Article

Lavandula pedunculata (Mill.) Cav. Aqueous Extract Antibacterial Activity Improved by the Addition of Salvia rosmarinus Spenn., Salvia lavandulifolia Vahl and Origanum compactum Benth

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Abstract: Lavender aqueous extracts are widely used in the Moroccan traditional medicine for their antibacterial properties. However, previous research have generally focused on investigating the antibacterial activity of lavender essential oils. The aim of this study is to evaluate the antibacterial activity of the Moroccan *Lavandula pedunculata* (Mill.) Cav. aqueous extract, alone, as well as in combination with extracts of other plant species known for their antibacterial activity: *Salvia rosmarinus* Spenn., *Salvia lavandulifolia* Vahl. and *Origanum compactum* Benth. We have tested the antibacterial activity of *L. pedunculata*, *S. rosmarinus*, *S. lavandulifolia* and *O. compactum* aqueous extracts individually and in combination against 34 strains using the agar dilution method. The combination effect was evaluated using the fractional inhibitory concentration (FIC). Polyphenol and tannin contents were determined using Folin-Ciocalteu reagent, and then some phenolic compounds were identified using UHPLC-MS. All the extracts displayed a large spectrum of antibacterial activity, especially against staphylococci, streptococci, *Mycobacterium smegmatis* and *Proteus mirabilis*. The minimum inhibitory concentration (MIC) values reached 0.15 ± 0.00 mg/mL for *Staphylococcus warneri* tested with *S. lavandulifolia* and 0.20 ± 0.07 mg/mL for *Staphylococcus epidermidis* tested with *L. pedunculata* or *S. rosmarinus*. Association of the *L. pedunculata* extract with *S. rosmarinus*, *S. lavandulifolia* and *O. compactum* showed synergistic effects (FIC ≤ 1). Moreover, the association of *L. pedunculata* with *S. lavandulifolia* was active against most of the Gram-negative strains resistant to the individual extracts. Determination of polyphenol and tannin contents showed the richness of the studied plants in these compounds. Additionally, chromatographic analysis demonstrated the high presence of rosmarinic acid in all the studied plant extracts. To our knowledge, this is the first study that shows the enhancing effect of the antibacterial activity of *L. pedunculata* aqueous

extract combined with *S. rosmarinus*, *S. lavandulifolia* and *O. compactum*. These results confirm the effectiveness of the plant mixtures commonly used by traditional healers in Morocco and suggest that *L. pedunculata* might be used as an antibacterial agent either alone or, more efficiently, in combination with *S. rosmarinus*, *S. lavandulifolia* and *O. compactum*.

Keywords: *Lavandula pedunculata* (Mill.) Cav.; *Salvia rosmarinus* Spenn.; *Salvia lavandulifolia* Vahl.; *Origanum compactum* Benth.; polyphenols; antibacterial activity

1. Introduction

Infections can cause moderate to severe damages and are a burden to global economies and public health [1]. These infections are increasing due to the emergence of higher virulence, such as the multidrug-resistant bacteria caused by multiple antibiotic treatments [2,3]. Some of the most problematic multidrug resistant organisms that are encountered currently include *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, extended-spectrum beta-lactamases (ESBL)-producing *Escherichia coli* and *Klebsiella pneumoniae*, vancomycin-resistant enterococci, methicillin-resistant *Staphylococcus aureus* and extensively drug-resistant *Mycobacterium tuberculosis* [2,4]. In addition to increasing resistance to existing agents, there is a lack of new antibiotics in development [5].

In order to find solutions for this important issue, more and more research is now made on medicinal plants trying to find new natural sources of antibiotics. Indeed, traditional phytotherapy has been the main source of remedy for various diseases in the past, and several studies have proved its efficacy. Researchers in this field are giving importance to the use of aromatic and medicinal plants as alternatives to antibiotics or as therapeutic complements. Because of their multiple modes of action, plant extracts could be as efficient as antibiotics, with lower risks of causing resistance or side effects. Plants contain several active compounds (secondary metabolites) belonging mainly to three classes: phenolic compound, terpenes and alkaloids [6,7]. In Morocco, 4200 plant species were identified, of which 600 are recognized for their aromatic and medicinal properties [8].

Among aromatic and medicinal plants, lavender is famous for the quality of its essential oils and aqueous extracts that have long been used in traditional medicine, perfumes, cosmetics, hygiene products, food industry and pharmacy [9,10]. In Morocco, lavender is one of the most used plants by the population for the treatment of several diseases [11,12]. Researchers have been investigating its properties and have proven its antidiabetic, antirheumatic, anti-inflammatory, antioxidant, antibacterial, antifungal, antidepressant and antispasmodic activities. It is also efficient in the treatment of respiratory and digestive diseases [13–20]. However, lavender is usually investigated for the activity of its essential oils, regardless of the large use of its aqueous extracts in traditional medicine. Moreover, Moroccan people tend to use lavender in combination with other plants for better results [12,21,22].

L. pedunculata is one of the lavender species widely used in traditional Moroccan medicine to treat several diseases [12,21]. However, few research studies have been performed to investigate and prove its efficacy. For these reasons, the aim of this study is to investigate the antibacterial activity of the aqueous extract of Moroccan *L. pedunculata* towards several microbial strains. Moreover, its combinations with *S. rosmarinus*, *S. lavandulifolia* and *O. compactum* from Morocco, known for their antibacterial activities [23–25], will also be tested. In addition, the chemical composition of the aqueous extracts of all the chosen plants will be determined using UHPLC-MS.

2. Materials and Methods

2.1. Chemicals and Reagents

The used standards are luteolin, herniarin and myricetin (purchased from Sarsyntex, Merignac, France), apigenin (obtained from Carl Roth, Karlsruhe, Germany), coumarin

(Behringer, Willich, Germany), cinnamic acid (Rhône-Poulenc, Paris, France), protocatechuic acid (Koch-Light Laboratories LTD, Bucks, UK), vanillic acid (Merck, Darmstadt, Germany), chlorogenic acid (Sigma-Aldrich, St. Louis, MO, USA), rosmarinic acid (Extrasynthèse, Genay, France) and gallic acid (Prolabo, Paris, France), as well as ferulic acid and caffeic acid purchased from Sigma (St. Louis, MO, USA). Formic acid (Carlo Erba Reagents^TM, Cornaredo, Italy) and methanol (Carlo Erba Reagents, Val-de-Reuil, France) were of HPLC grades. All other chemicals used were also of analytical grade.

2.2. Plant Material

L. pedunculata, *S. rosmarinus*, *S. lavandulifolia* and *O. compactum* were collected from different regions in Morocco (Table 1). The used organs were chosen according to the bibliographic data [22–24,26], and they were dried for thirteen days in the open air and in the shade.

Table 1. Information about the studied plants.

Plant Species	Family	Voucher Number	Used Organ	Harvest Region	Geographic Coordinates	Harvest Date
Lavandula pedunculata (Mill.) Cav.	*Lamiaceae*	RAB111854	Flowering tops	Taza	34°04′01.2″ N 4°07′42.5″ W	May 2019
Salvia rosmarinus Spenn.	*Lamiaceae*	RAB111855	Leaves	Oulad Ali	33°27′46.6″ N 3°58′34.4″ W	May 2017
Salvia lavandulifolia Vahl	*Lamiaceae*	RAB111857	Leaves	Oulad Ali	33°27′45.2″ N 3°58′39.8″ W	May 2017
Origanum compactum Benth.	*Lamiaceae*	RAB111858	Leaves and flowers	Bouyablane	33°39′02.4″ N 4°09′49.6″ W	June 2018

Plant identification was carried out at the Scientific Institute of Rabat (Rabat, Morocco) by Dr. Hamid Khamar.

2.3. Aqueous Extraction

L. pedunculata flowering tops, *S. rosmarinus* leaves, *S. lavandulifolia* leaves and *O. compactum* leaves and flowers were each mixed and heated with distilled water (1:20; w/v) for one hour at 75 ± 2 °C using a hot plate. The temperature was monitored using an electronic laboratory thermometer. The mixtures were then filtered, and the obtained filtrates were dried in the oven at 70 °C until obtaining dry extract powders. The latter were put in closed flasks away from light and humidity until further use.

