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Full-Spectrum Analysis of Bioactive Compounds in Rosemary (*Rosmarinus officinalis* L.) as Influenced by Different Extraction Methods

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Abstract: *Rosmarinus officinalis* is a potent antioxidant herb rich in polyphenols. Ultra-high-performance liquid chromatography, coupled with electrospray ionization and quadrupole-time of flight mass spectrometry (UHPLC-ESI-QTOF-MS), enables an exhaustive, full-spectrum analysis of the molecular constituents of natural products. The study aimed to develop a rapid UHPLC method to contribute new insights into the phytochemical composition of rosemary and to assess the performance of nine different procedures for extraction. These include fresh tissue homogenization, fresh and dry leaf decoction, and their respective fermentation, Soxhlet extraction, and sonication using water and methanol. Different extraction methods were found to recover quite different groups of polyphenols within 11 min during 20 min of analysis. Soxhlet extraction, yielded very high concentrations of rosmarinic acid (33,491.33 ± 86.29 µg/g), luteolin-7-*O*-glucoside (209.95 ± 8.78 µg/g), carnosic acid (2915.40 ± 33.23 µg/g), carnosol (22,000.67 ± 77.39 µg/g), and ursolic acid (5144.27 ± 28.68 µg/g). UHPLC-ESI-QTOF-MS enabled the detection of more than 50 polyphenols, including phenolic acids, flavonoids, and terpenoids in the various extracts. Of these, sagerinic acid ($[M - H]^-$ *m/z* 719.16), salvianolic acid A ($[M - H]^-$ *m/z* 493.11) and B ($[M - H]^-$ *m/z* 717.15), and a pentacyclic triterpenoid corosolic acid ($[M - H]^-$ *m/z* 471.34) were detected for the first time in rosemary. Soxhlet extraction was found to be the most efficient method, followed by dry leaf decoction. The UHPLC-ESI-QTOF-MS methodology for the analysis proved to be very efficient in the identification and characterization of targeted and untargeted bioactive molecules in the rosemary.

Keywords: rosemary; rosmarinic acid; ursolic acid; Soxhlet extraction; sonication; UHPLC-ESI-QTOF-MS

1. Introduction

Rosemary (*Rosmarinus officinalis* L.) is an evergreen perennial culinary herb belonging to the family Lamiaceae and is popularly used as a spice and medicine. The herb is traditionally used to treat memory-related disorders, hypertension, headache, insomnia, and diseases related to the respiratory system [1,2]. Rosemary is considered as a powerful cardiac stimulant, a strong antiseptic, antispasmodic, carminative, emmenagogue, and nervine tonic, and is used to cure arthritis, dandruff, and skin diseases [3,4]. The essential oil from its leaves is used as a natural antimicrobial, pesticide, and insect repellent [5]. The therapeutic properties of rosemary have been attributed to its phytochemical constituents, such as phenolic acids, flavonoids, and terpenoids [6,7].

Ultra-high-performance liquid chromatography and electrospray ionization, coupled with quadrupole-time of flight mass spectrometry (UHPLC-ESI-QTOF-MS), is improved technology for separation and investigation of complex polyphenols in food samples [8]. UHPLC provides rapid, high-resolution, along with higher selectivity and sensitivity, while ESI-QTOF-MS identifies multiple targeted and untargeted constituents of the sample in real-time. Characterization of unknown compounds in UHPLC is based on their exact mass (*m/z*) and *m/z* fragmentation pattern with high *m/z* resolution; further, this technology could also be used to distinguish isobaric compounds by exact mass with different elemental positions [8,9]. Hence, the study was conducted to develop a rapid analytical methodology to provide new insights into the range of phytochemicals present in rosemary and the relative amounts of these compounds.

There are only a few studies reported on the phytochemical profiling of rosemary, and these mainly describe alcohol-based extraction. So far, minimal effort has been made to evaluate the quality of the herb and extracts hereof, based on traditional and industrial methods. There is some evidence in Ayurvedic classics that fermentation enhances the therapeutic and biochemical properties of herbal drugs [10–12]. At the same time, ultrasound extraction for a short period (1–2 h) at low frequencies (40 kHz) is reported to increase the yield of alkaloids in herbal extracts and to significantly reduce extraction time and solvent consumption, resulting in comparable or superior extracts to those obtained using decoction and maceration [13,14]. Hence, in the present investigation, an effort has been made to assess several extraction methods in terms of efficiency and final concentrations of critical bioactive constituents of rosemary. The extraction methods evaluated included aqueous extraction, decoction, Soxhlet's extraction, Ayurvedic fermentation, and sonic extractions in rosemary.

2. Results and Discussion

2.1. Quantification of Bioactive Compounds by UHPLC-ESI-QTOF-MS

The concentrations of different bioactive compounds, caffeic acid, rosmarinic acid, luteolin-7-*O* glucoside, carnosic acid, ursolic acid, and carnosol (μg/g) analyzed through UHPLC-ESI-QTOF-MS as influenced by different extraction methods are presented in Table 1. Among all the extractions, Soxhlet extract (T_7) recorded significantly higher rosmarinic acid (33,491.33 ± 86.29 μg/g), luteolin-7-*O*-glucoside (209.95 ± 8.78 μg/g), carnosic acid (2915.40 ± 33.23 μg/g), carnosol (22,000.67 ± 77.39 μg/g), and ursolic acid (5144.27 ± 28.68 μg/g). Soxhlet extraction combined with methanol solvent might enhance the solubility of polyphenols, flavonoids, and other bioactive compounds present in herbs, maximizing the extraction of phytochemical constituents [15]. All the extractions in the study yielded considerable amounts of rosmarinic acid ranging from 0.26 μg/g to 33.49 mg/g, contributing substantially to the high antioxidant potential of the extracts. The results are in good agreement with previous studies, in which rosmarinic acid concentrations were reported in the range of 5.6 μg–2.34 mg/g in rosemary leaf extracts from Serbia and Iraq [16,17]; rather, various extraction procedures of our study in rosemary (T_4–T_7, T_9) recorded higher rosmarinic acid concentrations than the previous reports.

Table 1. Polyphenol and terpenoid content (μg/g) in different extraction of Rosemary analyzed by ultra-high-performance liquid chromatography and electrospray ionization, coupled with quadrupole-time of flight mass spectrometry (UHPLC-ESI-QTOF-MS). (Figures S1–S6)

Treatment	Polyphenol and Terpenoid Content (μg/g) in Rosemary					
	Caffeic Acid	**Rosmarinic Acid**	**Luteolon-7-*O*-Glucoside**	**Carnosic Acid**	**Carnosol**	**Ursolic Acid**
T_1	6.02 ± 0.08 ^{b}	1.51 ± 0.07 ^{a}	1.59 ± 0.22 ^{a}	0.64 ± 0.01 ^{a}	112.06 ± 0.61 ^{b}	5.32 ± 0.17 ^{a}
T_2	12.30 ± 0.33 ^{c}	1124.03 ± 13.62 ^{b}	7.14 ± 0.14 ^{bc}	1374.63 ± 7.72 ^{b}	171.52 ± 1.59 ^{b}	6.08 ± 0.17 ^{a}
T_3	13.03 ± 0.70 ^{c}	2.51 ± 0.35 ^{a}	ND	0.25 ± 0.01 ^{a}	0.54 ± 0.01 ^{a}	0.36 ± 0.01 ^{a}
T_4	38.56 ± 1.58 ^{e}	5428.47 ± 19.69 ^{c}	ND	0.97 ± 0.01 ^{a}	1.91 ± 0.04 ^{a}	2.36 ± 0.07 ^{a}
T_5	322.02 ± 3.39 ^{g}	13,310.13 ± 26.12 ^{d}	130.53 ± 5.41 ^{d}	2671.83 ± 20.03 ^{c}	417.21 ± 1.99 ^{c}	89.20 ± 1.92 ^{b}
T_6	106.83 ± 1.49 ^{f}	15,242.40 ± 43.62 ^{e}	4.67 ± 0.68 ^{ab}	5.39 ± 0.48 ^{a}	10.82 ± 0.59 ^{a}	6.09 ± 0.19 ^{a}
T_7	40.55 ± 0.03 ^{e}	33,491.33 ± 86.29 ^{g}	209.95 ± 8.78 ^{e}	2915.40 ± 33.23 ^{d}	22,000.67 ± 77.39 ^{d}	5144.27 ± 28.68 ^{d}
T_8	2.40 ± 0.06 ^{a}	0.26 ± 0.00 ^{a}	0.97 ± 0.01 ^{a}	ND	2.19 ± 0.19 ^{a}	10.37 ± 0.88 ^{a}
T_9	23.77 ± 1.63 ^{d}	15,944.00 ± 36.39 ^{f}	9.11 ± 0.35 ^{c}	6.97 ± 0.34 ^{a}	34.98 ± 1.10 ^{a}	1042.88 ± 11.33 ^{c}
Mean	62.83	9393.85	52.99	872.01	2527.99	700.77
F Test	**	**	**	**	**	**
SEM ±	0.85	21.02	0.81	7.61	14.90	5.95
CD at 1%	3.46	85.56	3.28	31.00	60.67	24.21

** Significant at 1% level, values followed by different letters indicate a significant difference between the treatments at $p < 0.01$; ND—Not detected. Treatment Details: T_1: Fresh tissue homogenization; T_2: Fresh leaf decoction; T_3: Fresh homogenized tissue extract fermentation; T_4: Fresh leaf decoction fermentation; T_5: Dry leaf decoction; T_6: Dry leaf decoction fermentation; T_7: Soxhlet extraction; T_8: Sonic extraction—aqueous; T_9: Sonic extraction—methanol.

