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Antioxidant Activity and Phenolic Composition of Lavandin (*Lavandula x intermedia* Emeric ex Loiseleur) Waste

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The phenolic content of lavandin waste obtained after the distillation of essential oils for the perfume industry was investigated to find an alternative use for this material. The antioxidant activity of different fractions as well as their total phenolic content were evaluated by different methods. Twenty-three phenolic compounds were identified by liquid chromatography coupled to ionspray mass spectrometry (LC/MS/MS), including phenolic acids, hydroxycinnamoylquinic acid derivatives, glucosides of hydroxycinnamic acids, and flavonoids, none of which have previously been reported in lavandin waste. Some structure–activity relationships were proposed by relating the type of scavenging activity of different fractions with the identified phenolic compounds. Contents of representative phenolic acids of Lamiaceae (chlorogenic and rosmarinic) were evaluated by high performance liquid chromatography-diode array detection (HPLC-DAD) and compared with those of other plant species.

KEYWORDS: Lavandin waste; antioxidant activity; free radical scavenging; phenolics; hydroxycinnamic acids; flavonoids; tandem mass spectrometry

1. INTRODUCTION

Lavandin (*Lavandula x intermedia* Emeric ex Loiseleur), a sterile hybrid of *Lavandula angustifolia* Mill. and *Lavandula latifolia* Medic., is a well-known Mediterranean aromatic plant. Its essential oil has been widely used in perfumery throughout history, and nowadays, it is still one of the most appreciated essences in the manufacture of "eau de cologne" and perfumes. Due to its high economical value (*1*), lavandin is intensively cultivated in many countries, and a large amount of waste material is produced after essential oil extraction by distillation.

Lavandin's aromatic qualities are mainly due to the volatile compounds that form its essential oil, which has already been well-studied by gas chromatography (GC) and gas chromatography coupled with mass spectrometry (GC/MS) (*2, 3*). These volatile compounds have been identified as terpenes, acetates, and alcohols of small molecular mass (*4, 5*).

In addition, it is known that many Lamiaceae plants contain phenolic compounds, which have a wide range of physiological properties, such as antiallergenic, antiatherogenic, anti-inflammatory, antimicrobial, antithrombotic, cardioprotective, and vasodilatory (*6*). Many of these properties are thought to be due to antioxidant activity, involving various mechanisms such as free radical scavenging, electron or hydrogen atom donation, or metal cation chelation (*7*). Since phenolic compounds such as hydroxycinnamic acids and flavonoids are not volatile, they remain in lavandin waste. There are some phenolic compounds that could be degraded with thermal treatments, such as anthocyanins and flavonols (*8*), but this kind of degradation is limited (*9*) and these compounds (or a part of them) remain in the waste material after distillation, as shown by a previous study of another aromatic plant such as fennel (*10*). Furthermore, thermal treatments can inhibit enzymes such as polyphenol oxidases (PPO) and peroxydases (POD), responsible for oxidative degradation of phenolic compounds (*8, 11*).

Preliminary screening of aromatic Mediterranean plants (*12*) has shown that lavandin waste exhibits antioxidant activity, but although some phenolic compounds have been identified in other lavender species (*13*), no such studies have been done in lavandin waste. Thus, the main aim of this work was to identify the particular phenolic compounds responsible for antioxidant activity in lavandin waste, for this material's further utilization.

The identification of antioxidant phenolic compounds was carried out for the first time in lavandin waste by liquid chromatography coupled to ionspray mass spectrometry (LC/MS/MS), which has proved to be a powerful tool for identifying and quantifying phenolic compounds in plant extracts by means

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of soft ionization of compounds with electrospray ionization (ESI) and MS/MS techniques (*14*, *15*).

Owing to the increasing interest in the structure–activity relationship of phenolic compounds, some connections were proposed between the phenolic compounds identified by means of LC/MS/MS in each fraction and the antioxidant activity revealed by the different assays.

Due to the particular relevance of chlorogenic and rosmarinic acids in Lamiaceae species (*6*), their contents were determined by HPLC-DAD, in order to make a comparison with previously reported values in other plants.

2. MATERIALS AND METHODS

2.1. Plant Material. Lavandin cv. Super (*Lavandula x intermedia* Emeric ex Loiseleur, Lamiaceae) was cultivated under agronomically controlled conditions in an experimental plot in Cetina (Zaragoza, Spain), and collected during the flowering period. Essential oils were distilled from plant material by steam distillation (*16*) at pilot plant scale in "La Alfranca" (Experimental Farm, Diputación General de Aragón), with a gauge pressure between 1.2 and 1.4 bar for 60 min. The steam temperature oscillated between 119 and 122 °C. The chemical composition of essential oils was determined by GC/MS, according to UNE standards (*17–19*) and literature (*4*). After distillation, the remaining material was air-dried at a temperature below 40 °C, and then powdered and stored at 4 °C.

