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Characterization and Antimicrobial Activity of Alkaloid Extracts from Seeds of Different Genotypes of *Lupinus* spp.

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Abstract: Alkaloid profiles of 22 lupin genotypes belonging to three different cultivated species, *Lupinus albus* L., *Lupinus luteus* L., and *Lupinus angustifolius* L., collected from different Italian regions and grown in Sicily, were studied by gas chromatography mass spectrometry (GC-MS) to determine alkaloid composition. More than 30 alkaloids were identified. The lowest alkaloid concentration was observed in the *L. albus* Luxor, Aster, and Rosetta cultivars, and in all the varieties of *L. luteus* and *L. angustifolius*. The highest content was observed in all the landraces of *L. albus*. Surprisingly, the white lupin Lublanc variety and the commercial seeds of cv Multitalia had a high alkaloid content. The tested species and the different genotypes exhibited different alkaloid profiles: lupanine, 13 α -hydroxylupanine, and albine were the main alkaloids in the analyzed *L. albus* seeds; angustifoline and 13 α -tigloyloxylupanine were well-represented in *L. albus* landraces; sparteine and lupanine were typical of *L. luteus*; and lupanine, 13 α -hydroxylupanine, and angustifoline were the main alkaloids in *L. angustifolius* seeds. The samples with the highest amounts of total alkaloids proved to be interesting from a pharmaceutical viewpoint. The alkaloid extracts showed significant activity on *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* clinical isolates.

Keywords: alkaloids; antimicrobial activity; germplasm; *Klebsiella*; landraces; lupanine; *Pseudomonas*; varieties

1. Introduction

The *Lupinus* genus belongs to the Fabaceae family, subfamily Papilionoideae, and includes about 170 species [1], but only four species are cultivated, three of which originate from the Mediterranean area: *Lupinus albus* L. (white lupin, chromosomally, $2n = 50$), *L. angustifolius* L. (narrow-leafed lupin, $2n = 40$), and *L. luteus* L. (yellow lupin, $2n = 52$). One, *L. mutabilis* Sweet, commonly known as pearl lupin or Tarwi ($2n = 48$) [2], originates from the Andean mountain. Narrow-leafed lupins are important for both animal feed and human foodstuff for the production of lupin flour and isolate proteins, whereas yellow lupin is only used in the livestock chain [3,4].

Lupinus species are mainly grown in Australia, Chile, and Eastern and Central Europe, but it is almost absent in the Mediterranean basin because it requires acid or sub-acid soils [5].

All *Lupinus* species produce quinolizidine and bipiperidine alkaloids, but the former are the main lupin alkaloids [2,6–10]. Lupin alkaloids are secondary metabolites that the plant stores in its

organs, including seeds, likely as chemical agents against insects, microorganisms, and herbivores [11]. Quinolizidine alkaloids displayed oral toxicity due to neurological effects. Therefore, minimizing the risk of high levels of alkaloid uptake is important. For this reason, the health authorities of some countries, such as Great Britain, France, Australia, and New Zealand, have fixed the maximum alkaloid content in lupin food and flour marketing at 200 mg/kg [12,13].

The pharmacological benefits of alkaloids have been reported, with activity on the circulatory system, metabolism against obesity, cardiac dysfunction, and skin disease. In some cases, they act as hypoglycemic and hypolipidemic agents, as well as antibiotic, antiviral, anti-hepatitis, anti-inflammatory, anti-oxidant, anti-cancer, and neuroprotective agents. Moreover, alkaloids have a sedative effect on the central nervous system [14–17]. Therefore, a complete knowledge of lupin alkaloid patterns is important, not only because of their potential toxicity but also for their potential pharmacological benefits.

The genetic breeding program, conducted for nearly a century mainly by Sengbusch in Germany, Gladstones in Australia, and Baer in Chile, has led to the selection of sweet mutants with low or no alkaloid content (0.01–0.05% versus 1–8% of landraces) [4,18]. However, bitter lupins are still used in some parts of the world where the new sweet varieties are not well suited for the climate [17]. A renewed interest in lupin has grown in relation to its interesting nutritional properties and potential health benefits [19–21]. Lupin seeds are a good source for animal feeding and human nutrition due to their high protein content of 40–48% [18,22]. Lupin-based foods, such as ice cream, baking products, snacks, and meat-free products, including steaks, chops, and cutlets, as well as food supplements such as flour added to bread, have been developed in which the *L. albus* is one of the main ingredients [23]. In addition, the increase in demand for Genetically Modified Organisms-free materials in livestock chains has led to the reconsideration of the national grain protein-species, such as lupin, as an alternative to soybean as a source of protein [4].

As the application of antibiotics is limited in some contexts, the pharmaceutical industry and researchers have evaluated alternative antibacterial and antifungal agents of natural origin for use against pathogens [24–26]. The quinolizidine alkaloids of lupin are included in these natural agents. In addition, the extensive use of antibiotics has rapidly increased bacterial antibiotic resistance.

The aim of this work was to analyze 22 lupin seed samples corresponding to different genotypes of three *Lupinus* species to assess their total alkaloid content and the differences in the alkaloid pattern of the analyzed genotypes. This characterization was aimed at identifying and selecting the landraces and, eventually, the cultivars with the highest alkaloid content to be used as a source of bioactive compounds for pharmaceutical applications. This work also aimed to test the antibacterial and antifungal activities of *Lupinus* spp. alkaloid extracts against type strains and clinical isolate strains of Gram positive and negative bacteria and yeasts, considering the need for new antimicrobial natural agents against foodborne and clinical pathogens.