The decoction from dry leaf powder (T_5) recorded significantly higher levels of caffeic acid (322.02 ± 3.39 μg/g) as compared to other treatments. Fresh leaf decoction also contained a considerable amount of rosmarinic acid, carnosic acid, and carnosol. Carrying out the decoction process using water helps to dissolve the maximum amounts of these water-soluble compounds [18]. Levels of polyphenols and terpenoid compounds were significantly higher in dry leaf decoction compared to fresh leaf decoction, primarily because the amount of biomass that could be extracted was immense. The conversion rate of fresh to dry rosemary was 33%. Among the traditional extraction methods, dry leaf decoction (T_5) and its fermentation (T_6) were found to yield higher levels of caffeic acid and rosmarinic acid. Fermentation significantly enhanced the rosmarinic acid levels in both T_4 and T_6. Fermentation also enhanced caffeic acid content in both fermented fresh homogenized tissue extract (T_3) and fresh leaf decoction (T_4). This may be due to the microbial transformation of chemical compounds and better extraction of herbal constituents due to the production of alcohol during fermentation. It may also be the case that extraction was facilitated by fermentation due to the release of bacterial enzymes that broke down cell walls of the rosemary plant, making compounds more accessible to extraction by a solvent [19,20]. In the present study, fermentation enhanced the phenolic acids; however, it reduced flavonoid content, luteolin-7-glucoside and diterpenoids, carnosic acid, and carnosol. It is likely that the oxidation of phenolic compounds during fermentation reduced the levels of certain polyphenols. Similar results were reported in *Centella asiatica* and *Orthosiphon aristatus* [21–23].

Ultrasound extraction using methanol (T_9) resulted in significantly higher concentrations of rosmarinic acid and ursolic acid as compared to other fresh extraction and fermentation procedures. Ultrasound is known to disrupt plant cell walls, thereby facilitating the release of extractable compounds and enhancing mass transport of solvent from plant cells into the solvent phase. This effect boosts compound recovery, mostly when an optimal solvent, in this case, methanol, was used [13,24]. In contrast to sonication with methanol, sonication with water yielded the lowest levels of phenolic acids and flavonoids of all extraction methods employed. This is not surprising since the complex structures of phenolic compounds cause them to be rather insoluble in aqueous media [25]. Among aqueous and methanol extraction, methanol extracts showed significantly higher polyphenols and terpenoids, especially in Soxhlet and sonic extracts. This might be due to the higher solubility of complex bioactive compounds in organic solvents than the aqueous base [25,26]. The herb was found to contain a considerable quantity of rosmarinic acid and ursolic acid in most of the extractions, responsible for its healing properties, supporting traditional usage for treating gastrointestinal inflammation, colitis, colon cancer, and nervous system inflammation [27].

2.2. Identification and Characterization of Bioactive Constituents in R. officinalis

Rapid separation polyphenol molecules were achieved within the first 11 min of 20 min of UHPLC analysis duration. More than 50 polyphenolic compounds have been identified by UHPLC-ESI-QTOF-MS under negative electrospray ionization conditions $[M - H]^-$ based on their retention times, molecular weights, and mass (*m/z*) fragmentation patterns. The study was focused on negative ionization mode $[M - H]^-$ because it is reported to be more sensitive for analysis of phenolic acids and flavonoids, compared to positive ionization mode [28–30]. The phenolic compounds in rosemary extracts were mostly flavonoids, phenolic acids, and terpenoids. The terpenoids included diterpenoids largely, along with a few triterpenoids. The data are presented in three groups: polyphenols in homogenous aqueous extraction (T_1) and its fermentation (T_3) (Table 2); fresh and dry leaf decoctions (T_2 and T_5), and their respective fermentations (T_4 and T_6) (Table 3); industrial extractions Soxhlet (T_7) and sonication with water and methanol (T_8 and T_9) (Table 4). Chromatograms depicting the intensity of polyphenols in different rosemary extracts (T_1–T_9) versus retention time (min) are presented in Figure 1a–i. The compounds without reference standards were identified tentatively by comparing the mass spectra data, ion fragmentation, and molecular weight (*m/z*) with data available in the literature [17,31] and the mass spectral library obtained from the National Institutes of Standards and Technology (NIST-2017), AOI (All-in-One) spectral

library from Sciex, MoNA (MassBank of North America), and HILIC (Hydrophilic Interaction Liquid Chromatography) library database from University of California, Davis.

