





Article

Untargeted Metabolomics by Using UHPLC–ESI–MS/MS of an Extract Obtained with Ethyl Lactate Green Solvent from *Salvia rosmarinus*

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Abstract: *Salvia rosmarinus* (Lamiaceae), previously known as *Rosmarinus officinalis*, is a plant cultivated worldwide, native to the Mediterranean region. Its leaves are traditionally used for cooking. This species possesses numerous biological activities, including antioxidant, antimicrobial, anticancer, anti-inflammatory, and hepatoprotective properties. These biological properties are due to the presence of phenolic compounds, including rosmarinic acid and phenolic diterpenoids, such as carnosic acid and carnosol. In this study, we investigated the chemical composition of a green extract obtained by maceration with ethyl lactate for the first time. Seventy-five compounds were tentatively identified by UHPLC–ESI–MS/MS, including six organic acids, six cinnamic acid derivatives, five fatty acids, eighteen flavonoids, and thirty-eight terpenoids. Thus, abietane-type diterpenoids from the ethyl lactate extract were the predominant diterpenoids in the Chilean *S. rosmarinus* species, in contrast to the Chinese species, in which labdane and isopimarane-type diterpenoids were found for the first time. Finally, our study confirms that the extraction of *S. rosmarinus* with green ethyl lactate as a solvent is efficient and sustainable for the identification of flavonoids, phenols, and terpenoids from leaves.

Keywords: abietane; environment; ethyl lactate; green chemistry; phenolic compounds; rosemary; terpenoids



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1. Introduction

Organic solvents are widely used for dissolving, diluting, and dispersing water-insoluble substances, and as a medium for organic synthesis and extraction in natural product chemistry [1]. Despite the warnings against their use due to exposure and environmental pollution, their continued use is inevitable. In recent decades, many researchers have focused on reducing the use of volatile organic solvents by introducing green solvents into their processes [2]. Green solvents have shown to be promising candidates and good alternatives to petrochemical solvents because they are derived from crops and are environmentally friendly [3]. Ethyl lactate (EL) is considered to be a green solvent derived from corn. Chemically, it is the ester of lactic acid. EL is biodegradable, non-corrosive, non-carcinogenic, and non-ozone depleting. It is mainly used in the food, pharmaceutical, and cosmetic industries. Therefore, if it is not possible to replace volatile organic solvents, their use should be optimized, and one should try to recycle them [4–7]. However, to our knowledge, there are very few studies on the use of ethyl lactate as an extracting solvent in

natural product chemistry. Therefore, we decided to use ethyl lactate for the extraction of secondary metabolites.

S. rosmarinus is commonly known as “rosemary”, whose distinctive feature is its aroma, culinary use, and wide application in traditional medicine, including the treatment of colds, body aches, postpartum pain, varicose veins, discolored teeth, rheumatism, kidney infections, mental disorders, and even cancer. Additionally, it is used by the local people as a lucky charm [8,9]. The current scientific name of *Salvia rosmarinus* was previously known as *Rosmarinus officinalis*. This change was made in 2017 based on phylogenetic evidence. In addition, although the plant is cultivated worldwide, it is native to the Mediterranean region [10]. The phytochemistry of *R. officinalis* includes the presence of 1,8-cineole, α -pinene, camphor, limonene, and trans-caryophyllene as the main volatile terpenes in its essential oil [11–13]. In addition, terpenoids (carnosic acid, carnosol, and ursolic acid), flavonoids, phenolic compounds (caffeic acid, rosmarinic acid), vitamins, and minerals have been detected [14–16]. Their main biological activities include antioxidant, antibacterial, analgesic, anti-ulcerogenic, neuroprotective, antifungal, anti-inflammatory, antiobesity, antiviral, antidepressant, antidiabetic, and anticancer properties [14,17–19].

UHPLC, coupled with orbitrap technology as a mass analyzer, provides high resolution, sensitivity, high mass accuracy, and a powerful separation of metabolites in natural extracts; therefore, it is the most commonly used form in metabolomic studies. It can also determine the elemental composition of parent and daughter ions in the structural elucidation of organic compounds [20,21]. For *R. officinalis*, some LC–MS/MS reports have previously been published, showing the presence of flavonoids and their glycosides, phenols, phenolic diterpenoids, and pentacyclic triterpenoids (Table 1).

Table 1. Metabolomic profiles of *R. officinalis* samples by using LC–MS reported.

Solvent Extraction	Plant Section	Extraction Technique	Compounds Identified	Technique Use	Reference
Water	Leaves	Ultrasound	24	UHPLC–ESI–QTOF–MS	[22]
Water	Leaves and Branches	Infusion	51	HPLC–ESI–QTOF–MS	[23]
Methanol	Leaves	Maceration	47	UHPLC–MS	[24]
Methanol	Leaves	Ultrasound	46	UHPLC–ESI–QTOF–MS	[22]
Methanol	Leaves	Ultrasound	47	UHPLC–QTOF–MS/MS	[25]
Methanol	Aerial parts	Maceration	18	HPLC–DAD–ESI–Q–MS	[26]
Aqueous methanol	-	Solid/liquid	37	LC–ESI–MS/MS	[27]
Methanol/water	Leaves	Microwave assisted	34	HPLC–ESI–QTOF–MS	[28]
Methanol and ethanol	Callus	Maceration	53	UHPLC–MS	[24]
Ethanol	Aerial parts	Hydrodistillation- ultrasound	24	LC–MS	[29]
Ethyl acetate	Leaves	Soxhlet	17	LC/DAD/ESI–MS	[30]
Acetone	Leaves	Ultrasound	57	UHPLC–ESI–MS	[31]
CO ₂	Leaves	Supercritical fluid	29	LC–Q/TOF–MS	[32]

This study aims to determine and evaluate the chemical composition of an *S. rosmarinus* extract, obtained by maceration in the green solvent ethyl lactate using UHPLC–ESI–MS/MS to initiate the transition from toxic organic solvents to green solvents.

