

Article

## ***Rosmarinus Officinalis* Leaves as a Natural Source of Bioactive Compounds**

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External Editor: Chang Won Choi

Received: 22 July 2014; in revised form: 23 October 2014 / Accepted: 31 October 2014 /

Published: 10 November 2014

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**Abstract:** In an extensive search for bioactive compounds from plant sources, the composition of different extracts of rosemary leaves collected from different geographical zones of Serbia was studied. The qualitative and quantitative characterization of 20 rosemary (*Rosmarinus officinalis*) samples, obtained by microwave-assisted extraction (MAE), was determined by high performance liquid chromatography coupled to electrospray quadrupole-time of flight mass spectrometry (HPLC–ESI-QTOF-MS). The high mass accuracy and true isotopic pattern in both MS and MS/MS spectra provided by the QTOF-MS analyzer enabled the characterization of a wide range of phenolic compounds in the extracts, including flavonoids, phenolic diterpenes and abietan-type triterpenoids, among others. According to the data compiled, rosemary samples from Sokobanja presented the highest levels in flavonoids and other compounds such as carnosol, rosmaridiphenol, rosmadial, rosmarinic acid, and carnosic acid. On the other hand, higher contents in triterpenes were found in the extracts of rosemary from Gložan (Vojvodina).

**Keywords:** *Rosmarinus officinalis*; phenolic compounds; HPLC; QTOF-MS

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## 1. Introduction

Rosemary (*Rosmarinus officinalis*, Lamiaceae) is a shrubby herb that grows wild in the Mediterranean basin. Today, this plant is cultivated worldwide due to its diverse uses as a common household culinary spice for flavoring. Furthermore, rosemary extracts have been widely used as a preservative in the food industry due to their inherent high antioxidant activity. In addition, it has been used as a medicinal herb for centuries, due to significant activities against many illnesses. In this sense, many major biological properties have been attributed to this plant, mainly hepatoprotective [1], antimicrobial [2,3], antithrombotic [4], diuretic [5], antidiabetic [6], anti-inflammatory [7], antioxidant [8], and anticancer [9–12]. Accordingly, it has been previously reported that rosemary extracts and their isolated components show inhibitory effects on the growth of breast, liver, prostate, lung, and leukemia cancer cells [13,14].

These potent biological activities have been attributed to the presence of many bioactive compounds in its composition. The major families found in rosemary are phenolic diterpenes including: carnosic acid, carnosol or rosmanol; flavonoids such as genkwanin, cirsimaritin or homoplantaginin; and triterpenes such as ursolic acid [15–17].

A type of compound present in this matrix that is currently receiving much attention are phenolic diterpenes due to a variety of health-promoting properties, such as antimicrobial [18], anti-inflammatory [19], neuroprotective [20], anti-oxidant [21], and anticancer properties [14]. In particular, carnosic acid and carnosol are two of the main antioxidant compounds present in this herb, which have been reported to have broad anticancer properties in several cell-line models, including prostate, breast, leukemia and others [12,13,22].

Another group of promising secondary plant metabolites found in rosemary is triterpenes, which present marked bioactivity, especially to treat cancer by several modes of action, among other activities. In the last decade, many studies have shown other effects that justify this expectation. In this sense, compounds such as oleanolic, betulinic, and ursolic acids, which are pentacyclic triterpenoids with anticancer, antihyperlipemic, hepatoprotective, and anti-inflammatory properties, should be highlighted [23,24].

Bioactive compounds such as flavonoids, phenolic diterpenes and triterpenes from plant sources have been traditionally extracted by a conventional solid-liquid extraction (SLE). Nevertheless, this extraction technique presents several disadvantages, mainly that it is an arduous time-consuming process, requires a high consumption of solvents, and in some cases provides low recovery. For that reason, in recent years new promising extraction methods are arising, which introduce some form of additional energy in order to facilitate the transfer of solutes from the sample to solvent in a faster process [25]. In that sense, microwave-assisted extraction (MAE) represents an alternative to conventional SLE, while improving the speed and efficiency of the extraction process and reducing the consumption of solvents [26]. MAE has been successfully used for the extraction of phenolic compounds from various plant materials, and in the case of rosemary microwaves have also been used for obtaining essential oil in steam distillation [27–31].

Therefore, rosemary represents an exceptionally rich source of different bioactive compounds. For this reason, the objective of this work was to study the composition of different rosemary leaves harvested in various geographical zones in Serbia in order to explore the presence of bioactive compounds. In this sense, the present study demonstrates that different extracts of this plant could be used as natural sources of several bioactive compounds, especially carnosol, carnosic acid and triterpenes, which could be useful ingredients in complementary alternative medicine and nutritional supplements, as well as natural antioxidants for food preservation.

## 2. Results and Discussion

The rosemary leaves harvested at different sites in Serbia were extracted by MAE and subsequently analyzed by high performance liquid chromatography coupled to electrospray quadrupole-time of flight mass spectrometry (HPLC–ESI–QTOF–MS). The main compounds were identified using a QTOF mass analyzer, which has proven to be a valuable detection system for characterizing phenolic compounds, since it provides mass accuracy and true isotopic pattern in both MS and MS/MS spectra. Afterwards, the compounds characterized were quantified in the extracts using commercialized standards whenever available or compounds with structure similarities.

### 2.1. Qualitative Characterization of Bioactive Compounds Present in Rosemary-Leaf Extracts

Figure 1 shows the Base Peak Chromatograms (BPC) of extracts of rosemary harvested in Sokobanja and Gložan, namely rosemary sample 2 and 10 (RS 2 and 10), respectively, as an example of the composition found in the different rosemary extracts.

**Figure 1.** (a) Base Peak Chromatogram (BPC) of rosemary sample RS 2 (Sokobanja); (b) BPC of rosemary sample RS 10 (Gložan).