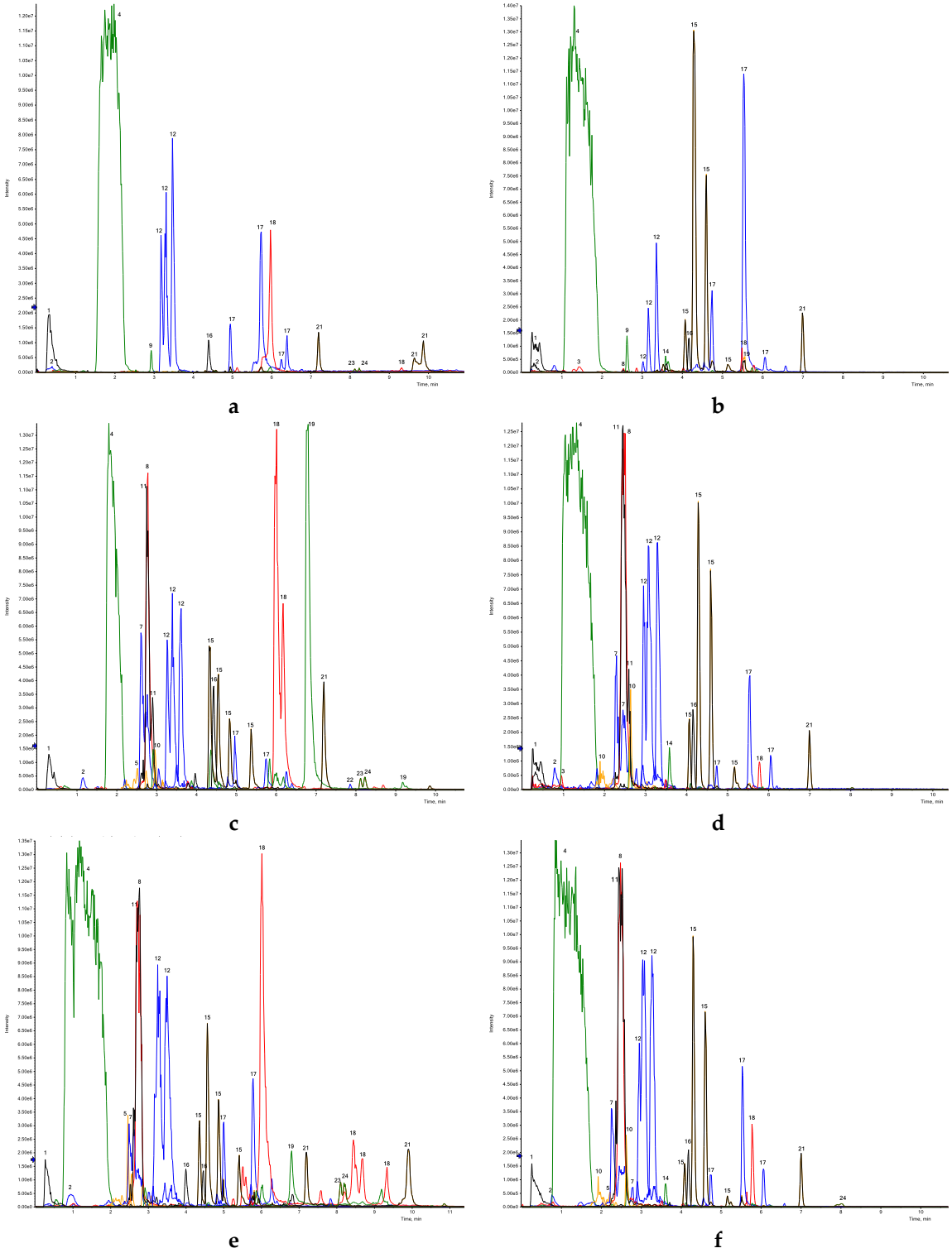

Figure 1. *Cont.*

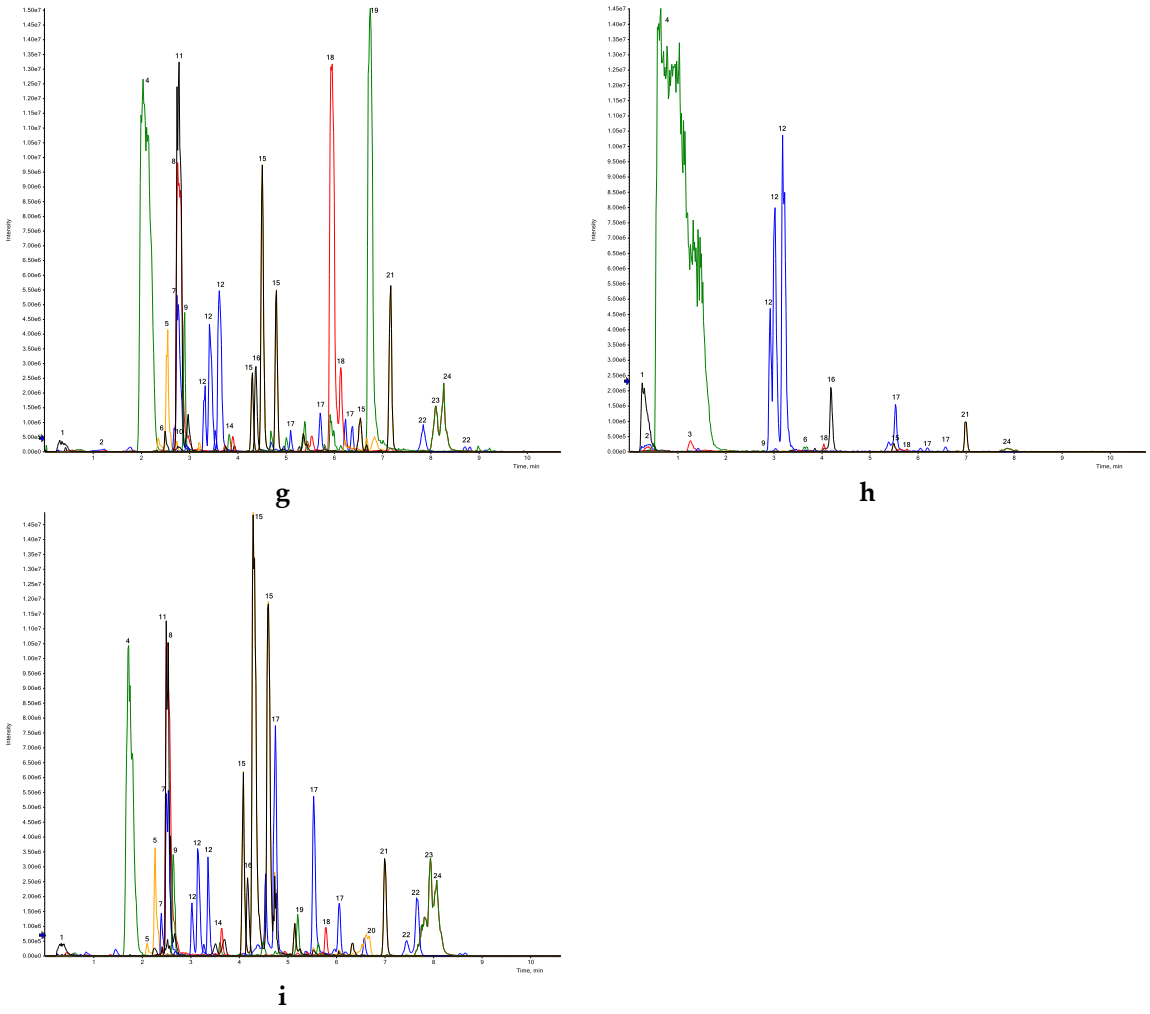

Figure 1. Chromatogram representing relative abundance of polyphenols and terpenoids in different extracts of rosemary leaves (T_1–T_9) analyzed through ultra-high-performance liquid chromatography with electrospray ionization and quadrupole-time of flight mass spectrometry (UHPLC-ESI-QTOF-MS) (intensity versus elution time); (**a**) Fresh tissue homogenization (T_1); (**b**) Fresh homogenized tissue extract fermentation (T_3); (**c**) Fresh leaf decoction (T_2); (**d**) Fresh leaf decoction fermentation (T_4); (**e**) Dry leaf decoction (T_5); (**f**) Dry leaf decoction fermentation (T_6); (**g**) Soxhlet extraction (T_7); (**h**) Sonication with water (T_8); (**i**) Sonication with methanol (T_9). Peak numbers refer to: 1—quinic acid; 2—caffeic acid; 3—coumaric acid; 4—gallocatechin; 5—rosmarinic acid-3-*O*-glucoside; 6—luteolin-7-*O*-glucoside; 7—salvianolic acid B; 8—rosmarinic acid; 9—hesperidin; 10—Salvianolic acid A; 11—Sagerinic acid; 12—Luteolin 3′-acetyl-*O*-glucuronide; 13—Apigenin; 14—Diosmetin; 15—Rosmanol; 16—Pectolinarigenin; 17—Rosmadial; 18—Carnosol; 19—Carnosic acid; 20—Corosolic acid; 21—12-methoxy-carnosic acid; 22—Micromeric acid; 23—Betulinic acid; 24—Ursolic acid.

Table 2. Bioactive compounds in fresh tissue homogenization (T_1) and its fermentation (T_3) of *Rosmarinus officinalis* identified by UHPLC-ESI-QTOF-MS.

Sl No	Compound	RT (min)	Mass $[M-H]^-$ (*m/z*)	Formula	Fragments	T_1	T_3
1.	Quinic acid	0.42	191.05681	$C_7H_{12}O_6$	85.0297 (42) *, 127.0401 (24), 59.0165 (13)	+	+
2.	Caffeic acid	0.78	179.03520	$C_9H_8O_4$	135.0438 (100), 134.0370 (21)	+	+
3.	*p*-Coumaric acid	1.44	163.04014	$C_9H_8O_3$	119.0500 (100)	+	+
4.	Gallocatechin	1.65	305.07057	$C_{15}H_{14}O_7$	225.1126 (69), 96.9597 (16), 98.9574 (8)	+	+
5.	Luteolin 7-*O*-rutinoside	2.18	593.15382	$C_{27}H_{30}O_{15}$	297.0740 (8), 285.0410 (6)	+	−
6.	Salvianolic acid B	2.29	717.14274	$C_{36}H_{30}O_{16}$	519.0900 (62), 339.0494 (33)	+	−
7.	Rosmarinic acid	2.58	359.07906	$C_{18}H_{16}O_8$	161.0236 (100), 197.0449 (72), 179.0347 (68), 135.0448 (7)	+	+
8.	Isorhamnetin-3-glucoside	2.80	477.10646	$C_{22}H_{22}O_{12}$	315.0695 (38)	+	−
9.	Apigenin-7-*O*-glucoside	2.88	431.109907	$C_{21}H_{20}O_{10}$	269.0449 (100)	+	−
10.	Hesperidin	2.92	609.18546	$C_{28}H_{34}O_{15}$	301.0695 (100)	+	+
11.	Hispidulin rutinoside	2.95	607.17094	$C_{28}H_{32}O_{15}$	301.0699 (100), 299.0559 (24)	+	+
12.	Hispidulin-7-*O*-glucoside	3.03	461.11113	$C_{22}H_{22}O_{11}$	283.0234 (13), 299.0561(8)	+	−
13.	6-Hydroxyluteolin-7-*O*-glucoside	3.10	463.08011	$C_{21}H_{20}O_{12}$	301.0350 (100)	+	−
14.	Luteolin	3.11	285.03995	$C_{15}H_{10}O_6$	133.0284 (12), 151.0029 (12), 175.0395 (9),199.0395 (8)	−	+
15.	Luteolin-7-*O*-glucuronide	3.11	461.07495	$C_{21}H_{18}O_{12}$	285.0385 (100)	+	+
16.	Isorhamnetin	3.20	315.05001	$C_{16}H_{12}O_7$	300.0255 (100), 301.0311 (39)	+	+
17.	Luteolin 3′-acetyl-*O*-glucuronide isomer I	3.29	503.08570	$C_{23}H_{20}O_{13}$	285.0381 (100), 443.0587 (100), 381.0606 (35), 399.0720 (28)	+	+
18.	Luteolin 3′-acetyl-*O*-glucuronide isomer II	3.38	503.08550	$C_{23}H_{20}O_{13}$	285.0366 (100)	+	+
19.	Apigenin	3.49	269.04559	$C_{15}H_{10}O_5$	117.0356 (12), 149.0356 (8), 225.0560 (5)	−	+
20.	Hesperetin	3.58	301.07074	$C_{16}H_{14}O_6$	242.0571 (87), 284.286.0468 (55), 164.0108(54), 151.0036 (35)	−	+
21.	Diosmetin	3.58	299.05595	$C_{16}H_{12}O_6$	284.0310 (100)	−	+

Table 2. *Cont.*

Sl No	Compound	RT (min)	Mass $[M-H]^-$ (*m/z*)	Formula	Fragments	T_1	T_3
22.	Luteolin 3′-acetyl-*O*-glucuronide	3.62	503.08594	$C_{23}H_{20}O_{13}$	285.4652 (100), 443.0598 (76)	+	+
23.	Rosmanol isomer	4.07	345.16874	$C_{20}H_{26}O_5$	301.1782 (100), 283.1673 (68), 284.1719 (29)	–	+
24.	Pectolinarigenin	4.15	313.07285	$C_{17}H_{14}O_6$	298.0464 (100), 283.0235 (52), 255.0285 (17), 163.0034 (10), 227.0344 (6), 117.0350 (4)	+	+
25.	Rosmanol	4.30	345.17145	$C_{20}H_{26}O_5$	301.1782 (100), 283.1673 (65), 284.1719 (32)	+	+
26.	Genkwanin	4.58	283.06224	$C_{16}H_{12}O_5$	268.0381 (100), 240.0431 (6)	+	+
27.	Rosmanol isomer	4.60	345.17190	$C_{20}H_{26}O_5$	284.1704 (100)	–	+
28.	Rosmadial isomer	4.98	343.15577	$C_{20}H_{24}O_5$	299.1618 (55), 243.1010 (9)	+	+
29.	Rosmanol methyl ether	5.08	359.14801	$C_{21}H_{18}O_5$	315.1577 (19)	–	+
30.	Rosmanol	5.15	345.16890	$C_{20}H_{26}O_5$	283.1669 (69)	–	+
31.	Carnosol isomer	5.49	329.17480	$C_{20}H_{26}O_4$	285.1825 (100)	–	+
32.	Rosmadial	5.61	343.15305	$C_{20}H_{24}O_5$	299.1623 (100)	+	+
33.	Trihydroxy-methoxyflavone	5.70	299.16397	$C_{16}H_{12}O_6$	284.0310 (100)	–	+
34.	Carnosol	5.75	329.17666	$C_{20}H_{26}O_4$	285.1834 (100)	+	+
35.	Carnosic acid	5.76	331.18358	$C_{20}H_{28}O_4$	287.1649 (100)	+	+
36.	Rosmaridiphenol	6.16	315.19780	$C_{20}H_{26}O3$	285.1843 (19)	+	+
37.	Rosmadial isomer	6.20	343.15249	$C_{20}H_{24}O_5$	299.1598 (56)	+	+
38.	Rosmadial isomer	6.56	343.15233	$C_{20}H_{24}O_5$	299.1602 (68)	+	+
39.	12-methoxy-carnosic acid	6.99	345.20823	$C_{21}H_{30}O_4$	301.2157 (100), 286.1923 (65)	+	+
40.	Betulinic acid	8.05	455.34934	$C_{30}H_{48}O_3$	–	+	–
41.	Ursolic acid	8.10	455.35307	$C_{30}H_{48}O_3$	–	+	+

T_1: Fresh tissue homogenization; T_3: Fresh homogenized tissue extract fermentation; * Fragmentation values are followed by their intensity % in parenthesis.

Table 3. Analysis of bioactive compounds in fresh leaf (T_2) and dry leaf decoction (T_5) and their respective fermented extracts (T_4 and T_6) of *R. officinalis* by UHPLC-ESI-QTOF-MS.

