Comparative phenolic fingerprint and LC-ESI+QTOF-MS composition of oregano and rosemary hydrophilic extracts in relation to their antibacterial effect

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ABSTRACT

Rosemary (*Rosmarinus officinalis*) and oregano (*Origanum vulgare*) are known aromatic plants used as spice, with good flavoring, preservative, antioxidant and antibacterial activity. Beside their known terpenoid content responsible for the antibacterial activity, the water-soluble compounds (phenolic derivatives) are of high interest not only for their antioxidant activity but as a good alternative or as a hydrophilic new antibacterial solution. Two hydrophilic extracts from each plant were obtained (15% plant in hot water) and the phytochemicals were fingerprinted by UV-Vis and FTIR spectrometry and quantified. The total phenolic content was higher in case of oregano (54.2 mg GAE/g DW) comparing to rosemary (15.35 mg GAE/g dw). By LC-ESI$^+$QTOF-MS analysis there were identified mainly, in both extracts, flavonoid and diterpene derivatives, mainly carnosol, carnosic acid, rosmarinic acid, kaempferol 3-O-glucuronide. Other flavonoid glucuronides were more specific to one or the other plant, e.g. luteolin 3'-(4''-acetylglucuronide) for rosemary and apigenin 7-O-glucuronide for oregano. Water favorized increased extraction of flavonoid derivatives and soluble diterpenes, but not non-soluble terpenes. The antibacterial activity of both extracts was tested against *B.cereus*, *L. monocytogenes*, *Salmonella*, *S. aureus* and *E.coli*. Both oregano and rosemary extracts showed a slight antibacterial activity, which can be related to the low concentration of terpenoids, known to have the most important antibacterial activity in these plants. Nevertheless, the antibacterial activity seems to be strain dependent, *Bacillus cereus* being the most sensitive bacterial strain comparing with the other four bacteria, the oregano extract having a slightly superior activity comparing to the rosemary extract.

Keywords: *antibacterial activity, bioactive phenols, disk diffusion method, oregano, rosemary.*

INTRODUCTION

Aromatic herbs and food spices represent a valuable reservoir of bioactive phytochemicals with antiviral, antibacterial or antioxidant properties, even anticarcinogenic potential (Proestos *et al*., 2005, 2006; Shan *et al*., 2007; Tajkarima *et al*., 2010). Only 5–15% from thousands of species of aromatic herbs and spices are screened for their composition in relation to their potential health benefits (Yáñez *et al*., 2013). Polyphenols are secondary metabolites (Leopoldini *et al*., 2011; Shahidi and Naczk, 2003) with specific roles in

the plant protection against pathogens and UV radiation (Wojdilo, 2007; Santos-Buelga *et al*., 2012; Tajkarimia *et al*., 2010; Vauzour *et al*., 2012). They act in food as flavours and preservatives with antioxidant power, essential in our diet, a key for their quality and application to the pharmaceutical and food industries.

Rosemary (*Rosmarinus officinalis*) and oregano (*Origanum vulgare*) are known for their flavoring properties, mainly due to volatiles (terpenoids) (Oniga, 2007) but also to their phenolic compounds (phenolic acids and flavonoids) (Chun *et al*., 2005; Bai *et al*., 2010; Yáñez *et al*., 2013). Carnosic acid and carnosol, diterpenes, are key antioxidant constituents having concentrations of 0.1% and 7% in rosemary with medicinal properties (Tounekti, 2012; Verma, 2012). Several studies reported the phenolic profile of *O. vulgarae* and *R. officinalis* (Hossain *et al*., 2011; Martins *et al*., 2014; Miron *et al.,* 2011; Vallverdú-Queralt *et al*., 2014), the different phenolic profiles being due to plant subspecies, geographic origin and different extraction protocols (Proestos *et al.*, 2005).