2. Materials and Methods

2.1. Chemicals

The green solvent used in this work was ethyl lactate (CAS 687-47-8), purchased from Sigma Aldrich (Santiago, Chile). UHPL–MS/MS solvents were purchased from Merck (Santiago, Chile).

2.2. Plant Material

Salvia rosmarinus L., was collected in Talca, VII Region, Chile. Leaves were dried at room temperature in darkness. *S. rosmarinus* was identified by Prof. O. Garcia, and a

voucher specimen (N° RO-2015/1215) was preserved in the Laboratory of Herbarium of Extreme Natural Products of the University of Chile.

2.3. Extract Preparation

An extract (80 mg) of *S. rosmarinus* was obtained by maceration from 1 g of dried and powdered leaves using 10 mL of ethyl lactate (three times, 10 mL each time, 3d/extraction) as an alternative food-grade solvent (Sigma-Aldrich, CAS 687-47-8, (-)-Ethyl (S)-2-hydroxypropionate). The extract was obtained by evaporation of the solvent in vacuum (2 mbar).

2.4. UHPLC–ESI–MS/MS Conditions for Analysis

The Ultimate 3000 UHPLC system (Thermo Scientific Dionex), equipped with a quaternary pump and Ultimate 3000 series TCC-3000RS column compartments, with a Ultimate 3000 series WPS-3000RS autosampler and a PDA detector controlled by Chromeleon 7.2 software (Thermo Fisher Scientific, Waltham, MA, USA, and Dionex Softron GmbH Part of Thermo Fisher Scientific, Bremen, Germany), coupled with a high-resolution Thermo Q Exactive focus mass spectrometer (Thermo, Bremen, Germany) were employed for analysis. The chromatographic unit was coupled with the MS with a heated electrospray ionization source II (HESI II). Nitrogen (purity > 99.999%) was used as both a collision and damping gas. Mass calibration was performed once a week, in both negative and positive modes, to ensure a working mass accuracy of 5 ppm or less. Caffeine and N-butylamine (Sigma Aldrich, Saint Louis, MO, USA) were the calibration standards for positive ions and bupirone hydrochloride, while sodium dodecyl sulfate and taurocholic acid sodium salt (Sigma Aldrich, Saint Louis, MO, USA) were used for mass-spectrometer calibration. These chemicals were dissolved in a mixture of acetic acid, acetonitrile, water, and methanol (Merck, Darmstadt, Germany) and were infused using a Chemyx Fusion 100 syringe pump (Thermo Fisher Scientific, Bremen, Germany). XCalibur 3.0 (Thermo Fisher Scientific, Bremen, Germany) and Trace Finder 3.2 (Thermo Fisher Scientific, San José, CA, USA) software were used for UHPLC control and data processing, respectively. Q Exactive 2.0 SP 2, from Thermo Fisher Scientific, was used to control the mass spectrometer.

Chromatography separations were performed using a UHPLC C18 column (Acclaim, 150 mm × 4.6 mm ID, 2.5 µm, Thermo Fisher Scientific, Bremen, Germany) at 25 °C. Analysis was monitored at 254, 280, 330, and 354 nm using a DAD range of 200 to 800 nm for peak characterization. Mobile phases A (0.1 % formic aqueous solution) and B (acetonitrile) were pumped at a flow rate of 1.00 mL min⁻¹, according to the following gradient pattern: (0.00, 5% B); (5.00, 5% B); (10.00, 30% B); (15.00, 30% B); (20.00, 70% B); (25.00, 70% B); (35.00, 5% B) for 12 min for column equilibration before each injection. For this study, 2 mg of an ethyl lactate extract was dissolved in 10 mL of ethanol and filtered (PTFE filter, Merck), and the injection volume was 10 µL. Then, the solutions were kept at 10 °C during storage in an autosampler.

The HESI parameters included a sheath gas flow rate of 75 units, an auxiliary gas flow rate of 20, a capillary temperature of 400 °C, an auxiliary gas heater temperature of 500 °C, a spray voltage of 2500 V (for ESI), and an S-lens at RF stage 30. Full scan data in the positive and negative regions were acquired at a resolving power of 70,000 FWHM (full width at half maximum) at *m/z* 200. A scan range of *m/z* 100–1000 was chosen for the compounds of interest: the automatic gain control (AGC) was set at 3 × 10⁶ and the injection time to 200 ms. The scan rate was set to 2 scans s⁻¹. External calibration was obtained with a calibration solution in positive and negative polarity. For confirmation, a targeted MS/MS analysis was performed using the mass inclusion list with a time window of 30 s, with the Orbitrap spectrometer operating in both positive and negative modes at 17,500 FWHM (*m/z* 200). The AGC target was set to 2 × 10⁵, and the maximum injection time was 20 ms. The precursor ions were filtered through the quadrupole, which operated with an isolation window of *m/z* 2. The pre-vacuum, high vacuum, and ultra-high vacuum were maintained at about 2 mbar, from 10⁵ and below 10¹⁰ mbar, respectively. The higher energy collisional dissociation (HCD) cell was operated at 30 kV. Detection was based on

the calculated exact mass and the retention time of the compounds. The mass tolerance window was set at 5 ppm for both modes.