Sl No	Compound	RT (min)	Mass $[M-H]^-$ (*m/z*)	Formula	Fragments	T_2	T_4	T_5	T_6
1.	Quinic acid	0.35	191.05670	$C_7H_{12}O_6$	85.0301 (39), 93.0354 (18), 127.0406 (15)	+	+	+	+
2.	Syringic acid	0.42	197.04643	$C_9H_{10}O_5$	135.0450 (100), 123.0450 (100), 72.9947 (84), 179.0349 (54)	+	+	+	+
3.	Chlorogenic acid	0.67	353.08621	$C_{16}H_{18}O_9$	191.0560 (28)	+	−	+	−
4.	Caffeic acid	0.78	179.03600	$C_9H_8O_4$	135.0444 (100), 134.0372 (19)	+	+	+	+
5.	4-*O*-Caffeoyl quinic acid	1.00	353.08940	$C_{16}H_{18}O_9$	173.0439 (100), 179.0329 (37), 135.0434 (14)	+	−	+	−
6.	*p*-Coumaric acid	1.30	163.04015	$C_9H_8O_3$	119.0509 (100)	+	+	+	+
7.	Gallocatechin	1.40	305.07127	$C_{15}H_{14}O_7$	225.1123 (49), 96.9595 (24)	+	+	+	+
8.	6-Hydroxyluteolin-7-*O*-glucoside	1.89	463.08849	$C_{21}H_{20}O_{12}$	286.0427 (100), 301.0350 (69), 285.7613 (44)	+	−	+	−
9.	Luteolin-7-*O*-glucoside	2.18	447.09508	$C_{21}H_{20}O_{11}$	285.0413 (53)	+	−	+	+
10.	Luteolin 7-*O*-rutinoside	2.18	593.15454	$C_{27}H_{30}O_{15}$	285.0431 (11)	+	+	+	+
11.	Scutellarin	2.21	461.07517	$C_{21}H_{18}O_{12}$	285.0405 (100), 113.0252 (9), 175.0252 (6)	−	−	−	+
12.	Rosmarinic acid-3-*O*-glucoside	2.23	521.13273	$C_{24}H_{26}O_{13}$	359.0792 (100), 324.0832 (78), 323.0785 (60)	+	+	+	+
13.	Salvianolic acid B	2.29	717.15054	$C_{36}H_{30}O_{16}$	519.0891 (100), 339.0500 (15)	+	+	+	+
14.	Isorhamnetin-3-*O*-glucoside	2.33	477.10584	$C_{22}H_{22}O_{12}$	315.0539(38)	+	+	+	+
15.	Sagerinic acid	2.52	719.16630	$C_{36}H_{32}O_{16}$	359.0761 (100), 179.0336 (20), 161.0223 (16)	+	+	+	+
16.	Rosmarinic acid	2.56	359.07835	$C_{18}H_{16}O_8$	161.0238 (100), 197.0447 (64), 179.0341 (57), 133.0290 (35), 72.9940 (6)	+	+	+	+
17.	Apigenin-7-*O*-glucoside	2.59	431.09779	$C_{21}H_{20}O_{10}$	269.0420 (100), 149.0969 (3)	−	−	+	−
18.	Hesperidin	2.63	609.18493	$C_{28}H_{34}O_{15}$	301.0714 (100)	+	+	+	+
19.	Salvianolic acid A	2.64	493.11382	$C_{26}H_{22}O_{10}$	295.0615 (100), 185.0224 (43), 109.0289 (11)	+	+	+	+
20.	Diosmin	2.66	607.17017	$C_{28}H_{32}O_{15}$	301.0704 (100), 299.0551 (59)	+	+	+	+
21.	Hispidulin-7-*O*-glucoside	2.68	461.11080	$C_{22}H_{22}O_{11}$	283.0235 (12), 299.0552 (9)	+	−	+	−
22.	Luteolin-7-*O*-glucuronide	2.79	461.07517	$C_{21}H_{18}O_{12}$	286.0430 (100), 285.0399 (38)	+	+	+	+
23.	Hesperetin	2.87	301.07245	$C_{16}H_{14}O_6$	286.0459 (12), 164.0100 (4)	−	−	+	−
24.	Methyl rosmarinate	2.99	373.09453	$C_{19}H_{18}O_8$	175.0403 (100), 357.0610 (61), 198.0477 (33), 179.0367 (22), 135.0465 (11)	+	+	+	+
25.	Luteolin 3′-acetyl-*O*-glucuronide isomer I	3.10	503.08463	$C_{23}H_{20}O_1$	399.0721 (100), 285.7547 (6)	+	+	+	+
26.	Luteolin	3.11	285.04114	$C_{15}H_{10}O_6$	133.0302 (18), 151.0051 (6), 175.0410 (5), 199.0414 (4)	+	+	+	+

Table 3. *Cont.*

Sl No	Compound	RT (min)	Mass $[M-H]^-$ (*m/z*)	Formula	Fragments	T_2	T_4	T_5	T_6
27.	Isorhamnetin	3.17	315.05133	$C_{16}H_{12}O_7$	300.0279 (100), 301.0332 (32)	+	+	+	+
28.	Luteolin 3′-acetyl-*O*-glucuronide isomer II	3.27	503.08550	$C_{23}H_{20}O_1$	286.0415 (100), 285.7547 (62), 443.0607 (60), 399.0721 (7)	+	+	+	+
29.	Apigenin	3.47	269.04675	$C_{15}H_{10}O_5$	117.0350 (8), 151.0043(7), 225.0576 (4)	+	+	+	+
30.	Luteolin 3′-acetyl-*O*-glucuronide	3.62	503.08530	$C_{23}H_{20}O_{13}$	286.0415 (100), 443.0607 (47), 285.7547 (38)	+	+	+	+
31.	Diosmetin	3.64	299.05647	$C1_6H_{12}O_6$	284.0339 (100)	+	+	+	+
32.	Rosmanol isomer	4.07	345.17252	$C_{20}H_{26}O_5$	301.1802 (100), 283.1698 (67)	+	+	+	+
33.	3,7 Dihydroxy-dimethoxyflavone	4.15	313.07320	$C_{17}H_{14}O_6$	298.0473 (100), 283.0243 (70), 255.0306 (19), 269.0464 (12)	–	–	+	–
34.	Pectolinarigenin	4.15	313.07258	$C_{17}H_{14}O_6$	298.0469 (100), 283.0240 (63)	+	+	–	+
35.	Rosmanol	4.30	345.17100	$C_{20}H_{26}O_5$	283.8834 (18)	+	+	+	+
36.	Pectolinarigenin isomer	4.37	313.07211	$C_{17}H_{14}O_6$	298.0471 (91), 283.0233 (63), 255.0290 (24)	+	–	–	–
37.	Rosmanol isomer	4.57	345.17200	$C_{20}H_{26}O_5$	284.1750 (13), 283.1706 (11)	+	+	+	+
38.	Genkwanin	4.58	283.06218	$C1_6H_{12}O_5$	268.0398 (79), 240.0434 (5)	+	+	+	+
39.	Rosmadial isomer	4.80	343.15636	$C_{20}H_{24}O_5$	299.1669 (32)	+	+	+	+
40.	Rosmanol isomer	5.17	345.17210	$C_{20}H_{26}O_5$	283.8769 (39)	+	+	+	+
41.	Rosmanol methyl ether	5.07	359.18598	$C_{21}H_{28}O_5$	283.1703 (100), 300.1747 (82)	+	+	&	&
42.	Asiatic acid	5.57	487.34312	$C_{30}H_{48}O_5$	&	+	&	&	&
43.	Rosmadial	5.69	343.15590	$C_{20}H_{24}O_5$	299.1645 (9)	+	+	+	+
44.	Trihydroxy-methoxyflavone	5.69	299.16340	$C1_6H_{12}O_6$	284.0333 (100)	&	+	&	&
45.	Carnosol	5.75	329.17690	$C_{20}H_{26}O_4$	286.1870 (100), 285.1845 (92)	+	+	+	+
46.	Carnosic acid	5.76	331.19253	$C_{20}H_{28}O_4$	287.2007 (100)	+	+	+	+
47.	Rosmadial isomer	6.04	343.17192	$C_{20}H_{24}O_5$	299.1653 (15)	-	+	&	+
48.	Rosmaridiphenol	6.16	315.19689	$C_{20}H_{28}O_3$	284.1860 (4)	+	+	+	+
49.	Carnosol isomer	6.17	329.17560	$C_{20}H_{26}O_4$	286.1880 (100), 285.1852 (58)	+	&	+	&
50.	12-methoxy-carnosic acid	6.99	345.20853	$C_{21}H_{30}O_4$	286.1943 (100), 301.2186 (81)	+	+	+	+
51.	Micromeric acid	7.64	453.33442	$C_{30}H_{46}O_3$	&	+	&	+	&
52.	Betulinic acid	8.05	455.34921	$C_{30}H_{48}O_3$	&	+	&	+	&
53.	Ursolic acid	8.10	455.35205	$C_{30}H_{48}O_3$	&	+	+	+	+

T_2: Fresh leaf decoction; T_4: Fresh leaf decoction fermentation; T_5: Dry leaf decoction; T_6: Dry leaf decoction fermentation.

Table 4. Analysis of bioactive constituents by UHPLC-ESI-QTOF-MS in Soxhlet extract (T_7) and sonicated extracts (water and methanol—T_8 and T_9) of *R. officinalis*.