2.4. Determination of Total Polyphenol Content

Total polyphenol concentrations in the different plant aqueous extracts were determined by the method described by Zhang and his collaborators using the Folin-Ciocalteu reagent with some modifications [27]. In a 96-well microplate, 25 μL of the extract at 0.5 mg/mL were introduced, then 125 μL of the Folin-Ciocalteu reagent (10%) were added, followed by 100 μL of sodium carbonate at 145 mg/mL. After 5 min of orbital stirring and 2 h incubation in the dark at 25 °C, the reading was made at 760 nm by a SPECTROstarNano spectrophotometer. The negative control was prepared according to the same protocol using 25 μL of distilled water instead of the extract.

A standard calibration curve was made from different concentrations of gallic acid. It was used for the calculation of polyphenol concentrations in the extracts. The total phenolic content is therefore expressed in milligrams equivalent of gallic acid per gram of the dry extract (mg GAE/gExt).

2.5. Determination of Total Tannin Content

Total tannin concentrations in the aqueous extracts were determined using the hide-powder method. It consists of the determination of polyphenol contents in the extracts after their contact with the hide-powder, which precipitates tannins [28].

1 mL of the 0.5 mg/mL extract was stirred with 10 mg of the hide-powder for 1 h. The mixture was then centrifuged at 2500 rpm for 5 min. The polyphenol content in the supernatant was then determined using the method described above (Section 2.4). The total tannin content in an extract corresponds, therefore, to the difference between the total polyphenol content in the extract and the polyphenol content after precipitating tannins with the hide-powder. This content is expressed in mg GAE/gExt.

2.6. UHPLC Analysis of Aqueous Extracts

Chromatographic analysis of the aqueous extracts was carried out on an AQUITY UPLC H-Class System (Waters Corporation, Manchester, UK) equipped with two independent pumps, an automatic injector, a controller, a diode array UV detector (DAD), a mass spectrometer with ESI ionization source and a quadrupole as an analyzer. The stationary phase is a reverse phase Waters® Acquity BEH C18 column (2.1 × 50 mm, 1.7 μm) connected to a 0.2 μm in-line filter. The mobile phase is composed of two solvents: (A) ultrapure water (Milli-Q® Integral 5, Merck™, Darmstadt, Germany) + 0.1% formic acid; (B) methanol + 0.1% formic acid. The elution gradient established was 0–5% B (1 min), 5–20% B (0.5 min), 20% B (3.5 min), 20–100% B (4 min), rinsing of the column 100% B (2 min) and re-equilibration 100–0% B (0.5 min), 0% B (2.5 min). Methanol (70%) was required for washing the system.

The aqueous extracts were solubilized in a methanol/water mixture (1:1, *v*/*v*) to obtain a concentration of 1 mg/mL, and then they were filtered through 0.2 μm PTFE filter. For each analysis, 0.004 mL of the extract was injected. The temperature was set at 30 °C, and the flow rate was set at 0.3 mL/min.

This analysis was carried out on few standards chosen according to bibliographic data. They were injected under the same conditions as those of the extracts. These standards are cinnamic acid, luteolin, apigenin, myricetin, ferulic acid, protocatechuic acid, vanillic acid, chlorogenic acid, caffeic acid, rosmarinic acid, gallic acid, herniarin and coumarin.

Chemical compounds of the extracts were identified by matching their retention time, UV spectrum and molecular weight to those of the used standards.

2.7. Antibacterial Activity of Aqueous Extracts

2.7.1. Preparation of Bacterial Suspensions

The chosen microorganisms, some of which are resistant to antibiotics, are involved in various opportunistic or nosocomial infections. They were cultured from suspensions of the strains contained in a liquid Brain Heart Infusion Agar medium in tubes containing a sloping Mueller-Hinton Agar (MHA) culture medium (MHB Oxoid™, Basingstoke, UK; Bacto™ Agar, Le Pont de Claix, France). The latter were incubated for 24 h at 37 °C, and then 10 mL of Ringer Cysteine (RC) liquid (Merck™, Darmstadt, Germany) was added into the tubes. A good mixing is necessary in order to suspend the cultured microorganisms. A drop of each suspension was collected to be added in a dilution tube containing 10 mL of RC solution. The turbidity of the obtained suspension that was used for the test was therefore estimated at 0.5 McFarland. An amount of 1 mL of suspension from each dilution tube was withdrawn to fill the wells of the inoculum replicator plate.

2.7.2. Activity of Individual Extracts

Antibacterial activity of the plants aqueous extracts was evaluated using the agar dilution method [29]. This method allows for determining the MIC of each extract towards 34 microorganisms in in vitro culture. The aqueous extracts were primarily dissolved in water/ethanol (7:3), and then they were mixed with MHA in Petri dishes. The final concentrations tested were 1.2, 0.6, 0.3, 0.15 and 0.075 mg/mL. The Petri dishes containing

the MHA-extract mixture were inoculated with the microorganisms using an inoculum replicator, and they were incubated for 24 h at 37 °C. Activity was assessed visually by the presence or absence of culture. MIC values were recorded as the lowest concentrations of extracts that completely inhibit the growth of a specific microbe. A negative control was tested using the solvent. Three antibiotics were used as positive controls: gentamicin, vancomycin and amoxicillin. The studied strains are considered to be susceptible to the used antibiotics when MIC $\leq$ 4 mg/L, and they are considered to be resistant to gentamicin when MIC > 8 mg/L and resistant to vancomycin and amoxicillin when MIC > 16 mg/L [29,30].

2.7.3. Activity of Extracts in Mixtures

In order to investigate the antibacterial activity of extracts mixtures, the checkerboard assay was used. This method consists in studying all the possible combinations in the range of the chosen concentrations (1.2, 0.6, 0.3, 0.15 and 0.075 mg/mL).

The MIC of an extract mixture is also determined by the agar dilution procedure. It corresponds to the lowest concentrations of mixtures inhibiting the growth of a microbe.

In order to determine the effect of a combination, the FIC is calculated. It is an indicator of the activity of a plant mixture against microbial strains [31]. It is calculated as follows for the combination of extracts A and B:

$$FIC = \sum FIC(X) = FIC(A) + FIC(B) \tag{1}$$

$$FIC(A) = \frac{MIC \text{ of A in combination}}{MIC \text{ of A alone}} \tag{2}$$

$$FIC(B) = \frac{MIC \text{ of B in combination}}{MIC \text{ of B alone}} \tag{3}$$

FIC value tells the effect of the combination:

- FIC < 1: synergistic effect;
- FIC = 1: commutative effect;
- 1 < FIC $\leq$ 2: indifferent effect;
- 2 < FIC: antagonistic effect.

2.8. Statistical Analysis

Data are presented as means $\pm$ standard deviations. Their statistical analysis was performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, California, USA. Multiple-group comparisons were analyzed using the one-way analysis of variance (ANOVA). Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Determination of Total Polyphenol and Total Tannin Contents

Polyphenol and tannin contents in *L. pedunculata*, *S. rosmarinus*, *S. lavandulifolia* and *O. compactum* aqueous extracts are shown in Figure 1. The highest polyphenol content was found in *S. rosmarinus* (290.63 $\pm$ 7.69 mg GAE/gExt), and it is significantly ($p < 0.01$) different from the three other contents, which are 248.05 $\pm$ 7.27, 252.67 $\pm$ 5.40 and 241.90 $\pm$ 16.95 mg GAE/gExt, respectively, for *L. pedunculata*, *S. lavandulifolia* and *O. compactum*.