3. Results and Discussion

3.1. Metabolomic Profiling Using UHPLC–ESI–MS/MS

Ethyl lactate was chosen because there is little information on an environmentally friendly extraction agent and to reduce the negative effects of the toxic organic solvents used. The high-resolution, accurate mass via Orbitrap used in this study yielded the identification and preliminary characterization of seventy-five compounds (Figure 1; Table 2), including organic acids, cinnamic acids, flavonoids, and terpenoids. As shown in Table 1, the solvents previously used in LC/MS studies are methanol, or their mixtures. Here, ethyl lactate was used for the first time for *S. rosmarinus*.

Table 2. Metabolomic profiles of ethyl lactate extract from *S. rosmarinus*.

Peak	Tentative Identification	[M-H] ⁻	λ _{max} (nm)	t _R (Min.)	Theoretical Mass (m/z)	Measured Mass (m/z)	Accuracy (ppm)	MS ² Ions
ORGANIC ACIDS								
1	Quinic acid	C ₇ H ₁₁ O ₆ ⁻	-	1.41	191.0556	191.0555	0.5	173.0450
2	Hydroxybenzoic acid	C ₇ H ₅ O ₃ ⁻	210	8.81	137.0239	137.0237	1.5	109.0285
7	Tuberonic acid glucoside isomer	C ₁₈ H ₂₇ O ₉ ⁻	212, 285	9.72	387.1655	387.1660	1.3	225.1125 (tuberonic acid)
9	Tuberonic acid glucoside	C ₁₈ H ₂₇ O ₉ ⁻	213, 290, 322	10.17	387.1655	387.1659	1.0	225.1126 (tuberonic acid)
10	Benzoic acid *	C ₇ H ₅ O ₂ ⁻	213, 281	10.49	121.0290	121.0288	1.7	-
12	Tuberonic acid *	C ₁₂ H ₁₇ O ₄ ⁻	213, 278	10.87	225.1127	225.1128	0.4	207.1023
UNIDENTIFIED								
4	Unknow	C ₂₅ H ₁₉ O ₇ ⁻	212	9.30	439.1757	439.1739	4.1	-
5	Unknow	C ₂₃ H ₁₉ O ₇ ⁻	214–265	9.44	407.1131	407.1113	4.4	-
CINNAMIC ACID DERIVATIVES								
3	Caffeic acid hexoside	C ₁₅ H ₁₇ O ₉ ⁻	210–326	9.16	341.0873	341.0877	1.2	179.0343 (caffeic acid); 161.0235; 135.0443
6	Caffeic acid hexoside isomer	C ₁₅ H ₁₇ O ₉ ⁻	212, 290, 322	9.51	341.0873	341.0877	1.2	179.0343 (caffeic acid); 161.0235 135.0444 191.0549; 179.0350
8	Chlorogenic acid	C ₁₆ H ₁₇ O ₉ ⁻	213, 310, 324	9.81	353.0873	353.0877	1.1	(caffeic acid); 161.0236; 135.0443 197.0447; 179.0343
17	Rosmarinic acid isomer	C ₁₈ H ₁₅ O ₈ ⁻	204, 277, 329	11.96	359.0767	359.0772	1.4	(caffeic acid); 161.0237; 133.0287 197.0449; 179.0342
19	Rosmarinic acid	C ₁₈ H ₁₅ O ₈ ⁻	198, 329	12.34	359.0767	359.0771	1.1	(caffeic acid); 161.0237; 133.0287
