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Essential Oils of *Lavandula stoechas* subsp. *luisieri* as Antifungal Agent against Fungi from Strawberry Tree Fruit

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Abstract: *L. stoechas* subsp. *luisieri* is one of the five spontaneous species of the genus *Lavandula* that occurs spontaneously in Portugal. The chemical profile and antifungal activity of *L. stoechas* subsp. *luisieri* essential oils were investigated. The essential oil of two phenological stages was isolated by hydrodistillation and their chemical components analyzed by GC-FID/GC-MS. The minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC) of both essential oils were determinate to verify antifungal activity against different strains of fungi isolated from strawberry tree. The fungi tested were *Aspergillus carbonarius*, *Rhizopus stolonifer*, *Penicillium brevicompactum*, *Aureobasidium pullulans* and *Saccothecium rubi*. Essential oils were characterized by a high percentage of oxygenated monoterpenes (46-64%) such as *trans*-α-necrodyl acetate (12.58%), fenchone (5.97%), 1,8-cineole (4.84%) and 5-methylene-2,3,4,4-tetramethylcyclopenten-2-enone (10.97%) were the major compounds in essential oil from dormancy stage, while the main compounds in flowering stage were *trans*-α-necrodyl acetate (26.90%), *trans*-α-necrodol (13.02%), lavandulyl acetate (6.53%) and linalool (5.82%). A strong antifungal activity of the essential oils was found against all strains, with MIC and MFC values ranged from 0.07-0.29 µL/mL and 0.58-9.33 µL/mL, respectively.

Key words: Portuguese short-stalked lavender, Hydrodistillation, Gas Chromatography, Chemical characterization, Minimum inhibitory concentration, Minimum fungicidal concentration.

1. Introduction

Lavandula stoechas subsp. *luisieri* (Rozeira) Rozeira or also called *Lavandula luisieri* (Rozeira) Rivas Mart. is an aromatic and medicinal shrub endemic to the Iberian Peninsula and common in the semi-arid regions and mountainous areas of Southern Portugal and Southwest Spain. This plant belongs to Lamiaceae family and *Stoechas* section [1, 2]. In Portugal *L. stoechas* subsp. *luisieri* grows spontaneously and abundantly in poor agricultural soils and neglected areas. This particular species has deserved a lot of attention due the production of necrodane derivatives with high biological potential [3, 4]. In the Plant kingdom, *L. stoechas* subsp. *luisieri* is the distinct species that produces these types of compounds, reported only previously in a defensive secretion from a beetle (*Necrodes surinamensis*), which suggests a potential defensive role for the plant [5].

Since ancient times *Lavandula* species were used in folk uses and traditional medicine, due to expectorant proprieties, for headaches, insomnia, and anxiety treatment, for blood circulation, heart-burn and seasickness. It is also used as a disinfectant and

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clothes perfume to protect it against insects [1, 6]. In the last years some studies have reported the high potential of *L. stoechas* subsp. *luisieri* essential oil as an antibacterial and antifungal agent [4, 7-9]. Many others biological activities have been described as antifeedant, nematicidal and ixodicidal effects, anti-inflammatory properties and promising results in the treatment of Alzheimer's disease [3, 10-13]. Due to the *L. stoechas* subsp. *luisieri* potential, it may be a viable species for use as an alternative to antifungal synthetic agents. Usually, the essential oil has a variable chemical composition due to extrinsic and intrinsic factors, and the response of plants to these factors influence the production of secondary metabolites [14]. Thus, different phenological stages can give rise to chemotype variation in essential oil, and this fact can be translated into distinct biological effects [15].

Fruits and vegetables play an important role in the human diet, providing a significant amount of essential nutrients. However, these foods are very vulnerable to microbial contamination, which reduces their shelf life. Also, due to the presence of fungi, in particular *Aspergillus* spp., the presence of mycotoxigenic strains can compromise human health [16, 17]. In recent years, some ways of preservation and stabilization methods have been improved such as smart packaging, modified atmosphere, antifungal agents or bioactive coatings. However, resistance to conventional antifungals has been reported. The application of essential oils seems to be a viable way to act as an antifungal due to its complex chemical composition. Normally, the antimicrobial potential of the essential oil is basically due to the synergism between the chemical compounds and not to a single compound, which is an advantage for inhibiting the resistance mechanisms of the microorganisms [18]. *Arbutus unedo* L. is a spontaneous shrub and its fruits have many food applications, becoming a species with a great economic impact in rural areas. The use of natural products as essential oils in the food industry to extend the shelf life of perishable foods becomes important. The main goal of the present study was the evaluation of the chemical profile and antifungal activity of *L. stoechas* subsp. *luisieri* essential oils, from dormancy and flowering stages, against fungi isolated from the fruits of the strawberry tree.