2.2. Chemicals and Reagents. All chemical reagents used for the radical scavenging essays and total phenolic content determinations were purchased from Sigma (St. Louis, MO), with the exception of the Folin–Ciocalteu reagent, which was purchased from Panreac (Barcelona, Spain). Methanol, ethyl acetate, hexane, and HPLC-grade acetonitrile were purchased from SDS (Peypin, France), and acetic acid was purchased from Merck (Darmstadt, Germany). Ultrapure water (Milli-Q, Waters, Milford, MA) was used. Phenolic standards were obtained as follows: luteolin, caffeic acid, and ferulic acid from Fluka (Buchs, Switzerland); kaempferol, chlorogenic acid, and cumaric acid from Sigma (St. Louis, MO), and apigenin, rosmarinic acid, and quercetin-3-*O*-glucoside (isoquercitrin) from Extrasynthese (Genay, France).

2.3. Extraction and Bioguided-Assay Fractionation. Lavandin waste (927 g dry weight) was extracted three times by maceration with methanol for 24 h at room temperature. The pooled supernatant phases were filtered and concentrated under a vacuum to dryness, obtaining 66.51 g of crude extract (CE), which was then redissolved in water and partitioned with hexane until the organic solvent was colorless. A dried hexane fraction (HxF) (3.44 g) was obtained after removing the organic solvent under a vacuum. The remaining defatted aqueous fraction was then filtered and partitioned with ethyl acetate. The ethyl acetate fraction was dried under a vacuum to obtain 9.48 g of dried ethyl acetate fraction (EAF). The aqueous fraction was freeze-dried, and 27.54 g of powder (AqF) was obtained.

After the total phenolic content and antioxidant activity were determined in CE, HxF, EAF, and EAq, EAF proved to be the most active fraction. Thus, it was selected for fractionation by gel filtration using a Sephadex LH-20 (Pharmacia, 5 cm × 50 cm) column, eluting with methanol at a flow-rate of 1.5 mL/min. One-hundred and seventy fractions of 2 mL each were collected and monitored by thin-layer chromatography (TLC) (Alugram Sil G/UV 254, Machery-Nagel), eluting with a mixture of EtOAc/AcOH/H_2O (10:2:3). The TLC plates were sprayed with 1% diphenylboric acid in MeOH for UV enhancement of phenolic compounds and then visualized under UV light at 254 and 365 nm. On the basis of their TLC profile, eluated fractions were combined to obtain three active fractions (from A to C), which were also evaluated in terms of their total phenolic content and antioxidant activity by the tests described below.

2.4. Total Phenolic Content and Antioxidant Activity. The total phenolic content (TPhC) was determined by the Folin–Ciocalteu method as described by Parejo et al. (*12*). Values are expressed as gallic acid equivalents (GAE)/mg of dry weight (DW). Since antioxidant activity of phenolic compounds is due to different mechanisms (*20*), it was evaluated by three different radical scavenging assays, using the DPPH$^\bullet$ free radical (DPPH$^\bullet$), the $^\bullet$OH/luminol chemioluminescence (CL), and the superoxide–nitroblue tetrazolium hypoxanthine/xanthine oxidase (XO) methods. The DPPH$^\bullet$ and CL results are expressed as inhibitory concentration IC_{50} (μg/mL), while XO results are expressed as percentage of inhibition. Radical scavenging activity experiments were carried out as described by Parejo et al. (*12*).

2.5. Qualitative Analysis by HPLC-DAD/ESI-MS/MS. Samples of B and C (the most active fractions of EAF) were analyzed by HPLC-DAD/ESI-MS/MS. They were prepared at a concentration of 1 mg/mL in MeOH, and filtered through a 0.45 μm poly-tetrafluoroethylene (PTFE) filter (Waters). LC analyses were carried out using a 1100 Agilent quaternary pump system (Waldbronn, Germany), equipped with an autosampler and diode array detector (DAD). A Luna C_{18} column (150 × 2.1 mm, 5 μm) (Phenomenex, Torrance, CA) was used for the separation of phenolic compounds. Gradient elution was performed with water/0.05% acetic acid (solvent A) and acetonitrile/0.05% acetic acid (solvent B) at a constant flow rate of 400 μL/min. An increasing linear gradient (v/v) of solvent B was applied: (*t* (min), %B): (0, 5), (10, 15), (30, 35), (40, 80), (45, 5). Chromatograms were recorded at 280 nm, with peak scanning between 200 and 600 nm. An API 3000 triple quadrupole mass spectrometer (Perkin-Elmer Sciex, Concord, ON, Canada) coupled online to LC as described above was used. The mass spectrometer was equipped with a Turbo Ionspray source operating in the negative mode with the following settings: capillary voltage, −3500 V; nebulizer gas (N_2), 10 (arbitrary units); curtain gas (N_2), 12 (arbitrary units); collision gas (N_2), 4 (arbitrary units); focusing potential, −200 V; entrance potential, 10 V; drying gas (N_2), heated to 400 °C and introduced at a flow rate of 8000 cm^3/min. The declustering potential (DP) was –60 and collision energy (CE) –35, although other DP and CE potentials were also assayed to differentiate between luteolin and kaempferol. Different MS/MS experiments, such as product ion scan, precursor ion scan, and neutral loss scan, were carried out in order to confirm the structure of compounds previously mass identified by full scan mode, and to detect and identify new compounds which were undectectable in this previous full scan mode. Full scan acquisition was performed scanning from *m*/*z* 100 to 800 u in a profile mode with a cycle time of 2 s, a step size of 0.1 u, and a pause between each scan of 2 ms. In the product ion scan experiments, MS/MS product ions were produced by collision-activated dissociation (CAD) of selected precursor ions in the collision cell of the triple quadrupole mass spectrometer (Q_2), and mass analyzed using the second analyzer of the instrument (Q_3). However, in the precursor ion scan experiments, Q_1 scanned over all possible precursors of the selected ion in Q_3 of the triple quadrupole. Finally, in neutral loss scan experiments, both Q_1 and Q_3 quadrupoles scanned for a pair of ions that differed by a characteristic mass difference (neutral mass) that is not ionizated, and thus not detected by quadrupole analyzers.