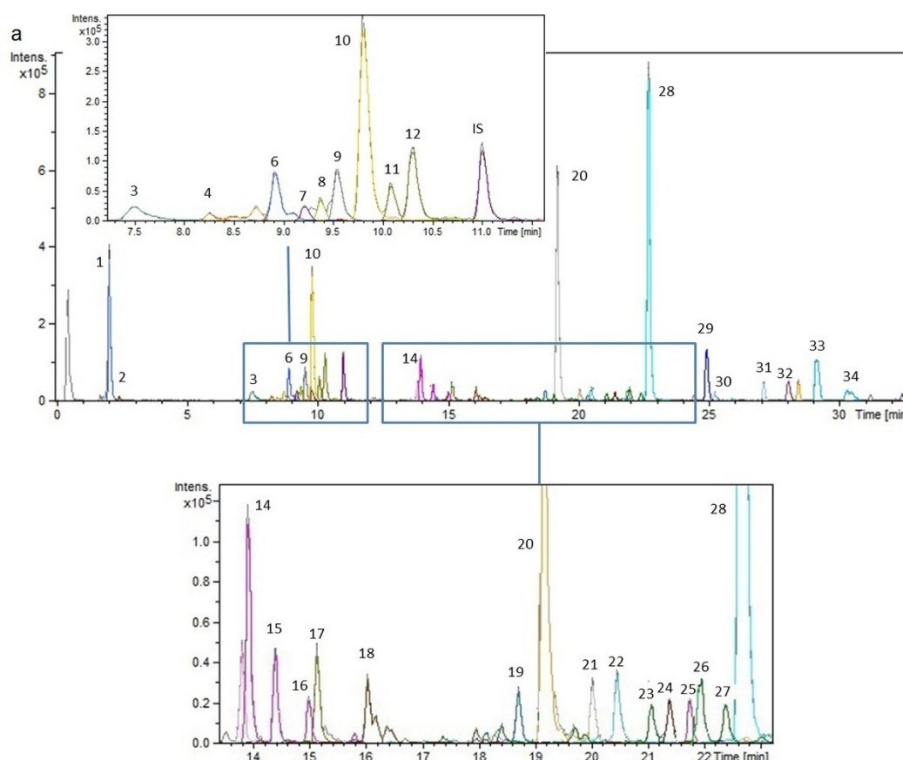
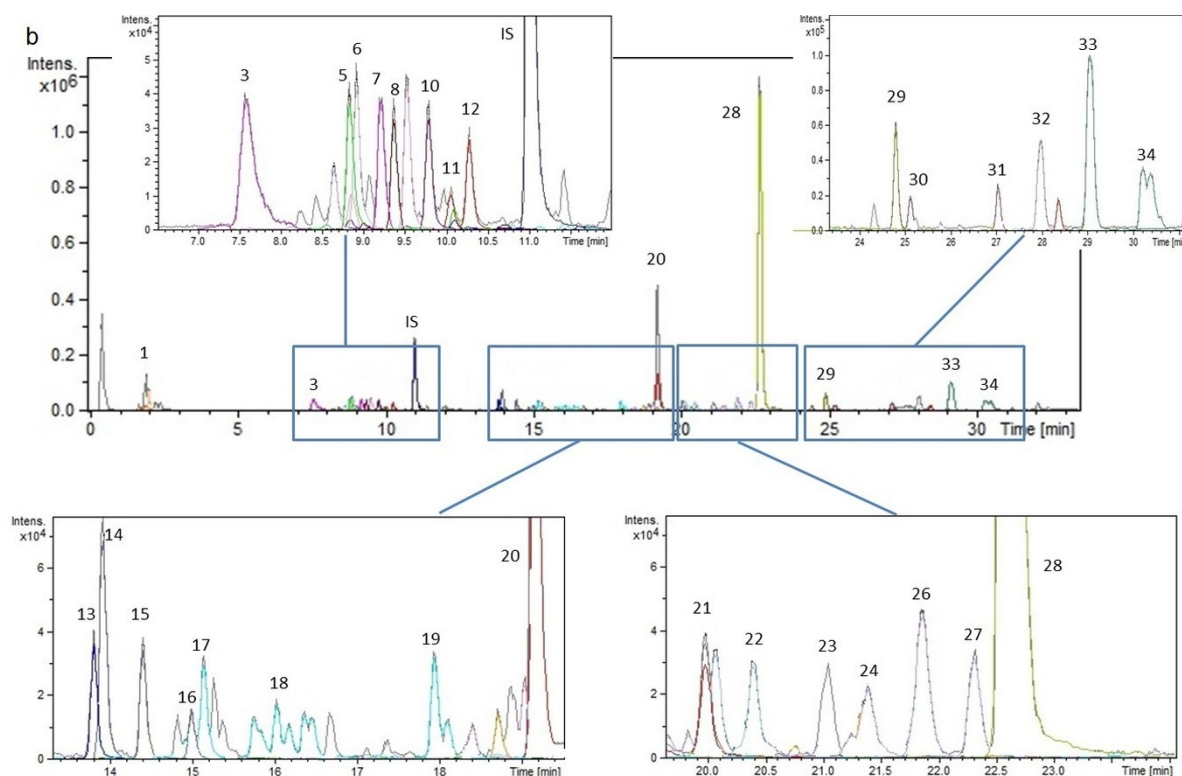


Figure 1. Cont.



The analysis of the extracts revealed the presence of 34 compounds, mainly flavonoids and phenolic diterpenes, although some organic acids and abietan-type triterpenoids were also found. The detected compounds were characterized by comparison of retention time and the MS and MS/MS spectra provided by the Q-TOF mass analyzer with those of authentic standards when available. The remaining identifications were performed by interpretation of the MS and MS/MS spectra of the detected compounds combined by the data from the literature and data bases.

Table 1 summarizes the MS data of the compounds identified, numbered according to their elution order, together with their retention times, theoretical  $m/z$ , molecular formulas, and main fragments derived from MS/MS analysis.

Most of the compounds detected in the extracts have been previously identified in rosemary leaves, such as carnosic acid, carnosol, rosmanol, its isomers epiisorosmanol and epirosmanol, as well as other derivative compounds such as methylcarnosate, epirosmanolmethylether, and 5,6,7,10-tetrahydro-7-hydroxyrosmaquinone.

Carnosic acid and carnosol were identified by comparing retention times and fragmentation patterns of those of authentic standards. Rosmanol and its isomers ( $m/z$  345), epiisorosmanol and epirosmanol, were identified on the basis of their retention times and MS/MS spectra. In these MS/MS spectra the fragments with  $m/z$  301 and 283 were observed, which correspond to the ions  $[M-H-CO_2]^-$  and  $[M-H-CO_2-H_2O]^-$ , although the first one was present only in rosmanol. These data agree with the literature concerning these compounds [32,33]. Moreover, methylcarnosate exhibited a characteristic fragmentation pattern with two ions, corresponding to the loss of  $CO_2$  and a subsequent loss of  $CH_3$  ( $m/z$  301 and 286, respectively) [34]. Furthermore, the MS/MS spectra acquired for epirosmanol methylether was previously reported in the literature [35]. The compound 5,6,7,10-tetrahydro-7-

hydroxyrosmaquinone showed ion fragments corresponding to the losses of water and the isopropyl group ( $m/z$  283 and 358, respectively), data in agreement with other authors [36].

Other compounds typically found in rosemary, e.g. rosmarinic acid, rosmadial, rosmaridiphenol or the flavonoids homoplantagin, cirsimaritin, genkwanin, galocatechin, nepetrin, hesperidin, 6-hydroxyluteolin-7-glucoside, luteolin-3'-glucuronide, and two isomers of luteolin-3'-*O*-(*O*-acetyl)- $\beta$ -D-glucuronide, were also detected in these extracts [15,17,32,37–39].

Rosmadial presented a fragmentation pattern corresponding to the losses of ethylene and propyl moieties, showing fragment ions of  $m/z$  315 and 299, respectively [34]. For the flavonoid cirsimaritin the fragmentation pattern presented two major fragment ions at  $m/z$  298 and 283, which are formed by two subsequent losses of methyl groups from the precursor ion [34]. The same losses were also observed for rosmaridiphenol, resulting in the fragment ion at  $m/z$  285. Respect to galocatechin, the fragmentation pattern presented ions at  $m/z$  97 and 225, which are consistent with data found in literature and data bases [34]. The fragment ions found for nepetrin and 6-hydroxyluteolin-7-glucoside at  $m/z$  315 and 301 respectively, were attributable to the loss of a glucose moiety. Similar results were found for hesperidin, which presented a major fragment ion at  $m/z$  301 due to the loss of rutinoside [35,40]. The compound luteolin-3'-glucuronide presented a major fragment at  $m/z$  285 from the loss of glucuronic acid [34].

Two peaks with  $m/z$  503 were detected at retention times of 10.07 and 10.28 min corresponding to isomers of luteolin-3'-*O*-(*O*-acetyl)- $\beta$ -D-glucuronide. The first isomer could be attributed to luteolin-3'-*O*-(2''-*O*-acetyl)- $\beta$ -D-glucuronide due to its fragmentation pattern, which presented fragment ions at  $m/z$  285 and 399 corresponding to  $[M-H-C_8H_{10}O_7]^-$  and  $[M-H-C_3H_4O_4]^-$ . Nevertheless, for the second isomer, it was not possible to characterize their identity because the isomers luteolin-3'-*O*-(3''-*O*-acetyl)- $\beta$ -D-glucuronide and luteolin-3'-*O*-(4''-*O*-acetyl)- $\beta$ -D-glucuronide presented the same fragmentation pattern and both of them have been previously found in rosemary. In this case, the MS/MS analysis revealed two main fragment ions at  $m/z$  443 and 285 corresponding to the loss of acetyl and acetyl-glucuronide moieties, respectively.

