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Essential Oils of Two Portuguese Endemic Species of *Lavandula* as a Source of Antifungal and Antibacterial Agents

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Abstract: Chemical investigations of *L. stoechas* subsp. *luisieri* and *L. pedunculata* essential oils were analyzed by GC-MS, and the antimicrobial activity was performed against bacteria and fungi isolated from food sources. The cytotoxicity of the essential oil was performed in NHDF cells using the MTT method. According to the results, the main compounds of *L. stoechas* subsp. *luisieri* essential oil were *trans*-α-necrodyl acetate (40.2%), lavandulyl acetate (11%), and *trans*-α-necrodol (10.4%), while fenchone (50.5%) and camphor (30.0%) in *L. pedunculata* essential oil. The antifungal activity of essential oils was confirmed with MIC values ranging from 1.2 to 18.7 µL/mL; for bacteria, it ranged from 4.7 to 149.3 µL/mL. Both the *Lavandula* species tested showed low or equal MIC and MBC/MFC values for *L. stoechas* subsp. *luisieri* essential oil, revealing greater efficacy in antimicrobial activity. The *L. stoechas* subsp. *luisieri* essential oil revealed cytotoxic effects (30 ± 2% of cell viability) in NHDF cells at all concentrations tested.

Keywords: *L. stoechas* subsp. *luisieri*; *L. pedunculata*; chemical profile; antimicrobial activity; cytotoxicity

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1. Introduction

Aromatic and medicinal plants have been intensively studied due to the biological potential of essential oils (EOs) and other extracts. Dispersed around the Mediterranean area, *Lavandula* species belong to the Lamiaceae (=Labiatae) family. Around 41 species are recognized in this genus [1]. Due to the geographical position and climate, this area represents a biodiversity hotspot, allowing the development of several endemic plant species. However, climate change and the high risk of fires are endangering Mediterranean species. *Lavandula stoechas* subsp. *luisieri* (Rozeira) Rozeira and *L. pedunculata* (Mill.) Cav. are perennial shrubs and endemic to the Iberian Peninsula. In Portugal, these plants are important resources for beekeeper activities and the extraction of their essential oils as a value-added product in poor agricultural regions. Both species produce valuable secondary metabolites, namely terpenes in their EOs, and phenolic compounds with great biological properties. Some ethnobotanical investigations reveal the uses of these species for healing indigestion, heartburn, headaches, blood circulation, and also act as a sedative, antidermatitis, asthma, and decongestive nasal bronchitis [1–3]. The chemical compounds of their EOs are mainly oxygenated monoterpenes (33–87%), followed by monoterpene hydrocarbons (0.1–17%), oxygenated sesquiterpenes (0.3–12%), and sesquiterpene hydrocarbons (0.5–10%) [4]. *L. stoechas* subsp. *luisieri* deserves particular interest due to the

composition of their EO that contains unique compounds: the necrodane derivatives, such as *trans*-α-necrodol and *trans*-α-necrodyl acetate [5–7]. The main compounds reported in *L. pedunculata* EO are fenchone, camphor, and 1,8-cineole [8,9]. The morphological aspects of both species and their respective main compounds are presented in Figure 1.

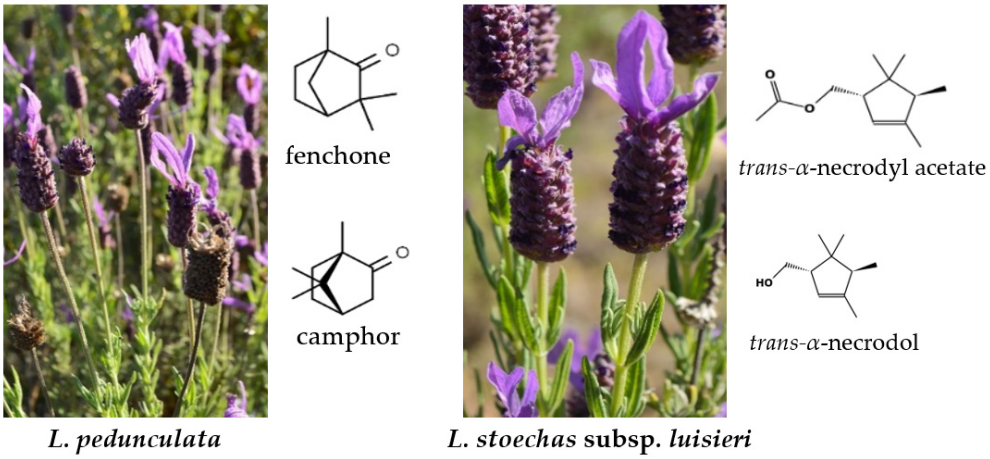

Figure 1. Morphological traits of *Lavandula pedunculata* and *L. stoechas* subsp. *luisieri* and its main compounds. The chemical structures were downloaded from Chemical Structure Search–ChemSpider (https://www.chemspider.com/StructureSearch.aspx, accessed on 23 March 2023).