2.6. Quantitative Analysis of Chlorogenic and Rosmarinic Acids by HPLC-DAD. Quantitative analyses were carried out with the same 1100 Agilent quaternary pump system (Waldbronn, Germany) used in qualitative analyses. The column, mobile phase, and solvent gradient were also the same as those used in the LC/MS/MS analysis (see section 2.5). The flow rate was 1 mL/min and the injection volume 25 μL. UV detection was performed at 280 nm. Two calibration curves were done with solutions of known concentrations of standard chlorogenic and rosmarinic acids. Plant material was prepared as follows: 250 mg of powdered lavandin waste was sonicated in 25 mL of a 50% solution of MeOH/H_2O, in an ultrasonic bath for 30 min. After centrifugation at 14000*g* for 10 min, the supernatant was adjusted to 25 mL in a measuring flask, and quantified immediately after extraction in order to avoid possible chemical alterations. Samples from 10 extractions were analyzed, and the coefficients of variation (%CV) were calculated for both retention time and peak area.

3. RESULTS AND DISCUSSION

3.1. Total Phenolic Content and Antioxidant Activity. Values of the total phenolic content and antioxidant activity of different fractions are shown in **Table 1**. These results confirm that lavandin waste has a clear antioxidant activity, as previously

Table 1. Total Phenolic Content and Radical Scavenging Activity of Different Fractions of Lavandin Waste (CE, HxF, EAF, and AqF) and of EAF Subfractions (A, B, and C), Compared to That of Reference Compounds BHA and Quercetin (SD Values Are Calculated by Means of 10 Replicates)

		antioxidant activity		
	TPhC (GAE/mg) ± SD	DPPH• IC_{50} ± SD (μg/mL)	CL IC_{50} ± SD (μg/mL)	XO AA (%) ± SD (500 μg/mL)
CE	59.96 ± 1.65	168.51 ± 18.49	69.49 ± 5.91	32.88 ± 4.86
HxF	41.56 ± 2.43	631.70 ± 91.20	109.36 ± 16.03	7.68 ± 3.31
EAF	240.01 ± 13.24	40.63 ± 5.91	10.82 ± 4.61	68.16 ± 2.61
AqF	32.56 ± 1.99	421.48 ± 42.73	187.88 ± 22.29	25.34 ± 5.68
A	124.53 ± 9.71	190.34 ± 23.27	19.10 ± 1.92	19.34 ± 6.49
B	423.82 ± 13.79	57.01 ± 5.72	5.25 ± 0.52	60.99 ± 1.67
C	783.36 ± 24.61	7.98 ± 0.97	7.67 ± 0.28	81.48 ± 1.70
BHA		9.70 ± 0.92	2.14 ± 0.01	67.51 ± 0.30
quercetin		6.11 ± 0.53	5.13 ± 0.12	97.45 ± 3.25