Furthermore, the analysis of the rosemary-leaf extracts showed the presence of triterpenes anemosapogenin, micromeric acid, betulinic acid and ursolic acid, which were previously described in the literature for this plant matrix [7,41–43].

Other compounds detected in the extract have been described in different plants belonging to the Lamiaceae family, such as quinic acid, syringic acid, rosmarinic acid-3-*O*-glucoside, [9]-shogaol and the triterpenic acids asiatic, benthamic, and augustic acids [44–51]. The MS/MS analysis of rosmarinic acid-3-*O*-glucoside showed the major fragments at  $m/z$  477, 359 and 323 corresponding to  $[M-H-COO]^-$ ,  $[M-H-C_6H_{10}O_5]^-$ , and  $[M-H-C_9H_{10}O_5]^-$  [52]. Lastly, two different isomers of [9]-shogaol were also characterized with the same fragmentation patterns, possessing two fragment ions for  $[M-H-OCH_3]^-$  and  $[M-H-C_{10}H_{19}]^-$  ( $m/z$  287 and 179, respectively).

**Table 1.** Compounds characterized in rosemary-leaf extracts.

Peak	Retention Time (min)	Theoretical <i>m/z</i>	Molecular Formula	Fragments	Proposed Compound
1	2.06	191.0561	C <sub>7</sub> H <sub>12</sub> O <sub>6</sub>	93.0338 (3.7), 127.0423 (10.2)	Quinic acid
2	2.45	197.0455	C <sub>9</sub> H <sub>10</sub> O <sub>5</sub>	135.0731 (100.0), 179.0516 (57.9)	Siringic acid
3	7.48	305.0666	C <sub>15</sub> H <sub>14</sub> O <sub>7</sub>	96.9595 (47.1), 225.1178 (100.0)	Gallocatechin
4	8.43	463.0882	C <sub>21</sub> H <sub>20</sub> O <sub>12</sub>	301.0414 (63.3)	6-Hydroxyluteolin-7-glucoside
5	8.85	521.1300	C <sub>24</sub> H <sub>26</sub> O <sub>13</sub>	323.0774 (68.7), 359.0801 (53.3), 477.1052 (100.0)	Rosmarinic acid-3- <i>O</i> -glucoside
6	8.90	477.1038	C <sub>22</sub> H <sub>22</sub> O <sub>12</sub>	315.0528 (36.5)	Nepetrin
7	9.20	609.1824	C <sub>28</sub> H <sub>34</sub> O <sub>15</sub>	301.0732 (100.0)	Hesperidin
8	9.36	461.1089	C <sub>22</sub> H <sub>22</sub> O <sub>11</sub>	161.0294 (32.8), 283.0258 (100.0), 297.0408 (14.3)	Homoplantaginin
9	9.53	461.0725	C <sub>21</sub> H <sub>18</sub> O <sub>12</sub>	285.0417 (100.0)	Luteolin-3'-glucuronide
10	9.79	359.0772	C <sub>18</sub> H <sub>16</sub> O <sub>8</sub>	123.0445 (19.9), 161.0244 (100.0), 179.0357 (29.6), 197.0463 (12.7)	Rosmarinic acid
11	10.07	503.0831	C <sub>23</sub> H <sub>20</sub> O <sub>13</sub>	285.0370 (29.1), 399.0737 (100.0)	Luteolin 3'- <i>O</i> -( <i>O</i> -acetyl)-β-D-glucuronide Isomer I
12	10.28	503.0831	C <sub>23</sub> H <sub>20</sub> O <sub>13</sub>	285.0418 (100.0), 443.0654 (20.0)	Luteolin 3'- <i>O</i> -( <i>O</i> -acetyl)-β-D-glucuronide Isomer II
13	13.81	313.0717	C <sub>17</sub> H <sub>14</sub> O <sub>6</sub>	283.0272 (100.0), 298.0503 (85.4)	Cirsimaritin
14	13.92	345.1707	C <sub>20</sub> H <sub>26</sub> O <sub>5</sub>	283.1718 (49.4), 301.1833 (100.0)	Rosmanol
15	14.40	345.1707	C <sub>20</sub> H <sub>26</sub> O <sub>5</sub>	283.1713 (48.0)	Epiisorosmanol
16	15.00	345.1707	C <sub>20</sub> H <sub>26</sub> O <sub>5</sub>	283.1712 (32.6)	Epirosmanol
17	15.14	283.0611	C <sub>16</sub> H <sub>12</sub> O <sub>5</sub>	268.0401 (100.0)	Genkwanin
18	16.04	487.3428	C <sub>30</sub> H <sub>48</sub> O <sub>5</sub>	–	Asiatic acid
19	18.69	359.1863	C <sub>21</sub> H <sub>28</sub> O <sub>5</sub>	283.1734 (35.2), 329.3651 (21.6)	Epirosmanol methyl ether
20	19.15	329.1758	C <sub>20</sub> H <sub>26</sub> O <sub>4</sub>	285.1885 (100.0)	Carnosol
21	20.00	329.1758	C <sub>20</sub> H <sub>26</sub> O <sub>4</sub>	285.1887 (100.0)	Carnosol isomer
22	20.32	343.1550	C <sub>20</sub> H <sub>24</sub> O <sub>5</sub>	299.1644 (12.9), 315.1634 (24.1)	Rosmadial
23	21.04	471.3479	C <sub>30</sub> H <sub>48</sub> O <sub>4</sub>	–	Anemosapogenin
24	21.35	315.1965	C <sub>20</sub> H <sub>28</sub> O <sub>3</sub>	285.1877 (42.8)	Rosmaridiphenol
25	21.83	301.1809	C <sub>19</sub> H <sub>26</sub> O <sub>3</sub>	258.6483 (42.6), 283.6915 (25.7)	2,3,4,4a,10,10a-Hexahidro-5,6-dihydroxy-1,1-dimethyl-7-(1-methylethyl)-9(1H)-Phenantrenone
26	21.91	471.3479	C <sub>30</sub> H <sub>48</sub> O <sub>4</sub>	–	Benthamic acid
27	22.35	471.3479	C <sub>30</sub> H <sub>48</sub> O <sub>4</sub>	–	Augustic acid
28	22.63	331.1914	C <sub>20</sub> H <sub>28</sub> O <sub>4</sub>	287.2078 (100.0)	Carnosic acid
29	24.84	345.2071	C <sub>21</sub> H <sub>30</sub> O <sub>4</sub>	286.1999 (76.1), 301.2239 (100.0)	12-metoxycarnosic acid
30	25.14	317.2122	C <sub>20</sub> H <sub>30</sub> O <sub>3</sub>	179.8164 (23.8), 287.2076 (60.5)	[9]-Shogaol isomer
31	27.05	317.2122	C <sub>20</sub> H <sub>30</sub> O <sub>3</sub>	179.7812 (19.7), 287.2079 (54.8)	[9]-Shogaol
32	27.99	453.3347	C <sub>30</sub> H <sub>46</sub> O <sub>3</sub>	–	Micromeric acid
33	29.05	455.3530	C <sub>30</sub> H <sub>48</sub> O <sub>3</sub>	–	Betulinic acid
34	30.25	455.3530	C <sub>30</sub> H <sub>48</sub> O <sub>3</sub>	–	Ursolic acid

## 2.2. Quantitative Characterization of the Compounds Present in Rosemary-Leaf Extracts

Standard calibration graphs of carnosol, carnosic acid, ursolic acid, rosmarinic acid, genkwanin, luteolin-7-O-glucoside, homoplantagin, epigallocatechin, quinic acid, syringic acid and neohesperidin were prepared using luteolin at a concentration of 5 ppm as an internal standard. The proposed method was validated with the sensitivity and precision parameters. Thus, Table 2 presents the analytical parameters: limits of detection (LODs), and quantification (LOQs), calibration range, calibration equations, and regression coefficient ( $R^2$ ). All the calibration curves showed good linearity for the analytes studied. LODs and LOQs for individual compounds in standard solutions were also calculated as  $S/N = 3$  and  $S/N = 10$ , respectively, where  $S/N$  is the signal-to-noise ratio.