Sl No	Compound	RT (min)	Mass $[M-H]^-$ (*m/z*)	Formula	Fragments	T_7	T_8	T_9
1.	Quinic acid	0.35	191.05628	$C_7H_{12}O_6$	85.0299 (34), 93.0353 (17), 127.0403 (12)	+	+	+
2.	Syringic acid	0.42	197.04569	$C_9H_{10}O_5$	135.0450 (100), 123.0450 (75), 72.9947 (60), 179.0349 (54)	+	-	+
3.	Chlorogenic acid	0.56	353.08506	$C_{16}H_{18}O_9$	191.0545 (26)	&	&	+
4.	Caffeic acid	0.82	179.03589	$C_9H_8O_4$	135.0441 (100), 134.0370 (23)	+	&	+
5.	4-*O*-Caffeoyl quinic acid	0.85	353.08949	$C_{16}H_{18}O_9$	173.0437 (100), 191.0544 (27), 179.0334 (6)	&	&	+
6.	*p*-Coumaric acid	1.28	163.04014	$C_9H_8O_3$	119.0509 (100)	+	+	+
7.	Gallocatechin	1.81	305.06932	$C_{15}H_{14}O_7$	96.9588 (65), 225.1109 (59)	+	+	+
8.	6-Hydroxyluteolin-7-*O*-glucoside	1.97	463.08518	$C_{21}H_{20}O_{12}$	301.0350 (69), 285.7613 (44)	&	&	+
9.	3-*p*-coumaroylquinic acid	2.1	337.10122	$C_{16}H_{18}O_8$	163.0397 (100), 119.0506 (32)	+	&	&
10.	Luteolin-7-*O*-glucoside	2.23	447.09286	$C_{21}H_{20}O_{11}$	285.0377 (29)	+	+	+
11.	Luteolin 7-*O*-rutinoside	2.24	593.15126	$C_{27}H_{30}O_{15}$	285.0380 (2)	+	&	+
12.	Rosmarinic acid-3-*O*-glucoside	2.25	521.12957	$C_{24}H_{26}O_{13}$	359.0740 (100), 323.0737 (89), 179.0337 (15)	+	&	+
13.	Apigenin-7-*O*-glucunoride	2.51	445.07602	$C_{21}H_{18}O_{11}$	269.0437 (100), 113.0255 (10), 175.0252 (9)	&	+	&
14.	Sagerinic acid	2.52	719.15575	$C_{36}H_{32}O_{16}$	359.0761 (100), 179 (50), 161.0223 (17)	+	&	+
15.	Rosmarinic acid	2.56	359.07651	$C_{18}H_{16}O_8$	161.0229 (100), 197.0434 (78), 179.0330 (60), 133.0285 (15), 72.9934 (8)	+	+	+
16.	Isorhamnetin-3-*O*-glucoside	2.56	477.10320	$C_{22}H_{22}O_{12}$	315.0466 (32), 300.0246 (5)	+	&	+
17.	Apigenin-7-*O*-glucoside	2.59	431.09762	$C_{21}H_{20}O_{10}$	269.0435 (100)	+	+	+
18.	Isoferulic acid	2.62	193.05102	$C_{10}H_{10}O_4$	134.0386 (89), 133.0295 (75), 178.0271 (12)	+	&	&
19.	Isorhamnetin-3-*O*-rutinoside	2.63	623.15851	$C_{28}H_{32}O_{16}$	315.0471 (9)	+	&	+
20.	Hispidulin-7-*O*-glucuronide	2.64	475.08642	$C_{22}H_{20}O_{12}$	299.0543 (100), 285.0367 (50), 283.0313 (3)	&	+	&
21.	Hesperidin	2.64	609.18172	$C_{28}H_{34}O_{15}$	301.0674 (100)	+	&	+
22.	Salvianolic acid A	2.64	493.11132	$C_{26}H_{22}O_{10}$	295.0615 (100), 185.0224 (43), 109.0289 (11)	+	&	&

Table 4. *Cont.*

Sl No	Compound	RT (min)	Mass $[M-H]^-$ (*m*/*z*)	Formula	Fragments	T_7	T_8	T_9
23.	Diosmin	2.66	607.16666	$C_{28}H_{32}O_{15}$	299.0527 (57), 284.0309 (4)	+	+	+
24.	Hispidulin-7-*O*-glucoside	2.69	461.11138	$C_{22}H_{22}O_{11}$	298.0477 (18), 283.0234 (7)	+	&	+
25.	Kaempferol-7-*O*-hexoside	2.72	447.09094	$C_{21}H_{20}O_{11}$	285.0382 (100)	+	&	+
26.	Luteolin-7-*O*-glucuronide	2.83	461.06888	$C_{21}H_{18}O_{12}$	285.7541 (100)	+	+	+
27.	Phlorizin	2.95	435.13210	$C_{21}H_{24}O_{10}$	273.0767 (100), 167.0349 (28), 125.0247 (5)	+	&	&
28.	Luteolin	3.12	285.04024	$C_{15}H_{10}O_6$	133.0300 (14), 151.0042 (10), 175.0409 (8), 199.0406 (6)	+	+	+
29.	Isorhamnetin	3.21	315.04996	$C_{16}H_{12}O_7$	300.0248 (100), 301.0286 (41)	+	&	+
30.	Luteolin 3′-acetyl-*O*-glucuronide isomer I	3.29	503.08148	$C_{23}H_{20}O_{13}$	399.0707(100), 285.4652 (9), 443.0598 (5)	&	+	&
31.	Methyl rosmarinate	3.30	373.08946	$C_{19}H_{18}O_8$	135.0441 (100), 175.0397 (100), 179.0346 (85), 197.0449 (79)	+	&	+
32.	Luteolin 3′-acetyl-*O*-glucuronide II	3.38	503.08230	$C_{23}H_{20}O_{13}$	286.0407 (100), 285.4649 (38)	+	+	+
33.	Apigenin	3.50	269.04386	$C_{15}H_{10}O_5$	117.0347 (11), 151.0036 (11), 225.0557 (5)	+	+	+
34.	Salvianolic acid B	2.29	717.14025	$C_{36}H_{30}O_{16}$	519.0912 (57), 339.0471 (37)	+	&	&
35.	Hesperetin	3.58	301.07242	$C_{16}H_{14}O_6$	164.0114 (11), 286.0827 (5)	&	+	&
36.	Luteolin 3′-acetyl-*O*-glucuronide	3.62	503.08259	$C_{23}H_{20}O_{13}$	443.0607 (100), 285.7547 (68)	+	+	+
37.	Diosmetin	3.64	299.05429	$C_{16}H_{12}O_6$	284.0298 (100)	+	+	+
38.	Rosmanol isomer	4.07	345.16839	$C_{20}H_{26}O_5$	301.1773 (100), 283.1668 (65)	+	&	+
39.	Pectolinarigenin	4.15	313.06990	$C_{17}H_{14}O_6$	298.0474 (100), 283.0237 (59), 255.0295 (24), 163.0037 (15), 117.0345 (7)	+	+	+
40.	Rosmanol	4.30	345.16852	$C_{20}H_{26}O_5$	301.1773 (100), 283.1668 (57)	+	&	+
41.	Pectolinarigenin isomer	4.37	313.06892	$C_{17}H_{14}O_6$	298.0471 (91), 283.0233 (63), 255.0290 (24)	+	&	&
42.	Triptolidenol	4.45	375.15421	$C_{20}H_{24}O_7$	331.1526 (13), 244.1082 (9), 313.1430 (7)	&	&	+
43.	Rosmadial isomer	4.54	343.15249	$C_{20}H_{24}O_5$	299.1610 (9)	+	&	+
44.	Genkwanin	4.58	283.06017	$C_{16}H_{12}O_5$	268.0379 (89), 117.0353 (5), 151.0039 (4)	+	+	+

Table 4. *Cont.*

Sl No	Compound	RT (min)	Mass $[M - H]^-$ (*m*/*z*)	Formula	Fragments	T_7	T_8	T_9
45.	Rosmanol isomer	4.59	345.16789	$C_{20}H_{26}O_5$	284.1687 (40), 283.8801 (23)	+	&	+
46.	Rosmanol isomer	4.80	345.16793	$C_{20}H_{26}O_5$	283.1668 (19)	+	&	+
47.	Asiatic acid	5.47	487.34312	$C_{30}H_{48}O_5$	&	+	&	&
48.	Rosmadial	5.71	343.15314	$C_{20}H_{24}O_5$	299.1616 (10)	+	+	+
49.	Rosmanol isomer	5.71	345.16811	$C_{20}H_{26}O_5$	283.1670 (12)	&	+	&
50.	Carnosol	5.75	329.17532	$C_{20}H_{26}O_4$	285.1833 (100)	+	+	+
51.	Epirosmanol methyl ether	5.85	359.18381	$C_{21}H_{28}O_5$	283.1665 (97), 329.1719 (16), 300.1713 (15)	+	&	+
52.	Rosmadial isomer	6.05	343.15233	$C_{20}H_{24}O_5$	299.1621 (11)	+	&	+
53.	Carnosic acid	6.58	331.19123	$C_{20}H_{28}O_4$	287.1982 (100)	+	&	+
54.	Corosolic acid	6.61	471.34212	$C_{30}H_{48}O_4$	&	&	&	+
55.	12-methoxy-carnosic acid	6.99	345.20630	$C_{21}H_{30}O_4$	301.2170 (100), 287.1938 (64)	+	&	+
56.	Micromeric acid	7.84	453.34261	$C_{30}H_{46}O_3$	&	+	&	+
57.	Betulinic acid	8.05	455.34934	$C_{30}H_{48}O_3$	&	+	&	+
58.	Ursolic acid	8.10	455.35011	$C_{30}H_{48}O_3$	&	+	+	+

T_7: Soxhlet extraction; T_8: Sonic extraction—aqueous; T_9: Sonic extraction—methanol.

As shown in Table 2, there were about 41 polyphenols detected in fresh homogenized tissue extraction (T_1) and its fermentation (T_3); among them, T_1 contained 30 and T_3 contained 33 polyphenols. The chromatogram in Figure 1a,b represents the relative intensity of phenolic compounds in T_1 and T_3, respectively. The fermented sample (T_3) was found to have a higher intensity of rosmanol and rosmadial compared to T_1, whereas the relative intensities of luteolin 3-acetyl-*O*-glucuronide and carnosol were high in T_1. Rosemary leaf decoctions (fresh and dry, T_2, and T_5, respectively) were found to be a more efficient extraction method for polyphenol content. In total, 54 phenolic compounds were identified in T_2 and T_5 and their fermented extracts (T_4 and T_6) (Table 3). The intensities of phenolic compounds and terpenoids were lower in the fermented decoctions compared to the fresh and dry leaf decoctions (Figure 1c–f). This is consistent with earlier reports with other herbs, indicating that prolonged fermentation can break down phenolic compounds resulting in decreased antioxidant potential [22].