Oregano is used worldwide both as spice and crude drug, which is mainly provided by species of *Origanum* genus. The terpenoid composition of the essential oils of 17 different populations of *Origanum vulgare L.* collected from wild populations were recently reported by GC/MS (Verma, 2010), identifying 51 components representing up to 99.94% of the total oil. The GC analysis of essential oil components revealed mainly differences between different species (*Origanum vulgare* subsp*. hirtum* and *Origanum majorana*, e.g. carvacrol and thymol, in the first one, while in marjoram major are terpinene-4-ol, gamma-terpinene and terpinolene (Novak, 2003).

Miron *et al.* (2011) studied the methanolic extract of oregano leafs originating from Romania and determined more phenolic acids (syringic acid, protocatechuic acid, chlorogenic acid, caffeic acid, rosmarinic acid and homovanilic acid) than flavonoids (luteolin, naringenin and apigenin). Martins *et al*. (2014) identified more flavonoids like glycosides of apigenin, myricetin, quercetin, luteolin, luteolin glucuronide, naringenin and erydictiol in oregano leaf decoct, beside protocatechuic acid, but also caffeoylquinic acid. By accelerated solvent extraction of rosemary and oregano leafs, rosmarinic, caffeic and gallic acids as phenolic acids were identified as well as glucosides of luteolin and apigenin (Afonso *et al*.**,** 2013).

The phenolic profile of rosemary and oregano ethanol extracts purified by SPE was represented by gallic acid, vanillic acid, caffeic acid, protocathechuic acid, homovanilic acid, coumaric acid, rosmarinic acid, ferulic acid and dicaffeoylquinic acid. The flavonoids apigenin, kaempferol and quercetin were detected as glycosylated derivatives (glucuronides, rutinosides or hexosides). Only hesperetin, apigenin and naringenin were identified in free form (Vallverdú-Queralt *et al*., 2014).

The antimicrobial effect of phenolic compounds (phenolic acids, flavonoids) is related to their cytotoxicity and ability to degrade cell membranes, due to the sulfhydryl groups which inhibit nutrients availability to the bacterial growth, as determined for pomegranate (Alzoreky *et al*., 2009). However, there are only a few reports that studied the relationship between the phenolic profile of these two aromatic plants and antibacterial activity, related to the extraction type. The sensitivity of bacterial strains towards water extracts of oregano and rosemary is related to the affinity of phenolics to bacterial membrane (Pistelli and Giorgi, 2012). G negative bacteria have a hydrophobic surface structure which exclude hydrophilic molecules, e.g. phenolic acids or flavonoids (Alakomi *et al*. 2007; Cueva *et al*. 2010; Terpson, 2009), while G positive bacteria have a peptidoglycan wall, accepting better hydrophilic molecules (Alakomi *et al.* 2007; Cueva *et al.* 2010; Terpson, 2009).

The present study aims to investigate comparatively the phenolic composition of rosemary (*Rosmarinus officinalis*) and oregano (*Origanum vulgare*) hydrophilic extracts by advanced LC-ESI$^+$QTOF-MS analysis, UV-Vis and infrared spectrometry and to relate the composition to their antibacterial activity.

MATERIALS AND METHODS

Plant extracts. Dried oregano *(Origanum vulgare*) and rosemary (*Rosmarinus officinalis*) were extracted in hot water acidified with 1% HCl, to a final concentration of 15% (w/w) plant in water. The extracts were sonicated 30 min, centrifuged and filtered, in order to obtain a clear extract. The extracts were kept in deep freezer until analysis.

Standards and reagents. Methanol, acetonitrile and acetic acid used for HPLC grade were obtained from Merck (Darmastadt, Germany). The phenolic standards (gallic acid, protocatehuic acid, gentisic acid, chlorogenic acid, catechin, caffeic acid, p-coumaric acid, rutin, naringin, ferulic acid, miricetin, tilirosid, quercetin, trans-cinamic acid, kaempherol) used for calibrations were purchased from Sigma Aldrich (St. Louis, MO, USA).

UV-Vis Spectrometry. The UV-Vis spectra was recorded (700-200 nm) for each plant extract using a Jasco V 530 spectrofotometer. There were identified the maxima wavelengths specific to polyphenols in the range 280-330 nm, to flavonoids and quinones resulting by polyphenols oxidation, between 390-420 nm.