**Table 2.** Analytical parameters of the proposed method.

Analyte	LOD ( $\mu\text{g/mL}$ )	LOQ ( $\mu\text{g/mL}$ )	Calibration Range ( $\mu\text{g/mL}$ )	Calibration Equations	$R^2$
Carnosic acid	0.018	0.06	LOQ – 70	$y = 94.036x + 0.0152$	0.9907
Carnosol	0.019	0.06	LOQ – 25	$y = 84.476x + 0.3537$	0.989
Ursolic acid	0.07	0.22	LOQ – 50	$y = 10^6x + 56483$	0.9763
Rosmarinic acid	0.035	0.09	LOQ – 15	$y = 40352x - 0.0142$	0.9909
Genkwanin	0.014	0.04	LOQ – 15	$y = 147.37x - 0.0399$	0.9803
Luteolin-7-O-glucoside	0.08	0.25	LOQ – 15	$y = 14.22x + 0.088$	0.9818
Homoplantagin	0.016	0.05	LOQ – 5	$y = 62.358x + 0.0308$	0.9912
Epigallocatechin	0.08	0.26	LOQ – 15	$y = 12.584x - 0.0429$	0.9887
Neohesperidin	0.03	0.1	LOQ – 15	$y = 17.158x - 0.0018$	0.9882
Quinic acid	0.08	0.3	LOQ – 15	$y = 15.223x - 0.0244$	0.9918
Syringic acid	0.24	0.8	LOQ – 15	$y = 1.8012x + 0.0022$	0.9909

Repeatability of the proposed method was measured as the relative standard deviation (RSD, %) in terms of concentration. Different rosemary-leaf extracts with a composition which covered all the compounds detected in the extracts were injected several times ( $n = 6$ ) on the same day (intraday precision) and 3 times on 2 consecutive days (interday precision,  $n = 12$ ). Intraday repeatability of the method developed for all the analytes was from 0.15% to 4.57%, whereas the interday repeatability ranged from 0.23% to 4.69%.

The compound concentrations were determined using the corrected area of each individual compound (three replicates) and by interpolation in the corresponding calibration curve. Carnosic acid, carnosol, ursolic acid, rosmarinic acid, genkwanin, homoplantagin, quinic acid and syringic acid were quantified by the calibration curves obtained from their respective commercial standards. The remaining compounds were tentatively quantified on the basis of calibration curves from other compounds with structural similarities. The carnosic acid standard curve was used for the quantification of methylcarnosate and 5,6,7-10-tetrahydro-7-hydroxyrosmariquinone. Rosmanol, its isomers epiisorosmanol and epirosmanol, epirosmanol methylether, rosmadial, and rosmaridiphenol were quantified using the carnosol calibration curve. Ursolic acid was used to quantify asiatic, augustic, benthamic, micromeric, and betulinic acids, as well as anemosapogenin. The compounds rosmarinic acid-3-O-glucoside and the isomers of [9]-shogaol were expressed as rosmarinic acid. Luteolin-7-O-glucoside calibration curve was used to estimate the content of several compounds, in particular 6-hydroxyluteolin-7-glucoside,

nepetrin, luteolin-3'-glucuronide, and the isomers luteolin-3'-*O*-(*O*-acetyl)- $\beta$ -D-glucuronide. Finally, the genkwanin standard was used for cirsimaritin quantification, gallic catechin was expressed as epigallocatechin, and lastly neohesperidin was used to estimate the hesperidin content. It should be taken into account that the response of the standards can differ from that of the analytes found in the extract, and consequently the quantification of these compounds is only an estimation of their actual concentrations. Nevertheless, it can be considered a useful approximation to quantify the compounds in rosemary-leaf extracts. Table 3 summarizes the quantitative results found by HPLC–ESI-QTOF-MS for the studied extracts.

The quantitative results showed that the most abundant compounds in the rosemary-leaf extracts were phenolic diterpenes and the triterpene acids, specifically carnosic acid, carnosol, micromeric acid, betulinic acid, and ursolic acid. Moreover, quinic and syringic acids were found in high quantities in some extracts, as well as some flavonoids, such as nepetrin and gallic catechin.

As mentioned above, the presence of quinic and syringic acids were found in only some extracts, although, in the extracts where they were detected, these compounds were found at high concentrations. In particular, quinic acid was detected in the extracts RS 1, 2, 3, 4, 10, 16, 18 and 19, and syringic acid in RS 1, 3 and 18.

The extracts RS 2, 3, and 4, collected in Sokobanja, showed the highest content for most of the compounds detected: flavonoids such as homoplantagin, gallic catechin, 6-hydroxyluteolin-7-glucoside, genkwanin, cirsimaritin, luteolin-3'-glucuronide or the isomers luteolin-3'-*O*-(*O*-acetyl)- $\beta$ -D-glucuronide; together with other compounds such as quinic acid, rosmarinic acid, rosmanol, asiatic acid, rosmaridiphenol, 2,3,4,4a,10,10a-hexahydro-5,6-dihydroxy-1,1-dimethyl-7-(1-methylethyl)-9(1H)-phenanthrene, carnosol, rosmadial, carnosic acid, 12-methoxycarnosic acid, and [9]-shogaol. Moreover, the RS 4 showed very high contents of micromeric acid, betulinic acid and ursolic acid. Simultaneously with those samples harvested in Sokobanja, the RS 1 collected in Kikinda showed a high concentration of syringic acid, luteolin-3'-glucuronide and rosmanol.

On the other hand, other compounds were found in the highest concentration in the extract RS 10 harvested in the province of Gložan. These compounds were nepitrin (together with the extract RS 9, harvested in Silbaš) and the triterpenes anemosapogenin, benthamic acid, augustic acid, betulinic acid, micromeric acid, and ursolic acid. The triterpene content was remarkably high in this extract compared with the rest of rosemary extracts and proved to be a very rich source of these types of compounds, which have proved to have anti-inflammatory and anticancer activities [24].

Moreover, the extract RS 12 coming from Bačko Petrovo Selo, presented high concentrations of gallic catechin, homoplantagin, cirsimaritin, carnosol, and the highest contents of rosmarinic acid-3-*O*-glucoside, epirosmanol, and epiisorosmanol.

Additionally, rosemary harvested in Stara Planina (RS 18) showed high contents in syringic acid, homoplantagin, rosmarinic acid, the isomers luteolin-3'-*O*-(*O*-acetyl)- $\beta$ -D-glucuronide and carnosol, together with the extracts of Sokobanja samples, as described previously.

On the other hand, the maximum contents of epirosmanol methylether and hesperidin were found in extracts collected from different provinces, specifically in extract RS 14 and 20 harvested in Rumenka and Niš, respectively.



**Table 3.** Concentrations of compounds in rosemary-leaf extracts. (A) Extracts RS 1–6; (B) Extracts RS 7–13; (C) Extracts RS 14–20. Value =  $X \pm SD$ , ND: non-detected, <LQ: below the limit of quantification.