Essential oils are promising antimicrobial products, both against human and animal pathogenic microorganisms and for food spoilage control. The complex mixture of chemical components in the EO makes this natural product an effective agent in the resistance of microorganisms, contrary to what occurs in common antibiotics, which are constituted by a single compound [10]. The remarkable efficacy of essential oils is not related to the presence of their main compounds but often to the synergistic effect among varied constituents [11]. Specific reports have demonstrated the antimicrobial action of *Lavandula* EOs and their main compounds, such as camphor and 1,8-cineole, which agree that the activity of the EOs is not due to merely one compound [8,12–14]. Despite several studies on the antimicrobial action of the EOs, the mechanism of action in microorganisms is not evident among investigators. For example, some studies have reported greater susceptibility of Gram-positive than Gram-negative bacteria. Gram-negative bacteria have a complex cell envelope that includes an outer membrane linked to the inner peptidoglycan layer via lipoproteins, and due to this structure, this kind of bacteria is more resistant. On the other hand, other researchers do not find antibacterial differences between both bacteria types [15]. Furthermore, the mode of action may depend on the chemical profile and their components ratio [16]. The mechanism of action of EOs may involve different events in the cell's outer membrane and within the cytoplasm. Among the different mechanisms, we enhance: the disintegration of the bacterial outer membrane; the alteration of the fatty acid composition; increase the membrane fluidity, resulting in leakage out of metabolites and ions; the interference with glucose uptake; and the inhibition of enzyme activity [15,17]. Concerning the major compounds found in each species, *L. pedunculata* EO is rich in fenchone and camphor [8,18]. Fenchone is a bicyclic monoterpene ketone, with a structure and odor like camphor, which is also a monoterpenoid ketone. Both compounds revealed high biological properties, such as antibacterial and antifungal activities [14,19]. However, other minor compounds may influence antimicrobial activity, such as α-pinene [20], limonene [19], and linalool [13]. Regarding the *L. stoechas* subsp. *luisieri* EO, the main compounds are irregular monoterpenoids with cyclopentenic structures, namely necrodane derivatives, such as *trans*-α-necrodol and *trans*-α-necrodyl acetate [5,7]. Eisner and Meinwald discovered these compounds in the defensive secretion of *Necrodes surinamensis* [21], and recently, these compounds were also discovered in *Evolvulus alsinoides* L. essential oils [22]. To understand the potential of these compounds, Zuzarte et al. (2012) studied two chemically distinct

profiles of *L. stoechas* subsp. *luisieri* EO, one with high amounts of necrodanes and one with low amounts of these compounds. The great antifungal activity was revealed in EO with high necrodane compounds [5]. Despite the great antimicrobial activity, the cytotoxicity of the EOs in human cells must be considered to avoid toxic effects on the organism [23]. The cytotoxicity studies of the EOs of *L. pedunculata* and *L. stoechas* subsp. *lusieri* are sparse, and for the *L. stoechas* subsp. *luisieri* EO, only two studies report no cytotoxicity effects at small concentrations (<3.2 mg/mL and <0.08 µL/mL) in human skin fibroblasts [24] and in mouse macrophage cell line (RAW 264.7) [5]. Additionally, for the *L. pedunculata*, aqueous and hydroalcoholic extracts were reported in two studies with no cytotoxic effects revealed against the porcine liver (PLP2) and human keratinocytes (HaCat) cell lines [25,26]. On the other hand, some studies have reported the anticancer potential of EOs and other extracts. Regarding cancer cells, anti-proliferative effects were observed in *L. pedunculata* extracts against breast adenocarcinoma (MCF-7), cervical carcinoma (HeLa), lung cancer (NCI-H460), and hepatocellular carcinoma (HepG2) [26]. Moreover, *L. stoechas* subsp. *lusieri* showed cytotoxic effects against HepG2 [24]. This study aims to contribute to disseminating the chemical variation of the EO of two important types of endemism of the Iberian Peninsula, *L. stoechas* subsp. *Luisieri*, and *L. pedunculata*, and to contribute to a broad spectrum of microorganisms, some of which are reported for the first time in the antimicrobial activity of these species. The cytotoxicity of *L. stoechas* subsp. *luisieri* EO against normal human cells was also performed.

2. Materials and Methods

2.1. Plant Collection and Essential Oils

Flowering *L. stoechas* subsp. *luisieri* and *L. pedunculata* were collected in Serra da Malcata (558 m, 40°12′06.741″ N; 7°06′22.085″ W), Portugal. A replica of each plant was deposited in the herbarium of the Biology Laboratory of IPCB-ESA (Polytechnic Institute of Castelo Branco—Agrarian School). The voucher numbers are ESACBMLS08 and ESACBMLP01, for *L. stoechas* subsp. *luisieri* and *L. pedunculata*, respectively. The EO from the fresh aerial parts was obtained by hydrodistillation for 2 h in a Clevenger-type apparatus according to the procedure described in the European Pharmacopoeia [27].