FTIR Spectrometry. Oregano and rosemary water extracts were analyzed using a Shimatsu FTIR spectrometer with Horizontal Attenuated Total Reflection (HATR). The Fourier Transform Infrared spectrum (FTIR) of each extract was recorded in the MIR region, from 4000 to 900 cm^{-1}, and then the fingerprint region (1000-1750 cm^{-1}) was selected for data analysis.

Total phenolic content. The total phenolic concentration was determined by Folin-Ciocâlteu method using gallic acid as standard for the calibration curve (R^2 = 0.9945). The results were expressed as gallic acid equivalents (GAE eq).

HPLC-DAD and LC-ESI$^+$QTOF-MS analysis. Each rosemary and oregano extract were diluted (1:1) with methanol and aliquots of 5 µl from each sample were subjected to HPLC coupled with photodiode array detection (HPLC-DAD) and liquid chromatography coupled with mass spectrometry (LC-ESI$^+$QTOF-MS) analysis, using a Thermo Scientific HPLC UltiMate 3000 system equipped with a quaternary pump delivery system Dionex and MS detection by a Bruker Daltonics MaXis Impact device. The plant metabolites were separated on the Thermo Scientific Acclaim C_{18} column (3µm, 2.1 X 50 mm) at 40°C. The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The flow rate was set at 0.5 $mL \cdot min^{-1}$. The gradient elution initial conditions were 5% B with linear gradient to 15% B from 0 to 3 min, followed by linear gradient to 50% B at 6 min, linear gradient to 95% B at 9 min, isocratic on 95% B for 6 min and then returned to initial conditions at 15 min and kept isocractic on 5%B for 5 min. The DAD detector was set at 270 nm. The separated molecules were introduced directly into the mass spectrometer by electrospray. The mass range was set between 50-1000m/z, using a nebulizing gas pressure set at 2 bar, the drying gas flow at 8 L/min, the drying gas temp at 180 ºC. Before each separation run, a calibration solution of sodium formate was injected. The control of the instrument and the data processing were done using TofControl 3.2 and Data Analysis 4.1 (Bruker Daltonics), respectively.

Antimicrobial assays (disk diffusion method). The selective growth mediums and the necessary supplements used for the antimicrobial assay were: MYP (CM 0929, Oxoid-UK and egg yolk emulsion supplement-X073, Lab M Limited), agar base Baird-Parker (CM 0275, Oxoid-UK and egg yolk emulsion with tellurite supplement-SR 00540, Oxoid-UK), Oxford (CM 0856, Oxoid-UK with selective supplement for *Listeria* 107006, Merck Milipore-Germania), TBX (CM 0945, Oxoid-UK) and XLD (IVD CM 0469 Oxoid-UK).

Five different bacterial strains were used: *Bacillus cereus*-ATCC 11778 (MediMark, Franţa), *Stafilococcus aureus*-ATCC 6538P (MediMark, Franţa), *Lysteria monocytogenes*-ATCC 35152 (Liofilchem, Italia), *Escherichia coli*-ATCC 25922 (Microbiologics, USA) and *Salmonella typhimurium*-ATCC 14028 (Microbiologics, USA).

The antimicrobial assay was performed following the methodology proposed by Roessner in 2013 with slight modifications. Volumes of 100 µl of bacterial suspension were spread on the Petri dishes containing selective growth medium for all the specified bacterial strains. After 30 minutes, sterile paper disks (6mm) were placed on the surface of the inoculated medium, well spaced out. A volume of 25 µl from each sample was added on the disk and then incubated overnight at 30^0C and 37^0C. Gentamicin was used as positive reference standard in order to determine the sensitivity of each bacterial strain. The antimicrobial effect represents the clear zone (no growth) around the disk in the plate.