2. Materials and Methods

2.1. Plant Material and Sampling

The seeds of 10 sweet lupin cultivars: 6 white lupin (*L. albus*: Aster, Lublanc, Lutteur, Luxor, Rosetta, and Multitalia 1), 3 yellow lupin (*L. luteus*: Dukat, Mister, and Taper), and 1 narrow-leaved lupin (*L. angustifolius*: Sonet), together with 9 Italian landraces of *L. albus*, were collected from different Italian regions and tested in Sicily, Italy. Note that Multitalia 1 is a historically bitter variety less selected by geneticists [27]. The samples were also compared with three other Italian Multitalia seed samples. Two of these were cultivated in two other areas in Southern Italy: Multitalia 2 and Multitalia 3 from Battipaglia, Campania and Acireale, Sicily, respectively; and one commercial certified seed, Multitalia 4, reproduced in Northern Italy. The names of the Italian landraces are the same as the region from where they were harvested, with the exception of Modica and Scicli, which were from Sicily. The trial was conducted in 2012–2013 on volcanic soil in East Sicily, Giarre, Italy. All seed samples were sowed in duplicated plots of 5 m² (2.5 × 2 m). Manual seeding was completed on

November 24, 2012. Fertilization was applied during sowing with 30 kg/ha of ammoniacal nitrogen (ammonium sulfate) and 60 kg/ha of mineral perphosphate (P_2O_5). Chemical weed control was applied at 200 mL/hL of Pendimetalin (Stomp 330, Basf, Ludwigshafen, Germany) pre-emergence and mechanical post-emergence. Flood irrigation was required from March to June. Aphicide treatment with 50 mL/hL of Imidacloprid (Confidor, Bayer CropScience, Milan, Italy) was applied in late March. The crop was harvested on 20 June 2013. Seeds of all studied genotypes were deposited within the germplasm collection of the Research Centre for Cereal and Industrial Crops (CREA), Laboratory of Acireale (Italy).

2.2. Alkaloid Extraction

Alkaloid extraction was performed as described by Erdemoglu et al. [18] with modifications. Lupin seeds were finely ground and 1 g of each sample was suspended in 10 mL of 0.5 N HCl. After stirring for 30 min at room temperature, the homogenate was centrifuged for 10 min at 4 °C and 10,000 rpm. For quantitative analysis, the pellet was suspended in 0.5 N HCl and centrifuged again. Both supernatants were then pooled and adjusted to pH 12 with 5 N NaOH. Alkaloids were extracted by solid phase extraction using Extrelut columns (NT20 Merck, Darmstadt, Germany). Total alkaloids were eluted after 20 min with CH_2Cl_2 (3×20 mL) and the solvent evaporated until dry under vacuum at 40 °C. The residue was diluted in 1.5 mL dichloromethane and analyzed by gas chromatography-mass spectrometry (GC-MS) apparatus. Each sample was independently extracted and analyzed at least three times.

2.3. GC-MS Analysis

The analyses were performed on an Agilent 6890 gas chromatograph equipped with an Agilent 5973 Network quadrupole mass selective spectrometer and an Agilent 7683B Series autosampler. The separation was achieved using a VF-5ms 5% phenyl, 95% methylpolysiloxane capillary column ($30\text{ m} \times 0.25\text{ mm}$, 0.25 μm film thickness, Varian). The GC-MS analysis was performed under the following conditions: the ion source temperature was 220 °C in EI mode at 70 eV, injector temperature was 250 °C, interface was 270 °C, carrier gas helium at 1 mL/min, split ratio 1/10, injection volume 1 μL , and mass range of 50 to 450 m/z. GC oven temperature was kept at 70 °C for 1 min, and programmed to 150 °C, heating at a rate of 40 °C/min, then to 300 °C at a rate of 6 °C/min, and kept constant for 1 min. Ionization was kept off during the first 3 min to avoid solvent overloading. The analyses were performed in full-scan mode. The retention index (RI) of the alkaloids was determined according to the Kovats method [13] by injecting a mixture of linear C8–C20 (cod. 04070, Sigma-Aldrich, Milan, Italy) and C21–C40 (cod. 04071, Sigma-Aldrich, Milan, Italy) alkanes. The compounds were identified by comparing their mass spectra with data in the NIST 05 MS Library Database [28] and the literature [11,29,30].

2.4. Alkaloid Quantification

The alkaloid quantification was performed using the external standard method, using (–)-sparteine (Sigma-Aldrich, St. Louis, MO, USA) as the standard. The calibration curve was prepared by injecting six solutions of sparteine at different concentrations in the range of 10 to 1000 mg/L and then a known amount (100 mg/L) of caffeine (Sigma-Aldrich, St. Louis, MO, USA) was added to each solution to check the response of the instrument. Since tetracyclic lupanine and sparteine are the most representative alkaloids in the seeds of lupins [6], and standards of most compounds are not commercially available, in the present work, the reported quantitative results are expressed as sparteine. The precision of the method was assessed by analyzing sparteine solutions within the same day and on different days, obtaining relative standard deviations (RSD %) of 2–3% and 4–5%, respectively. The limit of detection (LOD) of sparteine in the standard solutions was 0.1 mg/L.

2.5. Antimicrobial Activity

2.5.1. Microorganisms

The strains used in this study were type strains: *Candida albicans* DSM 1386, *Saccharomyces cerevisiae* DSM 1333, *Pseudomonas aeruginosa* DSM 1117, *Escherichia coli* DSM 1103, and *Trichophyton interdigitale* DSM 4870. Clinical isolates used were: *Candida krusei* (wound and vaginal tampon), *Staphylococcus aureus* (skin), *P. aeruginosa* (skin and ulcer), *Klebsiella pneumoniae* (skin and inguinal skin site), and *Proteus mirabilis* (skin). Clinical isolates were identified with vitek 2 (bioMerieux, Florence, Italy) in the Laboratory of Microbiology of University Hospital Policlinico Vittorio Emanuele (Catania, Italy).

2.5.2. Antibacterial and Antifungal Tests

The minimum inhibitory concentrations (MICs) of the extracts were determined following the Broth Microdilution Techniques according to the National Committee for Clinical Laboratory Standards [31,32]. Mueller-Hinton Broth (Merck, Darmstadt, Germany) and Sabouraud Broth (Oxford, UK) were used for growing and diluting the bacteria and fungi, respectively. Alkaloid extracts, obtained as per Section 2.2 from the two samples showing the highest alkaloid amount (Multitalia 4 and Calabria 2, as reported in Figure 1), after the evaporation to dryness under vacuum at 40 °C, were dissolved in dimethylsulfoxide (DMSO). Then the extracts, ranging from 3.75 to 1,000 µg/mL, were prepared for the test. Before the broth microdilution procedure, microorganism inocula were standardized to a turbidity of 0.5 McFarland standard (10⁶ yeasts or 10⁸ bacteria cells/mL). Final concentrations were approximately 10³ cells/mL for yeasts and 10⁴ cells/mL for bacteria. The microorganisms and pure media were placed in the wells of a microtiter plate together with the different concentration extracts. Proper blanks were tested simultaneously. Microtiter plates were incubated under atmospheric conditions at 37 °C for 24 h for bacteria, and at 25 °C for 48 h for the yeasts. Spectrophotometric lectures were performed at 600 nm. Each extract was tested in triplicate.