13	Methyl dihydro- <i>p</i> -coumaric acid *	C ₁₀ H ₁₁ O ₃ ⁻	212, 278	11.06	179.0708	179.0709	0.6	-
FLAVONOIDS								
11	Hydroxyluteolin-7- <i>O</i> -glucoside	C ₂₁ H ₁₉ O ₁₂ ⁻	281, 340	10.78	463.0877	463.0881	0.9	301.0349 (hydroxyluteolin)
14	Luteolin-7- <i>O</i> -glucoside	C ₂₁ H ₁₉ O ₁₁ ⁻	272, 334	11.32	447.0927	447.0933	1.3	285.0402 (luteolin)
15	Luteolin-3- <i>O</i> -glucuronide	C ₂₁ H ₁₇ O ₁₂ ⁻	268, 340	11.39	461.0720	461.0724	0.9	285.0396 (luteolin)
16	Nepitrin	C ₂₂ H ₂₁ O ₁₂ ⁻	273, 342	11.50	477.1033	477.1037	0.8	315.0508 (nepetin)
18	Hispidulin-7- <i>O</i> -glucoside	C ₂₂ H ₂₁ O ₁₁ ⁻	277, 331	12.05	461.1084	461.1088	0.9	299.0556 (hispidulin)
20	Feruloylnepitrin	C ₃₂ H ₂₉ O ₁₅ ⁻	215, 281, 332	12.71	653.1506	653.1501	0.8	477.1041 (nepitrin); 315.0510 (nepetin)
21	Luteolin acetyl- <i>O</i> -glucuronide	C ₂₃ H ₁₉ O ₁₃ ⁻	281, 326	12.85	503.0826	503.0829	0.6	285.0402 (luteolin)
22	Luteolin acetyl- <i>O</i> -glucuronide I	C ₂₃ H ₁₉ O ₁₃ ⁻	269, 335	13.14	503.0826	503.0829	0.6	285.0400 (luteolin)
23	Luteolin acetyl- <i>O</i> -glucuronide II	C ₂₃ H ₁₉ O ₁₃ ⁻	269, 336	13.45	503.0826	503.0829	0.6	285.0401 (luteolin)
25	Luteolin	C ₁₅ H ₉ O ₆ ⁻	340	14.05	285.0399	285.0404	1.8	-
26	Isorhamnetin	C ₁₆ H ₁₁ O ₇ ⁻	269, 344	14.16	315.0505	315.0509	1.3	300.0270; 85.0402;
29	Pectolinarigenin	C ₁₇ H ₁₃ O ₆ ⁻	285, 328	16.24	313.0712	313.0717	1.6	-
31	Apigenin	C ₁₅ H ₉ O ₅ ⁻	266, 335	16.78	269.0450	269.0454	1.5	201.0547; 151.0029
32	Methyl luteolin *	C ₁₆ H ₁₁ O ₆ ⁻	266, 334	17.04	299.0556	299.0560	1.3	284.0325
34	Methyl isorhamnetin *	C ₁₇ H ₁₃ O ₇ ⁻	274, 340	17.90	329.0661	329.0666	1.5	299.0194
42	Cirsimaritin	C ₁₇ H ₁₃ O ₆ ⁻	275, 334	19.46	313.0712	313.0716	1.3	-
48	Acacetin	C ₁₆ H ₁₁ O ₅ ⁻	270, 329	20.43	283.0606	283.0611	1.8	268.0372
49	Genkwanin	C ₁₆ H ₁₁ O ₅ ⁻	268, 334	20.52	283.0606	283.0610	1.4	268.0375
TERPENOIDS								
24	Hydroxyrosmanol *	C ₂₀ H ₂₅ O ₆ ⁻	-	13.83	361.1651	361.1656	1.4	317.1754
27	Nor-rosmanol *	C ₁₉ H ₂₃ O ₅ ⁻	-	14.83	331.1545	331.1551	1.8	287.1651
33	Rosmanol	C ₂₀ H ₂₅ O ₅ ⁻	283	17.73	345.1702	345.1707	1.4	301.1807
35	Hydroxyrosmadial *	C ₂₀ H ₂₃ O ₆ ⁻	-	18.20	359.1495	359.1500	1.4	331.1550; 315.1600; 287.1650
37	Hydroxyepirosmanol *	C ₂₀ H ₂₅ O ₆ ⁻	-	18.41	361.1651	361.1657	1.7	317.1757
39	Hydroxycarnosic acid *	C ₂₀ H ₂₇ O ₅ ⁻	283	18.84	347.1858	347.1864	1.7	303.1962