A large group of phenolic compounds was observed in Soxhlet and sonicated methanol extracts (T_7 and T_9, respectively). Of the 59 polyphenols, 11 were tentatively identified as phenolic acids and seventeen as terpenoids (Table 4). In methanolic samples, the intensity of terpenoid compounds, rosmanol, rosmadial, carnosol, carnosic acid, and ursolic acid, was found to be very high, as is depicted in their chromatograms in Figure 1g,i. Sonication of rosemary in water (T_8) resulted in a much lower number of polyphenols, compared to methanolic extraction (Figure 1h). This is likely due to the lower solubility of complex terpenoids and phenolic molecules in water compared to methanol [24]. Out of 11 identified phenolic compounds in methanol extracts, quinic acid, syringic acid, chlorogenic acid, caffeic acid, 4-*O*-caffeoylquinic acid, p-coumaric acid, and rosmarinic acid have been reported before [16,30]. However, isoferulic acid ($[M - H]^-$ *m/z* 193.05), sagerinic acid ($[M - H]^-$ *m/z* 719.16), and salvianolic acid A ($[M - H]^-$ *m/z* 493.11) and B ($[M - H]^-$ *m/z* 717.15) were reported herein for the first time in rosemary extracts, based on comparison of the *m/z* ion fragmentation pattern of the observed compounds compared to those in the NIST MS library. Sagerinic acid was found in very high intensities (T_2, T_4, T_5, T_6, T_7, and T_9—Figure 1), and it shared some (*m/z*) MS/MS ion fragments (359.08) with rosmarinic acid ($[M - H]^-$ *m/z* 359.08). Lu and Foo reported sagerinic acid as possible derivatives of rosmarinic acid, since they are structurally related [32]. Similarly, syringic acid ($[M - H]^-$ at *m/z* 197.05), 4-*O*-caffeoylquinic acid ($[M - H]^-$ at *m/z* 353.09), rosmarinic acid ($[M - H]^-$ at *m/z* 359.08), and methyl rosmarinate ($[M - H]^-$ at *m/z* 373.09) all shared many of the same MS/MS (*m/z*) ion fragments (179.03), since they were found to be dimers of caffeic acid.

A large group of flavonoids has been reported in this study, and most of them were derivatives of luteolin ($[M - H]^-$ at *m/z* 285.04), hesperidin ($[M - H]^-$ at *m/z* 609.18), and apigenin ($[M - H]^-$ at *m/z* 269.04). Similar results were obtained from LC/MS analysis of rosemary herb from the USA and Iraq [17,31]. Very high intensities of gallocatechin ($[M - H]^-$ at *m/z* 305.07) were observed in all rosemary extracts (Figure 1a–i), and this was reported in previous studies [6,17]. Gallocatechin is a flavan-3-ol found predominantly in fruit peels, and gallocatechin was reported to be responsible for the high antioxidant potential of the herb [33–35]. In the present study, some flavonoid compounds have been detected for the first time in rosemary, viz., phlorizin ($[M - H]^-$ at *m/z* 435.13) in Soxhlet extract (Table 4) and pectolinarigenin ($[M - H]^-$ at *m/z* 313.07) in all the extracts (T_1–T_9). Phlorizin was earlier found in tree barks of the Rosaceae family, and the studies indicated high antidiabetic property of the drug [36]. Pectolinarigenin was also reported before in rosemary as dimethoxyflavone with similar fragment ions, and it was found to have potent anti-inflammatory and anticancer properties [31,37,38]. Further, these newly detected flavonoids and phenolic acids can be confirmed by procuring respective standards or by using advanced techniques like nuclear magnetic resonance (NMR) spectroscopy for identification and confirmation of unknown molecules. The presence of three peaks for luteolin 3′-acetyl-*O*-glucuronide ($[M - H]^-$ at *m/z* 503.08) eluted at 3.29, 3.38, and 3.62 min with similar *m/z* fragments (443.06, 245.47) could be observed in chromatograms of all extracts (T_1–T_9). Previously, multiple peaks for luteolin 3′-acetyl-*O*-glucuronide in rosemary extract were reported by Borras-Linares [17]. These are probably due to the existence of multiple positional isomers of this compound in rosemary.

There were about 17 terpenoid compounds that have been tentatively identified in methanolic extracts of rosemary, out of which 12 were diterpenoids (Table 4). Rosmanol ($[M - H]^-$ at *m/z* 345.17),

rosmadial ($[M - H]^-$ at *m/z* 343.15), carnosol ($[M - H]^-$ at *m/z* 329.18), carnosic acid ($[M - H]^-$ at *m/z* 331.19), and 12-methoxy carnosic acids ($[M - H]^-$ at *m/z* 345.21) were the major diterpenoids present in higher intensities in T_2, T_3, T_4, T_5, T_6, T_7, and T_9 (Figure 1). The presence of more than one peak corresponding to the same molecular mass but different elution times was due to the presence of isomers, especially in rosmanol and rosmadial. Rosmanol ($[M - H]^-$ *m/z* 345.17) eluted at four different retention times, with the same ion fragmentation (MS^2 *m/z* fragments 301.1779, 183.1668). Rosmadial ($[M - H]^-$ *m/z* 343.15) and its isomers also resulted in three to four peaks with similar fragmentation patterns (MS^2 *m/z* 299.16). Similar peaks were obtained in rosmadial of sage and rosemary extracts during the chromatographic determination of polyphenols [31,39]. A diterpenoid, triptolidenol ($[M - H]^-$ at *m/z* 375.15), was detected only in T_9. Five pentacyclic triterpenoid compounds viz., asiatic acid ($[M - H]^-$ at *m/z* 487.33), corosolic acid ($[M - H]^-$ at *m/z* 471.34), micromeric acid ($[M - H]^-$ at *m/z* 453.34), betulinic acid, and ursolic acid ($[M - H]^-$ at *m/z* 455.35) were tentatively detected in methanolic samples (Table 4). Previously, betulinic acid, ursolic acid, and micromeric acids were determined in rosemary leaves [17,31]. Betulinic acid in the herbs was found to have potent antiviral activity against severe acute respiratory syndrome coronavirus [40]. A triterpenoid corosolic acid was tentatively identified for the first time in the rosemary extract of T_7. Asiatic acid was present in T_2 and T_7; micromeric acid and betulinic acid were detected in T_2, T_5, T_7, and T_9; ursolic acid was found in all extracts. Pentacyclic triterpenoids reported having several medicinal properties, especially anti-inflammatory, anticancer, and antidiabetic potential [41,42]. Even though ursolic acid and betulinic acid have the same pseudomolecular weight ($[M - H]^-$ at *m/z* 455.35), the former was identified through the reference standard, and the later molecule was confirmed by comparison to the NIST mass spectral library. Besides, several other compounds were detected in significant amounts in certain extracts that were not represented in the mass spectral databases available.

Even though comparison of high resolution, accurate mass, LC-MS/MS chromatograms and m/z fragmentation patterns of observed compounds with high-resolution mass spectral libraries is a very effective approach for the identification and characterization of known and previously unknown compounds, this approach is limited to those compounds represented in MS/MS libraries. Our analyses generated mass spectral data for a large number of yet-to-be-identified phenolic compounds present in the rosemary extracts, which we analyzed. As mass spectral libraries expand, the data that we have already gathered can be further analyzed to structurally identify additional polyphenols based on *m/z* fragmentation patterns. In addition, if further inspection of our data identifies unnamed compounds that are of particular interest, possibly due to a high abundance of other features of interest, then additional work can be done to isolate and identify those compounds using nuclear magnetic resonance (NMR) spectroscopy and other approaches.

3. Materials and Methods

3.1. Herb Collection

Rosemary (*Rosmarinus officinalis* L.) leaves after eight months of planting were harvested from the Regenerative Organic Farm, Maharishi University of Management, Fairfield, Iowa. Freshly harvested leaves were used for fresh extractions, whereas air-dried leaf powder was used for dry extractions. The sample was submitted to Ada Hayden Herbarium (ISC/IA), Iowa State University, Iowa, USA, and obtained the accession no. ISC-454695.

3.2. Chemicals

LCMS grade acetonitrile and methanol were purchased from Honeywell, Burdick, and Jackson, USA. LCMS grade formic acid and glacial acetic acid were procured from Merck, Germany. Caffeic acid, rosmarinic acid, carnosic acid, ursolic acid, and luteolin 7-glucoside were purchased from Toronto Research Chemicals, Canada. Carnosol and ^{13}C- caffeic acid were purchased from Cayman Chemical,

USA. Ultrapure water from the Milli-Q, A10 water purification system (Millipore Sigma, Madison, WI., USA) was used throughout the experiment.

3.3. Preparation of R. officinalis Extracts

There were nine different sample extraction methods used as treatments for liquid chromatographic analysis.

T_1: Fresh aqueous extraction by tissue homogenization—10 g of fresh leaf samples was macerated in 100 mL Milli-Q water at room temperature and fresh leaf juice was extracted by filtering through cellulose filter paper.

T_2: Fresh leaf decoction—10 g of fresh leaves was chopped into 1–2 cm pieces and boiled in 200 mL Milli-Q water at 100–110 °C temperature until the volume was reduced to 100 mL. The extract was cooled, filtered, and used for the analysis.

T_3: Fresh tissue homogenized extract fermentation—homogenized fresh leaf tissue extract (T_1) was fermented by adding 24% sugar and 10 mg of the activated wine yeast *Saccharomyces cerevisiae* for 60 days; the resultant clear fermented extract was filtered and used for analysis.

Preparation of yeast (*Saccharomyces cerevisiae*) inoculum: 10 mg of commercial wine yeast culture (Lalvin EC-1118 strain—produced in Canada from grape skin) was dissolved in 2 mL of warm water (43 °C) for 10 min; as the yeast activates at warm water, it starts producing small bubbles. A total of 2 mL of such activated *Saccharomyces cerevisiae* culture was added into the rosemary extracts for fermentation.