2.2. GC-MS Analysis

The volatile profiles of the EOs were obtained in triplicate by gas chromatography coupled with mass spectrometry (GC/MS SCION-SQ 456 GC, Bruker Corporation, Massachusetts, United States of America). The separation was achieved on an HP-5MS capillary column (30 m × 0.25 mm id × 0.25 µm film thickness, Agilent J&W, Folsom, CA, USA). Helium was the carrier gas used with a flow rate of 1 mL/min. The EO samples were injected with a volume of 1 µL, using a split ratio of 1:100, and analyzed using electron impact ionization mass spectrometry (EI-MS) at 70 eV. The compounds were identified in scan mode with the positive polarity of ions 20–300 m/z with a time of 250.0 ms. The initial oven temperature was programmed to 45 °C, gradually increasing 3 °C/min to 175 °C and 300 °C with a heating rate of 15 °C/min, and maintaining this temperature for 10 min. The transfer line and the ion source were programmed at 250 °C and 220 °C, respectively. The identification of the compounds was based on the retention index (RI) compared with the RI given by the MS library (NIST 17 version 2.3) and with RI calculated from the n-alkane series standards (C7–C18 and C19–C30) that were injected under the same chromatographic column and chromatographic conditions. The relative amount of each compound was expressed as a percentage of the relative peak area of the compound, relative to the total area of the peaks identified in the samples.

2.3. Microorganism Cultures

Nine fungal cultures were earlier isolated from fruits of *Arbutus unedo* L. [28] in the Microbiology Laboratory of IPCB-ESA, and these cultures were identified by molecular approach in the Micoteca da Universidade do Minho (MUM). These fungi were identified as

Alternaria section *Alternaria* (ESA.M.11), *Penicillium simile* (ESA.M.13), *Aspergillus tubingensis* (ESA.M.38), *Aspergillus niger* (ESA.M.45), *Meyerozyma guilliermondii* (ESA.M.47), *Penicillium crustosum* (ESA.M.48), *Penicillium glabrum* (ESA.M.54), *Aureobasidium* sp. (ESA.M.57), and *Hanseniaspora* sp. (ESA.M.99). Two ATCC reference strains, *Saccharomyces cerevisiae* ATCC 9763 and *Aspergillus brasiliensis* ATCC 16404, and a clinical isolate of *Candida albicans* ESALD/2016 were also tested. The reference cultures were acquired in ielab® (Alicante, Spain). Each culture was transferred to a potato dextrose agar (PDA, HiMedia Chemicals, Nagpur, India) medium, at 25 ± 2 °C during 48 h for yeasts and 4 to 5 days for molds (until the spore formation) and used for analysis after three subcultures. For molds, the spore suspensions were prepared according to Domingues et al. (2021) [6]. For yeasts, a suspension was prepared in 0.85% (*w*/*v*) NaCl (Applichem Panreac, Darmstadt, Germany) to match the turbidity of the 1.0 McFarland standard (bioMérieux, Lyon, France), representing approximately 3.0×10^7 yeasts/mL.

Nine bacterial cultures were used (Table 1), six Gram-negative bacteria, such as *Aeromonas hydrophila*, *Burkholderia* sp., *Chromobacterium violaceum*, *Pseudomonas aeruginosa*, *Salmonella* sp., and *Serratia marcescens*, and three Gram-positive bacteria such as *Bacillus cereus*, *Listeria monocytogenes*, and coagulase-positive *Staphylococcus*. *Pseudomonas aeruginosa* ATCC 27853 was used as reference strain. Bacterial cultures were obtained by growing the bacterial cultures for 18–24 h at 37 °C in tryptone soya yeast extract agar (TSA-YE, prepared with TSA (Oxoid, Chester, UK) and YE (Biokar, Beauvais, France)). The exception was *B. cereus* culture, which was grown for 15 h. *B. cereus* growth conditions were used to obtain non-sporulated cultures.

Table 1. Identification and characterization of bacterial cultures.

Cultures	Lab. Reference	Origin	Gram Staining [1]
Aeromonas hydrophila	SC-V-AP/2015	Untreated water	-
Burkholderia sp.	B-AM-Pa-3F/2014	Untreated water	-
Chromobacterium violaceum	SC-AF/2014	Untreated water	-
Pseudomonas aeruginosa ATCC 27853	ATCC 28753	ATCC	-
Pseudomonas aeruginosa	SC-V-AP/2015	Untreated water	-
Salmonella sp.		Food isolates	-
Serratia marcescens	A-LO-596/2018	Raw sheep's milk	-
Bacillus cereus	A-FL-PB/2013	Bread flour	+
Listeria monocytogenes	QD-LCP$_{24}$/2014	Raw goat's milk	+
Coagulase-positive *Staphylococcus*	CB-QM-L7/11/2018	Cheese	+

[1]: Gram-negative; +: Gram-positive.