As for tannin contents, *O. compactum* extract (148.20 $\pm$ 17.96 mg GAE/gExt) was significantly ($p < 0.05$) richer in tannins than *S. rosmarinus* (113.93 $\pm$ 6.16 mg GAE/gExt), but both extracts (*O. compactum* and *S. rosmarinus*) do not significantly differ from *L. pedunculata* (125.13 $\pm$ 13.26 mg GAE/gExt) and *S. lavandulifolia* (124.25 $\pm$ 6.07 mg GAE/gExt).
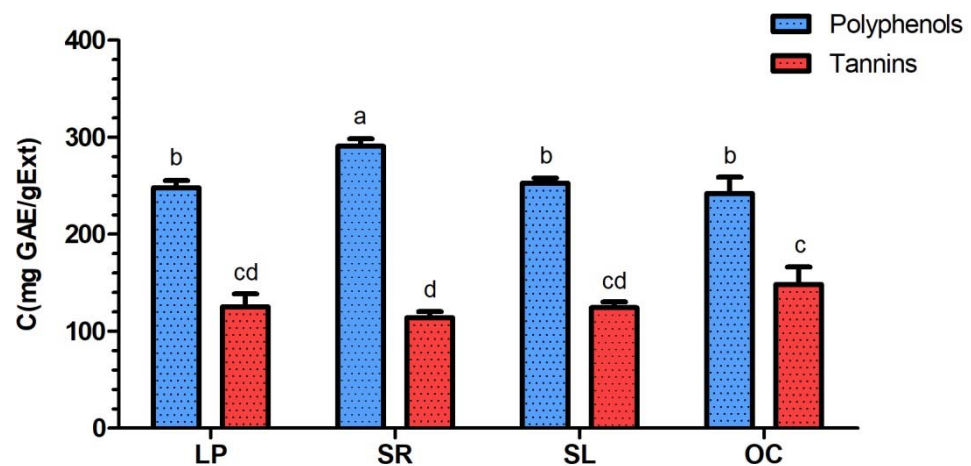
Figure 1. Total polyphenol and tannin contents in *L. pedunculata* (LP), *S. rosmarinus* (SR), *S. lavandulifolia* (SL) and *O. compactum* (OC) aqueous extracts. Contents with common letters (a, b, c and d) are not significantly different.

3.2. UHPLC Analysis of Aqueous Extracts

The chemical composition of the aqueous extracts was determined using UHPLC-MS. The results (Table 2) show that the most abundant compound in all the studied extracts is rosmarinic acid. Figure 2 shows the UHPLC-MS chromatograms of rosmarinic acid detected in the aqueous extracts.

Table 2. Results of UHPLC-MS analysis regarding the presence or the absence of some chemical compounds in *L. pedunculata*, *S. rosmarinus*, *S. lavandulifolia* and *O. compactum* aqueous extracts.
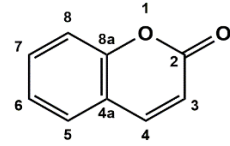
t_R (min)	λ_{max} (nm)	$[M - H]^-$ (m/z)	$[M + H]^+$ (m/z)	Compounds	LP	SR	SL	OC
6.869	278.1	WD	147	**Coumarin**	+	T	T	T
8.259	255.5, 297.2	147	149	**Cinnamic acid**	T	+	T	−
2.697	259.1, 293.6	153	155	**Protocatechuic acid**	+	+	+	+

Table 2. *Cont.*
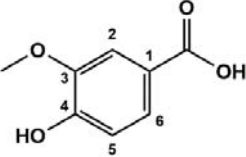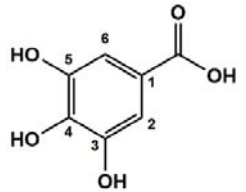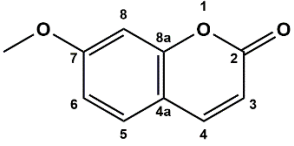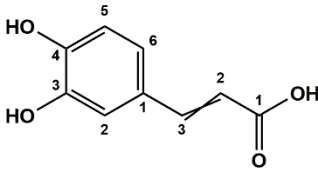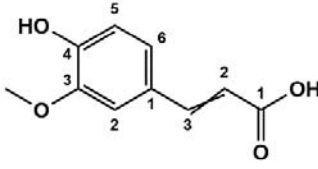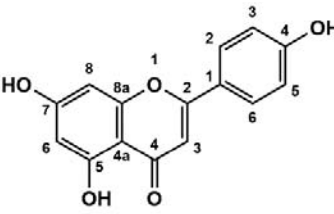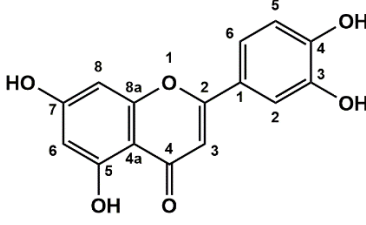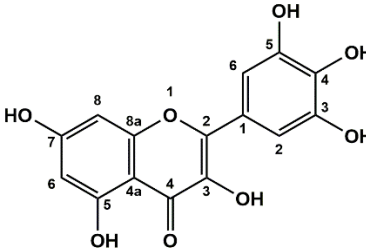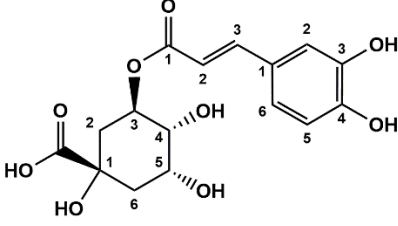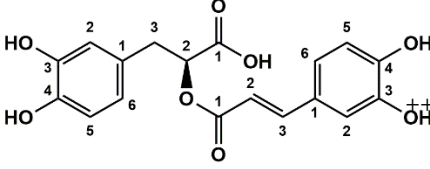
t_R (min)	λ_{max} (nm)	[M − H]$^-$ (*m/z*)	[M + H]$^+$ (*m/z*)	Compounds	LP	SR	SL	OC
3.923	260.3, 292.4	167	169	Vanillic acid	+	+	T	+
1.518	371.0	169	171	Gallic acid	+	T	T	T
7.761	307.9	WD	177	Herniarin	+	−	+	T
3.976	324.7	179	181	Caffeic acid	+	+	+	+
6.536	322.3	193	195	Ferulic acid	+	+	−	−
8.606	338.8	269	271	Apigenin	+	+	+	+

Table 2. *Cont.*

t_R (min)	λ_{max} (nm)	$[M-H]^-$ (*m/z*)	$[M+H]^+$ (*m/z*)	Compounds	LP	SR	SL	OC
8.368	254.3, 350.4	285	287	**Luteolin**	+	+	+	+
7.792	253.2, 372.1	317	319	**Myricetin**	+	−	+	T
3.680	240.1, 325.8	353	355	**Chlorogenic acid**	−	T	T	T
7.831	329.4	359	WD	**Rosmarinic acid**	++	++	++	++

(WD) weak detection; (T) traces; (−) absence; (+) presence; (++) high presence; (LP) *L. pedunculata*; (SR) *S. rosmarinus*; (SL) *S. lavandulifolia*; (OC) *O. compactum*.

Moreover, coumarin, apigenin, luteolin, protocatechuic acid, vanillic acid, gallic acid and caffeic acid are found in the different extracts but with a lower abundance or as traces. We can notice that herniarin and myricetin are present in all the plants except *S. rosmarinus*. As for ferulic acid, it is present only in *L. pedunculata* and *S. rosmarinus* extracts. Cinnamic acid is absent in *O. compactum* extract, and chlorogenic acid is absent in *L. pedunculata*.