T_4: Fresh leaf decoction fermentation—T_2 samples were fermented by adding 24% sugar and the activated wine yeast *Saccharomyces cerevisiae* culture for 60 days.

T_5: Dry leaf decoction—10 g of leaf powder was boiled in 200 mL Milli-Q water at 100–110 °C temperature until the volume was reduced to 100 mL, and the extract was cooled, filtered, and used for further analysis.

T_6: Dry leaf decoction fermentation—T_5 samples were fermented by adding 24% sugar and the activated wine yeast *Saccharomyces cerevisiae* culture for 60 days.

T_7: Soxhlet extraction—10 g of leaf powder was extracted using 250 mL LCMS grade methanol in the Soxhlet apparatus at 70 °C for 6 h, and the volume was further reduced to 100 mL by a vacuum evaporator and filtered through a 0.2 μ Nalgene filter unit from Thermo Fisher Scientific Inc. (Waltham, MA, USA).

T_8 and T_9: Sonic/ultrasound extraction in water and methanol, respectively—10 g of leaf powder was extracted in 100 mL Milli-Q water and methanol (50 °C) for 2 h with a frequency of 40 kHz in a Bransonic-52 ultrasonic bath unit from Branson, USA.

All extractions were made in triplicates and stored protected from light at −20 °C until chromatographic analysis.

3.4. UHPLC-ESI-QTOF-MS Method Development

R. officinalis samples were analyzed by ultra-high-performance liquid chromatography, electrospray ionization coupled with quadrupole-time of flight mass spectrometry (UHPLC-ESI-QTOF-MS). The analysis was carried out by reverse-phase UHPLC (Shimadzu Nexera, Kyoto, Japan) directly connected to a quadrupole Time-of-Flight (QTOF) Triple TOF 5600 mass spectrometer (AB SCIEX, Concord, ON, Canada). The autosampler (Shimadzu SIL30AC, Kyoto, Japan) was operated in direct injection mode, filling a 50 μL loop with 10 μL analyte for optimal sample delivery reproducibility. Samples were passed through the C_{18} column (Kinetex XB, 1mm I.D. × 5 cm, 2.6 μm, particle size, 100 Å) and eluted at a flow rate of 250 μL/min. Pumps (Shimadzu LC30AD, Kyoto, Japan) were operated in the following multi-step linear gradient with different proportion of mobile phase B: 0 min, 10% B; 10 min, 90% B; 12.5 min, 90% B; 15 min, 10% B; 20 min, 10% B, with a total runtime of 20 min including mobile phase equilibration. Mobile phases A and B used were 0.1% of acetic acid made in Milli-Q water and acetonitrile, respectively. The column oven (Shimadzu CTO30A, Kyoto, Japan) was set to 40 °C.

3.5. Identification and Quantification of Polyphenols

Mass spectra and tandem mass spectra data were recorded in electrospray ionization (ESI), "negative-ion" mode with a resolution of ~35,000 full-width half-maximum on the QTOF 5600. The ion spray needle voltage was at −4500 V with drying gas temperature 600 °C, and ion source Gas 1 (nebulizer) and Gas 2 (heater) values were 50 psi each. The collision-energy values for QTOF MS were at 5 eV and for MS/MS experiments at 25 eV with a spread of 15 eV. For collision-induced dissociation tandem mass spectrometry, the mass window for precursor ion selection of the quadrupole mass analyzer was set to ±1 *m/z*. The precursor ions were fragmented in a collision cell using nitrogen as the collision gas. Data independent acquisitions (DIA) with SWATH-MS^2 cover the mass range of *m/z* 50–1000 in 16 segments (15 × 48.5 ms), yielding a cycle time of 0.8268 s, which includes one 50 msec MS^1 scan. During the execution of the liquid chromatography method, the mass spectrometer was externally calibrated using a known mixture of masses from Sciex (P/N 4460134, AB SCIEX, Concord, ON, Canada).

Quantitative analysis was performed by diluting the extracted samples with 0.1% formic acid (1/10 to 1/10,000) in order to quantify the samples within the linearity range of the standard calibration curve, avoiding MS signal saturation. The method was validated for sensitivity and precision. The standard calibration curves were constructed for quantification of caffeic acid, rosmarinic acid, luteolin-7-*O*-glucoside, carnosol, carnosic acid, and ursolic acid. Table 5 represents calibration parameters, including limits of quantification (LOQ), calibration range, equations, and slope. All samples were extracted and analyzed in triplicate. Unknown polyphenolic compounds and flavonoids were identified based on their accurate mass (*m/z*) and molecular (*m/z*) ion fragmentation patterns using Peak view Software (ver.2.2, AB SCIEX, Concord, Canada), Master view, Library view (AB SCIEX, Concord, ON, Canada), National Institute of Standards and Technology (NIST), and the AOI database.

Table 5. Results of analysis of calibration curve and limits of quantification.

	Standard	Purity (%)	Formula	Molecular Weight	LOQ (ng/mL)	Calibration Range (ng/mL)	Calibration Equations	Slope (R^2)
1	Caffeic acid	98.0	$C_9H_8O_4$	180.00	6.0	6–250	$y = 0.00523x + 0.00157$	0.9993
2	Rosmarinic acid	98.0	$C_{18}H_{16}O_8$	360.31	6.0	6–250	$y = 0.00374x - 0.00269$	0.9998
3	Luteolin-7-*O*-glucoside	98.0	$C_{21}H_{20}O_{11}$	448.38	6.0	6–250	$y = 0.00347x - 0.00156$	0.9994
4	Carnosol	100.0	$C_{20}H_{26}O_4$	330.40	6.0	6–250	$y = 0.00673x + 0.03215$	0.9982
5	Carnosic acid	96.0	$C_{20}H_{28}O_4$	332.43	24.0	24–1000	$y = 9.06876 \times 10^{-5}x + 0.00303$	0.9984
6	Ursolic acid	97.0	$C_{30}H_{48}O_3$	456.70	24.0	24–1000	$y = 0.00147x - 0.03017$	0.9938

Limits of quantification (LOQ).

3.6. Statistical Analysis

The results of polyphenol quantification were expressed as mean ± SD. The data were analyzed statistically by using single-factor ANOVA in MS Excel software. The critical difference at 1% level of significance or Tukey's HSD (Honestly Significant Difference) test (at $p < 0.01$) was used to compare the significant difference between the treatments [43].

4. Conclusions

Rapid separation of most of the polyphenols was achieved within the first 11 min during 20 min of UHPLC analysis. Among all the extraction methods, Soxhlet extraction yielded significantly higher levels of polyphenols, both in terms of numbers of compounds and levels of these compounds. Dry leaf decoction was found to be the next best extraction method for rosemary, yielding significantly higher caffeic acid, rosmarinic acid, carnosol, carnosic acid, and flavonoids. This might be the best method for large-scale commercial extraction. Sonic extraction with methanol was found to be the second-best for the extraction of rosmarinic acid and ursolic acid. Most of the extractions in the study yielded a high concentration of rosmarinic acid up to 33.49 mg/g, contributing substantially to the high antioxidant potential of the extracts. As compared to previous studies, the rosemary extract of our study recorded a higher concentration of bioactive constituents, indicating the quality of the herb grown in Fairfield, Iowa, USA. The present study also helps to choose an efficient extraction method for obtaining maximum polyphenolic and terpenoid content, not only in rosemary but also in similar herb species. UHPLC-ESI-QTOF-MS methodology for the analysis proved to be very efficient in the identification and characterization of targeted and untargeted phenolic compounds present in the rosemary. However, there is substantial scope to investigate structurally and functionally the many potentially interesting but yet-unidentified phenolic compounds present in rosemary.

Supplementary Materials: Figure S1: QTOF-MS spectrum of caffeic acid, Figure S2: QTOF-MS spectrum of rosmarinic acid, Figure S3: QTOF-MS spectrum of luteolin-7-*O*-glucoside, Figure S4: QTOF-MS spectrum of carnosol, Figure S5: QTOF-MS spectrum of carnosic acid, Figure S6: QTOF-MS spectrum of ursolic acid.

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References

1. Orhan, I.; Aslan, S.; Kartal, M.; Sener, B.; Baser, K.H.C. Inhibitory effect of Turkish *Rosmarinus officinalis* L. on acetylcholinesterase and butyrylcholinesterase enzymes. *Food Chem.* **2008**, *108*, 663–668. [CrossRef] [PubMed]
2. Minaiyan, M.; Ghannadi, A.R.; Afsharipour, M.; Mahzouni, P. Effects of extract and essential oil of *Rosmarinus officinalis* L. on TNBS-induced colitis in rats. *Res. Pharm. Sci.* **2011**, *6*, 13–21. [PubMed]
3. Al Sereitia, M.R.; Abu-Amerb, K.M.; Sena, P. Pharmacology of rosemary (*Rosmarinus officinalis* Linn.) and its therapeutic potentials. *Indian J. Exp. Biol.* **1999**, *37*, 124–131.
4. Jordan, M.J.; Lax, V.; Rota, M.C.; Loran, S.; Sotomayor, J.A. Effect of bioclimatic area on the essential oil composition and antibacterial activity of *Rosmarinus officinalis* L. *Food Control.* **2013**, *30*, 463–468. [CrossRef]
5. Duke, J.A. *Hand Book of Medical Herbs*; CRC Press: Boca Raton, FL, USA, 2001; p. 677.
6. Bai, N.; He, K.; Roller, M.; Lai, C.S.; Shao, X.; Pan, M.H.; Ho, C.T. Flavonoids and Phenolic Compounds from *Rosmarinus officinalis*. *J. Agric. Food Chem.* **2010**, *58*, 5363–5367. [CrossRef]
7. Habtemariam, S. The Therapeutic Potential of Rosemary (*Rosmarinus officinalis*) Diterpenes for Alzheimer's Disease. *Evid. Based Complement Altern. Med.* **2016**. [CrossRef]