2.6. Statistical Analysis

SPSS software (version 21.0, IBM Statistics Corp., New York, NY, USA) was used for data processing. One-way analysis of variance (ANOVA) was used to test the effects of the different genotypes on the measured factors (total and each single alkaloid). Duncan's multiple range test was used to compare means when a significant variation was highlighted by the analysis of variance. The total amount of alkaloids and all single compounds were also analyzed using principal component analysis (PCA).

3. Results and Discussion

The mass spectral data of seed extracts from the different lupin samples, shown in Table 1, revealed and confirmed the presence of quinolizidine alkaloids previously reported by other authors [12,29,30,33].

Table 1. Mass spectral data of alkaloids and their distribution along the three *Lupinus* species. The alkaloids in bold letters are those shared by all the analyzed species. RI, retention index.

Peak n.	Alkaloid	RI	M+	Characteristic Ions	<i>Lupinus albus</i>	<i>Lupinus luteus</i>	<i>Lupinus angustifolius</i>
1	lupinine	1464	169	83-152-138		X	
2	unknown_a	1624	208	121-175		X	
3	gramine	1679	174	130-103-77		X	
4	genisteine (α -isosparteine)	1759	234	98-137		X	
5	sparteine	1827	234	137-98	X	X	
6	unknown_b	1879	232	134-232-98	X		
7	β -isosparteine	1883	234	137-98	X	X	

Table 1. Cont.

Peak n.	Alkaloid	RI	M+	Characteristic Ions	<i>Lupinus albus</i>	<i>Lupinus luteus</i>	<i>Lupinus angustifolius</i>
8	11,12-dehydrosparteine	1893	232	134	X	X	X
9	ammodendrine	1932	208	165-110	X	X	X
10	unknown_c	1951	208	166-136-110		X	
11	albine	1984	232	191-110	X	X	X
12	unknown_d	2030	232	191-110	X		
13	isoangustifoline	2127	234	193-112	X		X
14	tetrahydrhorombifoline	2135	248	207	X		X
15	angustifoline	2178	234	193-112	X		X
16	α -isolupanine	2206	248	136-248	X		X
17	5,6-dehydrolupanine	2225	246	98	X		X
18	unknown_e	2235	246	150-136-110	X		
19	lupanine	2273	248	136-149	X	X	X
20	11,12-dehydrolupanine	2296	246	134-246	X		
21	unknown_f	2308	248	110-191-149	X		
22	11,12-seco-12,13-didehydromultiflorine	2330	246	58-205	X		
23	unknown_g	2349	246	134	X		
24	3 β -hydroxylupanine	2361	264	136-44	X		
25	unknown_h	2429	262	150-164-96	X		
26	multiflorine	2441	246	134-246	X	X	X
27	unknown_i	2460	264	134-152-246	X		
28	17-oxolupanine	2482	262	150-110-262	X		
29	N-formylangustifoline	2502	262	193-112-221	X		
30	13 α -hydroxylupanine	2534	264	152-246	X		X
31	unknown_l	2570	264	152-246-134	X		
32	unknown_m	2581	262	245-150	X		
33	unknown_n	2609	260	260-148-112	X		
34	N-formylalbine	2688	260	219-96	X		
35	unknown_o	2712	262	149-150-148	X		
36	13 α -hydroxymultiflorine	2727	262	150	X		X
37	13 α -isovaleroyloxylupanine	2779	348	246-134-112	X		
38	13 α -angeloyloxylupanine	2858	346	246	X		
39	13 α -tigloyloxylupanine	2879	346	246	X		X
40	unknown_p	2912	348	246-134-231	X		
41	unknown_q	2930	348	246-134-112	X		
42	3 β -tigloyloxylupanine	2944	346	134	X		
43	unknown_r	3073	344	132-244-149	X		
44	feruloyllupanine	3097	345	152-151		X	
45	13 α -tigloyloxymultiflorine	3112	344	132	X		
46	unknown_s	3262	394	246-134-112	X		

The estimated total amount of alkaloids from the seeds of the 22 genotypes is shown in Figure 1. The post-hoc test revealed 11 homogeneous groups. Among the 10 selected cultivars belonging to the three species, Luxor, Aster, Dukat, Rosetta, Taper, Mister, and Sonet exhibited low and similar total alkaloid content, whereas Multitalia 1 and Lutteur contained higher amounts of alkaloids.

The commercial certified seed, Multitalia 4, was directly produced from the certified seed of the first generation, which was established and officially controlled with the purpose of producing certified second generation seed, marked with a red card, as provided by the Italian seed legislation [34]. Despite this fact, Multitalia 4 was one of the most alkaloid-rich samples along with Calabria 2 landrace, with 18,979 and 19,340 mg/kg, respectively. This seed was probably selected for genetic purity without a genetic selection for alkaloid content. The results obtained for Multitalia 2 and 3, and especially for Multitalia 4 and Lublanc, which were formerly sweet cultivars, indicated a genetic contamination occurred during the reproduction of those varieties. In fact, *L. albus* species demonstrated prevalent cross pollination, and for this reason, the alkaloid content increased over the years if the seeds with low alkaloid content were not selected and reproduced. However, Multitalia 1 showed levels of alkaloids in accordance with those reported by Calabrò et al. [27] and Gresta et al. [4] for cv

Multitalia. The amount of quinolizidine alkaloids can differ between years due to differing weather [35] or agronomic conditions [36]. Conversely, this problem was not observed in *L. angustifolius* and *L. luteus* since they are prevalently autogamous species. The most alkaloid-rich landraces may be of interest because they may be used for extracting pure alkaloids, as analytical standards of the majority of quinolizidine alkaloids are not commercially available [12]. Moreover, as mentioned above, the alkaloids possess several favorable pharmacological properties [37]. Therefore, the landraces could be practically and conveniently used in further studies of their properties and potential future applications in medicine and phytotherapy.