Table 2. Cont.

Peak	Tentative Identification	[M-H] ⁻	λ _{max} (nm)	t _R (Min.)	Theoretical Mass (m/z)	Measured Mass (m/z)	Accuracy (ppm)	MS ² Ions
40	Acetyl hydroxy rosmanol *	C ₂₂ H ₂₇ O ₇ ⁻	-	19.02	403.1757	403.1762	1.2	359.1863; 341.1757
41	Acetyl hydroxy rosmanol isomer *	C ₂₂ H ₂₇ O ₇ ⁻	-	19.21	403.1757	403.1763	1.5	-
43	Epirosmanol	C ₂₀ H ₂₅ O ₅ ⁻	283	19.54	345.1702	345.1706	1.2	301.1808; 283.1700
44	Carnosic acid isomer	C ₂₀ H ₂₇ O ₄ ⁻	216, 281	19.92	331.1909	331.1913	1.2	-
45	Epirosmanol isomer I	C ₂₀ H ₂₅ O ₅ ⁻	283	20.01	345.1702	345.1706	1.2	301.1807; 283.1697
46	Desoxy nor-carnosol *	C ₁₉ H ₂₃ O ₃ ⁻	-	20.25	299.1647	299.1651	1.3	255.1599
47	Rosmadiol	C ₂₀ H ₂₃ O ₅ ⁻	-	20.35	343.1545	343.1550	1.5	315.1605; 299.1650; 287.1648
50	Salvicanaric acid methyl ester *	C ₂₀ H ₂₇ O ₅ ⁻	-	20.65	347.1858	347.1863	1.4	329.1758; 287.1649
52	Hydroxyacetylcarnosol *	C ₂₂ H ₂₇ O ₆ ⁻	-	20.80	387.1808	387.1812	1.0	343.1913; 283.1707
53	Epirosmanol isomer II	C ₂₀ H ₂₅ O ₅ ⁻	-	20.91	345.1702	345.1707	1.4	301.1806; 287.1685
54	Rosmanol isomer I	C ₂₀ H ₂₅ O ₅ ⁻	-	21.18	345.1702	345.1707	1.4	301.1808
55	Prenylated dihydroxycarnosic acid *	C ₂₅ H ₃₇ O ₆ ⁻	-	21.25	433.2590	433.2595	1.2	317.1757
56	Dihydroxy methylcarnosic acid *	C ₂₁ H ₂₉ O ₆ ⁻	-	21.32	377.1964	377.1970	1.6	301.1809; 269.1541
57	Acetoxycarnosic acid *	C ₂₂ H ₂₉ O ₆ ⁻	-	21.49	389.1964	389.1970	1.5	345.2068; 303.1969; 285.1862
58	Prenylated dihydroxycarnosic acid derivative isomer	C ₂₅ H ₃₇ O ₆ ⁻	-	21.63	433.2590	433.2596	1.4	317.1757
59	Methoxy carnosol	C ₂₁ H ₂₇ O ₅ ⁻	277, 329	21.75	359.1858	359.1864	1.7	315.1963; 300.1731
60	Salvicanaric acid methyl ester isomer *	C ₂₀ H ₂₇ O ₅ ⁻	281	21.95	347.1858	347.1864	1.7	329.1758; 287.1651
61	Desoxy nor-carnosol *	C ₁₉ H ₂₃ O ₃ ⁻	-	22.00	299.1647	299.1646	0.3	-
62	Rosmanol isomer II	C ₂₀ H ₂₅ O ₅ ⁻	281	22.07	345.1702	345.1707	1.4	301.1830
63	Rosmanol methyl ether	C ₂₁ H ₂₇ O ₅ ⁻	218	22.14	359.1858	359.1863	1.4	283.1698
64	16-Hydroxy-20-deoxocarnosol *	C ₂₀ H ₂₇ O ₄ ⁻	218	22.21	331.1909	331.1914	1.5	313.1807
65	Carnosol	C ₂₀ H ₂₅ O ₄ ⁻	208	22.29	329.1753	329.1757	1.2	285.1858; 201.0917
66	Carnosol isomer I	C ₂₀ H ₂₅ O ₄ ⁻	217	22.64	329.1753	329.1758	1.5	285.1863; 201.0916
67	Rosmadiol isomer I	C ₂₀ H ₂₃ O ₅ ⁻	217, 269, 322	22.74	343.1545	343.1551	1.7	315.1599; 299.1653; 287.1654
68	Rosmadiol isomer II	C ₂₀ H ₂₃ O ₅ ⁻	218	23.05	343.1545	343.1551	1.7	315.1598; 299.1651
69	Rosmaridiphenol	C ₂₀ H ₂₇ O ₃ ⁻	218	23.28	315.1960	315.1965	1.6	285.1857
70	5,6,7,10-tetrahydro-7-hydroxyrosmariquinone	C ₁₉ H ₂₅ O ₃ ⁻	218, 278	23.49	301.1804	301.1809	1.7	265.1478
71	Carnosic acid	C ₂₀ H ₂₇ O ₄ ⁻	207, 285	24.04	331.1909	331.1913	1.2	287.2013; 244.1466
72	Methyl carnosate	C ₂₁ H ₂₉ O ₄ ⁻	216	25.54	345.2066	345.2070	1.2	286.1934
73	Desoxy carnosic acid derivative *	C ₂₀ H ₂₉ O ₃ ⁻	218	25.82	317.2117	317.2120	0.9	-
74	Desoxy carnosic acid derivative isomer *	C ₂₀ H ₂₉ O ₃ ⁻	218	27.73	317.2117	317.2120	0.9	-
75	Micromeric acid	C ₃₀ H ₄₅ O ₃ ⁻	216	28.95	453.3369	453.3373	0.9	-
FATTY ACIDS								
28	Trihydroxyoleic acid *	C ₁₈ H ₃₃ O ₅ ⁻	-	16.00	329.2328	329.2334	1.8	-
30	Trihydroxy-octadecadienoic acid *	C ₁₈ H ₃₁ O ₅ ⁻	-	16.53	327.2171	327.2177	1.8	-
36	Trihydroxyoleic acid isomer *	C ₁₈ H ₃₃ O ₅ ⁻	-	18.37	329.2328	329.2333	1.5	-
38	Hydroxyhexadecanedioic acid *	C ₁₆ H ₂₉ O ₅ ⁻	-	18.69	301.2015	301.2021	2.0	-
51	Dihydroxyoctadecadienoic acid *	C ₁₈ H ₃₁ O ₄ ⁻	-	20.74	311.2222	311.2227	1.6	-

* First report in the specie *S. rosmarinus*.