(A)							
Rt (min)	Compound	RS 1	RS 2	RS 3	RS 4	RS 5	RS 6
2.06	Quinic acid	121 ± 2	128 ± 6	154 ± 8	72 ± 5	ND	ND
2.45	Siringic acid	300 ± 20	ND	250 ± 30	ND	ND	ND
7.48	Gallocatechin	9.0 ± 0.6	11.1 ± 0.5	10.6 ± 0.3	31 ± 1	8.5 ± 0.2	4.8 ± 0.4
8.43	6-Hydroxyluteolin 7-glucoside	ND	0.81 ± 0.02	0.71 ± 0.05	ND	ND	ND
8.85	Rosmarinic acid-3- <i>O</i> -glucoside	ND	ND	ND	ND	6.14 ± 0.08	7.9 ± 0.7
8.90	Nepetrin	9.9 ± 0.1	10.0 ± 0.5	10.3 ± 0.1	3.22 ± 0.08	ND	ND
9.20	Hesperidin	2.2 ± 0.1	2.6 ± 0.1	2.8 ± 0.2	3.2 ± 0.2	1.88 ± 0.05	1.9 ± 0.2
9.36	Homoplantagin	1.28 ± 0.06	1.51 ± 0.03	1.71 ± 0.10	1.4 ± 0.1	0.57 ± 0.03	0.40 ± 0.03
9.53	Luteolin-3'-glucuronide	10.5 ± 0.3	9.2 ± 0.4	10.5 ± 0.5	6.2 ± 0.3	0.90 ± 0.02	0.68 ± 0.06
9.79	Rosmarinic acid	15.3 ± 0.5	25 ± 1	24.3 ± 0.5	6.3 ± 0.1	9.9 ± 0.6	5.6 ± 0.2
10.07	Luteolin 3'- <i>O</i> -( <i>O</i> -acetyl)-β-D-glucuronide Isomer I	4.5 ± 0.6	5.5 ± 0.2	6.8 ± 0.3	0.42 ± 0.01	ND	ND
10.28	Luteolin 3'- <i>O</i> -( <i>O</i> -acetyl)-β-D-glucuronide Isomer II	15 ± 1	17.9 ± 0.8	19.3 ± 0.6	4.8 ± 0.3	<LQ	0.3 ± 0.1
13.81	Cirsimaritin	0.47 ± 0.06	0.58 ± 0.02	0.70 ± 0.08	0.82 ± 0.09	0.32 ± 0.02	0.19 ± 0.01
13.92	Rosmanol	2.00 ± 0.04	1.49 ± 0.03	2.48 ± 0.09	1.69 ± 0.07	0.352 ± 0.006	0.173 ± 0.005
14.40	Epiisorosmanol	0.426 ± 0.010	0.95 ± 0.05	0.90 ± 0.03	1.1 ± 0.2	0.82 ± 0.05	0.19 ± 0.03
15.00	Epirosmanol	0.23 ± 0.01	0.41 ± 0.03	0.419 ± 0.002	0.6 ± 0.1	0.271 ± 0.003	<LQ
15.14	Genkwanin	0.44 ± 0.01	0.64 ± 0.03	0.75 ± 0.04	0.70 ± 0.02	0.234 ± 0.003	0.17 ± 0.02
16.04	Asiatic acid	ND	1.65 ± 0.08	3.3 ± 0.4	1.75 ± 0.05	0.907 ± 0.009	ND
18.69	Epirosmanol methyl ether	0.158 ± 0.005	0.70 ± 0.01	0.83 ± 0.07	0.62 ± 0.08	1.16 ± 0.03	0.159 ± 0.001
19.15	Carnosol	12 ± 1	22.1 ± 0.6	22 ± 1	18.8 ± 0.8	14.05 ± 0.02	5.3 ± 0.1
20.00	Carnosol isomer	0.75 ± 0.07	0.75 ± 0.10	0.80 ± 0.07	1.00 ± 0.03	0.17 ± 0.04	0.19 ± 0.01
20.32	Rosmadiol	0.30 ± 0.04	0.23 ± 0.02	0.298 ± 0.006	0.32 ± 0.02	<LQ	ND
21.04	Anemosapogenin	ND	0.457 ± 0.002	1.8 ± 0.1	2.9 ± 0.6	3.5 ± 0.2	ND

Table 3. Cont.

(A)							
Rt (min)	Compound	RS 1	RS 2	RS 3	RS 4	RS 5	RS 6
21.35	Rosmaridiphenol	0.256 ± 0.001	0.62 ± 0.02	0.49 ± 0.02	0.35 ± 0.02	0.18 ± 0.01	ND
21.83	2,3,4,4a,10,10a-hexahidro-5,6-dihydroxy-1,1-dimethyl-7-(1-methylethyl)-9(1H)-Phenantrenone	0.05 ± 0.02	0.24 ± 0.01	0.53 ± 0.01	0.49 ± 0.10	0.50 ± 0.01	ND
21.91	Benthamic acid	ND	3.7 ± 0.5	5.1292 ± 0.0002	6.1 ± 0.4	6.6 ± 0.2	ND
22.35	Augustic acid	ND	1.68 ± 0.05	2.0 ± 0.4	3.6 ± 0.1	3.6 ± 0.3	ND
22.63	Carnosic acid	24 ± 2	17.2 ± 0.8	19 ± 1	25 ± 1	2.9 ± 0.2	3.2 ± 0.5
24.84	12-metoxo-carnosic acid	2.9 ± 0.1	3.8 ± 0.1	4.0 ± 0.2	3.7 ± 0.3	0.64 ± 0.03	0.084 ± 0.005
25.14	[9]-Shogaol isomer	1.03 ± 0.02	1.41 ± 0.06	1.43 ± 0.04	1.7 ± 0.1	0.68 ± 0.01	ND
27.05	[9]-Shogaol	1.87 ± 0.08	3.4 ± 0.3	2.91 ± 0.07	1.58 ± 0.10	0.45 ± 0.02	ND
27.99	Micromeric acid	1.2 ± 0.2	8 ± 1	7.7 ± 1.0	33 ± 1	7.0 ± 0.7	ND
29.05	Betulinic acid	7.8 ± 0.6	77 ± 1	26 ± 1	70 ± 2	47 ± 2	ND
30.25	Ursolic acid	1.715 ± 0.008	21.9 ± 0.1	23 ± 1	40 ± 1	8.1 ± 0.3	ND

(B)								
Rt (min)	Compound	RS 7	RS 8	RS 9	RS 10	RS 11	RS 12	RS 13
2.06	Quinic acid	ND	ND	ND	12.97 ± 0.01	ND	ND	ND
2.45	Siringic acid	ND	ND	ND	ND	ND	ND	ND
7.48	Gallocatechin	5.1 ± 0.5	4.4 ± 0.4	4.0 ± 0.4	7.7 ± 0.5	10.2 ± 0.2	15.7 ± 0.2	ND
8.43	6-Hydroxyluteolin 7-glucoside	ND	ND	ND	ND	ND	ND	ND
8.85	Rosmarinic acid-3-O-glucoside	10.1 ± 0.6	6.7 ± 0.5	9.5 ± 0.5	0.99 ± 0.01	17.4 ± 0.1	27 ± 1	1.10 ± 0.08
8.90	Nepetrin	ND	ND	50 ± 1	57 ± 1	ND	ND	0.270 ± 0.006
9.20	Hesperidin	1.92 ± 0.07	1.35 ± 0.10	1.56 ± 0.09	2.15 ± 0.02	2.7 ± 0.2	4.2 ± 0.1	ND
9.36	Homoplantagin	0.66 ± 0.06	0.50 ± 0.03	0.49 ± 0.03	0.71 ± 0.03	0.71 ± 0.05	1.6 ± 0.2	0.45 ± 0.03
9.53	Luteolin-3'-glucuronide	1.31 ± 0.08	1.79 ± 0.05	0.44 ± 0.05	1.39 ± 0.08	2.6 ± 0.2	5.29 ± 0.09	0.11 ± 0.03

Table 3. Cont.