Table 2. List of Compounds Identified by LC/MS/MS in Lavandin Waste

					MS/MS experiments					
peak	compound	t_R (min)	fraction	$[M - H]^-$	product ion scan	fragments *(m/z (relative abundance, %))*	neutral loss scan	precursor ion scan	DP(v)	CE(v)
1	protocatechuic acid	5.69	C	153	153	109 (100)			−60	−35
2	coumaric acid-*O*-glucoside 1	13.65	B	325	325	119 (100), 163 (20)	162		−60	−35
3	caffeic acid	15.28	C	179	179	135 (100)			−60	−35
4	ferulic acid-*O*-glucoside 1	16.98	B	355	355	149 (100), 193 (20)	162		−60	−35
5	coumaric acid-*O*-glucoside 2	19.39	B	325	325	119 (100), 163 (20)	162		−60	−35
6	trihydroxycinnamic acid-*O*-glucoside	19.47	B	357	357	151 (30), 177 (20), 195 (100)	162		−60	−30
7	3-caffeoylquinic acid (chlorogenic acid)	20.50	B	353	353	119 (100), 173 (80), 179 (60), 191 (100)			−60	−35
8	caffeic acid-*O*-glucoside 1	21.63	B	341	341	135 (100), 179 (80)	162		−60	−35
9	ferulic acid-*O*-glucoside 2	21.75	B	355	355	149 (100), 193 (20)	162		−60	−35
10	eriodictyol-*O*-hexoside	22.42	B	449	449	135 (100), 151 (80), 287 (20)	162		−60	−30
11	caffeic acid-*O*-glucoside 2	22.90	B	341	341	135 (100), 179 (80)	162		−60	−35
12	quercetin-3-*O*-glucoside (isoquercitrin)	23.61	C	463	463	151 (<5), 179 (<5), 301 (100)	162	301	−60	−35
13	luteolin-*O*-hexoside 1	23.69	C	447	447	285 (100), 447 (10)	162	285	−60	−30
					285	133 (100), 151 (20), 285 (50)			−80	−40
14	chrysoeriol-*O*-hexoside	23.73	C	461	461	285 (100), 299 (80)	162	299	−60	−35
15	apigenin-*O*-hexoside	26.00	C	431	431	269 (100), 431 (10)	162		−60	−35
16	luteolin-*O*-hexoside 2	26.42	C	447	447	285 (100), 447 (10)	162	285	−60	−30
					285	133 (100), 151 (20), 285 (50)			−80	−40
17	chrysoeriol-*O*-glucuronide	28.86	C	475	475	285 (60), 299 (70)	176	299	−60	−30
18	luteolin-*O*-glucuronide 1	29.58	C	461	461	285 (100), 461 (10)	176	285	−60	−30
					285	133 (100), 151 (20), 285 (50)			−80	−40
19	rosmarinic acid	31.60	C	359	359	161 (100), 179 (25), 197 (20)			−60	−35
20	rosmarinic acid methylester	33.12	C	373	373	135 (80), 179 (100)			−60	−35
21	luteolin-*O*-glucuronide 2	34.57	C	461	461	285 (100), 461 (10)	176	285	−60	−30
					285	133 (100), 151 (20), 285 (50)			−80	−40
22	apigenin	35.20	C	269	269	117 (100), 151 (90)			−60	−35
23	luteolin-*O*-glucuronide 3	35.61	C	461	461	285 (100), 461 (10)	176	285	−60	−30
					285	133 (100), 151 (20), 285 (50)			−80	−40

reported by Parejo et al. (*12*). The EAF exhibited the highest antioxidant activity in all antiradical assays, showing an IC_{50} value of 40.63 μg/mL in DPPH•, an IC_{50} value of 10.82 μg/mL in CL, and 68.16% scavenging activity in XO. The total phenolic content of each fraction correlated positively with the antioxidant activity. EAF was also the fraction with the largest amount of phenolic compounds (240.01 GAE/mg). These results indicated that the EAF was the best fraction for the identification of phenolic compounds responsible for antioxidant activity.

Total phenolic content and antioxidant activity were also evaluated in the three final fractions (A, B, and C) resulting after the EAF fractionation. The highest amount of phenolic compounds was found in fraction C (783.36 GAE/mg), which also showed the highest activity in the DPPH• (IC_{50} value of 7.98 μg/mL) and XO (81.48% of scavenging activity) assays. This fraction also showed more antioxidant activity in the XO assay than the reference compound BHA (butylated hydroxyanisole) (see **Table 1**). In contrast, fraction B showed the highest •OH scavenging activity in the CL assay (IC_{50} value of 5.25 μg/mL), even though it has a lower amount of phenolic compounds than fraction C. These results suggest that the phenolic compounds contained in fraction B have some structural properties that let them react more easily with the hydroxyl radical, the most dangerous natural free radical.

3.2. Identification of Phenolic Compounds by LC-DAD/ESI-MS/MS. Since the full scan mass spectra of the chromatographically separated phenolics gave only deprotonated $[M - H]^-$ ions, MS/MS experiments were required for the identification. On the basis of the information referring to the chemical composition of this plant genus, different experiments of precursor ion scan, product ion scan, and neutral loss scan were carried out. Results of identifications as well as retention time and methods used are shown in **Table 2**. Two replicates were analyzed, and the CV of retention times of compounds in the different assays ranged from 0.081 to 4.088%. A total of 23 phenolic compounds were identified for the first time in

