavero et al. (2005) of the correlation studies between free radical scavenging activity and the concentration of compounds detected in the extracts showed that carnosic acid was the most correlated compound. Almela et al. (2006) have calculated the correlation coefficient between carnosic acid content and the free radical scavenging activity and obtained the value 0.49. However, in our study C_{SASA} and C_R values of vine leaf extracts also did not correlate with the contents of flavan-3-ols and flavonols. Balik et al. (2008) in their investigation correlated free radical scavenging activity and ferric reducing ability with the content of flavanols and determined correlation coefficients 0.681 and 0.0027, respectively.

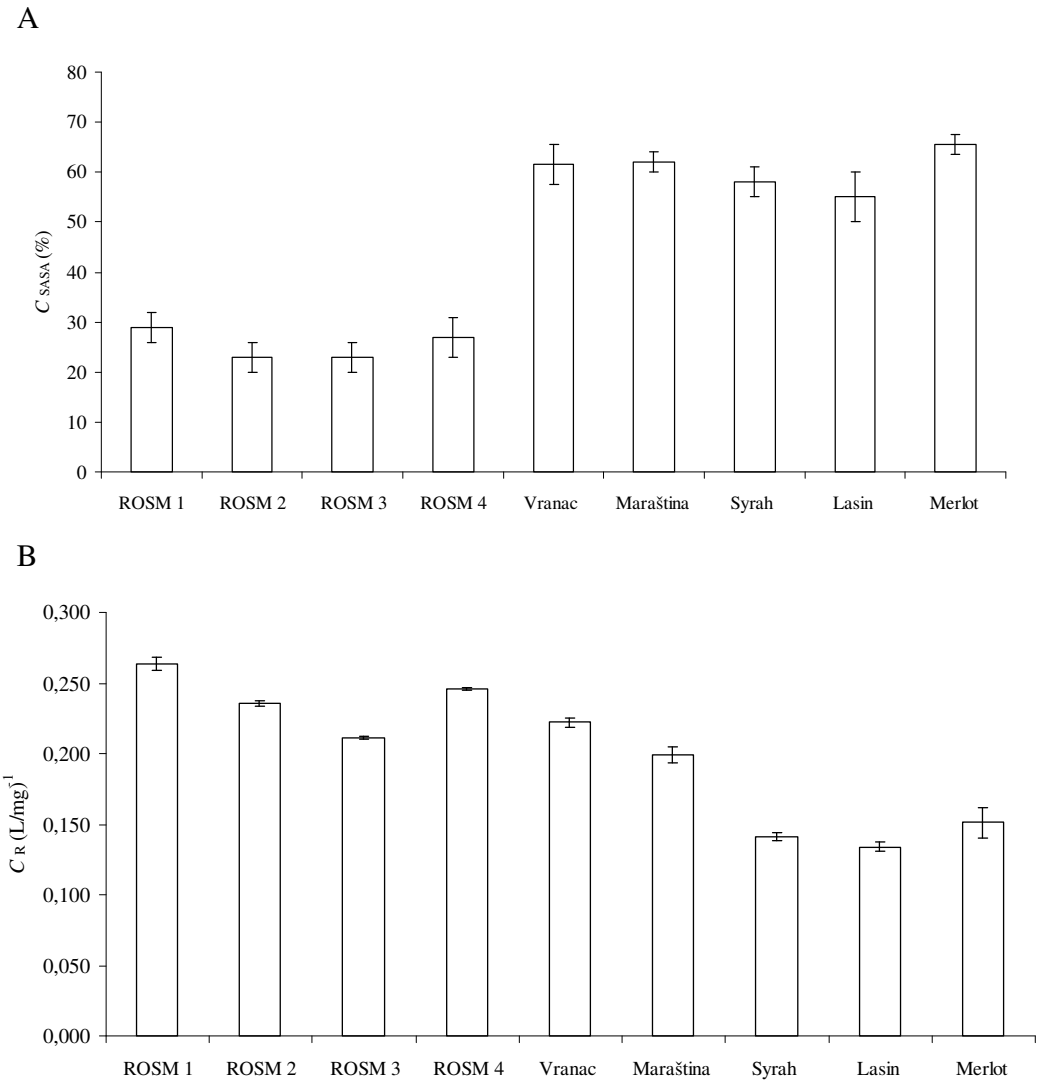


Fig. 1. The coefficient of superoxide radical scavenging activity (C_{SASA}) at a concentration of total phenolics in the reaction mixture of 5.0 mg/L (A) and the reducing power (C_R) (B) for rosemary and vine leaf extracts Values are expressed as mean ± standard deviation.