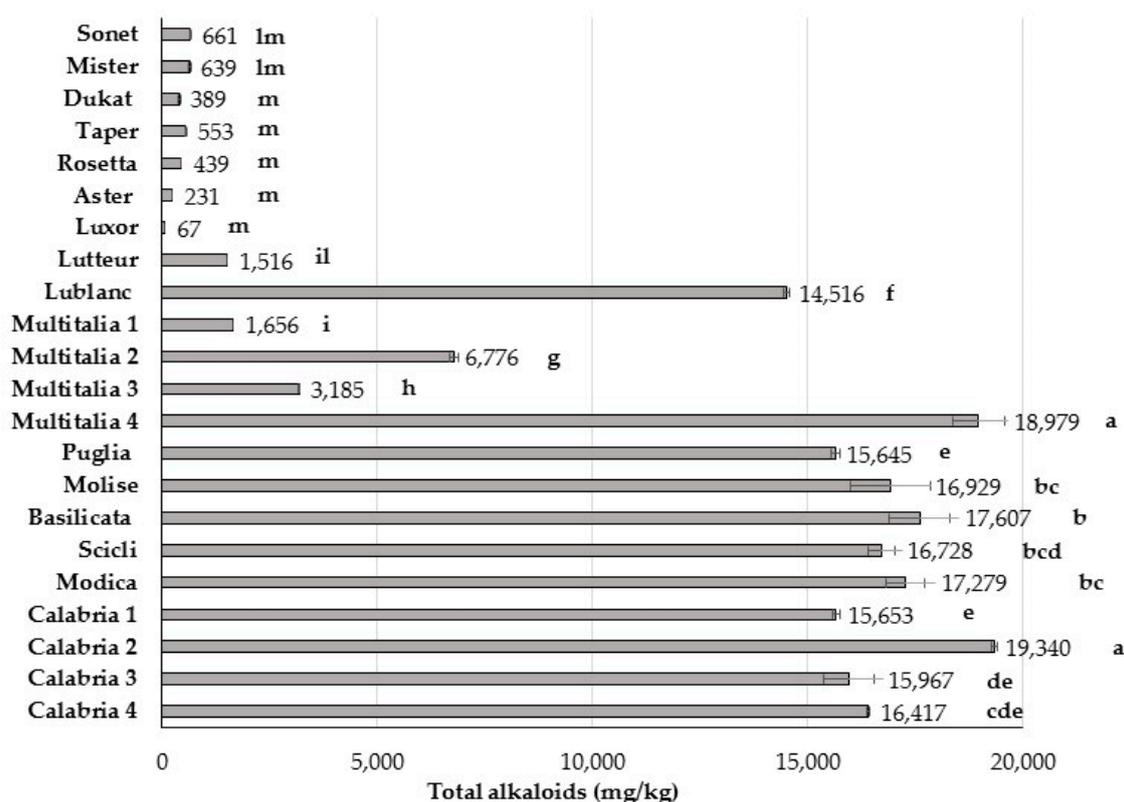


Figure 1. Total alkaloids in seed samples of the 22 genotypes belonging to the three analyzed lupin species. Mean values followed by different letters are significantly different (Duncan's post-hoc test).

In Table 2, the most representative alkaloids of the *L. albus* seed samples are shown. The seven compounds were also the only alkaloids shared by all *L. albus* samples, and the amounts of these compounds were significantly different among the genotypes ($p < 0.01$). Lupanine, albine, and 13α -hydroxylupanine were the main alkaloids were found in the highest amounts, but angustifoline and 13α -tigloyloxylupanine were also well-represented in *L. albus* landraces. Moreover, two landraces, Molise and Calabria 2, showed the presence of sparteine (data not shown), the main alkaloid found in *L. luteus* species [12]. Among the selected varieties, Lublanc had the largest number of alkaloids (36/46) and a different alkaloid pattern, possessing more than six compounds unshared with the other cultivars: β -isosparteine, 11,12-dehydrosparteine, 11,12-dehydrolupanine, 17-oxolupanine, *N*-formylangustifoline, *N*-formylalbine, and some unknown alkaloids (data not shown).

Table 2. Alkaloids shared by all the analyzed *Lupinus albus* genotypes.

Genotype	Albine	Tetrahydro-Rhombifoline	Angustifoline	Lupanine	13 α -Hydroxy-Lupanine	13 α -Tigloyloxy-Lupanine	3 β -Tigloyloxy-Lupanine
Aster	30.2 \pm 1.3 ⁿ	12.8 \pm 0.1 ^g	12.1 \pm 0.5 ^h	60.8 \pm 0.3 ^m	17.4 \pm 1.2 ^g	37.4 \pm 2.9 ^l	6.2 \pm 0.2 ^{c,d}
Lublanc	1012.4 \pm 38.5 ^g	24.1 \pm 0.8 ^{c,d}	268.4 \pm 6.1 ^e	11,218.6 \pm 82.5 ^{e,f}	491.7 \pm 3.8 ^{c,d}	102.0 \pm 1.7 ^{f,g}	16.6 \pm 1.1 ^a
Luxor	11.5 \pm 0.2 ⁿ	1.0 \pm 0.4 ⁿ	3.6 \pm 0.3 ^h	30.4 \pm 1.2 ^m	3.1 \pm 0.3 ^g	6.7 \pm 0.5 ^m	2.4 \pm 0.1 ^e
Lutteur	162.0 \pm 5.7 ^m	8.8 \pm 0.6 ⁱ	65.0 \pm 1.2 ^g	863.2 \pm 6.1 ^{i,l}	157.4 \pm 1.8 ^f	44.4 \pm 2.2 ^{i,l}	7.5 \pm 1.4 ^c
Rosetta	29.3 \pm 0.2 ⁿ	1.1 \pm 0.2 ^{m,n}	6.4 \pm 0.2 ^h	361.8 \pm 5.4 ^{l,m}	4.7 \pm 0.1 ^g	6.4 \pm 0.1 ^m	1.5 \pm 0.1 ^e
Calabria 1	1199.7 \pm 10.5 ^f	23.1 \pm 1.0 ^d	265.2 \pm 8.4 ^e	12,363.6 \pm 138.0 ^{c,d}	510.3 \pm 15.5 ^c	75.4 \pm 5.8 ^h	5.2 \pm 1.4 ^d
Calabria 2	1436.2 \pm 53.5 ^d	19.4 \pm 0.3 ^{e,f}	466.8 \pm 9.3 ^b	14,480.3 \pm 10.0 ^a	687.2 \pm 4.5 ^b	227.8 \pm 1.1 ^d	12.1 \pm 1.4 ^b
Calabria 3	1767.4 \pm 17.6 ^b	35.9 \pm 2.5 ^b	431.1 \pm 19.0 ^b	11,165.4 \pm 157.3 ^{e,f}	893.4 \pm 66.7 ^a	98.7 \pm 2.3 ^g	8.5 \pm 1.0 ^c
Calabria 4	1232.3 \pm 4.2 ^f	44.1 \pm 0.1 ^a	444.2 \pm 1.8 ^b	12,935.3 \pm 16.2 ^{b,c}	413.9 \pm 2.2 ^d	87.6 \pm 0.3 ^{g,h}	11.1 \pm 0.1 ^b
Modica	2596.2 \pm 2.2 ^a	18.8 \pm 0.8 ^d	518.0 \pm 15.0 ^a	11,358.3 \pm 140.4 ^{e,f}	747.4 \pm 25.0 ^b	124.5 \pm 7.6 ^e	12.4 \pm 0.6 ^b
Scicli	1367.6 \pm 60.6 ^e	44.1 \pm 1.6 ^a	382.2 \pm 13.6 ^c	11,916.5 \pm 130.1 ^{d,e}	506.2 \pm 41.9 ^c	308.4 \pm 1.6 ^c	8.5 \pm 0.1 ^c
Basilicata	1451.2 \pm 55.9 ^d	23.5 \pm 0.4 ^d	454.0 \pm 34.6 ^b	11,583.0 \pm 170.8 ^{e,f}	864.6 \pm 66.5 ^a	419.6 \pm 38.1 ^a	10.7 \pm 0.3 ^b
Molise	668.2 \pm 30.1 ⁱ	10.1 \pm 0.5 ^{h,i}	315.8 \pm 13.0 ^d	13,243.8 \pm 161.9 ^b	562.3 \pm 36.7 ^c	315.0 \pm 15.3 ^c	12.0 \pm 1.0 ^b
Puglia	845.0 \pm 1.2 ^h	21.1 \pm 1.1 ^e	322.6 \pm 7.7 ^d	10,919.9 \pm 6.6 ^f	548.5 \pm 70.1 ^c	347.8 \pm 2.2 ^b	17.0 \pm 2.4 ^a
Multitalia 1	136.8 \pm 12.3 ^m	2.9 \pm 0.2 ^m	20.7 \pm 1.8 ^h	1377.9 \pm 7.0 ⁱ	12.4 \pm 0.3 ^g	11.8 \pm 1.1 ^m	1.5 \pm 0.3 ^e
Multitalia 2	838.1 \pm 10.4 ^h	11.1 \pm 0.3 ^{g,h}	124.1 \pm 4.2 ^f	4744.2 \pm 88.0 ^g	305.3 \pm 4.1 ^e	65.5 \pm 0.4 ^{h,i}	10.9 \pm 0.5 ^b
Multitalia 3	375.1 \pm 3.0 ^l	6.5 \pm 0.3 ^l	41.9 \pm 50.3 ^{g,h}	2161.2 \pm 11.8 ^h	157.5 \pm 1.2 ^f	51.4 \pm 0.1 ^{i,l}	6.5 \pm 1.0 ^{c,d}
Multitalia 4	1647.4 \pm 12.3 ^c	25.9 \pm 0.4 ^c	431.5 \pm 15.7 ^b	13,588.0 \pm 188.3 ^b	730.9 \pm 10.7 ^b	121.1 \pm 4.5 ^{e,f}	18.0 \pm 0.7 ^a
Sig.	**	**	**	**	**	**	**