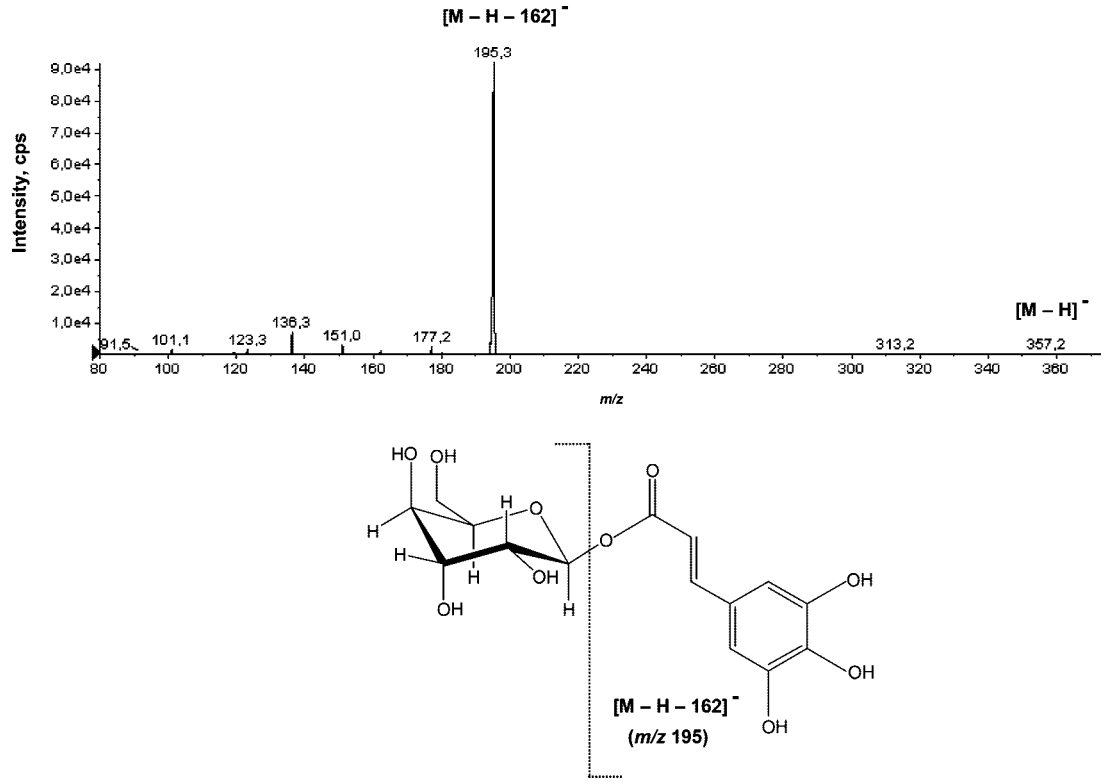

Figure 1. Product ion scan experiment of $[M - H]^-$ of trihydroxycinnamic acid-*O*-glucoside (357), with characteristic fragments (195, 177) obtained after fragmentation and the possible compound structure.

lavandin waste. They had not been previously reported in lavandin with the exception of rosmarinic acid, which was found in lavandin cell cultures (*21*).

3.2.1. Phenolic Acids and Hydroxycinnamoylquinic Acid Derivatives. The two phenolic acids found in fraction C (peaks 1 and 3) were tentatively identified as protocatechuic acid (a benzoic acid) and caffeic acid (a hydroxycinnamic acid). Peak 1 showed a $[M - H]^-$ ion at *m*/*z* 153 with an ion fragment of 109 *m*/*z*, which corresponds to the pattern fragmentation of protocatechuic acid, with loss of 44 u corresponding to the carboxylic group. Peak 3 exhibited a $[M - H]^-$ ion at 179 *m*/*z*, with a characteristic ion fragment at *m*/*z* 135, corresponding to the pattern fragmentation of caffeic acid (*22*, *23*). Peak 7, found only in fraction B, had a $[M - H]^-$ ion at *m*/*z* 353, which after fragmentation showed an MS^2 base peak of 191 *m*/*z*, and a secondary prominent ion at *m*/*z* 179. Consequently, this peak was identified as 3-caffeoylquinic acid (chlorogenic acid) according to Clifford et al. (*24*). Other caffeic acid derivatives were also tentatively identified: rosmarinic acid (peak 19) and rosmarinic acid methyl ester (peak 20). These compounds of $[M - H]^-$ ions of 359 and 373 *m*/*z*, respectively, are characteristic of Lamiaceae, and have a common fragment ion at *m*/*z* 179, characteristic of caffeic acid derivatives (*25*).

3.2.2. Glucosides of Hydroxycinnamic Acids. All seven glucosides of hydroxycinnamic acids found in lavandin waste were identified in fraction B. Neutral loss of 162 u revealed the existence of these glucosides, and the product ion scan of the corresponding $[M - H]^-$ ions confirmed their identities. This neutral loss could also mean a loss of galactose rather than of glucose, but to our knowledge, there are no galactosides of hydroxycinnamic acids reported in the bibliography, so these glycosides are most probably glucosides (*26*). Thus, peaks 2 and 5 were tentatively identified as glucosides of coumaric acid, both with a $[M - H]^-$ ion at *m*/*z* 325 and fragment ions at *m*/*z* 163 and 119; peaks 4 and 9 were identified as glucosides of ferulic acid, both with a $[M - H]^-$ ion at *m*/*z* 355 and fragment ions at *m*/*z* 193 and 149; and peaks 8 and 11 were identified as caffeic acid glucosides with a $[M - H]^-$ ion of 341 *m*/*z* and fragment ions at *m*/*z* 179 and 135. All of these compound fragmentation patterns corresponded to those reported by Sánchez-Rabaneda et al. (*14*).