2.4. Microdilution Method for MIC and MFC/MBC Determination

For bacterial cultures, a suspension was prepared in 0.85% (*w*/*v*) NaCl (Applichem Panreac, Darmstadt, Germany) to match the turbidity of the 0.5 McFarland standard (bioMérieux, Lyon, France), representing about 1.5×10^8 cells/mL. For molds and yeasts, the EOs were diluted in a potato dextrose broth (PDB, VWR Chemicals Prolabo, PA, USA) medium supplemented with 0.8% (*v*/*v*) of tween 80 (VWR Chemicals Prolabo, PA, USA). For the bacteria, the EOs were diluted in a Müeller–Hinton broth (MHB, Oxoid, Chester, UK) medium supplemented with 0.8% (*v*/*v*) of tween 80 (VWR Chemicals Prolabo, PA, USA). The MIC of the EOs was fulfilled according to the Clinical and Laboratory Standards Institute, CLSI (2002) method, with some modifications. Each microplate well was completed with 150 µL in total volume. The test wells were completed with 140 µL of EO/medium and 10 µL of inoculum. Negative control wells were completed with 150 µL of EO/medium, and the positive control wells were completed with 140 µL of medium and 10 µL of inoculum. A culture medium control was also made. The microplates were incubated under the optimum conditions for fungal and bacterial cultures under humid air. After incubation, all microplate wells were inoculated in PDA (HiMedia Chemicals, Nagpur, India) and nutritive agar (NA, Oxoid, Chester, UK) plates for fungi (MFC) and

bacteria (MBC), respectively. For MFC and MBC determinations, 10 µL loops were used. The MFC/MBC values matched the lowest EO concentration, of which no growth was observed after incubation. Afterward, 30 µL of resazurin (VWR Chemicals Prolabo, PA, USA) was added to each microplate well. Then, the microplates were incubated for 2 h for bacteria and fungi until the positive control changed color. According to Tulio et al. (2006) and The et al. (2017), the results were visually assessed by comparing the color of the inoculated wells with the color of the positive and negative control wells [29,30]. The MIC value matched the lowest EO concentration in which the color was similar to the negative control. All experiments were performed in triplicate and repeated whenever the results of each triplicate did not agree.

2.5. Cell Viability

The evaluation of cell viability was performed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, as described by Santos et al. [31]. The cytotoxicity of the *L. stoechas* subsp. *luisieri* EO was determined after 24 h of incubation. Normal human dermal fibroblasts (NHDF) cell lines were maintained in a Roswell Park Memorial Institute (RPMI-1640) culture medium supplemented with 10% fetal bovine serum (FBS), 1% antibiotic/antimycotic mixture, 0.02 M of L-glutamine, 0.01 M of HEPES, and 0.001 M of sodium pyruvate. The cells were incubated at 37 °C in an air incubator in a 5% CO_2-humidified atmosphere. They were seeded in 96-well plates (5×10^3 cells/well), which, after reaching confluence, were exposed to the samples dissolved in a RPMI-1640 culture medium. Supplemented RPMI-1640 culture medium was added to the negative control wells. At the end of incubation, the medium in the wells was removed and replaced by the MTT solution (0.5 mg/mL) and incubated again at 37 °C for 3 h. Afterward, the MTT solution was removed and formazan crystals were dissolved in 0.5% DMSO; the absorbances were recorded using a microplate reader at 570 nm.

3. Results

3.1. Chemical Profile of the Essential Oils

The constituents of the EOs of both species are listed in Table 2, according to their elution order, in an HP-5MS column.

Table 2. Identification and relative amounts of compounds of *L. stoechas* subsp. *luisieri* (LSL) and *L. pedunculata* (LP) essential oils.