Data are expressed as mg/kg of means \pm SD. Different superscript letters indicate statistical differences within the same column (** Significance at $p < 0.01$).

For this reason, Lublanc seems to be the most interesting, together with the landraces, for further pharmaceutical studies. Table 3 shows the alkaloids observed in the three *L. luteus* seed samples.

Table 3. Alkaloids shared by the three analyzed *Lupinus luteus* cultivars.

Alkaloid	Dukat	Mister	Taper	Sig.
Lupinine	177.1 ± 30.5 ^b	281.4 ± 24.5 ^a	194.6 ± 21.0 ^b	*
Sparteine	139.0 ± 1.3 ^b	233.9 ± 9.5 ^a	120.0 ± 0.8 ^c	**
β-Isosparteine	4.5 ± 0.1	5.7 ± 0.9	4.2 ± 0.4	n.s.
Ammodendrine	26.8 ± 0.4 ^{a,b}	31.2 ± 0.3 ^a	22.7 ± 2.5 ^c	*
Unknown_c	7.6 ± 0.2	8.5 ± 0.7	7.6 ± 0.7	n.s.
Lupanine	8.7 ± 0.5 ^b	36.6 ± 4.7 ^a	6.4 ± 0.6 ^b	**
Feruloyllupinine	12.8 ± 0.8 ^a	13.1 ± 0.5 ^a	9.1 ± 0.2 ^b	*

Data are expressed as mg/kg of means ± SD. Different superscript letters indicate statistical differences within the same row (** Significance at $p < 0.01$; * Significance at $p < 0.05$; n.s., not significant).

Lupinine and sparteine were the main quinolizidine alkaloids, as confirmed by Aniszewski [6]. The Mister cultivar showed the highest amount of each of the seven shared alkaloids. Lupanine, the main alkaloid of *L. albus* species, was also observed in *L. luteus*, but at much lower concentrations, as reported by other authors [4]. Although gramine and other indole alkaloids are not usually detected in lupin seeds, in the present work, gramine was detected in the Dukat and Taper samples at 7 and 190 mg/kg, respectively (data not shown). This result agrees with previous findings [7].

The principal component analysis score plot was completed using all alkaloids and their total amounts as measured factors (Figure 2a). The first two principal components (PCs) explained 63.9% of the total variability (PC1 = 46.18%; PC2 = 17.70%). The score plot highlighted three sample sets, the first of which included Lublanc and Multitalia 4. Those two samples were different from each other and they were positively related to both the PCs. Lublanc and Multitalia 4 appeared to be related to the largest number of unknown alkaloids and to the identified compounds located in the upper right side of the loading plot (Figure 2b), such as N-formylalbine, N-formylangustifoline, 11,12-dehydrosparteine, and 17-oxolupanine. The second cluster included all nine *L. albus* landraces, all samples were positively related to PC2 and negatively related to PC1. The landraces were principally related to unknown_d, isoangustifoline, multiflorine, 13α-hydroxymultiflorine, 13α-angeloyloxylupanine, and 13α-tigloyloxymultiflorine, which were observed in all landrace profiles. Moreover, the landraces were related to the total alkaloid content, as confirmed by Figure 1, in which all landraces showed a similar total alkaloid amount. The third sample set highlighted the genotypes that were positively related to PC1 and negatively to PC2. That group should be sub-clustered into two groups: the *L. luteus* group (Mister, Dukat, and Taper), and the *L. albus* varieties and cv Sonet (*L. angustifolius*). All *L. albus* varieties and cv Sonet had almost a zero PC1 value and positive PC2 values, whereas the PC2 value of the *L. luteus* was higher than that of the *L. albus* group. All compounds located in the upper left side of the loading plot (Figure 2b) were typical of *L. luteus* samples, with the exception of sparteine, which was also found in some *L. albus* landraces. The position of *L. albus* cultivars in the plot discriminated them from the landraces in terms of typical alkaloids and their total amount.