2. Materials and Methods

2.1 Plant Material and Essential Oils

To ensure the natural regeneration and propagation of the species, the aerial parts (leaves and flowers) of *L. stoechas* subsp. *luisieri* were collected according to good agricultural and collection practices [19]. Wild plants were collected during the dormancy and flowering season in Serra da Malcata (558 m, 40°12'06.741'' N; 7°06'22.085'' W), Portugal. A copy of the collected plant was deposited in the herbarium of the Biology Laboratory of IPCB-ESA (Polytechnic Institute of Castelo Branco Agrarian School). According to the procedure described in the European Pharmacopoeia [20], the essential oils were isolated by hydrodistillation for 2 h, using a *Clevenger*-type apparatus. The essential oils were stored in dark vials at 4 ºC for further assays.

2.2 Essential Oil Analysis (GC-FID and GC-MS)

Gas chromatographic analyses were performed using a Perkin Elmer Clarus 400 gas chromatograph (Perkin Elmer, Shelton, CT, USA) equipped with two flame ionization detectors (FIDs), a data handling system and a vaporizing injector port into which two columns of different polarities were installed: a DB-1 fused-silica column (100% Dimethylpolysiloxane, 30 m × 0.25 mm i.d., film thickness 0.25 μm; J & W Scientific Inc.) and a DB-17HT fused-silica column ((50%-Phenyl)-methylpolysiloxane, 30 m × 0.25 mm i.d., film thickness 0.15 μm; J & W Scientific Inc.). The oven temperature was programmed, 45-175 °C, at 3 °C/min, subsequently at 15 °C/min up to 300 °C, and then held isothermal for 10 min; injector and detector temperatures, 280 °C and 300 °C, respectively;

hydrogen was the carrier gas (30 cm/s). The samples were injected using the split ratio 1:50. The volume of injection was 0.1 µL of a pentane/essential oil solution (1:1, v/v). The percentage composition of the volatiles was computed, by the normalization method from the GC peak areas, calculated as the mean value of two injections, for each sample.

For identification of compounds was used a Perkin Elmer Clarus 600 gas chromatograph, equipped with DB-1 fused-silica column (100% Dimethylpolysiloxane, 30 m × 0.25 mm i.d., film thickness 0.25 µm; J & W Scientific, Inc.), and interfaced with Perkin-Elmer Clarus 600 T mass spectrometer (software version 5.4.2.1617, Perkin Elmer, Shelton, CT, USA). The injector and oven temperatures were as above; the transfer line temperature was 280 °C; ion source temperature was 220 °C; helium was the carrier gas (30 cm/s), with a split ratio of 1:50; ionization energy, 70 eV; scan range, 40–300 u; scan time, 1 s. The identification of the components was assigned by comparison of their retention indices, relative to C9-C21 n-alkane and GC-MS spectra from a lab-made library created with reference essential oil samples, laboratory-synthesized components, laboratory isolated compounds and commercially available standards.

2.3 Fungal Spore Suspension and Inoculum Preparation

Five cultures were previously isolated from strawberry tree fruits in the Microbiology Laboratory of IPCB-ESA and molecularly identified in the *Micoteca da Universidade do Minho* (MUM), a Portuguese fungal culture collection. The fungi were identified as *Aspergillus carbonarius* (ESA.M.51), *Rhizopus stolonifer* (ESA.M.44), *Penicillium brevicompactum* (ESA.M.60), *Aureobasidium pullulans* (ESA.M.64) and *Saccothecium rubi* (ESA.M.89). The generated sequences of the isolates were deposited on the GenBank (https://www.ncbi.nlm.nih.gov/genbank/), under the accession numbers MT374850, MT374851, MT375026, MT375027, MT375028 and MT375029. Beyond the isolated cultures, a reference culture of *Penicillium roquefortii* PRB6 HYP5D (Choozit™, Danisco, Copenhagen, Denmark) was also tested. *P. roqueforti* was selected due to its probable presence in fruits and its ability to grow at lower temperatures, belonging to the important group of cold storage fungi. Fungi cultures grew in PDA (Potato Dextrose Agar, HiMedia Chemicals, Maharashtra, India) medium at 25 ± 2 ºC during 48 h for yeasts and 3-5 days for moulds (until the spore formation). For yeasts cultures, 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, Marcy-l'Étoile, France), representing approximately 3.0×10^7 CFU/mL (colony forming units per mL). Following Alizadeh-Salteh et al. [21] method, spore suspensions of mould cultures were filtered through six layers of gauze, to remove the mycelium. The absorbance of a filtered solution was adjusted between 0.150-0.170 in a Genesys 10 UV-VIS spectrophotometer at 570 nm (Thermo Fisher Scientific, Waltham, USA) using 0.85% NaCl saline solution as blank. The moulds and yeasts final cell suspension was used to inoculate the microplates (Deltalab, Barcelona, Spain) wells. The inoculum was quantified for all cultures by inoculating appropriate dilutions of the moulds and yeasts suspensions in PDA plates, following by an enumeration of colonies and the expression of the final result (CFU/mL).