(B)								
Rt (min)	Compound	RS 7	RS 8	RS 9	RS 10	RS 11	RS 12	RS 13
9.79	Rosmarinic acid	5.0 ± 0.6	7.0 ± 0.6	6.3 ± 0.4	0.855 ± 0.003	12.5 ± 0.1	20.57 ± 0.04	5.4 ± 0.3
10.07	Luteolin 3'-O-(O-acetyl)-β-D-glucuronide Isomer I	ND	ND	ND	<LQ	<LQ	ND	ND
10.28	Luteolin 3'-O-(O-acetyl)-β-D-glucuronide Isomer II	1.2 ± 0.2	0.7 ± 0.1	0.28 ± 0.08	0.33 ± 0.02	3.0 ± 0.2	8.1 ± 0.8	ND
13.81	Cirsimaritin	0.24 ± 0.02	0.27 ± 0.02	0.24 ± 0.01	0.2935 ± 0.0009	0.44 ± 0.02	0.745 ± 0.009	ND
13.92	Rosmanol	0.42 ± 0.02	0.308 ± 0.008	0.37 ± 0.03	0.65 ± 0.02	0.46 ± 0.01	1.08 ± 0.03	0.110 ± 0.006
14.40	Epiisorosmanol	0.57 ± 0.02	0.83 ± 0.02	0.97 ± 0.01	0.31 ± 0.02	0.57 ± 0.02	2.41 ± 0.07	<LQ
15.00	Epirosmanol	0.20 ± 0.02	0.257 ± 0.003	0.45 ± 0.02	0.106 ± 0.006	0.195 ± 0.003	1.02 ± 0.01	ND
15.14	Genkwanin	0.210 ± 0.007	0.26 ± 0.02	0.168 ± 0.006	0.275 ± 0.002	0.38 ± 0.02	0.476 ± 0.005	ND
16.04	Asiatic acid	ND	ND	ND	1.4 ± 0.1	<LQ	ND	ND
18.69	Epirosmanol methyl ether	0.59 ± 0.02	1.128 ± 0.001	0.385 ± 0.010	0.113 ± 0.003	0.57 ± 0.04	1.00 ± 0.07	ND
19.15	Carnosol	10 ± 2	11.7 ± 0.9	4.8 ± 0.1	5.5 ± 0.3	16.0 ± 0.2	18.5 ± 0.4	1.8 ± 0.1
20.00	Carnosol isomer	ND	ND	0.13 ± 0.02	0.39 ± 0.03	0.31 ± 0.02	0.8 ± 0.2	ND
20.32	Rosmadial	ND	ND	ND	0.116 ± 0.010	ND	0.22 ± 0.02	ND
21.04	Anemosapogenin	ND	ND	ND	4.60 ± 0.06	ND	ND	ND
21.35	Rosmaridiphenol	ND	0.15 ± 0.01	ND	0.132 ± 0.001	0.20 ± 0.01	0.3572 ± 0.0009	ND
21.83	2,3,4,4a,10,10a-hexahydro-5,6-dihydroxy-1,1-dimethyl-7-(1-methylethyl)-9(1H)-Phenanthrenone	ND	0.27 ± 0.03	0.27 ± 0.02	ND	0.15 ± 0.01	0.16 ± 0.05	ND
21.91	Benthamic acid	ND	1.2 ± 0.1	ND	8.3 ± 0.2	3.6 ± 0.2	2.80 ± 0.04	ND
22.35	Augustic acid	ND	0.235 ± 0.007	ND	4.8 ± 0.2	2.2 ± 0.1	0.9 ± 0.1	ND
22.63	Carnosic acid	2.6 ± 0.6	2.11 ± 0.02	3.4 ± 0.4	14 ± 1	5.8 ± 0.8	17 ± 1	1.1 ± 0.1
24.84	12-metoxycarnosic acid	0.30 ± 0.02	0.40 ± 0.04	0.287 ± 0.007	0.62 ± 0.02	0.52 ± 0.03	1.12 ± 0.01	ND
25.14	[9]-Shogaol isomer	ND	ND	ND	0.59 ± 0.02	0.63 ± 0.03	1.21 ± 0.03	ND

Table 3. Cont.

(B)								
Rt (min)	Compound	RS 7	RS 8	RS 9	RS 10	RS 11	RS 12	RS 13
27.05	[9]-Shogaol	ND	ND	ND	0.779 ± 0.007	0.66 ± 0.07	1.264 ± 0.008	ND
27.99	Micromeric acid	1.7 ± 0.3	2.5 ± 0.2	ND	16.2 ± 0.9	11 ± 1	6.8 ± 0.6	ND
29.05	Betulinic acid	6.207 ± 0.001	7.2 ± 0.4	0.93 ± 0.05	76 ± 2	51.7 ± 0.3	39 ± 2	ND
30.25	Ursolic acid	3.0 ± 0.4	4.35 ± 0.06	0.11 ± 0.04	42 ± 1	26 ± 1	18.7 ± 0.4	ND

(C)								
Rt (min)	Compound	RS 14	RS 15	RS 16	RS 17	RS 18	RS 19	RS 20
2.06	Quinic acid	ND	ND	14.2 ± 0.8	ND	46 ± 3	16.8 ± 0.5	ND
2.45	Siringic acid	ND	ND	ND	ND	210 ± 10	ND	ND
7.48	Gallocatechin	3.1 ± 0.1	6.9 ± 0.5	6.5 ± 0.5	12.2 ± 0.6	6.6 ± 0.2	3.7 ± 0.1	9.6 ± 0.7
8.43	6-Hydroxyluteolin 7-glucoside	ND	ND	ND	ND	0.19 ± 0.04	ND	ND
8.85	Rosmarinic acid-3- <i>O</i> -glucoside	2.2 ± 0.1	12.4 ± 0.8	ND	16 ± 1	ND	0.90 ± 0.02	10.4 ± 0.6
8.90	Nepetrin	0.82 ± 0.08	ND	2.3 ± 0.2	ND	9.7 ± 0.3	3.7 ± 0.3	ND
9.20	Hesperidin	1.2 ± 0.1	1.93 ± 0.05	1.3 ± 0.1	2.93 ± 0.06	2.29 ± 0.08	1.01 ± 0.05	4.4 ± 0.2
9.36	Homoplantagin	0.417 ± 0.009	0.64 ± 0.04	0.59 ± 0.05	0.95 ± 0.09	1.4 ± 0.1	0.59 ± 0.03	1.10 ± 0.07
9.53	Luteolin-3'-glucuronide	1.24 ± 0.09	1.6 ± 0.1	7.3 ± 0.8	5.5 ± 0.4	9.3 ± 0.9	3.34 ± 0.04	3.2 ± 0.3
9.79	Rosmarinic acid	1.02 ± 0.02	10.1 ± 0.5	7.5 ± 0.6	9.7 ± 0.4	23 ± 1	9.0 ± 0.6	5.6 ± 0.2
10.07	Luteolin 3'- <i>O</i> -( <i>O</i> -acetyl)-β-D-glucuronide Isomer I	ND	ND	3.2 ± 0.2	0.367 ± 0.004	5.2 ± 0.3	1.46 ± 0.05	ND
10.28	Luteolin 3'- <i>O</i> -( <i>O</i> -acetyl)-β-D-glucuronide isomer II	0.67 ± 0.01	0.74 ± 0.06	10.1 ± 0.7	8.8 ± 0.9	16.6 ± 0.3	5.8 ± 0.3	0.89 ± 0.08
13.81	Cirsimaritin	0.17 ± 0.01	0.27 ± 0.02	0.31 ± 0.01	0.41 ± 0.05	0.51 ± 0.03	0.276 ± 0.009	0.53 ± 0.02
13.92	Rosmanol	0.10 ± 0.02	0.32 ± 0.02	0.69 ± 0.04	0.421 ± 0.008	1.44 ± 0.04	0.39 ± 0.01	0.78 ± 0.02
14.40	Epiisorosmanol	0.84 ± 0.04	0.51 ± 0.05	0.34 ± 0.01	0.26 ± 0.01	0.865 ± 0.004	0.492 ± 0.003	1.5 ± 0.1

Table 3. Cont.