Taking into account that the first products eluted from the column have an acid group, it seems that in the first hydroxycinnamic derivatives eluted (2, 4, and 8) the acid group of the hydroxycinnamoyl moiety is free, whereas in compounds 5, 9, and 11 this group is esterified.

Peak 6 showed a $[M - H]^-$ ion at *m*/*z* 357 and fragment ions at *m*/*z* 195, 177, and 151. The neutral loss of 162 u confirmed that this compound was a glucoside, while the fragmentation of the acidic part was characteristic of 3,4,5-trihydroxycinnamic acid (*27*), allowing a tentative identification of peak 6 as a 3,4,5-trihydroxycinnamic acid glucoside, as shown in **Figure 1**. Although there are no references to the detection of this compound by LC/MS, the related trihydroxycinnamoyl quinic acid has been found in dried plums (*27*). The presence of this glucoside in lavandin could suggest an alternative mechanism for the synthesis of gallic acid by a β-oxidation of trihydroxycinnamic acid (*28*). Additionally, trihydroxylated derivatives have been found to display a greater cytotoxic and antiproliferative effect than dihydroxylated derivatives, as well as a higher level of radical scavenging activity (*29*). These effects are further increased by the presence of a double bond in the side chain, which a compound like gallic acid lacks (*30*).

The fact that all of the glucosides of hydroxycinnamic acids were identified in fraction B, which is the most active against the $^{\bullet}$OH radical (CL test), could suggest that this type of compound is more efficient than flavonoids (mainly present in fraction C) at scavenging $^{\bullet}$OH radicals.

3.2.3. Flavonoids. All flavonoids were found in fraction C with the exception of eriodictyol-*O*-hexoside (peak 10), which

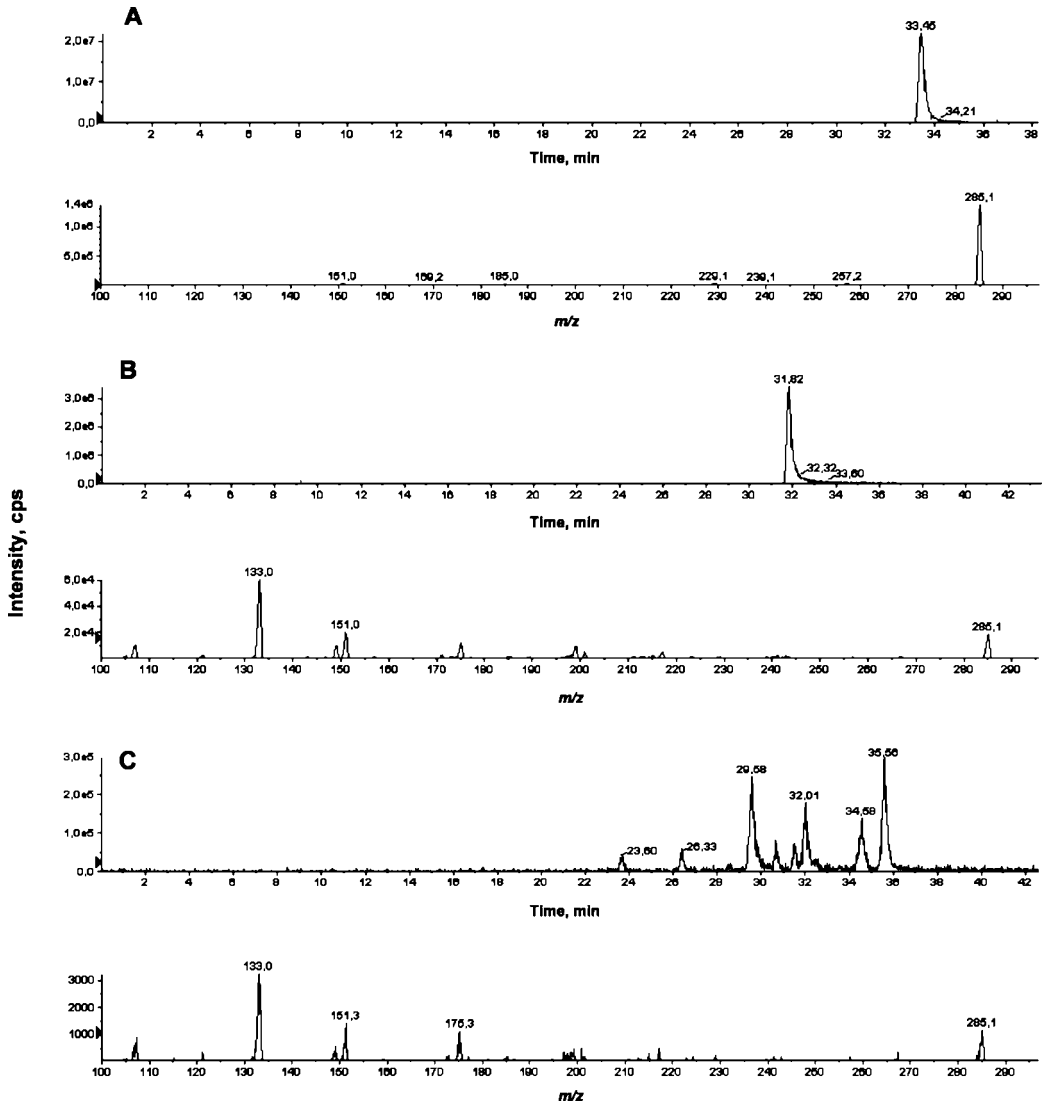

Figure 2. Chromatograms and mass spectra of product ion scan experiments of $[M - H]^-$ 285 *m/z* at DP 60 and CE 40 of standard kaempferol (A), standard luteolin (B), and fraction C (C). The fragmentation intensity of fraction C corresponds with that of luteolin.