2.4 Minimum Inhibitory Concentration and Minimum Fungicidal Concentration

The minimum inhibitory concentration (MIC) of the essential oils was performed according to the Clinical and Laboratory Standards Institute (CLSI) reference protocols M27-A3 [22] and M38-A2 [23], for yeasts and filamentous fungi, respectively, with slight modifications. Essential oils (EO) were diluted in PDB (Potato Dextrose Broth, VWR Chemicals Prolabo) medium supplemented with 0.8% (v/v) of

Tween 80 (VWR Chemicals Prolabo). For each EO, nine concentrations ranging from 0.04-9.33 µL/mL were prepared, diluting it in the medium. Each microplate well was filled with a total volume of 150 µL. Testing wells were filled with 140 µL of EO/medium and 10 µL of inoculum (three replicates were made for each EO, concentration and fungi culture). Negative control wells were filled with 140 µL of EO/medium and 10 µL of the NaCl sterile saline solution. Positive control wells were filled with 140 µL of medium solution and 10 µL of inoculum. The medium control was also tested. Microplates were incubated under the optimum conditions for fungi cultures at 25 ± 2 ºC, under humid atmosphere.

After incubation, all microplate wells were inoculated in PDA plates for determination of MFC using 10 µL loops. The MFC value matched the lowest EO concentration in which it was not observed any growth after incubation. Afterwards, 30 µL of resazurin (VWR, Chemicals Prolabo) was added to each microplate well and then, the microplates were incubated until the color change of the positive control. The results were visually assessed comparing the color of the inoculated wells with the color of the control wells. The MIC value matched the lowest EO concentration in which the color was like to the negative control.

2.5 Statistical Analysis

For calculating the average values and standard deviation of the obtained data, MS Excel (Microsoft Office Professional Plus 2010, Microsoft, USA) was used.

3. Results and Discussion

3.1 Chemical Composition of the Essential Oils

Constituents of both essential oils are listed in Table 1, according to their elution order in a dimethylpolysiloxane column. A total of 24 compounds were identified. We report 3 unidentified compounds (NI B, C and D) because they are always present in samples of *L. stoechas* subsp. *luisieri* essential oils from other works in the lab (data unpublished). The essential oils of both samples were characterized by high amounts of oxygenated monoterpenes (46-64%), followed by oxygenated sesquiterpenes (3.50-4.90%). Some irregular oxygenated monoterpenes characterized by a cyclopentenic nucleus, also called as necrodane derivatives were found in both phenological stages. Necrodane derivatives were reported for the first time by Garcia-Vallejo [25] in essential oils of *L. stoechas* subsp. *luisieri* from southern Spain. These compounds are not found in any other plant species, becoming this specie with an interesting value [13].

In our study, compounds as 3,5-dimethylene-1,4,4-trimethylcyclopentene, 3,4,4-trimethyl-2-cyclohexenone, *trans*-α-necrodol, *cis*-α-necrodol,5-methylene-2,3,4,4-tetramethylcyclop -enten-2-enone, *trans*-α-necrodyl acetate and *cis*-α-necrodyl acetate were identified and quantified in GC-MS. Significant quantitative differences were found between both phenological stages. The major components in essential oil of *L. stoechas* subsp. *luisieri* from the dormancy stage were *trans*-α-necrodyl acetate (12.58%), followed by 5-methylene-2,3,4,4-tetramethylcyclopenten-2-enone (10.97%), fenchone (5.97%) and *trans*-α-necrodol (5.22%). While the main compounds in the essential oil of plants from flowering stage were *trans*-α-necrodyl acetate (26.90%), followed by *trans*-α-necrodol (13.02%), lavandulyl acetate (6.53%) and both 3,5-dimethylene- -1,4,4-trimethylcyclopentene and linalool (5.82% each one). Similar results were reported by Pombal et al. [26], who compared the chemical profile of *L. stoechas* subsp. *luisieri* essential oils from different phenological stages. The highest concentration of *trans*-α-necrodyl acetate was also found in the full flowering (20.3%), followed by early flowering (16.6%), revealing the lowest concentration in the dormancy stage. Although significant quantitative