(C)								
Rt (min)	Compound	RS 14	RS 15	RS 16	RS 17	RS 18	RS 19	RS 20
15.00	Epirosmanol	0.103 ± 0.002	0.177 ± 0.009	0.126 ± 0.003	0.094 ± 0.007	0.41 ± 0.03	0.198 ± 0.008	0.636 ± 0.009
15.14	Genkwanin	0.16 ± 0.02	ND	0.42 ± 0.03	0.34 ± 0.04	0.56 ± 0.05	0.31 ± 0.02	0.531 ± 0.009
16.04	Asiatic acid	ND	ND	ND	ND	2.4 ± 0.3	ND	ND
18.69	Epirosmanol methyl ether	3.0 ± 0.2	0.534 ± 0.009	0.34 ± 0.01	0.15 ± 0.02	0.67 ± 0.03	0.67 ± 0.04	1.10 ± 0.04
19.15	Carnosol	3.7 ± 0.5	17.977 ± 0.002	11 ± 1	9.8 ± 0.3	18.2 ± 0.5	10 ± 1	22 ± 1
20.00	Carnosol isomer	ND	0.41 ± 0.05	0.281 ± 0.004	0.45 ± 0.01	0.57 ± 0.04	ND	0.34 ± 0.03
20.32	Rosmadial	ND	0.20 ± 0.03	ND	ND	0.226 ± 0.009	ND	0.26 ± 0.01
21.04	Anemosapogenin	ND	ND	ND	ND	0.84 ± 0.01	ND	ND
21.35	Rosmaridiphenol	ND	ND	0.206 ± 0.008	0.158 ± 0.007	0.383 ± 0.007	0.19 ± 0.01	0.37 ± 0.03
21.83	2,3,4,4a,10,10a-Hexahidro-5,6-dihydroxy-1,1-dimethyl-7-(1-methylethyl)-9(1H)-phenantrenone	ND	ND	ND	ND	ND	ND	ND
21.91	Benthamic acid	ND	1.0 ± 0.1	ND	1.3 ± 0.2	3.86 ± 0.07	ND	1.37 ± 0.07
22.35	Augustic acid	ND	<LQ	ND	0.48 ± 0.04	1.10 ± 0.03	ND	<LQ
22.63	Carnosic acid	ND	8.5 ± 0.6	6.5 ± 0.6	10.3 ± 0.6	13.7 ± 0.6	1.6 ± 0.1	4.8 ± 0.1
24.84	12-Metoxo-carnosic acid	0.13 ± 0.03	0.52 ± 0.04	4.05 ± 0.08	0.73 ± 0.09	3.15 ± 0.01	1.02 ± 0.03	1.06 ± 0.03
25.14	[9]-Shogaol isomer	ND	0.76 ± 0.05	ND	0.60 ± 0.03	1.14 ± 0.03	ND	1.11 ± 0.03
27.05	[9]-Shogaol	ND	0.85 ± 0.01	0.79 ± 0.05	0.88 ± 0.03	2.53 ± 0.08	0.51 ± 0.02	1.05 ± 0.04
27.99	Micromeric acid	ND	4.47 ± 0.06	ND	15 ± 1	5.4 ± 0.7	ND	4.1 ± 0.4
29.05	Betulinic acid	ND	26 ± 1	ND	58 ± 2	40 ± 1	ND	17 ± 1
30.25	Ursolic acid	ND	5.2 ± 0.7	ND	27 ± 1	5.5 ± 0.7	ND	3.61 ± 0.05

Finally, it can be concluded that Serbian rosemary samples harvested in Sokobanja, Bačko Petrovo Selo and Stara Planina, are very rich sources of flavonoids and typical compounds of rosemary, such as carnosic acid, carnosol, rosmadial, rosmaridiphenol, and rosmarinic acid, which are compounds with many biological properties, especially antioxidant. On the other hand, the triterpenoids as anemosapogenin, benthamic acid, augustic acid or ursolic acid were more abundant in the RS 10 collected in Gložan. These triterpenes are highly valued for their potent anticancer and anti-inflammatory activities.

The quantity, composition and ratio of plants metabolites are influenced by numerous internal and external factors, such as the plant age, climate, soil type or stress conditions that may inhibit or trigger the synthesis of specific compounds. Analyzed samples of *Rosmarinus officinalis* were collected in different geographical zones of Serbia, encompassing the altitudes from 72 to 764 m. Nevertheless, due to the fact that some compounds are in high concentration in samples collected in different altitudes, it can be assumed that this factor does not seem to seriously affect the concentration of bioactive compounds. The covered geographical area was relatively small, so the climate was very similar for all the cultivars, nevertheless the soil type for samples collected in the Northern and the Southern parts of Serbia was different further contributing to specificity of plants chemical profiles. In addition, even for the same soil type, slight shift in soil pH and composition reflected biochemical pathways in plants. Taking into consideration that analyzed rosemary samples were exposed to similar climate during their grow, it can be assumed that reported relatively modest variations in the altitude, as well as different soil composition and type, may have had significant effects of plant metabolites. This aspect should be studied in depth in future research.

### 3. Experimental Section

#### 3.1. Chemicals

All chemicals were of analytical reagent grade and used as received. Methanol for the MAE extraction of rosemary leaves was supplied by Centrohem (Stara Pazova, Serbia). Formic acid and acetonitrile for analytical chromatography were purchased from Fluka, Sigma-Aldrich (Steinheim, Germany) and Fisher Scientific (Madrid, Spain), respectively. Water was purified by a Milli-Q system from Millipore (Bedford, MA, USA). Ursolic acid, rosmarinic acid, genkwanin, luteolin, luteolin-7-*O*-glucoside, epigallocatechin and neohesperidin were from Extrasynthese (Genay, France). Carnosol, carnosic acid, and syringic acid were obtained from Fluka, Sigma-Aldrich (Steinheim, Germany). Quinic acid was supplied from Acros Organics (Geel, Belgium) and homoplantaginin from Chengdu Biopurity Phytochemicals (Chengdu, China). The stock solutions containing these analytes were prepared in dimethyl sulfoxide (DMSO) and methanol (Fisher Scientific, Madrid, Spain) and stored at  $-80^{\circ}\text{C}$  until used.

#### 3.2. Samples

The rosemary leaves used in this study were collected by applying non-probability haphazard sampling strategy in different geographical zones in Serbia (Figure 2), covering southern Serbia and Vojvodina and encompassing the altitudes from 72 to 764 m. The sample code, geographical origin and altitude for each sample are recovered in Table 4. From collected composite samples (~3 kg), leaves were removed and separated, participating further in the formation of representative samples. The leaves were

distributed in a layer and placed in darkness at room temperature for drying, which lasted two weeks. Then, dried samples were milled and particle size was determined by sieving the ground plant material to the appropriate size (between 500 and 999  $\mu\text{m}$ ). The samples were stored in the freezer until used.