Table 4 shows the antimicrobial results of the two tested extracts. Two species were affected differently by the alkaloid extracts. Among the tested strains, only the two clinical isolates *Pseudomonas aeruginosa* (skin and ulcer) and *Klebsiella pneumoniae* (skin and inguinal skin site) were inhibited by the lupin alkaloids. Antibacterial activity is considered by Erdemoglu et al. [18,38] as significant when the MIC is less than or equal to 100 µg/mL, and moderate when the MIC is 100–500 µg/mL. According to this classification, both alkaloid extracts showed significant activity on *K. pneumoniae* and significant moderate activity on *P. aeruginosa*. The extract of the landrace Calabria 2 had the highest activity.

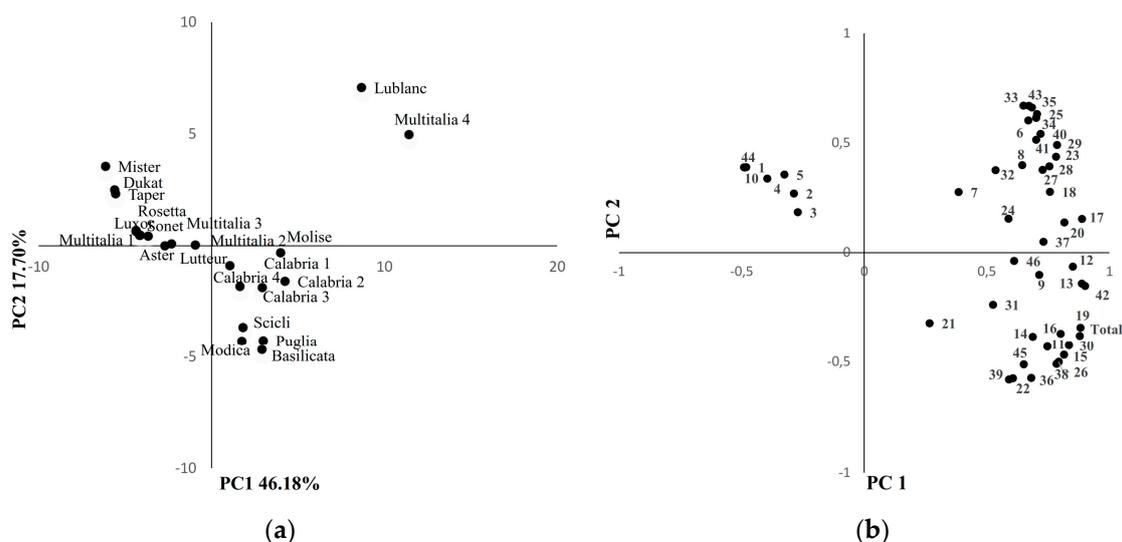


Figure 2. Score plot of (a) Principal Component Analysis showing the distribution of lupin genotypes along principal components PC1 and PC2, and (b) loading plot showing the distribution of the variables (total amount of alkaloids and all individual alkaloid compounds; each variable number refers to Table 1).

Table 4. Antimicrobial activity of alkaloid extracts of Multitalia 4 and Calabria 2 (*Lupinus albus*) seeds.

Microorganism	Multitalia 4	Calabria 2
<i>Pseudomonas aeruginosa</i> (from skin)	128	67
<i>Klebsiella pneumoniae</i> (from inguinal skin)	16	67

Data are expressed as minimum inhibitory concentrations (MICs, $\mu\text{g}/\text{mL}$).

The opportunistic pathogen *K. pneumoniae* can cause severe nosocomial infections, such as septicemia, pneumonia, urinary tract infections, and soft tissue infections. The indiscriminate use of antibiotics has resulted in a considerable increase in outbreaks caused by microorganisms resistant to antimicrobial drugs. Moreover, an alert was recently released due to the increasing evidence of the ability of *K. pneumoniae* to form biofilm, mostly on medical devices, thus new approaches are needed to control infection [39]. *P. aeruginosa* is a notoriously difficult organism to control with antibiotics or disinfectants due to its low antibiotic susceptibility. This resistance is attributable to a concerted action of multidrug efflux pumps with chromosomally-encoded antibiotic resistance genes and resistance developed due to mutation of chromosomally-encoded genes [40].

The present results about the antibacterial property of *Lupinus* extracts indicate that compounds from the plants of this genus could be used against common pathogens, as previously reported by other authors [18,41]. However, these extracts must be studied in animal models to determine their in vivo efficacy and potential toxicity, and to elucidate their mechanisms of action, as in vitro activity does not necessarily correspond to in vivo efficacy.

4. Conclusions

The three tested lupin species exhibited different alkaloid profiles, with some typical alkaloids present as the main compounds of each species. Lupanine, 13 α -hydroxylupanine, and albine were determined as the main alkaloids in the *L. albus* seed samples; angustifoline and 13 α -tigloyloxylupanine were also well-represented in *L. albus* landraces; whereas sparteine and lupanine were typical of *L. luteus*.

Finally, lupanine, 13 α -hydroxylupanine, and angustifoline were the main alkaloids of *L. angustifolius* seeds. Some alkaloids were shared by all three species: 11,12-dehydrosparteine, ammodendrine, albine, lupanine, and multiflorine.

The *L. luteus* and *L. angustifolius* samples, together with most of the *L. albus* varieties, had a lower alkaloid amount, thus supporting their use as a human foodstuff and/or animal feed (white lupin and narrow-leafed lupin), and yellow lupin for the livestock chain only. Conversely, all landraces and the Lublanc cultivar (*L. albus*) proved to be interesting from a pharmaceutical viewpoint due to their several unknown alkaloids and for having the highest total amount of alkaloids. The alkaloid extracts from landrace Calabria 2 showed high activity on *K. pneumoniae* and moderate activity on *P. aeruginosa* clinical isolates.

Additional studies are needed to test the potential pharmacological effects and the in vivo antibacterial properties of lupin extracts.