differences may occur in the amounts of the main compounds, necrodane derivatives are always present. Comparing the essential oils of the two phenological stages, the total of necrodane derivative compounds represents about 55% and 39%, in the flowering and dormancy stages, respectively. The greatest amount of necrodane derivatives found in the flowering stage may mean that the production of these compounds is stimulated or increased with the pollination season. Due to high polymorphism and the hybridization ability of this species in relation to others *Lavandula* species, the presence of necrodanes derivatives can be considered a chemotaxonomic marker of this species [27].

Table 1 Identification and relative amounts of compounds of both *L. stoechas* subsp. *luisieri* essential oils.

Compounds	RI[a]	RI[b]	% Peak area	
			Dormancy	Flowering
3,5-Dimethylene-1,4,4-trimethylcyclopentene	930	924*	3.81 ± 0.13	5.82 ± 0.64
α-Pinene	930	931	2.29 ± 0.07	1.56 ± 1.56
Camphene	938	938	0.33 ± 0.32	t
β-Pinene	963	962	1.12 ± 0.02	t
p-Cymene	1003	1004	0.29 ± 0.29	t
1,8-Cineole	1005	1010	4.84 ± 0.11	1.30 ± 0.11
3,4,4-Trimethyl-2-cyclohexenone	1038	*1055*	1.45 ± 0.06	0.93 ± 0.05
cis-Linalool oxide	1045	*1055*	1.56 ± 0.02	0.32 ± 0.32
NI B *L. luisieri*	1050		t	0.70 ± 0.03
Fenchone	1050	1067	5.97 ± 0.11	2.79 ± 0.13
trans-Linalool oxide	1059	*1070*	0.57 ± 0.57	t
Linalool	1074	1089	t	5.82 ± 0.20
Camphor	1102	*1118*	3.19 ± 0.02	1.35 ± 0.03
trans-Pinocarveol	1106	*1121*	1.02 ± 0.01	t
trans-*α*-Necrodol	1114	*1130*	5.22 ± 0.07	13.02 ± 0.24
NI C *L. luisieri*	1137		0.60 ± 0.00	0.31 ± 0.14
cis-*α*-Necrodol	1147		3.48 ± 0.03	3.89 ± 0.10
5-Methylene-2,3,4,4-tetramethylcyclopenten-2-enone	1152	1160*	10.97 ± 0.47	2.93 ± 0.98
Verbenone	1164	*1177*	0.99 ± 0.04	t
Bornyl acetate	1265	1270	1.60 ± 0.03	0.48 ± 0.48
trans-*α*-Necrodyl acetate	1265	*1265*	12.58 ± 0.26	26.90 ± 1.05
NI D *L. luisieri*	1267		4.15 ± 0.07	2.78 ± 0.03
Lavandulyl acetate	1278	*1269*	4.05 ± 0.08	6.53 ± 0.06
cis-*α*-Necrodyl acetate	1285	*1281*	1.90 ± 0.04	1.60 ± 0.15
δ-Cadinene	1505	1508	t	0.82 ± 0.04
Viridiflorol	1569	1583	2.98 ± 0.14	2.13 ± 0.19
Ledol	1580	1586	1.91 ± 0.09	1.34 ± 0.12
Identification (%)			72.05	79.48
Grouped components (%)				
Monoterpene hydrocarbons			4.00	1.60
Oxygenated monoterpenes			46.90	64.00
Sesquiterpene hydrocarbons			0.00	0.82
Oxygenated sesquiterpenes			4.90	3.50
Others			16.20	9.70

Results are expressed as mean values ± standard deviation (SD). Compounds listed in order of elution from the DB‐1 column. RI[a]: Retention index calculated relative to C9‐C21 *n*‐alkanes on the DB‐1 column; RI[b] Literature retention indexe on similar phase column (100% Dimethylpolysiloxane); Regular font RI[b] values from reference [24], italic values from reference [4] and * values from reference [7]. t: trace (< 0.05%); NI B, C, D: unidentified compounds.

Table 2 Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of two *L. stoechas* subsp. *luisieri* essential oils against six fungi cultures.