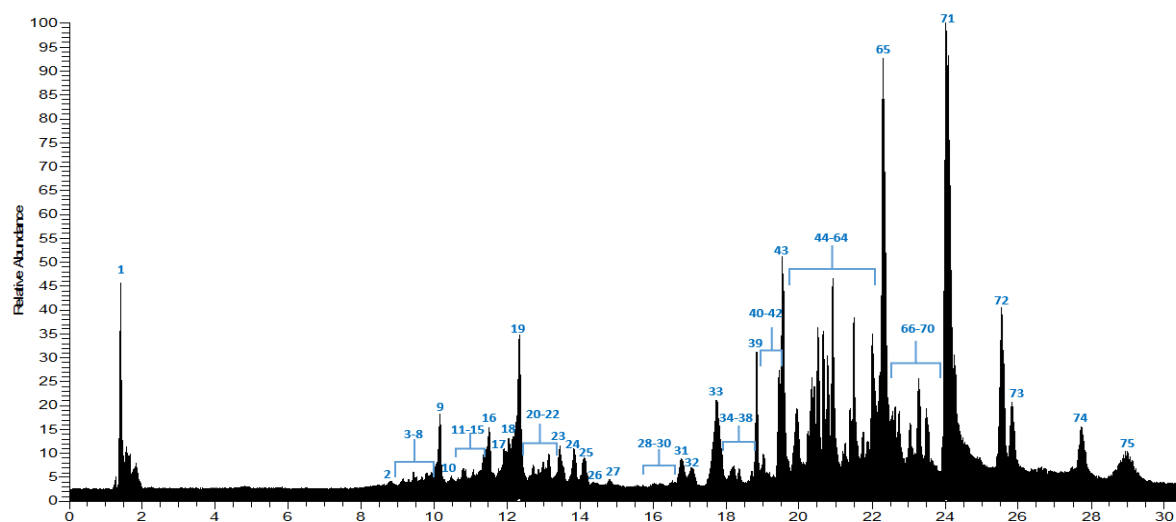


Figure 1. Chromatogram of *S. rosmarinus* (Ethyl lactate extract).

3.1.1. Organic Acids

Peak 1 was identified as quinic acid ($C_7H_{11}O_6^-$) [23], and peaks 2 and 10 as hydroxybenzoic acid ($C_7H_5O_3^-$) [27] and benzoic acid ($C_7H_5O_2^-$), respectively. Peaks 7 and 9 with an $[M-H]^-$ ion at m/z : 387.1655 were assigned to tuberonic acid glucoside and their isomer ($C_{18}H_{27}O_9^-$) [33], while its aglycone at peak 12 was identified as tuberonic acid ($C_{12}H_{17}O_4^-$).

3.1.2. Cinnamic Acid Derivatives

Peaks 3 and 6 with an $[M-H]^-$ ion at m/z : 341.0873 were assigned to isomers of caffeic acid hexoside ($C_{15}H_{17}O_9^-$), peak 8 to chlorogenic acid ($C_{16}H_{17}O_9^-$), and peaks 17 and 19 to rosmarinic acid and its isomer [25,27]. Peak 13 was tentatively assigned to methyl dihydro-*p*-coumaric acid with an $[M-H]^-$ ion at m/z : 179.0708.

3.1.3. Flavonoids

Many flavonoid glycosides and their aglycones were tentatively identified using UV and HRMS. Peaks 11, 14, 15, 25, and 32 were identified as hydroxyluteolin-7-*O*-glucoside ($C_{21}H_{19}O_{12}^-$), luteolin-7-*O*-glucoside ($C_{21}H_{19}O_{11}^-$), luteolin-3-*O*-glucuronide ($C_{21}H_{17}O_{12}^-$), Luteolin ($C_{15}H_9O_6^-$), and methyl luteolin ($C_{16}H_{11}O_6^-$), respectively. Peaks 21, 22 and 23 at m/z : 503.0829 ($[M-H]^-$) were assigned to luteolin acetyl-*O*-glucuronide isomers ($C_{23}H_{19}O_{13}^-$) [25–27]. Peak 18 was identified as hispidulin-7-*O*-glucoside ($C_{22}H_{21}O_{11}^-$) [26], and peaks 16 and 20 were assigned to nepitrin ($C_{22}H_{21}O_{12}^-$), previously isolated by Karim et al. [34], and its derivative feruloylnepitrin ($C_{32}H_{29}O_{15}^-$), which was also isolated by Bai et al. [35]. Peaks 26 and 34 were isorhamnetin ($C_{16}H_{11}O_7^-$) and methyl isorhamnetin ($C_{17}H_{13}O_7^-$), respectively, while peaks 29, 31, and 42 represented the flavonoid aglycones pectolinarigenin ($C_{17}H_{13}O_6^-$), apigenin ($C_{15}H_9O_5^-$), and cirsimaritin ($C_{17}H_{13}O_6^-$), respectively [25,27,36]. Finally, peaks 48 and 49 were identified as the isomers acacetin [27,36] and genkwanin [25,26,37], respectively, both with an $[M-H]^-$ ion at m/z : 283.0611 ($C_{16}H_{11}O_5^-$). A proposed biosynthetic relationship between luteolin (peak 25) and its derivatives detected by UHPLC–ESI–MS/MS is shown in Figure 2.

