

COMPOSITION OF ALKALOIDS IN SEEDS OF *LUPINUS MEXICANUS* (FABACEAE) AND ANTIFUNGAL EVALUATION OF THE ALKALOID EXTRACT

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ABSTRACT

Capillary gas chromatography-mass spectrometry (CG-MS) was used to analyze the composition of alkaloids in seeds of *Lupinus mexicanus*. In addition, an *in vitro* study was made of the antifungal activity of the crude extract of alkaloids. The antifungal action of the extract was evaluated based on the inhibition of the mycelial growth of the phytopathogenic fungi *Sclerotium rolfsii*, *Rhizoctonia solani* and *Fusarium oxysporum*. The analysis of alkaloids from the extract revealed the presence of lupanine and 3 β -hydroxylupanine, multiflorine, aphylline, epiaphylline and α -isolupanine as majority compounds. The extract at the concentration of 3 mg mL⁻¹ significantly inhibited the mycelial growth of *R. solani* by 87.7%, whereas the mycelial growth of *S. rolfsii* was only inhibited by 72.5% with the highest concentration lupanine was the most abundant alkaloid (76.2%) in the extract of seeds of *L. mexicanus*.

KEYWORDS

Lupinus, biological activity, quinolizidine alkaloids, phytopathogenic fungi

INTRODUCTION

Insects, phytopathogenic microorganisms and weeds are responsible for the reduction in yields in agricultural crops. According to Mohamed *et al.* (1996), economic losses due to diseases caused by fungi in fruits can be from 5 to 50% or more. Although these losses can be reduced through the use of resistant varieties, crop rotation or sanitary practices, the use of synthetic pesticides to protect crops is the most important factor for maximizing yields (Carpinella *et al.* 2003). However, the indiscriminant use of these products causes diverse problems, such as the elimination of natural enemies, surging of resistant organisms, accumulation of toxic residues in agricultural products and the contamination of the environment (Duke *et al.* 2000). This has motivated the search for alternative products in the protection of crops against the action of phytopathogenic organisms and weeds, whose activity, selectivity and environmental safety are adequate.

Natural products are less aggressive for the environment and represent an alternative source of natural pesticides in agriculture. The quinolizidine alkaloids are an important group of natural compounds in the genus *Lupinus* (Fabaceae), these secondary metabolites are a defense mechanism against phytopathogenic microorganisms, herbivores and against other plant species that cause competition (Wink, 1998). Preliminary studies have been made to know the fungicidal activity of powders and crude extracts obtained from seeds of *L. campestris*, *L. montanus* and *L. exaltatus* (Bravo *et al.* 2000; Zamora *et al.* 2002). In *L. mexicanus*, a wild species with wide distribution in Jalisco, Mexico, and with high content of alkaloids in its seeds, the biological activity of these compounds has not been studied. Therefore, the objective of the present investigation was to determine the composition of alkaloids in seeds of *L. mexicanus* and to evaluate *in vitro* the fungicidal activity of the extract of alkaloids.

MATERIALS AND METHODS

PLANT MATERIAL

The seeds of *L. mexicanus* were collected in May of 2007 in the municipality of Lagos de Moreno, Jalisco, Mexico. The identity of the species was determined with the support of the herbarium of the Instituto de Botánica of the Universidad de Guadalajara (Voucher No 853). The seeds were ground and the flour thus obtained was degreased with hexane in soxhlet equipment.

EXTRACTION OF ALKALOIDS FOR ANALYSIS

The extraction of alkaloids was made according to the technique described by Muzquiz *et al.* (1994): 500 mg of flour were homogenised with 5 mL of trichloroacetic acid at 5% for 1 min. The mixture was centrifuged 15 min at 2400 x g and the supernatant was decanted. The extraction was repeated twice and the volume of the three supernatants was transferred to funnels for alkanisation with 0.8 mL of NaOH 10 M. The alkaloids were extracted with dichloromethane

Table 1. Fragmentation pattern of the major quinolizidine alkaloids detected in *L. mexicanus*.

Alkaloids	M ⁺	Characteristic ions (% abundance)					
Lupanine	248	136(100)	149(52)	98(28)	150(34)	248(32)	110(12)
3-β-hydroxylupanine	264	136(100)	264(41)	134(48)	150(41)	98(38)	110(22) 84(21)
Multiflorine	246	136(100)	246(61)	110(35)	148(17)	97(10)	
Aphylline	248	136(100)	97(48)	96(44)	84(35)	220(22)	248(21) 124(23)
Epiaphylline	248	136(100)	137(51)	96(38)	220(37)	247(29)	248(33)
α-isolupanine	248	136(100)	149(52)	248(33)	150(31)	110(19)	84(16)

M⁺ = Molecular Ion.