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References

1. Gresta, F.; Wink, M.; Prins, U.; Abberton, M.; Capraro, J.; Scarafoni, A.; Hill, G. Lupins in European cropping systems. In *Legumes in Cropping Systems*; Murphy-Bokern, D., Stoddard, F., Watson, C., Eds.; CABI: Wallingford, Oxfordshire, UK, 2017; pp. 88–108, ISBN 9781780644981.
2. Carvajal-Larenas, F.E.; Van Boekel, M.J.A.S.; Koziol, M.; Nout, M.J.R.; Linnemann, A.R. Effect of processing on the diffusion of alkaloids and quality of *Lupinus mutabilis* sweet. *J. Food Process. Preserv.* **2014**, *38*, 1461–1471. [[CrossRef](#)]
3. Annicchiarico, P.; Manunza, P.; Arnoldi, A.; Boschin, G. Quality of *Lupinus albus* L. (White lupin) seed: Extent of genotypic and environmental effects. *J. Agric. Food Chem.* **2014**, *62*, 6539–6545. [[CrossRef](#)] [[PubMed](#)]
4. Gresta, F.; Abbate, V.; Avola, G.; Magazzù, G.; Chiofalo, B. Lupin seed for the crop-livestock food chain. *Ital. J. Agron.* **2010**, *4*, 333–340. [[CrossRef](#)]
5. FAOSTAT. Statistics Database of the Food and Agriculture Organization of the United Nations. Food and Agriculture Organization of the United Nations: Rome. Available online: <http://faostat3.fao.org/home/E> (accessed on 18 September 2017).
6. Aniszewski, T. *Alkaloids: Chemistry, Biology, Ecology, and Applications*, 2nd ed.; Elsevier: Amsterdam, The Netherlands, 2015; ISBN 9780444594334.
7. Magalhães, S.C.; Fernandes, F.; Cabrita, A.R.; Fonseca, A.J.; Valentão, P.; Andrade, P.B. Alkaloids in the valorization of European *Lupinus* spp. seeds crop. *Ind. Crops Prod.* **2017**, *95*, 286–295. [[CrossRef](#)]
8. Musco, N.; Cutrignelli, M.I.; Calabrò, S.; Tudisco, R.; Infascelli, F.; Grazioli, R.; Lo Presti, V.; Gresta, F.; Chiofalo, B. Comparison of nutritional and antinutritional traits among different species (*Lupinus albus* L.; *Lupinus luteus* L.; *Lupinus angustifolius* L.) and varieties of lupin seeds. *J. Anim. Physiol. Anim. Nutr.* **2017**, *101*, 1227–1241. [[CrossRef](#)] [[PubMed](#)]
9. Muzquiz, M.; Cuadrado, C.; Ayet, G.; de la Cuadra, C.; Burbano, C.; Osagie, A. Variation of alkaloid components of Lupin seeds in 49 genotypes of *Lupinus albus* L. from different countries and locations. *J. Agric. Food Chem.* **1994**, *42*, 1447–1450. [[CrossRef](#)]
10. Reinhard, H.; Rupp, H.; Sager, F.; Streule, M.; Zoller, O. Quinolizidine alkaloids and phomopsins in lupin seeds and lupin containing food. *J. Chromatogr. A* **2006**, *1112*, 353–360. [[CrossRef](#)] [[PubMed](#)]
11. Wink, M.; Meibner, C.; Witte, L. Pattern of quinolizidine alkaloids in 56 species of the genus *Lupinus*. *Phytochemistry* **1995**, *38*, 139–153. [[CrossRef](#)]