was found in fraction B. Neutral loss scan mode experiments of 162 u revealed the presence of five other flavonoid hexosides, identified as isoquercitrin (peak 12), two *O*-hexosides of luteolin (peaks 13 and 16), chrysoeriol-*O*-hexoside (peak 14), and apigenin-*O*-hexoside (peak 15). Neutral loss experiments of 176 u revealed the presence of four flavonoid glucuronides: a chrysoeriol-*O*-glucuronide (peak 17) and three luteolin-*O*-glucuronides (peaks 18, 21, and 23). Fraction C was also found to contain an aglicone, identified as apigenin (peak 22). All flavonoid derivatives were tentatively identified by comparison with standards and bibliography data. Ion fragments were confirmed by product ion scan mode experiments and O cleavages by the absence of 120 and 90 *m/z* fragment ions, which are characteristic of C cleavages.

Since luteolin and kaempferol derivatives have the same $[M - H]^-$ ion at *m/z* 285, and the same resulting product ion scan fragments, they were differentiated by comparing the intensities of product ion scan fragments of standard compounds. Luteolin derivatives are much more fragmented at the same DP and CE intensities (**Figure 2**).

The presence of eriodictyol derivatives and the absence of kaempferol derivatives, seems to indicate that, in this plant, quercetin originates from eriodictyol and dihydroquercetin, as shown in **Figure 3**. It is also interesting to observe the important presence of flavones (apigenin, luteolin, and chrysoeriol) in this plant, particularly of luteolin. Additionally, as the majority of flavonoids identified, with the exception of apigenin, require the enzyme flavonoid-3′-hydroxylase (F3′H) for their synthesis, it seems that this enzyme could have a high activity in lavandin.

3.3. Relationship between Chemical Structure and Antioxidant Activity. Antioxidant activity can take place in several ways. Flavonoids, for example, can inhibit oxidative enzymes such as xanthine oxidase owing to their coplanar structure, and they also have free radical scavenging properties (*31*), while hydroxycinnamic acids show antioxidant activity only by scavenging reactive oxygen species. In the latter case, although the mechanism and relationship between structure and activity are not clear, the increasing number of hydroxyl groups in the aromatic ring increases free radical scavenging activity, as does the presence of a methylenic ($-CH_2-$) spacer group, an ethylenic ($-CH_2CH_2-$) group, and an unsaturated chain ($-CH=CH-$) (*32*).

Fraction C from EAF, which was found to contain the majority of the flavonoid derivatives present in lavandin waste, was also the richest in phenolic compounds and the most active against the $DPPH^{\bullet}$ radical and the XO assay, where flavonoids can act either by scavenging the $SO^{\bullet -}$ radical, or by inhibiting