3.3. Antibacterial Activity of Aqueous Extracts

L. pedunculata, *S. rosmarinus*, *S. lavandulifolia* and *O. compactum* aqueous extracts were tested individually and in association against 34 bacteria. Moreover, different antibiotics (gentamicin, vancomycin and amoxicillin) were tested against the same bacteria. The different results of their antibacterial activities are shown in Tables 3–6 and Figures 3–5.
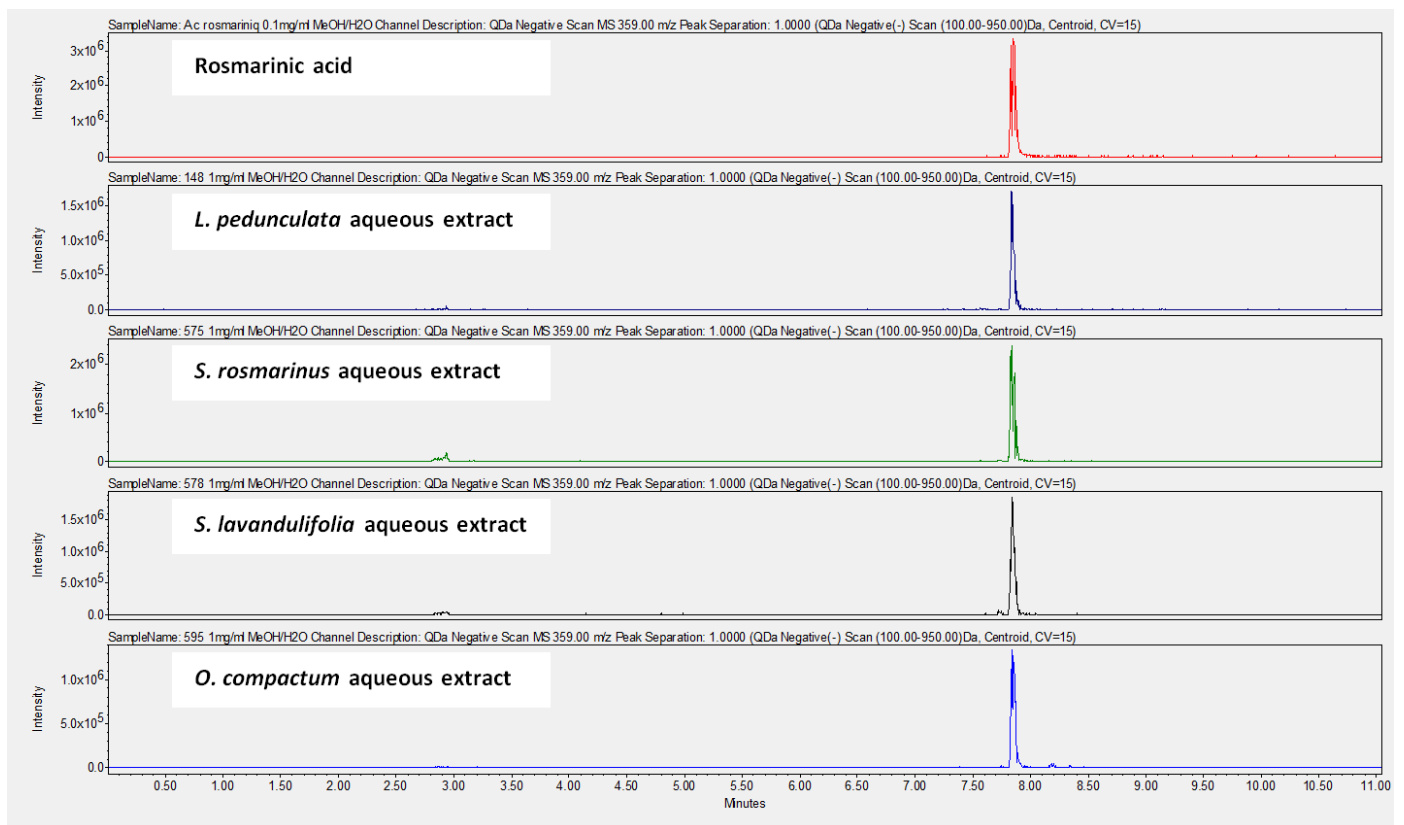
Figure 2. UHPLC-MS chromatograms at 359.00 *m/z* (negative scan) showing the presence of rosmarinic acid in *L. pedunculata*, *S. rosmarinus*, *S. lavandulifolia* and *O. compactum* aqueous extracts.
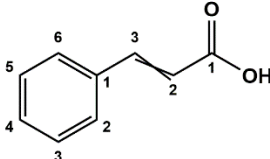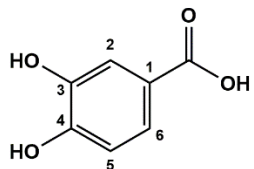
Table 3. MIC of antibiotics (gentamycin, vancomycin and amoxicillin) in mg/L.

Microorganisms		Reference	Antibiotics (MIC Values in mg/L)		
			Gentamicin	Vancomycin	Amoxicillin
Gram +	*Enterococcus faecalis*	C159-6	2	0.5	64
	Enterococcus sp.	8153	2	4	2
	Mycobacterium smegmatis	5003	0.03	0.5	1
	Staphylococcus aureus	8146	0.5	1	4
	Staphylococcus aureus	8241	0.5	1	16
	Staphylococcus aureus	ATCC 6538	0.25	1	0.125
	Staphylococcus aureus	T28-1	0.5	1	2
	Staphylococcus aureus	T17-4	0.5	1	1
	Staphylococcus epidermidis	T46A1	0.06	2	1
	Staphylococcus epidermidis	T19A1	32	2	16
	Staphylococcus epidermidis	T21A5	0.06	2	16
	Staphylococcus warneri	T12A12	0.06	4	1
	Staphylococcus warneri	T26A1	0.06	2	0.25
	Staphylococcus pettenkoferi	T47.A6	0.06	2	0.25
	Streptococcus agalactiae	T38.2	ND	ND	ND
	Streptococcus agalactiae	T53C9	0.5	0.25	0.03
	Streptococcus pyogenes	16138	0.125	0.25	0.03
	Streptococcus pyogenes	16135	0.125	0.25	0.03
	Corynebacterium striatum	T40A3	0.06	0.5	1

Table 3. *Cont.*

Microorganisms		Reference	Antibiotics (MIC Values in mg/L)		
			Gentamicin	Vancomycin	Amoxicillin
Gram −	*Citrobacter freundii*	11041	0.25	NA	2
	Citrobacter freundii	10268	ND	ND	ND
	Escherichia coli	ATCC 25922	0.5	NA	16
	Escherichia coli	T20A1	0.25	NA	NA
	Escherichia coli	8138	0.5	NA	NA
	Escherichia coli	8157	0.5	NA	NA
	Enterobacter aerogenes	9004	0.5	NA	NA
	Klebsiella pneumoniae	10270	8	NA	NA
	Klebsiella pneumoniae	11016	0.25	NA	NA
	Proteus mirabilis	11060	0.5	NA	2
	Proteus mirabilis	T28-3	0.25	NA	1
	Pseudomonas aeruginosa	8131	1	NA	NA
	Pseudomonas aeruginosa	ATCC 27583	2	NA	NA
	Pseudomonas aeruginosa	8129	0.03	NA	NA
	Salmonella sp.	11033	0.25	NA	2

(ND) not determined; (NA) Not active.

Table 4. MIC values of *L. pedunculata* and *S. rosmarinus* aqueous extracts individually and in combination (in mg/mL). The association of *L. pedunculata* with *S. rosmarinus* is active against 30 strains among 34.

Microorganisms		Reference	MIC ± SD (mg/mL)				
			LP	SR	Combination		FIC
					LP	SR	
Gram +	*Enterococcus faecalis*	C159-6	NA	NA	1.00 ± 0.28	0.70 ± 0.37	-
	Enterococcus sp.	8153	NA	NA	NA	NA	-
	Mycobacterium smegmatis	5003	0.43 ± 0.25	0.70 ± 0.37	0.13 ± 0.04	0.40 ± 0.14	0.87
	Staphylococcus aureus	8146	0.60 ± 0.00	0.60 ± 0.00	0.18 ± 0.09	0.30 ± 0.00	0.79
	Staphylococcus aureus	8241	0.50 ± 0.14	0.60 ± 0.00	0.15 ± 0.11	0.25 ± 0.07	0.72
	Staphylococcus aureus	ATCC 6538	0.60 ± 0.00	0.50 ± 0.14	0.18 ± 0.09	0.25 ± 0.07	0.79
	Staphylococcus aureus	T28-1	0.40 ± 0.14	0.50 ± 0.14	0.20 ± 0.07	0.13 ± 0.04	0.75
	Staphylococcus aureus	T17-4	0.60 ± 0.00	0.80 ± 0.28	0.08 ± 0.00	0.40 ± 0.14	0.63
	Staphylococcus epidermidis	T46A1	0.20 ± 0.07	0.20 ± 0.07	0.10 ± 0.04	0.10 ± 0.04	1.00
	Staphylococcus epidermidis	T19A1	0.25 ± 0.07	0.25 ± 0.07	0.18 ± 0.09	0.08 ± 0.00	1.00
	Staphylococcus epidermidis	T21A5	0.30 ± 0.00	0.25 ± 0.07	0.13 ± 0.04	0.10 ± 0.04	0.82
	Staphylococcus warneri	T12A12	0.30 ± 0.00	0.25 ± 0.07	0.13 ± 0.04	0.13 ± 0.04	0.92
	Staphylococcus warneri	T26A1	0.30 ± 0.00	0.25 ± 0.07	0.13 ± 0.04	0.13 ± 0.04	0.92
	Staphylococcus pettenkoferi	T47.A6	0.30 ± 0.00	0.25 ± 0.07	0.10 ± 0.04	0.13 ± 0.04	0.83
	Streptococcus agalactiae	T38.2	0.75 ± 0.45	0.75 ± 0.45	0.38 ± 0.23	0.60 ± 0.00	1.30
	Streptococcus agalactiae	T53C9	NA	1.20 ± 0.00	0.65 ± 0.43	0.50 ± 0.14	-
	Streptococcus pyogenes	16138	1.20 ± 0.00	1.20 ± 0.00	0.11 ± 0.04	0.38 ± 0.23	0.41
	Streptococcus pyogenes	16135	0.90 ± 0.30	0.60 ± 0.00	0.15 ± 0.00	0.45 ± 0.15	0.92
Gram −	*Corynebacterium striatum*	T40A3	NA	NA	0.80 ± 0.28	0.35 ± 0.19	-
	Citrobacter freundii	11041	NA	NA	0.80 ± 0.28	0.60 ± 0.00	-
	Citrobacter freundii	10268	1.20 ± 0.00	NA	0.80 ± 0.28	0.45 ± 0.21	-
	Escherichia coli	ATCC 25922	NA	NA	NA	NA	-
	Escherichia coli	T20A1	NA	NA	1.20 ± 0.00	1.20 ± 0.00	-
	Escherichia coli	8138	NA	NA	1.20 ± 0.00	1.20 ± 0.00	-
	Escherichia coli	8157	NA	NA	NA	NA	-
	Enterobacter aerogenes	9004	NA	NA	1.00 ± 0.28	0.70 ± 0.37	-
	Klebsiella pneumoniae	10270	NA	NA	1.20 ± 0.00	1.20 ± 0.00	-
	Klebsiella pneumoniae	11016	NA	NA	NA	NA	-
	Proteus mirabilis	11060	1.20 ± 0.00	1.20 ± 0.00	0.35 ± 0.19	0.33 ± 0.22	0.56
	Proteus mirabilis	T28-3	1.00 ± 0.28	0.60 ± 0.00	0.35 ± 0.19	0.33 ± 0.22	0.89
	Pseudomonas aeruginosa	8131	NA	NA	0.65 ± 0.43	0.80 ± 0.28	-
	Pseudomonas aeruginosa	ATCC 27583	1.20 ± 0.00	1.20 ± 0.00	0.70 ± 0.37	0.28 ± 0.23	0.81
	Pseudomonas aeruginosa	8129	0.50 ± 0.14	0.60 ± 0.42	0.20 ± 0.07	0.15 ± 0.11	0.65
	Salmonella sp.	11033	NA	NA	1.20 ± 0.00	0.80 ± 0.28	-