The antibacterial effect of extracts was confirmed by end-point analysis using the broth microdilution method. MICs, expressed in mg of total phenols per mL of growth medium, ranged between 0.02 - 1.42 mg/mL for different organisms and extracts (Table 2). Rosemary leaf extracts showed a stronger antimicrobial activity in the range of 0.02 to 0.06 mg/mL for gram-positive and to 1.03 mg/mL for gram-negative bacteria, which could be explained by the presence of carnosic acid as the main bioactive antimicrobial compound in rosemary extracts (Moreno et al., 2006). As we previously reported, carnosic acid is more efficient against gram-positive bacteria than rosmarinic acid (Klančnik et al., 2009). From these results, it is obvious that the rosemary leaf extracts have different modes of action and exhibited stronger biological activity against gram-positive bacteria. The best antibacterial activity determined by the broth microdilution method was seen against *B. cereus* and the lowest activity against *S.* Infantis. The main reason for differences in bacterial

susceptibility could be due to the outer membrane surrounding the cell wall in gram-negative bacteria and the periplasmatic space containing enzymes which are capable of breaking down foreign molecules introduced from outside (Vaara, 1992). *Campylobacter* appears to be more sensitive than other gram-negative bacteria, i.e. *S.* Infantis and *E. coli* O157:H7. However, vine leaf extracts were much less efficient against all the tested bacteria. No significant differences were found in the susceptibility of gram-positive and gram-negative bacteria to vine leaf extracts, also indicating their activity against usually more resistant food-borne bacteria like *Salmonella* and *Escherichia*. Differences in the efficiency of phenolics from different varieties were seen against all test organisms; the results of antimicrobial activity for vine leaf extracts ranged between 0.38 and 1.37 mg/mL (Table 2). Among vine leaf extracts, the extract of the Vranac variety was the most efficient against gram-positive bacteria.

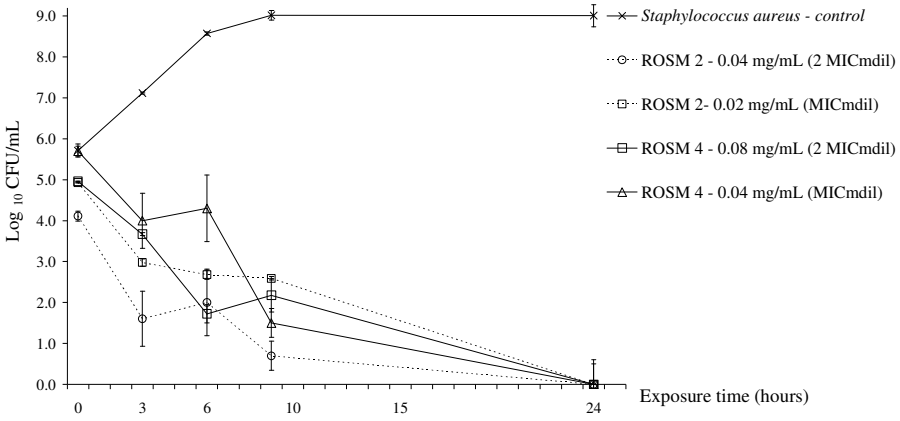
Table 2. Antimicrobial activity of rosemary and vine leaf extracts

	MIC (mg/mL)[a]					
Extract	*B. cereus* WSBC–10530	*S. aureus* ATCC25923	*L. monocytogenes* ZM58	*C. jejuni* ATCC 33560	*E. coli* O157:H7 ZM129	*S.* Infantis ZM9
ROSM 1	0.02 ± 0.005	0.02 ± 0.005	0.02 ± 0.005	0.09 ± 0.01	0.69 ± 0.15	0.69 ± 0.15
ROSM 2	0.02 ± 0.005	0.02 ± 0.005	0.02 ± 0.005	0.04 ± 0.01	0.51± 0.10	1.03 ± 0.20
ROSM 3	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.15 ± 0.03	0.59 ± 0.15	0.59 ± 0.15
ROSM 4	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.09 ± 0.02	0.70 ± 0.15	0.70 ± 0.15
Lasin	0.65 ± 0.16	0.98 ± 0.16	1.30 ± 0.16	0.65 ± 0.16	0.98 ± 0.16	1.30 ± 0.16
Maraština	0.69 ± 0.18	1.04 ± 0.18	1.42 ± 0.18	0.69 ± 0.18	1.04 ± 0.18	1.04 ± 0.18
Merlot	0.45 ± 0.23	0.91± 0.23	0.91± 0.23	0.91 ± 0.23	1.36 ± 0.23	1.36 ± 0.23
Syrah	0.46 ± 0.23	0.91 ± 0.23	0.91± 0.23	0.91 ± 0.23	1.37 ± 0.23	1.37 ± 0.23
Vranac	0.38 ± 0.19	0.76 ± 0.19	0.76 ± 0.19	0.76 ± 0.19	1.13 ± 0.19	1.13 ± 0.19