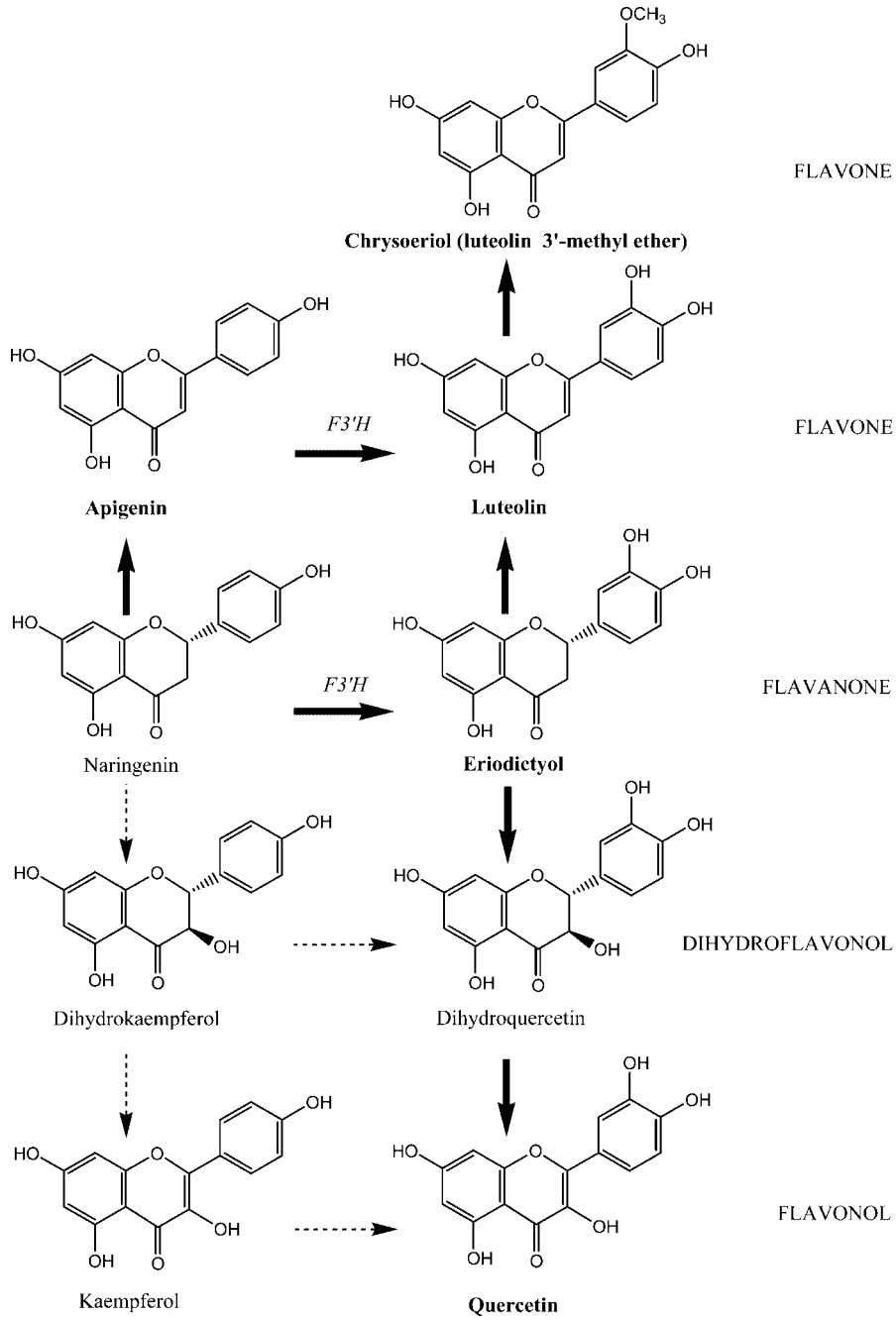

Figure 3. Metabolic pathways of the main flavonoid aglycones, with those proposed for lavandin drawn in bold arrows.

xanthine oxidase. In contrast, there were no flavonoids in fraction B, and all identified compounds were glucosides and other derivatives of hydroxycinnamic acids, only acting as $SO^{\bullet-}$ radical scavengers in the XO assay. This fraction proved to be the most active in the CL assay, which evaluates the capacity of compounds to scavenge the hydroxyl radical, although it was not the richest in phenolic compounds, as the results from the Folin–Ciocalteu assay show. This fact could suggest that the structure of glucosides and other derivatives of hydroxycinnamic acids is more suitable for scavenging hydroxyl radicals than that of flavonoids.

3.4. Quantification of Chlorogenic and Rosmarinic Acids. There are references to the presence of chlorogenic acid in various species of lavender but not in lavandin. In this work, chlorogenic acid has been found to be one of the main phenolic compounds in lavandin waste, with a concentration of 215 mg/100 g of DW and a CV (%) of 3.18. Comparing this chlorogenic acid content with that of other plant species shown in **Table 3**, it can be seen that lavandin waste is particularly rich in this compound, only exceeded by *Coffea pseudozanguebariae*.

Table 3. Chlorogenic Acid Content in Different Plant Materials (DW = Dry Weight)

plant material	mg/100 g of DW	ref
Coffea pseudozanguebariae Bridson	780	(*33*)
Lavandula x intermedia (lavandin) waste	215	
Foeniculum vulgare L.	208	(*34*)
Prunus domestica L.	123	(*35*)
Cynara scolymus L.	15	(*36*)

Although rosmarinic acid is known as one of the main phenolic compounds in Lamiaceae species (*6*), its presence in lavandin waste has been found to be comparatively low, with a concentration of 124 mg/100 g of DW and a CV (%) of 9.06

Table 4. Rosmarinic Acid Content in Different Plant Materials (DW = Dry Weight)

plant material	mg/100 g DW	ref
Lepechinia graveolens Willd.	2800	(*25*)
Origanum vulgare L.	2562	(*6*)
Salvia officinalis L.	2186	(*6*)
Mentha canadensis L.	1908	(*6*)
Lavandula latifolia L.	700	(*37*)
Rosmarinus officinalis L.	550	(*38*)
Lavandula x intermedia (lavandin) waste	124	

(**Table 4**). However, this result does not agree with López-Arnaldos et al. (*21*), who had previously reported a rosmarinic acid content of 2000–3000 mg/100 g of DW in lavandin cell cultures that demonstrated an ability to produce rosmarinic acid continuously in vitro.

In summary, it can be seen that lavandin waste is a relatively poor source of rosmarinic acid but has high levels of chlorogenic acid and glucosides of hydroxycinnamic acids, which are particularly active in scavenging the hydroxyl radical.

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