(NA) not active; (LP) *L. pedunculata*; (SR) *S. rosmarinus*.

Table 5. MIC values of *L. pedunculata* and *S. lavandulifolia* aqueous extracts individually and in combination (in mg/mL). The association of *L. pedunculata* with *S. lavandulifolia* is active against 32 strains among 33.

| | Microorganisms | Reference | MIC ± SD (mg/mL) | | | | FIC |
| | | | LP | SL | Combination | | |
					LP	SL	
Gram +	*Enterococcus faecalis*	C159-6	NA	NA	0.10 ± 0.04	0.83 ± 0.53	-
	Enterococcus sp.	8153	NA	NA	0.85 ± 0.49	1.20 ± 0.00	-
	Mycobacterium smegmatis	5003	0.43 ± 0.25	0.50 ± 0.14	0.10 ± 0.04	0.33 ± 0.22	0.89
	Staphylococcus aureus	8146	0.60 ± 0.00	0.60 ± 0.00	0.08 ± 0.00	0.30 ± 0.00	0.63
	Staphylococcus aureus	8241	0.50 ± 0.14	0.60 ± 0.00	0.08 ± 0.00	0.30 ± 0.00	0.65
	Staphylococcus aureus	ATCC 6538	0.60 ± 0.00	0.50 ± 0.14	0.15 ± 0.11	0.23 ± 0.11	0.70
	Staphylococcus aureus	T28-1	0.40 ± 0.14	0.50 ± 0.14	0.15 ± 0.11	0.23 ± 0.11	0.83
	Staphylococcus aureus	T17-4	0.60 ± 0.00	0.60 ± 0.00	0.13 ± 0.04	0.30 ± 0.00	0.71
	Staphylococcus epidermidis	T46A1	0.20 ± 0.07	0.25 ± 0.07	0.10 ± 0.04	0.13 ± 0.04	1.00
	Staphylococcus epidermidis	T19A1	0.25 ± 0.07	0.25 ± 0.07	0.13 ± 0.04	0.10 ± 0.04	0.90
	Staphylococcus epidermidis	T21A5	0.30 ± 0.00	0.25 ± 0.07	0.10 ± 0.04	0.10 ± 0.04	0.73
	Staphylococcus warneri	T12A12	0.30 ± 0.00	0.25 ± 0.07	0.13 ± 0.04	0.10 ± 0.04	0.82
	Staphylococcus warneri	T26A1	0.30 ± 0.00	0.15 ± 0.00	0.13 ± 0.04	0.08 ± 0.00	0.92
	Staphylococcus pettenkoferi	T47.A6	0.30 ± 0.00	0.20 ± 0.07	0.13 ± 0.04	0.10 ± 0.04	0.92
	Streptococcus agalactiae	T38.2	0.75 ± 0.45	ND	ND	ND	-
	Streptococcus agalactiae	T53C9	NA	1.20 ± 0.00	0.18 ± 0.09	0.60 ± 0.00	-
	Streptococcus pyogenes	16138	1.20 ± 0.00	1.20 ± 0.00	0.20 ± 0.07	0.50 ± 0.14	0.58
	Streptococcus pyogenes	16135	0.90 ± 0.30	1.00 ± 0.28	0.18 ± 0.09	0.30 ± 0.21	0.49
	Corynebacterium striatum	T40A3	NA	NA	0.25 ± 0.25	0.90 ± 0.42	-
Gram −	*Citrobacter freundii*	11041	NA	NA	1.00 ± 0.28	0.25 ± 0.25	-
	Citrobacter freundii	10268	1.20 ± 0.00	NA	0.90 ± 0.42	0.28 ± 0.23	-
	Escherichia coli	ATCC 25922	NA	NA	1.20 ± 0.00	1.20 ± 0.00	-
	Escherichia coli	T20A1	NA	NA	1.00 ± 0.28	1.20 ± 0.00	-
	Escherichia coli	8138	NA	NA	1.20 ± 0.00	0.80 ± 0.28	-
	Escherichia coli	8157	NA	NA	NA	NA	-
	Enterobacter aerogenes	9004	NA	NA	1.20 ± 0.00	0.10 ± 0.04	-
	Klebsiella pneumoniae	10270	NA	NA	1.20 ± 0.00	1.20 ± 0.00	-
	Klebsiella pneumoniae	11016	NA	NA	1.20 ± 0.00	1.20 ± 0.00	-
	Proteus mirabilis	11060	1.20 ± 0.00	1.00 ± 0.28	0.20 ± 0.07	0.50 ± 0.14	0.67
	Proteus mirabilis	T28-3	1.00 ± 0.28	0.90 ± 0.30	0.30 ± 0.00	0.30 ± 0.00	0.63
	Pseudomonas aeruginosa	8131	NA	NA	0.55 ± 0.46	0.85 ± 0.49	-
	Pseudomonas aeruginosa	ATCC 27583	1.20 ± 0.00	1.20 ± 0.00	0.50 ± 0.14	0.60 ± 0.00	0.92
	Pseudomonas aeruginosa	8129	0.50 ± 0.14	0.50 ± 0.14	0.15 ± 0.00	0.15 ± 0.00	0.60
	Salmonella sp.	11033	NA	NA	1.20 ± 0.00	0.60 ± 0.00	-

(ND) not determined; (NA) not active; (LP) *L. pedunculata*; (SL) *S. lavandulifolia*.