RESULTS AND DISCUSSIONS

Spectral fingerprinting by UV-Vis spectra

The UV-Vis spectra of both extracts were similar (data not shown) and characterized by three peaks of absorption in UV region, at 280 nm, 330 nm and 400 nm. These absorption

bands are related to the presence of phenolic acids derivatives, flavonoids and/or chinone derivatives (Robbins, 2003; Bohm, 1998). The intensities corresponding to rosemary extracts were slightly superior, comparing to oregano, but not statistically significant.

FTIR analysis

Fig. 1 represents the comparative FTIR fingerprint of the same rosemary and oregano extracts. The fingerprint in the region 600-1750 cm^{-1} was generally similar for both extracts, the main differences being observed in peak intensities, the rosemary extract having higher intensities of peaks from 1112 to 1591 cm^{-1}.

Differences between rosemary and oregano extract fingerprints were observed for bands 1070 cm^{-1}, 1112 cm^{-1} (stretching vibration C-O); 1253 cm^{-1} (carbonyl groups from esters and C-O, O-H from phenols); 1392 cm^{-1} (amides C-O); 1516 cm^{-1}, 1591 cm^{-1} (aromatic domain, N-H bending vibrations, C-C stretching alkenes) (Socaciu *et al*., 2009). Also, a weak band at 815 cm^{-1} corresponding to aromatic domain was observed, more intense for rosemary extract, in agreement to reported data (Adinew *et al*., 2014).

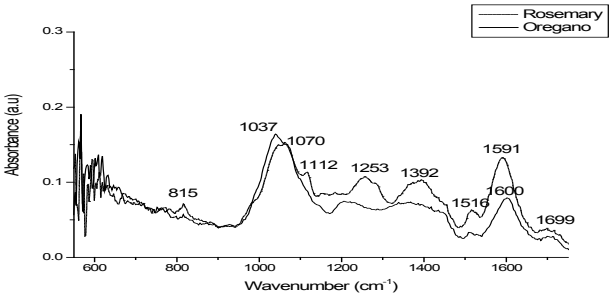

Fig. 1. Comparative FTIR spectra of oregano and rosemary hydrophilic extracts in the fingerprint region (600-1700 cm^{-1}).

Total phenolic content

The rosemary extract had a total phenolic content of 15.35 mg GAE/g dry plant, similar to results obtained by Afonso *et al. (*2013) (16.67 mg/g DW), while oregano had a mean value of 54.25 mg GAE/g dry plant, similar to the results obtained by Chun *et al*., 2005 and Spiridon *et al*., 2011 (39.4 mg GAE/g DW respectively 67.8 mg/g DW).

The phenolic profile determined by HPLC-DAD

Fig. 2 represents the HPLC-DAD chromatogram of the rosemary extract, registered at 280 and 330 nm. Two major peaks were identified, both

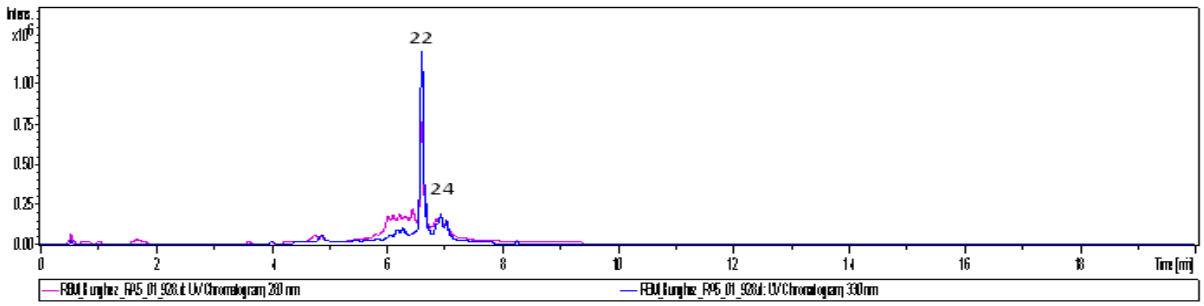

Fig. 2. HPLC-DAD chromatogram of rosemary extract registered at 280 and 330 nm.
Peaks: 22- Kaempferol 3-O-glucuronide, 24- Luteolin 3'-(3''-acetylglucuronide).