Compounds	Chemical Class [a]	RI [b]	RI [c]	% Peak Area	
				LSL	LP
α-Pinene	MH	925	936	2.8 ± 0.1	7.0 ± 0.2
Camphene	MH	939	950	0.1 ± 0.0	1.1 ± 0.1
β-Myrcene	MH	982	989	-	0.1 ± 0.0
p-Cymene	MH	1016	1024	0.1 ± 0.0	0.4 ± 0.0
Limonene	MH	1020	1030	0.1 ± 0.0	2.1 ± 0.0
1,8-Cineole	OM	1023	1032	3.2 ± 0.0	0.1 ± 0.0
trans-β-Ocimene	MH	1032	1038	-	0.7 ± 0.0
cis-Linalool oxide	OM	1068	1075	0.8 ± 0.0	-
3,4,4-Trimethyl-2-cyclohexene-1-one	OT	1076	1055 *	1.1 ± 0.0	-
Fenchone	OM	1084	1088	3.6 ± 0.0	50.5 ± 0.3
Linalool	OM	1099	1099	5.6 ± 0.1	0.9 ± 0.0
Fenchol	OM	1113	1115	-	0.6 ± 0.0
α-Campholenal	OM	1128	1124	-	0.3 ± 0.0
Camphor	OM	1146	1143	2.7 ± 0.0	30.0 ± 0.2
trans-α-Necrodol	OM	1151	1130 *	10.4 ± 0.1	-
Pinocarvone	OM	1168	1161	-	0.1 ± 0.0
Borneol	OM	1171	1166	-	0.2 ± 0.0
NI C *L. luisieri*		1175		0.8 ± 0.0	-
cis-α-Necrodol	OM	1182		2.0 ± 0.0	-
5-Methylene-2,3,4,4-tetramethylcyclopenten-2-enone	OT	1198	1160 **	0.8 ± 0.0	-
Terpinen-4-ol	OM	1185	1177	-	0.2 ± 0.0
p-Cymen-8-ol	OM	1194	1184	-	0.4 ± 0.0
α-Terpineol	OM	1200	1190	-	0.2 ± 0.0

Table 2. *Cont.*

Compounds	Chemical Class [a]	RI [b]	RI [c]	% Peak Area	
				LSL	LP
Myrtenal	OM	1206	1192	-	0.1 ± 0.0
Verbenone	OM	1220	1206	-	0.5 ± 0.0
Fenchyl acetate	OM	1233	1220	-	0.4 ± 0.0
trans-α-Necrodyl acetate	OM	1296	1265 *	40.2 ± 0.1	-
Bornyl acetate	OM	1305	1284	-	0.8 ± 0.0
Lavandulyl acetate	OM	1312	1289	11.0 ± 0.1	0.2 ± 0.0
cis-α-Necrodyl acetate	OM	1324		1.7 ± 0.0	-
NI D *L. luisieri*		1333		1.1 ± 0.0	-
Valencene	SH	1498	1492	-	0.3 ± 0.0
Caryophyllene oxide	OS	1602	1581	0.4 ± 0.0	-
Viridiflorol	OS	1610	1591	2.2 ± 0.1	-
Isovalencenol	OS	1788	1782	-	1.5 ± 0.1
NI E *L. luisieri*		1818		1.5 ± 0.1	-
NI F *L. luisieri*		1821		1.1 ± 0.1	-
Yield (%, *v*/*w*)				0.8 ± 0.1	2.1 ± 0.2
Identification (%)				88.8	98.7
Monoterpene hydrocarbons (%)				3.1	11.4
Oxygenated monoterpenes (%)				81.2	85.5
Sesquiterpene hydrocarbons (%)				0.0	0.3
Oxygenated sesquiterpenes (%)				2.6	1.5
Others (%)				1.9	-

Results are expressed as mean values ± standard deviation. Compounds are listed in order of elution from the HP-5MS column. [a] Chemical class: MH—monoterpene hydrocarbons; OM—oxygenated monoterpenes; SH—sesquiterpene hydrocarbons; OS—oxygenated sesquiterpenes; [b] retention index calculated relative to n-alkanes series standards (C7–C18 and C19–C30) on the HP-5MS column; [c] literature retention index on similar phase column (DIMS5P) [32]; * RI values consulted in [5]; ** RI values consulted in [33]. NI C, D, E, F: unidentified compounds.

Thirty-three compounds were identified in samples and four unidentified compounds (NI C, D, E, and F) were observed in *L. stoechas* subsp. *luisieri* EO, which are always present in the EO of this species as observed from other lab work (data unpublished). Good identification of chemical compounds was obtained with 88.8% in *L. stoechas* subsp. *luisieri* and 98.7% in *L. pedunculata*. The *L. stoechas* subsp. *luisieri* and *L. pedunculata* EOs are characterized by high amounts of oxygenated monoterpenes (81.2% and 85.5%, respectively). The main compounds in the *L. stoechas* subsp. *luisieri* EOs were *trans*-α-necrodyl acetate (40.2%), lavandulyl acetate (11%), and *trans*-α-necrodol (10.4%). Significant amounts of linalool (5.6%), fenchone (3.6%), and 1,8-cineole (3.2%) were also found. As it has been described, the *L. stoechas* subsp. *luisieri* EO is singularly characterized by the presence of irregular oxygenated monoterpenes called necrodanes, which are absent in the remaining *Lavandula* species [4]. These compounds becoming *L. stoechas* subsp. *luisieri* as an interesting biological value and could be a chemotaxonomic marker of this species. In our work, compounds such as *trans*-α-necrodol, *cis*-α-necrodol, *trans*-α-necrodyl acetate, and *cis*-α-necrodyl acetate were identified by GC-MS. According to the geographic distribution and chemical studies of EO, *L. stoechas* subsp. *luisieri* is only reported in the Iberian Peninsula. The major compounds reported in the *L. stoechas* subsp. *luisieri* EO are camphor (1.1–74%), *trans*-α-necrodyl acetate (1.8–48%), fenchone (0.1–22%), and 1,8-cineole (1.3–21%) in plants from Portugal and Spain [5,6,18,33–41]. The chemical variability of EOs appears to be common between *Lavandula* populations; Zuzarte et al. (2012) noticed significant variations in the chemical composition of EO among plants from central and southern Portugal. From the central region, the essential oil was characterized by *trans*-α-necrodyl acetate (17%), *trans*-α-necrodol (7%), and 1,8-cineol (6%), contrasting to the southern plants, whereby 1,8-cineole (34%) and fenchone (18%) were the main compounds. Although necrodane compounds were not present as major compounds, the following compounds were reported at low concentrations: *trans*-α-necrodyl acetate (3.2%) and *trans*-α-necrodol (4.5%) [5]. According to this study, it would seem that abiotic factors have a crucial influence on chemical compound production [42].