Table 6. MIC values of *L. pedunculata* and *O. compactum* aqueous extracts individually and in combination (in mg/mL). The association of *L. pedunculata* with *O. compactum* was active against 25 strains among 34.

| | Microorganisms | Reference | MIC ± SD (mg/mL) | | | | FIC |
| | | | LP | OC | Combination | | |
					LP	OC	
Gram +	*Enterococcus faecalis*	C159-6	NA	NA	1.20 ± 0.00	0.90 ± 0.42	-
	Enterococcus sp.	8153	NA	NA	NA	NA	-
	Mycobacterium smegmatis	5003	0.43 ± 0.25	1.00 ± 0.28	0.15 ± 0.11	0.45 ± 0.21	0.80
	Staphylococcus aureus	8146	0.60 ± 0.00	1.00 ± 0.28	0.15 ± 0.11	0.50 ± 0.14	0.75
	Staphylococcus aureus	8241	0.50 ± 0.14	0.80 ± 0.28	0.18 ± 0.09	0.23 ± 0.11	0.63
	Staphylococcus aureus	ATCC 6538	0.60 ± 0.00	1.00 ± 0.28	0.30 ± 0.00	0.15 ± 0.11	0.65
	Staphylococcus aureus	T28-1	0.40 ± 0.14	0.80 ± 0.28	0.13 ± 0.04	0.30 ± 0.00	0.69
	Staphylococcus aureus	T17-4	0.60 ± 0.00	0.80 ± 0.28	0.23 ± 0.11	0.25 ± 0.25	0.69
	Staphylococcus epidermidis	T46A1	0.20 ± 0.07	0.35 ± 0.19	0.10 ± 0.04	0.10 ± 0.04	0.79
	Staphylococcus epidermidis	T19A1	0.25 ± 0.07	0.40 ± 0.14	0.08 ± 0.00	0.20 ± 0.07	0.80
	Staphylococcus epidermidis	T21A5	0.30 ± 0.00	0.40 ± 0.14	0.08 ± 0.00	0.20 ± 0.07	0.75
	Staphylococcus warneri	T12A12	0.30 ± 0.00	0.50 ± 0.14	0.10 ± 0.04	0.20 ± 0.07	0.73
	Staphylococcus warneri	T26A1	0.30 ± 0.00	0.60 ± 0.00	0.13 ± 0.04	0.20 ± 0.07	0.75
	Staphylococcus pettenkoferi	T47.A6	0.30 ± 0.00	0.50 ± 0.14	0.13 ± 0.04	0.15 ± 0.00	0.72
	Streptococcus agalactiae	T38.2	0.75 ± 0.45	NA	0.64 ± 0.56	0.19 ± 0.11	-
	Streptococcus agalactiae	T53C9	NA	NA	1.00 ± 0.28	0.33 ± 0.22	-
	Streptococcus pyogenes	16138	1.20 ± 0.00	1.20 ± 0.00	0.45 ± 0.15	0.60 ± 0.00	0.88
	Streptococcus pyogenes	16135	0.90 ± 0.30	1.20 ± 0.00	0.08 ± 0.00	0.90 ± 0.30	0.83
	Corynebacterium striatum	T40A3	NA	NA	0.80 ± 0.28	0.45 ± 0.21	-

Table 6. *Cont.*

		MIC ± SD (mg/mL)					
					Combination		
Microorganisms		Reference	LP	OC	LP	OC	FIC
Gram −	*Citrobacter freundii*	11041	NA	NA	1.20 ± 0.00	0.33 ± 0.22	-
	Citrobacter freundii	10268	1.20 ± 0.00	NA	1.20 ± 0.00	0.23 ± 0.11	-
	Escherichia coli	ATCC 25922	NA	NA	NA	NA	-
	Escherichia coli	T20A1	NA	NA	NA	NA	-
	Escherichia coli	8138	NA	NA	NA	NA	-
	Escherichia coli	8157	NA	NA	NA	NA	-
	Enterobacter aerogenes	9004	NA	NA	1.20 ± 0.00	0.38 ± 0.23	-
	Klebsiella pneumoniae	10270	NA	NA	NA	NA	-
	Klebsiella pneumoniae	11016	NA	NA	NA	NA	-
	Proteus mirabilis	11060	1.20 ± 0.00	1.20 ± 0.00	0.60 ± 0.00	0.25 ± 0.07	0.71
	Proteus mirabilis	T28-3	1.00 ± 0.28	1.20 ± 0.00	0.43 ± 0.25	0.35 ± 0.19	0.72
	Pseudomonas aeruginosa	8131	NA	NA	NA	NA	-
	Pseudomonas aeruginosa	ATCC 27583	1.20 ± 0.00	NA	0.65 ± 0.43	0.70 ± 0.37	-
	Pseudomonas aeruginosa	8129	0.50 ± 0.14	0.80 ± 0.28	0.18 ± 0.09	0.33 ± 0.22	0.76
	Salmonella sp.	11033	NA	NA	NA	NA	-