8. Ghisoni, S.; Chiodelli, G.; Rocchetti, G.; Kane, D.; Lucini, L. UHPLC-ESI-QTOF-MS screening of lignans and other phenolics in dry seeds for human consumption. *J. Funct. Foods* **2017**, *34*, 229–236. [CrossRef]
9. Li, H.; Yao, W.; Liu, Q.; Xu, J.; Bao, B.; Shan, M.; Cao, Y.; Cheng, F.; Ding, A.; Zhang, L. Application of UHPLC-ESI-Q-TOF-MS to identify multiple constituents in processed products of the herbal medicine Ligustri Lucidi Fructus. *Molecules* **2017**, *22*, 689. [CrossRef]
10. Sayyad, S.F.; Randive, D.S.; Jagtap, S.M.; Chaudhari, S.R.; Panda, B.P. Preparation and evaluation of fermented Ayurvedic formulation: Arjunarishta. *J. Appl. Pharm. Sci.* **2012**, *5*, 122–124.
11. Valiathan, M.S. *The Legacy of Susruta*; Orient Longman Pvt Ltd.: Hyderabad, India, 2007.
12. Sabu, A.; Haridas, M. Fermentation in ancient Ayurveda: Its present implications. *Front. Life Sci.* **2015**, *8*, 324–331. [CrossRef]
13. Azwanida, N.N. A Review on the Extraction Methods Use in Medicinal Plants, Principle, Strength and Limitation. *Med. Aromat. Plants* **2015**, *4*, 3–8.
14. Vinatoru, M. An overview of the ultrasonically assisted extraction of bioactive principles from herbs. *Ultrason. Sonochem.* **2001**, *8*, 303–313. [CrossRef]
15. Pandey, A.; Tripathi, S. Concept of standardization, extraction and pre phytochemical screening strategies for herbal drug. *J. Pharmacogn. Phytochem.* **2014**, *115*, 115–119.
16. Hussain, M.H. Fast high-performance liquid chromatography and ultraviolet method for determination of phenolic antioxidants in fresh rosemary leaves. *J. Nat. Sci. Res.* **2015**, *5*, 89–92.
17. Borras-Linares, I.; Stojanovic, Z.; Quirantes-Pine, R.; Arraez-Roman, D.; Svarc-Gajic, J.; Fernandez-Gutierrez, A.; Segura-Carretero, A. *Rosmarinus officinalis* leaves as a natural source of bioactive compounds. *Int. J. Mol. Sci.* **2014**, *15*, 20585–20606. [CrossRef]
18. Tandon, S.; Rane, S. Decoction and Hot Continuous Extraction Techniques. In *Extraction Technologies for Medicinal and Aromatic Plants*; Handa, S.S., Khanuja, S.P.S., Longo, G., Rakesh, D.D., Eds.; ICS-UNIDO: Trieste, Italy, 2008; pp. 93–106.
19. Mishra, A.K.; Gupta, A.; Gupta, V.; Sand, R.; Bansal, P. Asava and arishta: An Ayurvedic medicine—An overview. *Int. J. Pharm. Biol. Arch.* **2010**, *1*, 24–30.
20. Mulay, S.; Khale, A. Asavarishtas through improved fermentation technology. *Int. J. Pharm. Sci. Res.* **2011**, *2*, 1421–1425.
21. Ariffin, F.; Heong, C.S.; Bhupinder, K.; Karim, A.A.; Huda, N. Antioxidant capacity and phenolic composition of fermented *Centella asiatica* herbal teas. *J. Sci. Food. Agric.* **2011**, *91*, 2731–2739. [CrossRef]
22. Heong, C.S.; Bhupinder, K.; Huda, N.; Karim, A.A.; Fazilan, A. Effect of fermentation on the composition of *Centella asiatica* teas. *Am. J. Food Technol.* **2011**, *6*, 581–593. [CrossRef]
23. Hunaefi, D.; Smetanska, I. The effect of tea fermentation on rosmarinic acid and antioxidant properties using selected in vitro sprout culture of *Orthosiphon aristatus* as a model study. *SpringerPlus* **2013**, *2*, 1–14. [CrossRef]
24. Li, H.; Pordesimo, L.; Weiss, J. High intensity ultrasound—Assisted extraction of oil from soybeans. *Food Res. Int.* **2004**, *37*, 731–738. [CrossRef]
25. Do, Q.D.; Angkawijaya, A.E.; Tran-Nguyen, P.L.; Huynh, L.H.; Soetaredjo, F.E.; Ismadji, S.; Ju, Y. Effect of extraction solvent on total phenol content, total flavonoid content, and antioxidant activity of *Limnophila aromatica*. *J. Food Drug Anal.* **2014**, *22*, 296–302. [CrossRef]
26. Dhawan, D.; Gupta, J. Comparison of Different Solvents for Phytochemical Extraction Potential from *Datura metel* Plant Leaves. *Int. J. Biol. Chem.* **2017**, *11*, 17–22.
27. Qiang, Z.; Ye, Z.; Hauck, C.; Murphy, P.A.; McCoy, J.A.; Widrlechner, M.P.; Reddy, M.; Hendrich, S. Permeability of rosmarinic acid in *Prunella vulgaris* and ursolic acid in *Salvia officinalis* extracts across Caco-2 cell monolayers. *J. Ethnopharmacol.* **2011**, *137*, 1107–1112. [CrossRef]
28. Madala, N.E.; Piater, L.; Dubery, I.; Steenkamp, P. Distribution patterns of flavonoids from three Momordica species by ultra-high-performance liquid chromatography quadrupole time of flight mass spectrometry: A metabolomics profiling approach. *Rev. Bras. Farmacogn.* **2016**, *26*, 507–513. [CrossRef]
29. Mercolini, L.; Protti, M.; Saracino, M.A.; Mandrone, M.; Antognoni, F. Analytical profiling of bioactive phenolic compounds in argan (*Argania spinosa*) leaves by combined microextraction by packed sorbent (MEPS) and LC–DAD-MS/MS. *Phytochem. Anal.* **2016**, *27*, 41–49. [CrossRef] [PubMed]
30. Kumar, S.; Singh, A.; Kumar, B. Identification and characterization of phenolics and terpenoids from ethanolic extracts of Phyllanthus species by HPLC-ESI-QTOF-MS/MS. *J. Pharm. Anal.* **2017**, *2*, 214–222. [CrossRef]

31. Mena, P.; Cirlini, M.; Tassotti, M.; Herrlinger, K.A.; Dall'Asta, C.; Ri, D.D. Phytochemical profiling of flavonoids, phenolic acids, terpenoids, and volatile fraction of a rosemary (*Rosmarinus officinalis* L.) extract. *Molecules* **2016**, *21*, 1576. [CrossRef]
32. Lu, Y.; Foo, L.Y. Rosmarinic acid derivatives from *Salvia officinalis*. *Phytochemistry* **1999**, *51*, 91–94. [CrossRef]
33. Plumb, G.W.; Pascual-Teresa, S.; Santos-Buelga, C.; Rivas-Gonzalo, J.C.; Williamson, G. Antioxidant properties of gallocatechin and prodelphinidins from pomegranate peel. *Redox. Rep.* **2002**, *7*, 41–46. [CrossRef]
34. Someya, S.; Yoshiki, Y.; Okubo, K. Antioxidant compounds from bananas (Musa Cavendish). *Food Chem.* **2002**, *79*, 351–354. [CrossRef]
35. Pandey, K.B.; Rizvi, S.I. Plant Polyphenols as Dietary Antioxidants in Human Health and Disease. *Oxid. Med. Cell Longev.* **2009**, *2*, 270–278. [CrossRef] [PubMed]
36. Londzin, P.; Siudak, S.; Cegieła, U.; Pytlik, M.; Janas, A.; Waligóra, A.; Folwarczna, J. Phloridzin, an apple polyphenol, exerted unfavorable effects on bone and muscle in an experimental model of type 2 diabetes in rats. *Nutrients* **2018**, *10*, 1701. [CrossRef] [PubMed]
37. Lim, H.; Son, K.H.; Chang, H.W.; Bae, K.; Kang, S.S.; Kim, H.P. Anti-inflammatory activity of pectolinarigenin and pectolinarin isolated from *Cirsium chanroenicum*. *Biol. Pharm. Bull.* **2008**, *31*, 2063–2067. [CrossRef] [PubMed]
38. Patel, K.; Gadewar, M.; Tahilyani, V.; Patel, D.K. A review on pharmacological and analytical aspects of diosmetin: A concise report. *Chin. J. Integr. Med.* **2013**, *19*, 792–800. [CrossRef] [PubMed]
39. Zimmermann, B.F.; Walch, S.G.; Tinzoh, L.N.; Stühlinger, W.; Lachenmeier, D.W. Rapid UHPLC determination of polyphenols in aqueous infusions of *Salvia officinalis* L. (sage tea). *J. Chromatogr. B* **2011**, *879*, 2459–2464. [CrossRef]
40. Wen, C.C.; Kuo, Y.S.; Jan, J.T.; Linag, P.H.; Wang, S.Y.; Liu, H.G.; Lee, C.K.; Chang, S.T.; Kuo, C.J.; Lee, S.S.; et al. Specific plant terpenoids and lignoids possess potent antiviral activities against severe acute respiratory syndrome coronavirus. *J. Med. Chem.* **2007**, *50*, 4087–4095. [CrossRef]
41. Laszczyk, M.N. Pentacyclic triterpenes of the lupane, oleanane and ursane group as tools in cancer therapy. *Planta Med.* **2009**, *75*, 1549–1560. [CrossRef]
42. Alqahtani, A.; Hamid, K.; Kam, A.; Wong, K.H.; Abdelhak, Z.; Razmovski-Naumovski, V.; Chan, K.; Li, K.M.; Groundwater, P.W.; Li, G.Q. The pentacyclic triterpenoids in herbal medicines and their pharmacological activities in diabetes and diabetic complications. *Curr. Med. Chem.* **2013**, *20*, 908–931.
43. Panse, V.G.; Sukhatme, P.V. *Statistical Methods for Agricultural Workers*; ICAR Publications: New Delhi, India, 1985.

Sample Availability: Samples of rosemary extracts are available from the authors.