Regarding *L. pedunculata* EO, the main compounds were fenchone (50.5%), camphor (30%), and α-pinene (7%). This species is widely distributed in the Mediterranean region and is abundant throughout Portugal [1]. The main compounds found in *L. pedunculata* EO are corroborated by other chemical studies; however, according to research conducted in these geographical regions, different chemotypes in this species have been found. Zuzarte et al. (2009) revealed considerable differences in the major compound between geographical origins, 1,8-cineol (34%) in northern region plants, fenchone (45%) in central region plants, and the chemotype camphor/1,8-cineol (34%/25%) in central–north region plants [8]. Another chemotype, camphor/fenchone (42%/37%), was reported in southern plants [9,18]. As previously described, identifying chemotypes in the *L. pedunculata* EO demonstrates the strong influence of extrinsic factors in producing chemical compounds [4,43].

3.2. Antifungal Activity of the Essential Oils

The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of the EOs of both *Lavandula* species (Table 3) were evaluated against nine cultures isolated from *Arbutus unedo* L. fruits, *C. albicans* (clinical isolate), and two reference cultures (*A. brasiliensis* ATCC 16404 and *S. cerevisiae* ATCC 9763). Concerning the antifungal activity of *L. stoechas* subsp. *Luisieri*, the scientific information is sparse. MIC and MFC values varied from 1.2 to 74.7 µL/mL. Generally, the concentration to inhibit a microorganism is lower than the concentration of lethality. However, the MIC value was the same in some microorganisms as in the MFC. Other studies have also verified this behavior, which reveals the fungicidal effects of these EOs [5,8,40]. The most sensible cultures to the action of EOs were *Aureobasidium* sp., *Hanseniaspora* sp., and *S. cerevisiae* ATCC 9763 for *L. stoechas* subsp. *luisieri* essential oil, with 1.2 µL/mL and 2.3 µL/mL MIC and MFC values, respectively. On the other hand, the most resistant microorganisms were *Alternaria* sp. section *Alternaria*, *A. brasiliensis* ATCC 16404, and *P. glabrum*. For these cultures, it was impossible to determine the MFC value for both species; the maximum concentration tests revealed the growth of the microorganisms. Özcan et al. (2018) also revealed the strong resistance of *A. alternaria* to the action of *L. stoechas* EO [44]. The *L. stoechas* subsp. *luisieri* EO revealed greater effectiveness than *L. pedunculata* due to the MIC or MFC values always being inferior or equal. Baptista et al. (2015) also reported the higher effectiveness of *L. stoechas* subsp. *luisieri* EO compared to the *L. pedunculata*. These authors evaluated the EOs of both species against *A. niger*, *C. albicans*, and *S. cerevisiae*, reporting higher MIC values (15.5 µg/mL, >100 µg/mL, and 31 µg/mL, respectively) [45]. In spite of the different analytical methods, Zuzarte et al. (2009) reported lower MIC and MFC values of *L. pedunculata* EO against *C. albicans* (2.5 and 5 µL/mL, respectively), but higher MFC values were revealed against *A. niger* (≥20 µL/mL) [8]. Regarding *L. stoechas* subsp. *luisieri* EO, Zuzarte et al. (2012) tested the antifungal activity against *A. niger* ATCC 16404 with a very low MIC value (0.32 µL/mL) but a high MFC value (20 µL/mL) [5].

3.3. Antibacterial Activity of Essential Oils

The *Lavandula* sp. EOs were also tested against several bacterial cultures, most potentially pathogenic. The antibacterial activity demonstrated similar behavior (Table 4). This means the *L. stoechas* subsp. *luisieri* showed a greater effectiveness against most microorganisms compared to *L. pedunculata* EO.