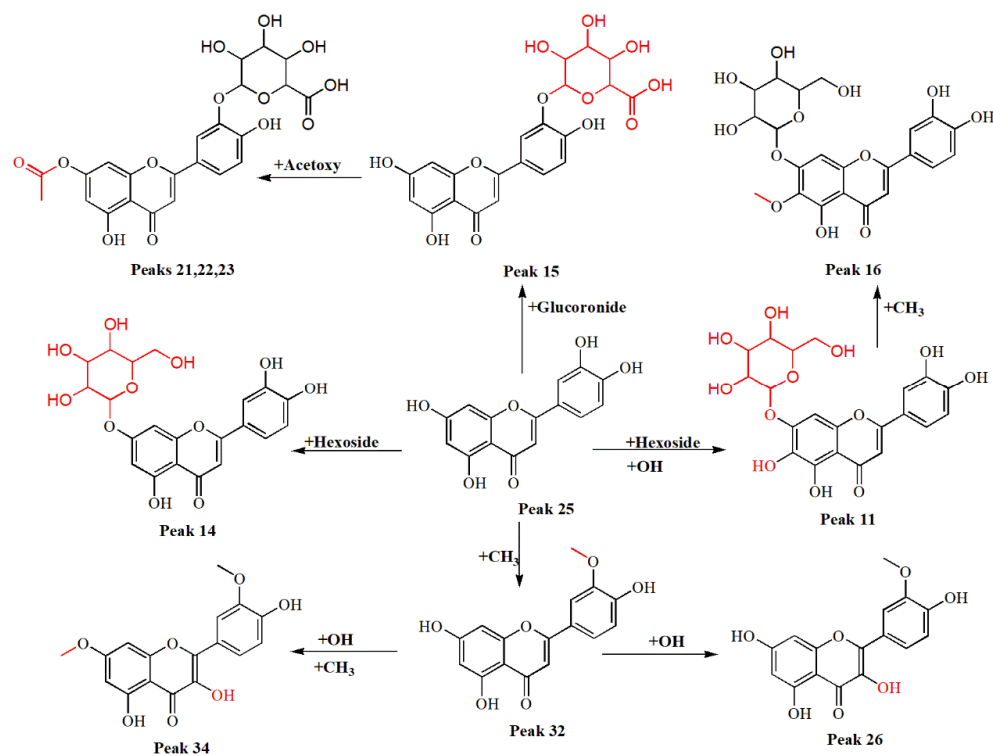


Figure 2. Proposed biosynthetic connection for the luteolin derivatives.

3.1.4. Terpenes and Their Derivatives

It is known that numerous phenolic diterpenoids have been isolated from *R. officinalis*. Among them, carnosic acid, carnosol, rosmanol, and epirosmanol have been reported. Peaks 43, 45, and 53 were tentatively identified as epirosmanol and its isomers ($C_{20}H_{25}O_5^-$) [25,27]. Peaks 33, 54, and 62 were assigned to rosmanol and its isomers ($C_{20}H_{25}O_5^-$) [23]. Peak 27 was considered to be a new rosmanol derivative and it tentatively designated as nor rosmanol ($C_{19}H_{23}O_5^-$). Acetylated hydroxyrosmanol was assigned to two other peaks, namely 40 and 41 ($C_{22}H_{27}O_7^-$), peak 63 was considered to be rosmanol methyl ether ($C_{21}H_{27}O_5^-$) [26] and peaks 24 and 37 were considered to be the hydroxy rosmanol isomers [38]. Rosmadiol and its isomers ($C_{20}H_{23}O_5^-$) were detected and tentatively identified in peaks 47, 67, and 68 [37], while peaks 35 and 69 were identified as hydroxyrosmadiol ($C_{20}H_{23}O_6^-$) and rosmaridiphenol ($C_{20}H_{27}O_3^-$), respectively [38].

The known phenolic diterpenoid carnosic acid and its isomer ($C_{20}H_{27}O_4^-$) were assigned to peaks 44 and 71 [25–27,37]. In addition, some carnosic acid derivatives were detected at peaks 56, 57, and 72, which were identified as dihydroxy methylcarnosic acid ($C_{21}H_{29}O_6^-$), acetoxycarnosic acid ($C_{22}H_{29}O_6^-$), and methyl carnosate ($C_{21}H_{29}O_4^-$), respectively [25,27,38]. Peaks 39 ($C_{20}H_{27}O_5^-$), 55 ($C_{25}H_{37}O_6^-$) and 58 ($C_{25}H_{37}O_6^-$) were assigned to hydroxycarnosic acid [38] and prenylated dihydroxycarnosic acid and its isomer, while peaks 73 and 74 were assigned to desoxy carnosic acid isomers ($C_{20}H_{29}O_3^-$) and considered as new diterpenoids. Peaks 65 and 66 corresponded to diterpenoid carnosol and its isomer ($C_{20}H_{25}O_4^-$), respectively [25–27,37]. Peak 59 was assigned to methoxycarnosol ($C_{21}H_{27}O_5^-$) and peak 64 to 16-hydroxy-20-deoxocarnosol ($C_{20}H_{27}O_4^-$) [38], while peaks 52, 46, and 61 were assigned to hydroxyacetylcarnosol ($C_{22}H_{27}O_6^-$) and desoxy norcarnosol isomers ($C_{19}H_{23}O_3^-$), respectively, which could be considered as the first time diterpenoids have been detected in this species. Finally, peaks 50 ($C_{20}H_{27}O_5^-$), 60 ($C_{20}H_{27}O_5^-$), 70 ($C_{19}H_{25}O_3^-$), and 75 ($C_{30}H_{45}O_3^-$) were tentatively assigned to salvicanaric acid methyl isomer [39], 5,6,7,10-tetrahydro-7-hydroxyrosmariquinone [36], and the triterpenoid micromeric acid [25]. A proposed biosynthetic pathway for the carnosic acid derivatives detected by UHPLC–ESI–MS/MS is shown in Figure 3.

3.1.5. Fatty Acids

Five fatty acids were detected and tentatively identified. Peaks 28 and 36 were assigned to trihydroxyoleic acid and its isomer ($C_{18}H_{33}O_5^-$). Peaks 36, 38, and 51 were identified as trihydroxyoctadecadienoic acid ($C_{18}H_{31}O_5^-$), hydroxyhexadecanedioic acid ($C_{16}H_{29}O_5^-$), and dihydroxyoctadecadienoic acid ($C_{18}H_{31}O_4^-$), respectively.