**Table 4.** Description of sample code, geographical area and altitude for each rosemary sample.

Sample Code	Geographical Area	Altitude
RS 1	Kikinda (Vojvodina)	73
RS 2	Sokobanja 1 (sur de Serbia)	400
RS 3	Sokobanja 2 (sur de Serbia)	415
RS 4	Sokobanja 3 (sur de Serbia)	350
RS 5	Bačka Palanka (Vojvodina)	80
RS 6	Bačka Palanka (Vojvodina)	80
RS 7	Novi Sad 1 (Vojvodina)	72
RS 8	Novi Sad 2 (Vojvodina)	80
RS 9	Silbaš (Vojvodina)	85
RS 10	Gložan (Vojvodina)	83
RS 11	Čelarevo (Vojvodina)	76
RS 12	Bačko Petrovo Selo 1 (Vojvodina)	86
RS 13	Bačko Petrovo Selo 2 (Vojvodina)	86
RS 14	Rumenka (Vojvodina)	88
RS 15	Fruška Gora (Vojvodina)	539
RS 16	Zrenjanin (Vojvodina)	80
RS 17	Vranje (sur de Serbia)	487
RS 18	Stara Planina (sur de Serbia)	764
RS 19	Leskovac (sur de Serbia)	225
RS 20	Niš (sur de Serbia)	194

**Figure 2.** Map of Serbia where the harvesting area of each sample is marked in the corresponding geographical area.



### 3.3. Microwave-Assisted Extractions of Rosemary Leaves

Microwave-assisted extraction was performed by using a home-made modified domestic microwave oven (LG Electronics, Seoul, Korea). The system operated as an open-vessel multimode extraction system, allowing random dispersion of microwave radiation within the microwave cavity, so that every zone in the cavity and sample were irradiated [53]. Open cells were quartz vessels topped by a vapor condenser. The solvent was heated and refluxed through the sample and the microwaves allowed very efficient heating.

The rosemary extracts were obtained by MAE using an optimized method as described by Švarc-Gajić *et al.* [54]. In brief, the optimized procedure consisted of two pre-heating steps of 1 min in duration at 160 W and 320 W, respectively, followed by two extraction cycles with fresh solvent at 800 W for 5 min. Between all heating steps short breaks (15 s) were made in order to avoid local overheating and the risk of consistent decomposition or chemical transformation of the phenolic compounds. The solvent that provided the highest extraction yield was methanol-water 70:30 (v/v).

Dried samples (2 g) were transferred to the extraction cuvettes and 25 mL of extraction solvent was added in each extraction step. The extracts obtained from two extraction cycles were joined, filtered, and evaporated to dryness using a rotary vacuum evaporator (Rotavapor R, Eph lavelle, Switzerland) and stored at  $-80\text{ }^{\circ}\text{C}$  until analyzed.

### 3.4. HPLC–ESI–QTOF–MS Analysis

The rosemary-leaf extracts obtained by MAE were analyzed by HPLC–ESI–QTOF–MS. The extracts were dissolved in methanol–water 50:50 (v/v) at a concentration of 800  $\mu\text{g/mL}$ . Finally, the solutions were filtered through a 0.25- $\mu\text{m}$  filter and stored at  $-80\text{ }^{\circ}\text{C}$  to avoid possible degradation before the HPLC analysis.

Analyses were made using a UPLC Acquity (Waters, Millford, MA, USA), equipped with a thermostat-controlled standard autosampler. The HPLC column was a Zorbax Eclipse Plus C18 (4.6 mm  $\times$  150 mm, 1.8  $\mu\text{m}$ ). The injection volume in the HPLC system was 5  $\mu\text{L}$  and the autosampler temperature was set at 4  $^{\circ}\text{C}$  in order to avoid thermal degradation. Mobile phases A and B were water with 0.1% formic acid and acetonitrile, respectively. The separation was carried out at room temperature with a gradient elution programmed at a flow rate of 0.8 mL/min. The following multi-step linear gradient with different proportion of mobile phase B was applied: 0 min, 5% B; 12 min, 50% B; 17 min, 75% B; 22 min, 95% B; 25 min, 5% B. The initial conditions were maintained for 5 min.

The HPLC system was coupled to a microTOF-Q II mass spectrometer (Bruker Daltoniks, Bremen, Germany) via an ESI interface (Bruker Daltoniks, Bremen, Germany) operating in negative ion mode. The flow rate under chromatographic conditions was set at 0.8 mL/min. For a stable spray and consequently reproducible results, the effluent from the HPLC had to be split. In this work, a “T” type splitter was employed, and thus the flow was reduced from 0.8 to 0.2 mL/min. For all the experiments the detection was made while considering a mass range of 50–1100  $m/z$  and using nitrogen as nebulizing and drying gas. The optimum values of the ESI–QTOF parameters were: capillary voltage, +4 kV; drying gas temperature, 210  $^{\circ}\text{C}$ ; drying gas flow, 9 L/min, nebulizing gas pressure, 2 bar; funnel 1 RF, 150.0 Vpp; funnel 2 RF, 200.0 Vpp; hexapole RF, 100.0 Vpp; transfer time, 70  $\mu\text{s}$ ;



pre-pulse storage, 7  $\mu$ s. The collision-energy values for MS/MS experiments were adjusted as follows:  $m/z$  100, 20 eV;  $m/z$  500, 35 eV;  $m/z$  1000, 50 eV.

During the execution of the HPLC method, the mass spectrometer was externally calibrated using a sodium formate cluster solution containing 10 mM sodium hydroxide and 0.1% formic acid in water:isopropanol (1:1, v/v). The mixture was injected at the beginning of each run and all the spectra were calibrated prior to compound identification. Due to the compensation of temperature drifts in the instrument, this external calibration provided accurate mass values for a complete run.

The accurate mass data of the molecular ions were processed using Data Analysis 4.0 software (Bruker Daltonics, Bremen, Germany), which provides a list of possible elemental formulas via the Generate Molecular Formula Editor. This editor used a CHNO algorithm, which provided standard functionalities such as minimum/maximum elemental range, electron configuration, and ring-plus double-bonds equivalents, as well as a sophisticated comparison of the theoretical with the measured isotope pattern (Sigma value) for increased confidence in the suggested molecular formula.

#### 4. Conclusions

In the present work, 20 rosemary plants harvested in different geographical zones of Serbia were studied in order to determine the composition of bioactive extracts. The first step was an extraction by MAE with a previously optimized procedure. Afterwards, these extracts were qualitatively and quantitatively characterized by HPLC–ESI-QTOF-MS, where the QTOF mass analyzer proved to be a valuable detection system for characterizing the phenolic compounds present in these extracts, since it provides mass accuracy and true isotopic pattern in both MS and MS/MS spectra. This coupling has enabled the tentative characterization and quantification of more than 30 different phenolic compounds, including flavonoids, phenolic diterpenes, and abietan-type triterpenes. These results highlight that extracts from Sokobanja presented the highest levels in flavonoids and other compounds such as carnosol, rosmaridiphenol, rosmadial, rosmarinic acid, and carnosic acid. On the other hand, higher contents in triterpenes were found in the extract from the rosemary collected in Gložan (Vojvodina). In conclusion, these extracts are of interest for their possible uses as ingredients in complementary alternative medicine and nutritional supplements, as well as natural antioxidants for food preservation.

#### Acknowledgments

This work was financially supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia through a national project (Project Number TR 31014) and through scientific and technological cooperation between the Republic of Serbia and Spain (Project Number RE451-03-02635/2011-14/4). The authors are also grateful to the Spanish Ministry of Science and Innovation for the project AGL2011-29857-C03-02 and the grant FPI BES-2009-028128, Andalusian Regional Government Council of Innovation and Science for the Excellence Projects P09-CTS-4564, P10-FQM-6563 and P11-CTS-7625.

## Author Contributions

Isabel Borrás-Linares carried out the extraction process of the rosemary samples, realized the analysis of the extracts, performed the data treatment and wrote the manuscript. Zorica Stojanović realized the sample collection and supervised the extraction process. Rosa Quirantes-Piné and David Arráez-Román supervised the analytical research. Jaroslava Švarc-Gajić, Alberto Fernández-Gutiérrez and Antonio Segura-Carretero designed and supervised the study. All authors contributed to the active revision of the manuscript.

## Conflicts of Interest

The authors declare no conflict of interest.

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