12. Boschini, G.; Annicchiarico, P.; Resta, D.; D'Agostina, A.; Arnoldi, A. Quinolizidine Alkaloids in Seeds of Lupin Genotypes of Different Origins. *J. Agric. Food Chem.* **2008**, *56*, 3657–3663. [[CrossRef](#)] [[PubMed](#)]
13. Resta, D.; Boschini, G.; D'Agostina, A.; Arnoldi, A. Evaluation of total quinolizidine alkaloids content in lupin flours, lupin-based ingredients, and foods. *Mol. Nutr. Food Res.* **2008**, *52*, 490–495. [[CrossRef](#)] [[PubMed](#)]
14. Canu Boido, C.; Tasso, B.; Boido, V.; Sparatore, F. Cytisine derivatives as ligands for neuronal nicotine receptors and with various pharmacological activities. *Farmaco* **2003**, *58*, 265–277. [[CrossRef](#)]
15. Guo, C.; Zhang, C.; Li, L.; Wang, Z.; Xiao, W.; Yang, Z. Hypoglycemic and hypolipidemic effects of oxymatrine in high-fat diet and streptozotocin-induced diabetic rats. *Phytomedicine* **2014**, *21*, 807–814. [[CrossRef](#)] [[PubMed](#)]
16. Lin, Z.; Huang, C.F.; Liu, X.S.; Jiang, J. In Vitro Anti-Tumour Activities of Quinolizidine Alkaloids Derived from *Sophora flavescens* Ait. *Basic Clin. Pharmacol.* **2010**, *108*, 304–309. [[CrossRef](#)] [[PubMed](#)]
17. Trugo, L.C.; von Baer, E.; von Baer, D. Breeding of grains: Lupin Breeding. *Ref. Mod. Food Sci.* **2016**, 1–8. [[CrossRef](#)]
18. Erdemoglu, N.; Ozkan, S.; Tosun, F. Alkaloid profile and antimicrobial activity of *Lupinus angustifolius* L. alkaloid extract. *Phytochem. Rev.* **2007**, *6*, 197–201. [[CrossRef](#)]
19. Arnoldi, A.; Boschini, G.; Zanoni, C.; Lammi, C. The health benefits of sweet lupin seed flours and isolated proteins. *J. Funct. Foods* **2015**, *18*, 550–563. [[CrossRef](#)]
20. Fontanari, G.G.; Batistuti, J.P.; da Cruz, R.J.; Saldiva, P.H.N.; Arêas, J.A.G. Cholesterol-lowering effect of whole lupin (*Lupinus albus*) seed and its protein isolate. *Food Chem.* **2012**, *132*, 1521–1526. [[CrossRef](#)] [[PubMed](#)]
21. Sirtori, C.R.; Lovati, M.R.; Manzoni, C.M.; Castiglioni, S.; Duranti, M.; Magni, C.; Moranti, S.; D'Agostina, A.; Arnoldi, A. Proteins of white lupin seed, a naturally isoflavone-poor legume, reduce cholesterolemia in rats and increase LDL receptor activity in HepG2 cells. *J. Nutr.* **2004**, *134*, 18–23. [[CrossRef](#)] [[PubMed](#)]
22. Nasar-Abbas, S.M.; Jayasena, V. Effect of lupin flour incorporation on the physical and sensory properties of muffins. *Qual. Assur. Saf. Crops Foods* **2012**, *4*, 41–49. [[CrossRef](#)]
23. Spina, A.; Cambrea, M.; Platania, A.; Roccasalva, D.; Rapisarda, P. Germoplasma di lupino (*Lupinus albus* L., *Lupinus angustifolius* L. e *Lupinus luteus* L.) in collezione presso il CRA-ACM di Acireale. In *Conservazione, Biodiversità, Gestione Banche Dati e Miglioramento Genetico*; D'Andrea, F., Ed.; Edizioni Nuova Cultura: Roma, Italy, 2013; pp. 271–279. [[CrossRef](#)]
24. Campolo, O.; Romeo, F.V.; Malacrino, A.; Laudani, F.; Carpinteri, G.; Fabroni, S.; Rapisarda, P.; Palmeri, V. Effects of inert dusts applied alone and in combination with sweet orange essential oil against *Rhyzopertha dominica* (Coleoptera: Bostrichidae) and wheat microbial population. *Ind. Crop. Prod.* **2014**, *61*, 361–369. [[CrossRef](#)]
25. Li Destri Nicosia, M.G.; Pangallo, S.; Raphael, G.; Romeo, F.V.; Strano, M.C.; Rapisarda, P.; Droby, S.; Schena, L. Control of postharvest fungal rots on citrus fruit and sweet cherries using a pomegranate peel extract. *Postharvest Biol. Technol.* **2016**, *114*, 54–61. [[CrossRef](#)]
26. Romeo, F.V.; Ballistreri, G.; Fabroni, S.; Pangallo, S.; Li Destri Nicosia, M.G.; Schena, L.; Rapisarda, P. Chemical characterization of different sumac and pomegranate extracts effective against *Botrytis cinerea* rots. *Molecules* **2015**, *20*, 11941–11958. [[CrossRef](#)] [[PubMed](#)]
27. Calabrò, S.; Cutrignelli, M.; Lo Presti, V.; Tudisco, R.; Chiofalo, V.; Grossi, M.; Infascelli, F.; Chiofalo, B. Characterization and effect of year of harvest on the nutritional properties of three varieties of white lupine (*Lupinus albus* L.). *J. Sci. Food Agric.* **2015**, *95*, 3127–3136. [[CrossRef](#)] [[PubMed](#)]
28. NIST Chemistry WebBook 2015. Available online: <http://webbook.nist.gov/> (accessed on 05 May 2016).
29. El-Shazly, A.; Ateya, A.M.M.; Wink, M. Quinolizidine Alkaloid Profiles of *Lupinus varius orientalis*, *L. albus albus*, *L. hartwegii*, and *L. densiflorus*. *Z. Naturforsch. C* **2001**, *56*, 21–30. [[CrossRef](#)] [[PubMed](#)]
30. Torres, K.B.; Quintos, N.R.; Herrera, J.M.; Tei, A.; Wink, M. Alkaloid profiles of leaves and seeds of *Lupinus aschenbornii* Schauer from Mexico. In *Lupin, an Ancient Crop for the New Millennium, Proceedings of the 9th International Lupin Conference, Klink/Miiritz, Germany, 20–24 June 1999*; van Santen, E., Wink, M., Weissmann, S., Roemer, P., Eds.; International Lupin Association Publisher: Canterbury, New Zealand, 1999; pp. 301–304, ISBN 0-86476-123-6.
31. CLSI. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard. In *Clinical and Laboratory Standards Institute Document M7-A7*, 7th ed.; CLSI: Wayne, PA, USA, 2006; ISBN 1-56238-587-9.

32. CLSI. Reference method for broth dilution antifungal susceptibility testing of yeasts; Approved standard. In *Clinical and Laboratory Standards Institute document M27-A3*, 3rd ed.; CLSI: Wayne, PA, USA, 2008; ISBN 1-56238-666-2.
33. Hernández, E.M.; Rangel, M.L.C.; Corona, A.E.; Cantor del Angel, A.E.; Sánchez López, J.A.; Sporer, F.; Wink, M.; Torres, K.B. Quinolizidine alkaloid composition in different organs of *Lupinus aschenbornii*. *Rev. Bras. Farmacogn.* **2011**, *21*, 824–828. [[CrossRef](#)]
34. Law 1096 of the 25 November 1971 and Subsequent Amendments and Additions. pp. 1–28. Available online: <http://scs.entecra.it/leggiEdisposizioni/NORM-NAZIONALI/Legge-1096-del-25-11-71.pdf> (accessed on 18 September 2017).
35. Jansen, G.; Jürgens, H.U.; Ordon, F. Effects of temperature on the alkaloid content of seeds of *Lupinus angustifolius* cultivars. *J. Agron. Crop Sci.* **2009**, *195*, 172–177. [[CrossRef](#)]
36. Barlóg, P.K. Effect of magnesium and nitrogenous fertilisers on the growth and alkaloid content in *Lupinus angustifolius* L. *Aust. J. Agric. Res.* **2002**, *53*, 671–676. [[CrossRef](#)]
37. Jayasena, V.; Nasar-Abbas, S.M. Development and quality evaluation of high-protein and high-dietary-fiber pasta using lupin flour. *J. Texture Stud.* **2012**, *43*, 153–163. [[CrossRef](#)]
38. Erdemoglu, N.; Ozkan, S.; Duran, A.; Tosun, F. GC-MS analysis and antimicrobial activity of alkaloid extract from *Genista vuralii*. *Pharm. Biol.* **2009**, *47*, 81–85. [[CrossRef](#)]
39. Vuotto, C.; Longo, F.; Balice, M.P.; Donelli, G.; Varaldo, P.E. Antibiotic resistance related to biofilm formation in *Klebsiella pneumoniae*. *Pathogens* **2014**, *3*, 743–758. [[CrossRef](#)] [[PubMed](#)]
40. Lambert, P.A. Mechanisms of antibiotic resistance in *Pseudomonas aeruginosa*. *J. R. Soc. Med.* **2002**, *95*, 22–26. [[PubMed](#)]
41. Khan, M.K.; Karnpanit, W.; Nasar-Abbas, S.M.; Huma, Z.E.; Jayasena, V. Phytochemical composition and bioactivities of lupin: A review. *Int. J. Food Sci. Technol.* **2015**, *50*, 2004–2012. [[CrossRef](#)]



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