3.1.6. Unidentified Compounds

Peak 4 ($C_{25}H_{19}O_7^-$) and peak 5 ($C_{23}H_{19}O_7^-$) were not identified.

Many toxic organic solvents have been identified as causing negative environmental impacts, pollution, and potential harm to humans. Fortunately, natural product extraction processes consider environmental safety by using a combination of environmentally friendly technology and solvents. The use of ethyl lactate as a solvent in the extraction of natural products for the untargeted analysis of extracts is not widely used, but it is considered to be a potential green solvent for the extraction of hydrophilic and lipophilic phytonutrients [40]. In some studies, ethyl lactate was used as a solvent for the decaffeination of green tea, preserving the content of catechins [41,42]. In addition, it has shown a higher extraction capacity of α -mangotin in *Garcinia mangostana* [43], carotenoids in dried tomatoes, luteolin, and β -carotene from powders of white corn and carrots [44]. Other studies have shown that ethyl lactate is an efficient solvent for the extraction of polyphenols, such as caffeic acid, protocatechuic acid, kaempferol, quercetin, chrysin, orientin, and apigenin, as well as the alkaloid lupamine, which has high antioxidant and antibacterial activity [45].

It has been reported that phenolic diterpenoids with abietane skeleton possess several biological activities, including diterpenoids, such as methyl carnosate with antibacterial and antioxidant properties [46,47], carnosol with anticancer, anti-inflammatory, antimicrobial, and gastroprotective activities [48–51], and carnosic acid with antioxidant, antimicrobial, antitumor, and anti-SARS-CoV-2 properties [47,50,52–54]. On the other hand, rosmarinic acid, a well-known phenolic acid found in many Lamiaceae species, has shown a wide range of biological activities, such as anti-SARS-CoV-2, anti-inflammatory, anti-ageing, antidepressant, anti-inflammatory, anticancer, antidiabetic, antioxidant, and antimicrobial properties, as well as the inhibition of tau protein fibrillization [50,52,54–56].

Some studies based on green strategies have been applied to *R. officinalis*. Wang et al. [57] studied ultrasound-assisted extraction coupled with high-speed countercurrent chromatography (HSCCC) separation using hydrophobic deep eutectic solvents (DESs). They found that, among the studied DESs, D,L-menthol: D,L-lactic acid, 1:2 was the best extraction agent, but not as a HSCCC solvent. Similarly, Vladimir-Knezevic et al. [58] showed that DESs, such as choline chloride (ChCl): ethylene glycol (EG), 1:3 at 50% water, gave the same yields as 70% ethanol for phenolic acid extraction. Meanwhile, 70% ethanol was most effective for flavonoid extraction from *R. officinalis* compared to water, 70% ChCl: EG and 50% ChCl: EG. Kessler et al. [59] compared two extraction methods from Portuguese *R. officinalis*: hydrodistillation (HD) and supercritical fluid extraction (SFE)-CO₂. They demonstrated and confirmed that the essential oils from the SFE-CO₂ extraction were higher than those obtained from the HD extraction and, after defining the safety profile, they can be used to improve bread odor with these green extracts. Finally, Chen et al. [60] reported that they used SFE-CO₂ as the first step of the purification of *R. officinalis*. Then, the remaining solid was subjected to an isolation process, which revealed the presence of labdane (six) and isopimarane (five) diterpenoids for the first time. Among them, seven diterpenoids were identified as new diterpenoids after nuclear magnetic resonance (NMR) and MS/MS analyses. In addition, rosmarinusin J; M; O; labda-8(14), 12E, 15-triene-18-acid and (E)-geranylferulic acid showed cytoprotective activity against H₂O₂-induced oxidative damage to SH-SY5Y cells. In this study, neither labdane nor isopimarane diterpenoids were found in the Chilean species.

Our study confirms that the extraction of *S. rosmarinus* with the green solvent ethyl lactate is efficient and sustainable for the identification of flavonoids, phenols and terpenoids from the leaves.

4. Conclusions

Green solvents are a good alternative to toxic organic solvents because they are environmentally friendly. Among them, ethyl lactate, which is considered a green solvent, is biodegradable, non-corrosive, non-carcinogenic, and non-ozone depleting. In this work, a green extract of the plant *Salvia rosmarinus*, formerly known as *Rosmarinus officinalis*, was prepared by maceration as a conventional technique, in combination with ethyl lactate. Then, the chemical composition of this extract was investigated for the first time by UHPLC–ESI–MS/MS. The obtained results showed that seventy-five compounds were tentatively identified by untargeted metabolomics study, including six organic acids, six cinnamic acid derivatives, five fatty acids, eighteen flavonoids, one triterpene, and thirty-seven phenolic diterpenes. This result shows that the extraction of phenolic diterpenoids with ethyl lactate is better than that with toxic organic solvents (Table 1). Many diterpenoids, such as hydroxyrosmanol, hydroxyrosmadial, hydroxyepirosmanol, hydroxycarnosic acid, salvicanaric acid methyl ester, acetoxycarnosic acid, hydroxydeoxocarnosol, desoxycarnosic acid, and prenylated dihydroxycarnosic acid, were detected for the first time in this species. Further isolation efforts should be made to confirm the molecular structures of these diterpenoids. Finally, ethyl lactate could be used for the extraction of secondary compounds as an alternative to toxic solvents to enable more sustainable extraction processes.

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