The exception was against *Salmonella* sp., where the *L. pedunculata* EO showed a minimum bactericidal concentration (MBC) value (37.3 µL/mL) lower than *L. stoechas* subsp. *luisieri* (74.7 µL/mL). The greater antimicrobial activity of *L. stoechas* subsp. *luisieri* compared with other *Lavandula* sp. was also revealed in other studies [45,46]. This antimicrobial potential may be due to the presence of necrodane derivatives in their essential oil/non-polar extracts, which have been reported as compounds with high biological properties [18,35]. The most sensitive Gram-negative bacteria was *C. violaceum* for both EOs with MBC at 9.3 µL/mL. The most resistant Gram-negative bacteria was *P. aeruginosa*

ATCC 27853 with the same MIC and MBC values (149.3 µL/mL) for both *Lavandula* EOs. The isolate *P. aeruginosa* SC-V-AP/2015 also demonstrated high resistance to the action of both EOs. However, the maximum concentration tested of *L. pedunculata* (149.3 µL/mL) did not show lethality against this culture. Some studies reveal the high resistance of *P. aeruginosa* to the action of *L. stoechas* subsp. *luisieri* and *L. pedunculata* [24,33,46,47]. Gram-negative bacteria are known for their strong resistance to antibacterial agents due to the external membrane surrounding the cell wall restricting the diffusion of hydrophobic compounds through the lipopolysaccharides [17].

Concerning Gram-positive bacteria, the most resistant was *B. cereus*, despite low MIC values of 4.7 and 9.3 µL/mL, for *L. stoechas* subsp. *luisieri* and *L. pedunculata*, respectively. The MBC of both EOs was higher than 149.3 µL/mL. This high MBC value must be caused by their ability to produce spores that are highly resistant to adverse conditions [48]. Other studies with high inhibitory concentrations reported this strong resistance of *B. cereus* [24,33]. *Listeria monocytogenes* showed a lower MBC value (18.7 µL/mL) to the action of *L. stoechas* subsp. *luisieri* EO. For *L. stoechas* EO of plants from the Morocco region, this culture showed a very low MIC value of 2.5 µL/mL [49]. Regarding coagulase-positive *Staphylococcus*, the MIC and MBC values were the same for both EOs (37.3 µL/mL). Pombal et al. also tested the antibacterial potential of *L. stoechas* subsp. *luisieri* EO against *Staphylococcus aureus*, where the MIC and MBC also had the same values [40].

Table 3. Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of *L. stoechas* subsp. *luisieri* (LSL) and *L. pedunculata* (LP) essential oils.

Fungi	*Lavandula* sp.	Essential Oil Concentration (µL/mL)								
		74.7	**37.3**	**18.7**	**9.3**	**4.7**	**2.3**	**1.2**	**0.6**	**0.3**
Alternaria section *Alternaria* (ESA.M.11)	LSL	MFC>					MIC			
	LP	MFC>					MIC			
Aspergillus brasiliensis ATCC 16404	LSL	MFC>				MIC				
	LP	MFC>		MIC						
Aspergillus niger (ESA.M.45)	LSL					MIC MFC				
	LP				MIC MFC					
Aspergillus tubingensis (ESA.M.38)	LSL						MIC MFC			
	LP				MIC MFC					
Aureobasidium sp. (ESA.M.57)	LSL						MFC	MIC		
	LP					MFC	MIC			
Candida albicans (ESALD/2016)	LSL				MBC	MIC				
	LP			MBC	MIC					
Hanseniaspora sp. (ESA.M.99)	LSL						MFC	MIC		
	LP					MIC MFC				
Meyerozyma guilliermondii (ESA.M.47)	LSL				MFC	MIC				
	LP			MFC	MIC					
Penicillium crustosum (ESA.M.48)	LSL					MFC	MIC			
	LP				MFC	MIC				
Penicillium glabrum (ESA.M.54)	LSL	MFC>						MIC		
	LP	MFC>					MIC			
Penicillium simile (ESA.M.13)	LSL				MIC MFC					
	LP	MFC>		MIC						
Saccharomyces cerevisiae ATCC 9763	LSL						MFC	MIC		
	LP					MFC	MIC			

3.4. Cytotoxicity of the Essential Oil

Due to the greater antimicrobial activity, the *L. stoechas* subsp. *luisieri* EO was selected for cytotoxicity evaluation. The NHDF cells were exposed to different concentrations of the EO (0.25 to 10 µL/mL) for 24 h. According to the results presented in Figure 2, the viability of the cells with the presence of *L. stoechas* subsp. *luisieri* EO was significantly reduced with mean values of around 30 ± 2%, even at a lower concentration of EO tested (0.25 µL/mL).

Table 4. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *L. stoechas* subsp. *luisieri* (LSL) and *L. pedunculata* (LP) essential oils.