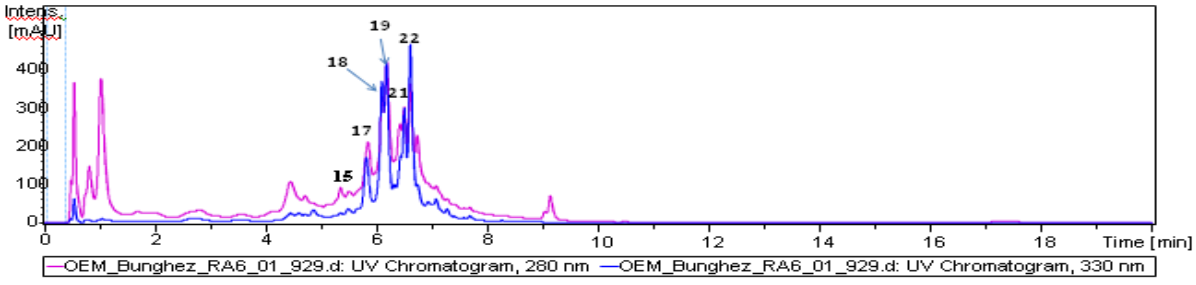

Fig. 3. HPLC-DAD chromatogram of oregano extract registered at 280 and 330 nm. Peaks: 15-p-coumaric acid, 17- quercetin-3'-glucuronide, 18-6,8-dihydroxykaemferol, 19- kaempferol 3-glucuronide, 21- apigenin 7-O-glucuronide, 22-carnosic acid derivative.

corresponding to flavonoids. Peak 22 corresponds to kaempferol 3-O-glucuronide and peak 24 to luteolin 3'-(3''-acetylglucuronide), confirmed by mass spectrometry.

Fig. 3 represents the HPLC-DAD chromatogram of oregano extract, registered at 280 and 330 nm. Different peaks were identified, corresponding to phenolic acids (t_R=1-2 min and 4.2-5.8 min) and peaks 17-22, corresponding to flavonoids (17- quercetin-3'-glucuronide, 18- 6,8-dihydroxykaemferol, 19- kaempferol 3-glucuronide, 21- apigenin 7-O-glucuronide, 22-carnosic acid derivative).

Phenolic profile and molecular identification by LC-ESI$^+$QTOF-MS analysis

Fig. 4 and Fig. 5 shows the base peak chromatograms obtained by LC-ESI$^+$QTOF-MS analysis of rosemary and oregano extracts. The molecules were identified by their m/z values released as protonated ions $[M+H]^+$. The identification was based also on comparisons with similar data found in phenol explorer (www.phenol-explorer.eu) and HMDB database (www.hmdb.ca). Data are presented in Tab. 1.

The main peaks in rosemary extract (Fig. 4) correspond to kaempferol 3-O-glucuronide (19), carnosol (39), luteolin 3'-(3''-acetylglucuronides) (21-22) and carnosic acid (41) while rosmarinic acid (26) and *p*-coumaric acid ethyl ester represented around 3 % (see Tab.1).

The major peaks in oregano extract corresponded to carnosol (56) and carnosic acid (58), corresponding to more than 14% % of peak area fractions, followed by 6, 8-dihydroxykaempferol (4.53% from area fraction), while rosmarinic acid represented 1.74% from area fraction. Carnosol, carnosic acid, rosmarinic acid and kaempferol 3-O-glucuronide represented the most common compounds identified in both rosemary and oregano extracts (see Tab.1). Other flavonoid glucuronides were more specific to one or the other plant. In both extracts, the majority of the detected compounds belong to flavonoids family. Among terpenoid compunds found in these plants, the hydrophilic extract contained only diterpenes like carnosic acid derivatives, the other terpenes being insoluble in water. A similar study (Suk-Kim *et al.,* 2011) using hot water extracts obtained as well, a flavonoid rich extract.