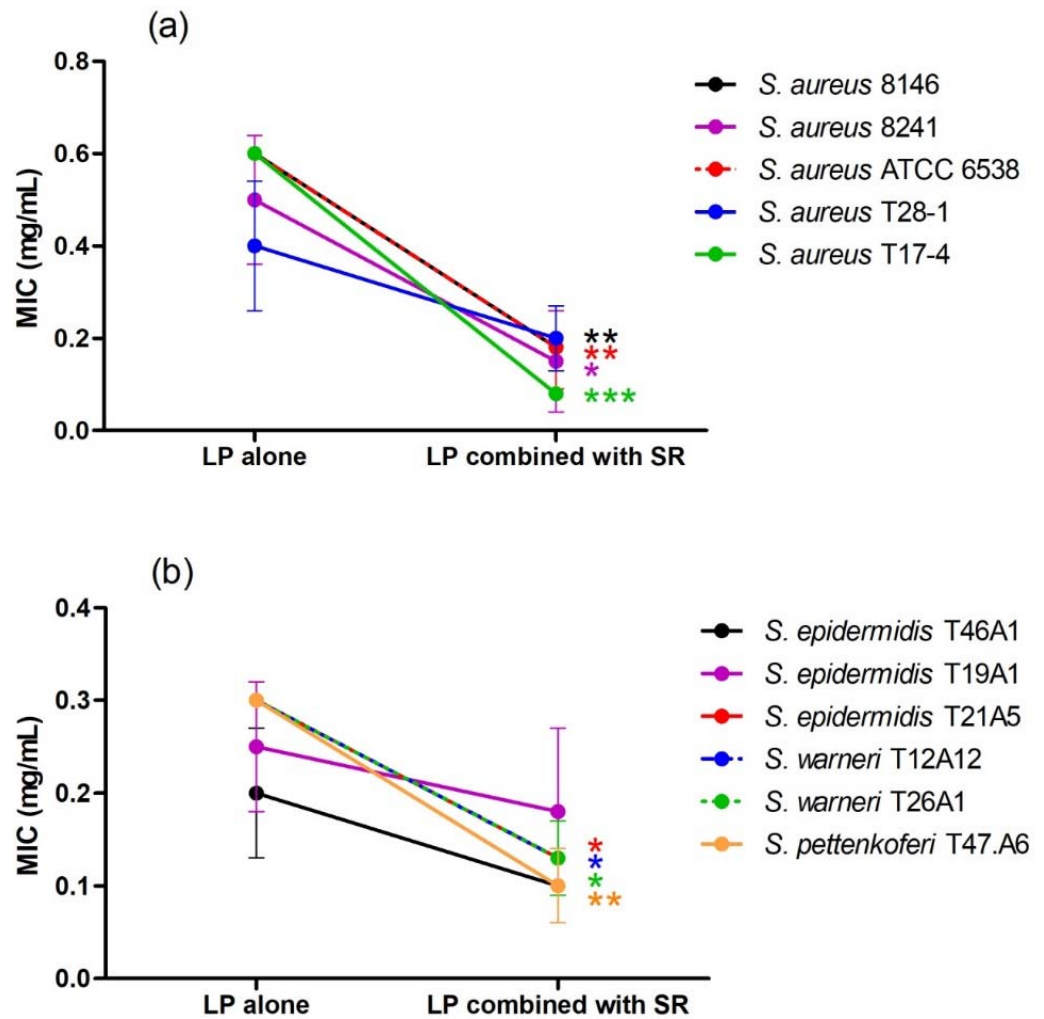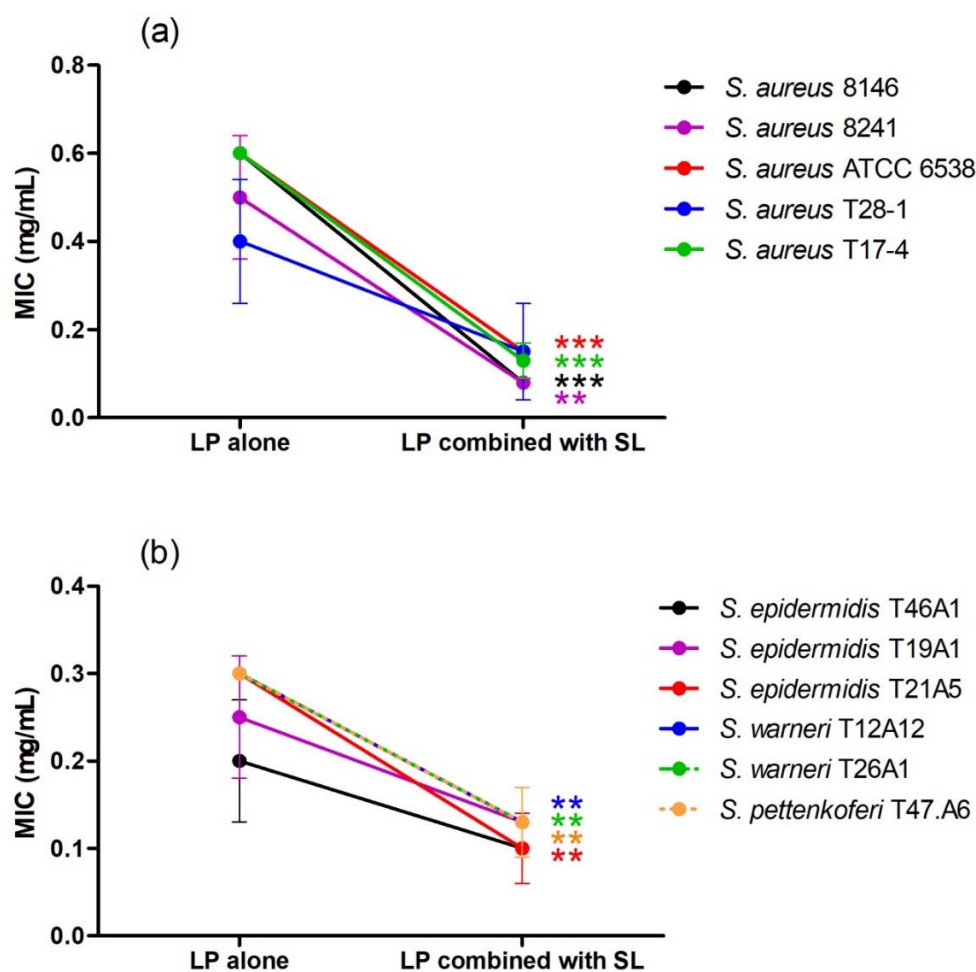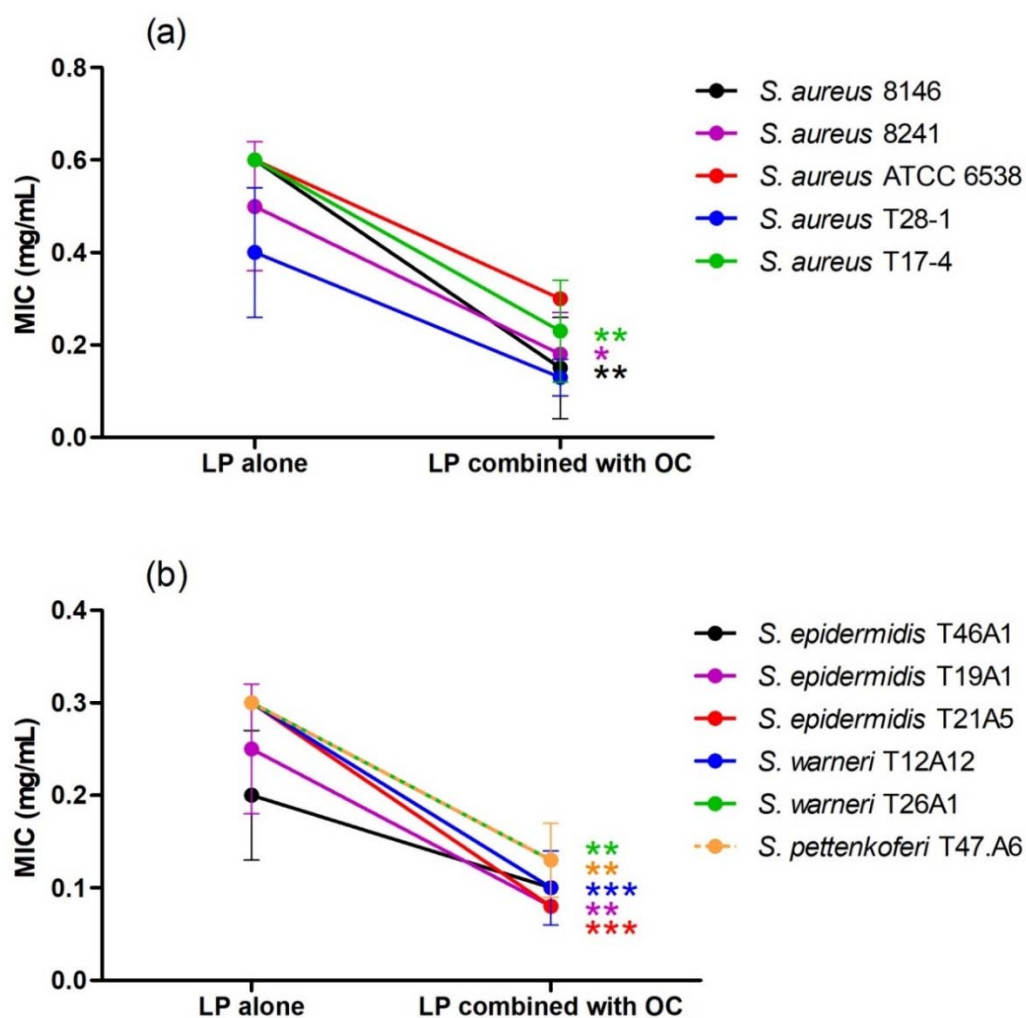(NA) not active; (LP) *L. pedunculata*; (OC) *O. compactum*.

Figure 3. Effect of the association of *L. pedunculata* (LP) with *S. rosmarinus* (SR) on MIC values of LP ((**a**) = *S. aureus* strains, (**b**) = coagulase negative staphylococci). After combination, a remarkable improvement of the antibacterial activity was observed. MIC values of LP combined with SR are significantly lower than MIC values of LP alone * *p* < 0.05; ** *p* < 0.01: *** *p* < 0.001.

Figure 4. Effect of the association of *L. pedunculata* (LP) with *S. lavandulifolia* (SL) on MIC values of LP ((**a**) = *S. aureus* strains, (**b**) = coagulase negative staphylococci). After combination, MIC values of LP combined with SL are significantly lower than MIC values of LP alone ** $p < 0.01$: *** $p < 0.001$.

All the individual extracts are active against staphylococci and streptococci, as well as against *Proteus mirabilis*, *Mycobacterium smegmatis* and *Pseudomonas aeruginosa*. *L. pedunculata* extract is also active against one of the two tested strains of *Citrobacter freundii*. The lowest MIC obtained after using *L. pedunculata* and *S. rosmarinus* extracts was 0.20 ± 0.07 mg/mL against *S. epidermidis* T46A1 (Table 4), and 0.35 ± 0.19 mg/mL is the lowest MIC obtained by *O. compactum* against the same strain (Table 6). As for *S. lavandulifolia*, the lowest MIC value is 0.15 ± 0.00 mg/mL, and it is obtained against *S. warneri* T26A1 (Table 5).

After combining *L. pedunculata* extract with *S. rosmarinus*, *S. lavandulifolia* and *O. compactum*, a remarkable improvement of the antibacterial activity was observed. On one hand, additive and synergistic effects were noticed on the majority of the strains that are susceptible to the individual extracts. On the other hand, we noticed that extract mixtures were active against several strains that are resistant to the individual extracts, especially Gram-negative strains such as *E. coli*, *E. aerogenes*, *K. pneumoniae* and *Salmonella* sp. Moreover, the association of *L. pedunculata* with *S. lavandulifolia* was active against 32 strains among 33 (the strain *S. agalactiae* T38.2 was not tested because of some experimental issues), and it was the most effective combination. The association of *L. pedunculata* with *S. rosmarinus* came in the second position by being active against 30 strains, followed by the mixture of *L. pedunculata* with *O. compactum* that was active against 25 strains. It is remarkable that some plant mixtures were active against the majority of the Gram-negative strains often multidrug-resistant (Table 3).