Bacteria	*Lavandula* spp.	Essential Oil Concentration (µL/mL)								
		149.3	**74.7**	**37.3**	**18.7**	**9.3**	**4.7**	**2.3**	**1.2**	**0.6**
Aeromonas hydrophila (SC-V-AP/2015)	LSL				MBC	MIC				
	LP				MIC MBC					
Burkholderia sp. (B-AM-Pa-3F)	LSL			MIC MBC						
	LP			MIC MBC						
Chromobacterium violaceum (SC-AF/2014)	LSL					MIC MBC				
	LP					MIC MBC				
Pseudomonas aeruginosa (ATCC 27853)	LSL	MIC MBC								
	LP	MIC MBC								
Pseudomonas aeruginosa (SC-V-AP/2015)	LSL	MBC		MIC						
	LP	MBC>	MIC							
Salmonella sp.	LSL		MBC				MIC			
	LP			MBC			MIC			
Serratia marcescens (A-LO-596/2018)	LSL	MBC	MIC							
	LP	MIC MBC								
Bacillus cereus (A-FL-PB/2013)	LSL	MBC>					MIC			
	LP	MBC>				MIC				
Listeria monocytogenes (QD-LCP$_{24}$/2014)	LSL				MIC MBC					
	LP			MBC	MIC					
Coagulase-positive *Staphylococcus* (CB-QM-L7/11/2018)	LSL			MIC MBC						
	LP			MIC MBC						

The cell viability did not differ statistically between different concentrations tested, which means that in these concentrations, the EO has a high potential of in vitro cytotoxicity in NHDF cells. As far as we know, the cytotoxicity of *L. stoechas* subsp. *luisieri* EO was only noticed by Zuzarte et al. (2012) [5]. The authors tested the cytotoxic effects of *L. stoechas* subsp. *luisieri* EO in a mouse macrophage cell line (RAW 264.7), and they demonstrated that the EO with higher percentages of necrodanes compounds (17% of *trans*-α-necrodyl acetate) had minor percentages of cell viability with values around 45% at 0.64 µL/mL. Only 0.08 µL/mL of EO did not affect the cell viability. However, we should not compare these values due to considerable differences, such as the kind of cell line, analytical methods, and the origin of plants/EO. We believe that the chemical profile of *L. stoechas* subsp. *luisieri* EO with high percentages of necrodanes compounds strongly contributes to this high cytotoxicity in fibroblast cells. On the other hand, cell lines have different behavior according to their origin; for example, the same study that used two mouse cell models, RAW 264.7 and fibroblasts, verified that fibroblasts cells were more resistant to the action

of the same cytotoxic agents compared to macrophage cells [50]. Due to high cytotoxicity revealed by *L. stoechas* subsp. *luisieri* EO in NHDF cells, its direct application in topical uses is not recommended. However, new EO nanoencapsulation strategies could be considered as a way to reduce their toxicity, such as liposomes, emulsions, and biopolymeric nanoparticles [51].

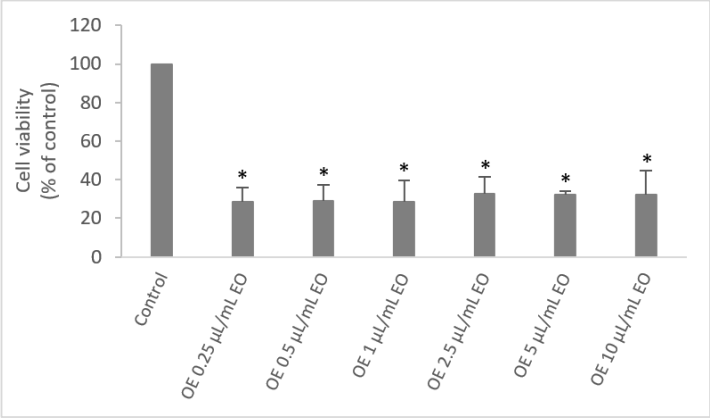

Figure 2. Effect of *Lavandula stoechas* subsp. *luisieri* essential oil on NHDF cell viability (MTT assay). Results are expressed as a percentage of MTT reduction by control cells maintained in a culture medium. Each value represents the mean ± SD from triplicates (* $p < 0.01$ compared to control).

4. Conclusions

In conclusion, our results reveal the great antimicrobial activity of the *L. stoechas* subsp. *luisieri* and *L. pedunculata* EOs against Gram-positive and Gram-negative bacteria, and also against yeasts and filamentous fungi. Comparing both species, the greater antimicrobial activity is attributed to the *L. stoechas* subsp. *luisieri* EO. According to these results, the EOs of both species are promising natural products to be used as antibacterial and antifungal agents against foodborne and potential pathogenic human and animal strains. Considering the microorganisms' resistance to conventional antimicrobial agents, the use of these natural products could be applied in antimicrobial formulations. Regarding cytotoxicity, *L. stoechas* subsp. *luisieri* EO revealed low cell viability in NHDF cells. In order to explore the potential application by the food and pharmaceutical industries, more cytotoxic studies of the EOs are to be investigated.

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