Similar results were obtained by Hossain *et al*. (2011) who identified glycosylated luteolin in oregano and rosemary extracted by accelerated solvent extraction, as well glycosylated apigenin and rosmarinic acid in oregano. Martins *et al*., (2014) and Vallverdú-Queralt *et al*. (2014) identified also flavonoids in rosemary (kaempferol, luteolin and quercetin in glycosilated forms) and oregano (quercetin, kaempferol, glycosilated apigenin and rosmarinic acid). Miron *et al. (*2011) identified apigenin 7-O-glucuronide, quercetin

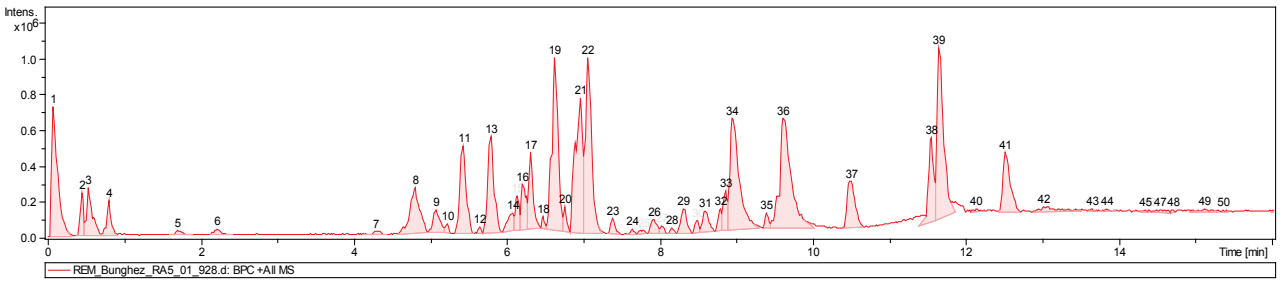

Fig. 4. Base peak chromatogram of rosemary extract by LC-ESI$^+$QTOF-MS.

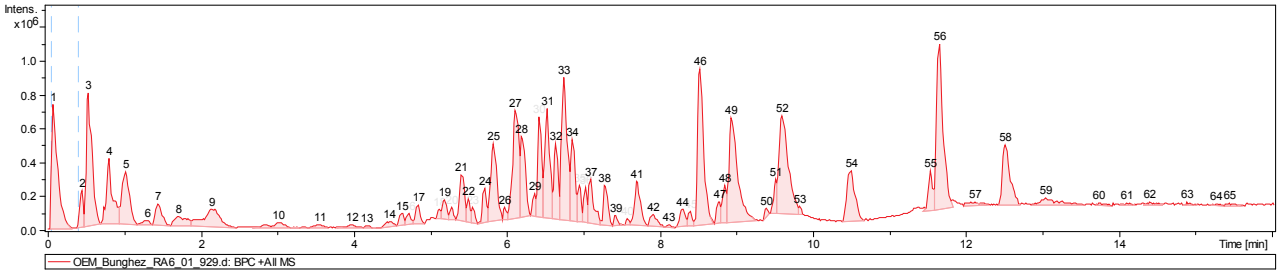

Fig. 5. Base peak chromatogram of oregano extract by LC-ESI$^+$QTOF-MS.

and rosmarinic acid in oregano hydrophilic extract originating from Romania.

Antibacterial activity

The antibacterial screening was assessed on three Gram positive (*Bacillus cereus, Stafilococcus aureus, Lysteria monocytogenes)* and two Gram negative (*Escherichia coli* and *Salmonella typhimurium*) bacterial strains. No antibacterial activity was observed against *Salmonella typhimurium, E.coli, Stafilococcus aureus* and *Lysteria monocytogenes*, either for rosemary or oregano extracts.