Figure 5. Effect of the association of *L. pedunculata* (LP) with *O. compactum* (OC) on MIC values of LP ((**a**) = *S. aureus* strains, (**b**) = coagulase negative staphylococci). After combination, MIC values of LP combined with OC are significantly lower than MIC values of LP alone * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

4. Discussion

Lavender species are widely used for their antibacterial activity demonstrated in several studies [32,33]. However, their essential oils are usually studied regardless of the large use of their aqueous extracts in the traditional medicine [34–36]. Besides, the use of essential oil is very limited in human and animal pharmacy due to the potential occurrence of side effects and toxicity [37].

In the present study, we tested the antibacterial effect of *L. pedunculata* aqueous extract that showed a strong antibacterial activity mainly against Gram-positive strains. Lopes and his collaborators investigated the antibacterial activity of *L. pedunculata* aqueous extract from Portugal using the microdilution method. They found MIC values ranging from 0.10 to 0.45 mg/mL for *E. coli*, and from 0.15 to 0.45 mg/mL for *S. aureus*, *P. aeruginosa* and S. Typhimurium. Some of these data are close to the results of our study, where *L. pedunculata* was active against *S. aureus* with MIC values ranging from 0.4 to 0.6 mg/mL and against *P. aeruginosa* with MIC values ranging from 0.5 to 1.2 mg/mL. However, our extract was not active against *Salmonella* sp. and *E. coli* [26].

In addition to *L. pedunculata*, the aqueous extracts of three other plant species, *S. rosmarinus*, *S. lavandulifolia* and *O. compactum*, were also tested in this study and were active against several bacterial strains, especially the Gram-positive ones. In a study carried out by Ramdan et al. (2018), the antibacterial activity of the hydroethanolic ex-

tracts of *S. rosmarinus* and *O. compactum* from Marrakech (Morocco) was evaluated by the broth microdilution method. The respective MIC values obtained for *S. rosmarinus* and *O. compactum* were 25 and 12.5 mg/mL for *Salmonella enterica*, 50 and 25 mg/mL for *E. coli* and *P. aeruginosa*, and 12.5 mg/mL for both extracts against *S. aureus*. These values are very high in comparison with our study [38]. Giner and his collaborators conducted a study on the hydroalcoholic extract mixture of *S. lavandulifolia* with *S. rosmarinus* and *Thymus mastichina*. They showed that it is active against *E. coli* and *E. aerogenes* with a MIC value of 12.8 mg/mL. This mixture was also active against *S. enterica* and *S. aureus*, with respective MIC values of 6.4 and 0.4 mg/mL. These results are very different from the ones obtained in this work, except for *S. aureus* which is quite similar [39].

The three species were not only tested individually, but also in association with *L. pedunculata*, thus giving promising activities that were found for the first time. In fact, some plant mixtures, such as the mixture of *L. pedunculata* with *S. lavandulifolia*, were active against the majority of the Gram-negative strains, often multidrug-resistant, while the individual extracts were not active. Moreover, activities of the plant mixtures against the Gram-positive strains were boosted. One of the most problematic Gram-positive strains was *S. aureus*. It is a human pathogen that possesses a high adaptability and tenacity, making it abundant in the environment. It is capable of colonizing various human organs, and it is a source of a variety of virulence factors. The multidrug-resistant form of this microorganism, especially the methicillin-resistant *S. aureus*, is one of the major microorganisms responsible for bloodstream infections that cause high levels of mortality worldwide. Since *S. aureus* has succeeded in developing resistance against practically all antibiotics, finding a new alternative is urgently needed, of which includes the importance of plant extracts such as the ones tested in this study and that showed strong activities [40,41].

Polyphenol and tannin contents of *L. pedunculata*, *S. rosmarinus*, *S. lavandulifolia* and *O. compactum* aqueous extracts were determined during the present study using the Folin-Ciocalteu method. The highest polyphenol content corresponded to *S. rosmarinus* extract (290.63 ± 7.70 mg GAE/gExt). This result was significantly high while compared to the ethanolic extract of the same plant species from Taiwan that has a polyphenol content of 161.07 ± 3.12 mg GAE/gExt [42]. Moreover, ethanolic extract of *S. rosmarinus* from different regions in Morocco also had lower polyphenol contents ranging from 74.15 to 146.63 mg GAE/gExt [43]. As for *L. pedunculata*, *S. lavandulifolia* and *O. compactum* aqueous extracts studied in the present work, their respective polyphenol contents are 248.03 ± 7.30, 252.67 ± 5.41 and 241.90 ± 16.96 mg GAE/gExt. The polyphenol content of *Salvia officinalis* aqueous extract from Portugal was determined by Afonso et al. (2019), and it is similar to *S. lavandulifolia* studied in this work (229.0 ± 44.0 mg GAE/gExt) [44]. Furthermore, a study was conducted on the ethanolic extracts of *O. compactum* aerial parts from Ouezzane and Taounate (Morocco), and their polyphenol contents were found to be lower than in our extract, with respective values of 117.60 ± 1.12 and 117.56 ± 2.74 mg GAE/gExt [23,45].

As for the tannin contents, the obtained values represent almost the half of the polyphenol contents, with respective values of 125.13 ± 13.24, 113.93 ± 6.15, 124.23 ± 6.08 and 148.20 ± 17.97 mg GAE/gExt for *L. pedunculata*, *S. rosmarinus*, *S. lavandulifolia* and *O. compactum*. Indeed, tannins are known for their antibacterial properties [46,47]; therefore, they might be the compounds that are responsible for the observed activities of the studied plant extracts.

UHPLC analysis of the plant extracts demonstrated their high content in rosmarinic acid. In fact, rosmarinic acid is found to be a good antibacterial agent [48]. This compound has the ability of damaging the cell membrane [49]. Other compounds were also detected in the studied extracts that are known for their antibacterial and antifungal activities such as coumarin, apigenin and caffeic acid [49–51].

Lopes et al. (2018) analyzed the phenolic compounds of *L. pedunculata* aqueous extract from 13 different natural populations in Portuguese regions using HPLC-DAD-ESI/MSn. They found that phenolic acids represent the major phenolic compounds present in these extracts. Salvianolic acid B and rosmarinic acid were present in large concentrations and

caffeic acid in smaller ones. Concerning flavonoids, the main present compound was luteolin-7-O-glucuronide [26]. In another study also conducted on *L. pedunculata* from Portugal, different extracts were analyzed using HPLC/DAD. The obtained results showed that these extracts contain high concentrations of rosmarinic acid and smaller ones of luteolin. Moreover, apigenin was not quantified in the aqueous extracts, but it was present in the ethanolic and hydroethanolic ones [52]. Some studies conducted on the methanolic and ethanolic extracts of *S. rosmarinus* showed the presence of caffeic acid, rosmarinic acid, vanillic acid and ferulic acid [24,53]. Furthermore, in a review concerning polyphenolic compounds of *Salvia* species, it was mentioned that *S. lavandulifolia* contains rosmarinic acid, apigenin and luteolin [54]. All of these studies are in concordance with the results we found in this research work.

5. Conclusions

The results obtained in the present study demonstrated that *L. pedunculata* aqueous extract from Morocco exerts an important antibacterial activity mainly against Gram-positive bacteria. Moreover, this activity is boosted when *L. pedunculata* extract is used in mixtures with *S. rosmarinus*, *S. lavandulifolia* and *O. compactum*. Some active compounds were investigated, and all the extracts were shown to contain high amounts of polyphenols and tannins. These results represent a first step in investigating the use of *L. pedunculata* aqueous extracts by the Moroccan population. Furthermore, these results showed the effectiveness of the alternative and combinative polyphytotherapy. This suggests that *L. pedunculata* aqueous extract could be used as a new potential source of natural antibacterial agents either alone or in combination with *S. rosmarinus*, *S. lavandulifolia* and *O. compactum*. It might be an effective solution for resistant bacteria that cause damage around the world, such as *S. aureus*. However, clinical studied are needed to confirm these activities on real microbial infection cases. Ultimately, this could contribute to the valorization of biodiversity and resources of Morocco and could generate new sources of income for the population.

Author Contributions: Conceptualization, S.B. and B.E.; methodology, M.B. and H.M.; software, A.D. and F.R.; validation, J.S. and C.N.; formal analysis, B.G., F.K.E. and L.N.; writing—original draft preparation, S.B.; writing—review and editing, A.S. and O.A.k.; supervision, T.Z. and S.S. All authors have read and agreed to the published version of the manuscript.

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