Fungi cultures	Phenological stage	Essential oil concentration (μL/mL)								
		9.33	4.67	2.33	1.17	0.58	0.29	0.15	0.07	0.04
Aspergillus carbonarius ESA.M.51	D	MFC					MIC			
	F	MFC					MIC			
Aureobasidium pullulans ESA.M.64	D			MFC			MIC			
	F			MFC			MIC			
Penicillium brevicompactum ESA.M.60	D			MFC			MIC			
	F			MFC			MIC			
Penicillium roqueforti PRB6 HYP5D	D				MFC				MIC	
	F					MFC			MIC	
Rhizopus stolonifer ESA.M.44	D		MFC						MIC	
	F	MFC							MIC	
Saccothecium rubi ESA.M.89	D				MFC		MIC			
	F				MFC		MIC			

D: Dormancy stage, F: Flowering stage.

These compounds were also reported in *L. stoechas* subsp. *luisieri* essential oils from Spain [7, 27] and from central and southern Portugal [3, 4, 12]. For most of reports, namely for plants from Portugal, the main chemical compound present in essential oil of *L. stoechas* subsp. *luisieri* is *trans*-α-necrodyl acetate followed by 1,8-cineole, fenchone or camphor in minor concentrations. Another interesting aspect is related to the chemical variability that it seems to be very common among populations. Zuzarte et al. [4] found considerable differences in the chemical composition of essential oils between plants from central and southern Portugal. In the first place, the essential oil was characterized by *trans*-α-necrodyl acetate (17%), *trans*-α-necrodol (7%) and 1,8-cineol (6%), while the main compounds of essential oils from of southern plants were 1,8-cineole (34%) and fenchone (18%), where the concentration of necrodane compounds was very low. In recent years the biological potential of necrodane-essential oil of *L. stoechas* subsp. *luisieri* has been demonstrated, namely the antimicrobial activity [10, 11, 13].

3.2 Antifungal Activities of Essential Oils

According to results presented in Table 2, the MIC obtained for both yeasts was the same, 0.29 μL/mL, however the MFC was superior for *A. pullulans* (2.33 μL/mL) while for *S. rubi* was 1.17 μL/mL, also for both essential oils (D and F). Regarding moulds, the most susceptible microorganisms to the inhibition of the essential oils were *R. stolonifer* and *P. roqueforti,* for which the lowest MIC values (0.07 μL/mL) were observed. However, the MFC values for *R. stolonifer*, for both essential oils, were found in two higher concentrations of essential oils tested (4.67 and 9.33 μL/mL, respectively for dormancy and flowering stages). Muszewska et al. [28] also reported the greater resistance to fungicidal agents of Zygomycota filo, compared with Ascomycota ones. Comparing the antifungal activity of the two tested species of *Penicillium*, *P. roqueforti* was more susceptible than *P. brevicompactum*. The great resistance of *P. brevicompactum* to the action of essential oils and plant extracts in comparison with several species of *Penicillium* was also verified by other authors [29, 30]. The most resistant species tested was *A. carbonarius* with MIC and MFC values of 0.29 and 9.33 μL/mL. These results were also corroborated in other studies, where the culture more resistant to the action of essential oils was *Aspergillus* spp. [4, 17].

In relation to the antifungal activity of the *L. stoechas* subsp. *luisieri* essential oil, the information available is reduced. Zuzarte et al. [4] evaluated the antifungal activity of two essential oils of *L. stoechas* subsp. *luisieri* (one them rich in necrodane compounds) against reference strains of *A. flavus*, *A. fumigatus* and *A. niger*, with the MIC values ranged 0.32-10 μL/mL, while MFC (minimum fungicidal concentration) values ranged 10-20 μL/mL. A relevant aspect was that the essential oil rich in necrodane compounds was more active against *Aspergillus* spp. Baptista et al. [31] also reported similar MIC values, with 15.5 μg/mL of *L. stoechas* subsp. *luisieri* essential oil needed to inhibit *A. niger*. In two more studies a strong antifungal activity was observed in *L. stoechas* subsp. *luisieri* essential oil against *Candida* spp. and *Trichophyton* spp. [7, 8]. According to the results, the essential oils studied may prove to be an excellent option for the development of new antifungal agents. As they are a mixture of several compounds, they do not act on a single target in fungal cells, and no resistance or adaptation to essential oils has been reported [32].

4. Conclusions

In both essential oils was confirmed the presence of necrodane derivates compounds at high percentages. Our results showed that the essential oils of dormancy and flowering phenological stages of *L. stoechas* subsp. *luisieri* have a strong antifungal activity (0.07-9.33 μL/mL). According with results, *L. stoechas* subsp. *luisieri* essential oils are promising natural products to be used as antifungal agents against spoilage strains. To explore its potential for application in the food industry, cytotoxicity studies should be considered to assess the practical effect.

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Conflict of Interest

There is no conflict of interest declared by the authors or by writing.

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