[a]MIC: minimal inhibitory concentration expressed in mg of total phenols per mL of growth medium in the broth microdilution test

To study the kinetics of inactivation of rosemary leaf extracts during a 24 h treatment, we used the broth macrodilution method. The growth, survival and death curves for *S. aureus, B. cereus* and *C. jejuni* at various concentrations of phenolics are shown in Fig. 2. These examples clearly demonstrate that MIC values determined by the microdilution method for the ROSM 2 and ROSM 4 extracts were really the concentrations of phenolics that inhibited bacterial growth. As visible for gram-positive *S. aureus*, inhibitory concentrations were mostly simultaneously bactericidal. The results provide an example where all the concentrations tested inhibited growth of *B. cereus*, but culturable cell reduction was only minor. For *C. jejuni* it was confirmed a bacterial but not bactericidal effect, using the macrodilution method at a concentration determined as the MIC by the microdilution (MIC mdil) method.

A



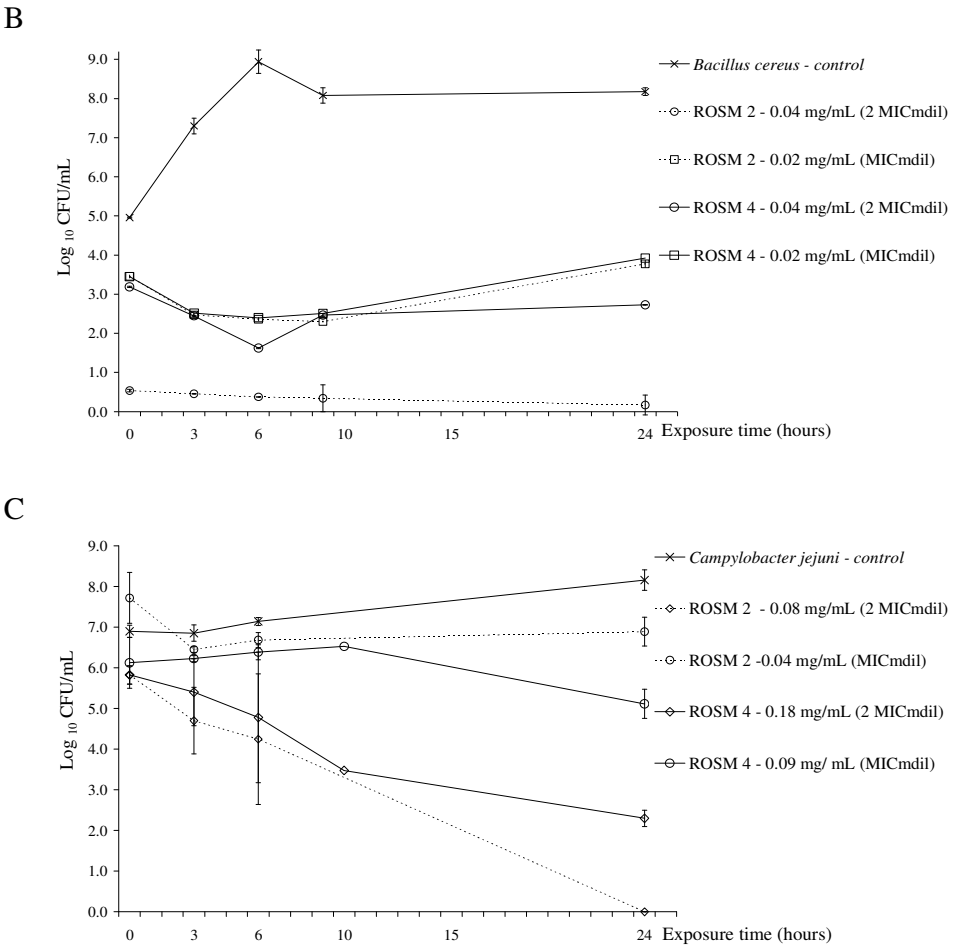


Fig. 2. *Staphylococcus aureus* (A), *Bacillus cereus* (B) and *Campylobacter jejuni* (C) survival and death curves on exposure to rosemary leaf extracts ROSM 2 and ROSM 4. Values are expressed as mean ± standard deviation

Conclusions

The results indicate the protective activity of rosemary and vine leaf extracts and support their use as natural antimicrobial and antioxidant agents. Vine leaf extracts containing predominately flavonoids were weaker in superoxide radical scavenging and stronger in reducing power than rosemary extracts containing predominately phenolic diterpenes. The antioxidant and antimicrobial activities of the investigated extracts did not correlate with the contents of their main constituents, suggesting that activity is achieved through interactions among the various phenolic constituents. Differences in activity against gram-positive and gram-negative bacteria were not found for phenolics from vine leaves.

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