Meanwhile, a slight antibacterial activity of both rosemary and oregano extracts was observed against *Bacillus cereus* (G+), with an inhibition diameter of 0.75 mm and 0.5 mm for 37.5 µg oregano and rosemary extract, respectively. Our findings are in agreement with Shan *et al*. (2007) who reported a superior antibacterial activity for oregano, comparing to rosemary. Proestos *et al.* (2005, 2006) suggested also a slight antimicrobial activity against *Bacillus cereus* for rosemary and no antibacterial activity against *Salmonella typhimurium, Stafilococcus aureus* and *Lysteria monocytogenes*.

The low bacterial inhibition induced by hydrophilic extracts of rosemary and oregano can be due to the low concentration of terpenoids which have the most important antibacterial activity in these plants. The antibacterial activity of the major flavonoid derivatives found in the hydrophilic extracts seems to be inferior in the tested conditions, beside their low diffusion rate in the agar medium. The antibacterial activity seems to be strain dependent, *Bacillus cereus* being the most sensitive bacterial strain, oregano extract having a slight superior activity against rosemary extract.

CONCLUSION

The spectral fingerprint of rosemary and oregano hydrophilic extracts, as determined by UV-Vis and FTIR analysis, showed similar compositions, containing mainly flavonoid and phenolic acid derivatives. The intensity of FTIR peaks in the fingerprint area (1000-1750 cm^{-1}) was more intense for rosemary than oregano, especially at 1253 cm^{-1} (corresponding to esters' carbonyl groups and phenol groups), at 1392 cm^{-1} (amides) and 1516 - 1591 cm^{-1} (aromatic and N-H bending vibrations).

Tab.1. Phenolic compounds identified in rosemary and oregano extracts by LC-ESI^+QTOF-MS

Peak no	Molecular identification	t_R (min)	$[M+H]^+$ m/z	Area fraction (%)
	Rosemary			
8	*p*-coumaric acid ethyl ester	4.8	193	3.28
16	kaempferol 3-glucuronide	6.2	463	1.71
17	quercetin 3-O-glucuronide	6.3	479	2.56
19	kaempferol 3-O-glucuronide	6.6	463	8.03
21, 22	luteolin 3'-(3''-acetylglucuronide) luteolin 3'-(4''-acetylglucuronide)	7	505	7.62; 8.03
26	rosmarinic acid	7.8	361	3.06
39	carnosol	11.7	331	7.8
41	carnosic acid	12.6	333	5.6
	Oregano			
25	quercetin-3'-glucuronide	5.8	481	3.14
27	6,8-dihydroxykaemferol	6.1	319	4.53
28	kaempferol 3-glucuronide	6.2	463	2.63
31, 32	apigenin 7-O-glucuronide	6.5, 6.7	447	3.55; 2.12
41	rosmarinic acid	7.7	361	1.74
56	carnosol	11.7	331	8.8
58	carnosic acid	12.6	333	5.9

Significant differences were noticed between the total phenolic concentrations of the two dried plants in hot water (15%), namely 15.35 mg GAE/g dry matter of rosemary and 54.25 mg GAE eq./g dry matter of oregano, a more rich source of phenolics.

The LC-ESI$^+$QTOF-MS analysis shows fingerprints of different complexity, for rosemary and oregano. In both extracts, the majority of the detected compounds belong to flavonoids family. Carnosol, carnosic acid, rosmarinic acid, kaempferol 3-O-glucuronide represented the most common compounds identified in both rosemary and oregano extracts (see Tab.1). Other flavonoid glucuronides were more specific to one or the other plant, e.g. luteolin 3'-(4''-acetylglucuronide) for rosemary and apigenin 7-O-glucuronide for oregano. Out of terpenoids known to be found in these plants, the hydrophilic extract contained only diterpenes like carnosic acid derivatives, the other terpenes being insoluble in water.

Both oregano and rosemary hydrophilic extracts showed a slight antibacterial activity, which can be due to the low concentration of terpenoids known to have the most important antibacterial activity in these plants. Nevertheless, the antibacterial activity seems to be strain dependent, *Bacillus cereus* being the most sensitive bacterial strain comparing with the other four bacteria, the oregano extract having a slightly superior activity comparing to the rosemary extract.

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