Table 2. Composition of alkaloids in the extract obtained from the seeds of *L. mexicanus*.

Alkaloids	Content (mg g ⁻¹)	Abundance (%)
Lupanine	21.2	76.2
3β-hydroxylupanine	2.61	9.2
Multiflorine	1.09	3.9
Aphylline	0.79	2.8
Epiaphylline	0.48	1.7
α-isolupanine	0.36	1.3
11,12-Dehidrolupanina	0.19	0.07
17-Oxolupanine	0.14	0.05
Ammodendrine	0.13	0.04
11,12-dehydro-oxosparteine	0.13	0.04
Total alkaloid content	27.1	

(3 x 15 mL). The crude extracts were concentrated in a rotavapor at 40°C until dried. The residue was dissolved in 1 mL of methanol and passed through Millipore filters of 0.45 μm.

CAPILLARY GAS CHROMATOGRAPHY-MASS SPECTROMETRY

After being filtered, 0.5 mL of the extract was mixed with 0.5 mL of methanol and 1 μL of sample was injected to Perkin- Elmer Autosystem 1XL capillary gas chromatograph with SPB-1 (30 m x0.25 mm d.i.) capillary column, coupled to a mass spectrometer (Perkin-Elmer Turbomass Gold). The gas carrier was He with a flow of 1 mL min⁻¹; the temperatures of the injector and of the detector were 240°C and 300°C. The temperature program was 180°C 2 min, isothermic, 180-300°C at 5°C min, 300°C 10 min, isothermic. The relative abundance of alkaloids was calculated considering the areas of the peaks of the chromatogram, whereas to identify the alkaloids, a comparison was made of the spectra of masses obtained with those of software library and with those reported by Meißner and Wink (1991).

OBTAINMENT OF THE CRUDE EXTRACT OF ALKALOIDS FOR THE BIOASSAYS

One hundred grams of previously degreased flour were weighed, and homogenised with 400 mL of

trichloroacetic acid at 5% in constant agitation for 12 h; the mixture was centrifuged 15 min at 2400 x g. The supernatant was alkalised with 10 mL of sodium hydroxide 10M in a separation funnel, the alkaloids were extracted with dichloromethane (3X50mL), the organic phase was recovered and was brought to dryness at 40°C with rotavapor. The extract was re-suspended in methanol, was transferred to amber jars, and after the solvent was evaporated, it was stored at 4°C until being used in the bioassays.

BIOASSAYS

The in vitro antifungal activity of the extract was evaluated based on the capacity of inhibition of the mycelia growth of the phytopathogenic fungi *Sclerotium rolfsii*, *Rhizoctonia solani* and *Fusarium oxysporum*. The extract of alkaloids dissolved in distilled water was added to 100 mL of potato dextrose culture medium at concentrations of 0, 1, 2, 3, 4 and 5 mg mL⁻¹. The medium was sterilised 15 min in autoclave at 1.05 kg cm⁻² of pressure and was transferred to Petri dishes (8.5 cm diameter). The dishes were inoculated with discs of mycelia (0.5 cm diameter) and were incubated in darkness at 25°C. When the mycelium of the fungus covered the surface of the medium in the control, all of the dishes were removed

from the oven and the diameter of the mycelia was measured. The percentage of inhibition was calculated with the following equation: $I = [(C - T)/C] \times 100$, where I is the inhibition (%), C is the diameter of the mycelium in the dish of the control (0 mg mL⁻¹), and T is the diameter of the mycelium in the dish of the treatment (1, 2, 3, 4 and 5 mg mL⁻¹).

STATISTICAL ANALYSIS

The experimental design was completely randomised with five replicates per treatment and the variables were as follows: mycelial growth, and seed germination. An analysis of variance was made and the means were compared with the Tukey test ($p \leq 0.05$).

RESULTS AND DISCUSSION

ANALYSIS OF ALKALOIDS

The Table 1 shows the fragmentation pattern of the majority alkaloids in the extract of *L. mexicanus*, which were identified when the spectra of masses were compared with the spectra of the data base of the program and those of the bibliography (Meibner and Wink, 1991).

Some minority alkaloids were also identified: 11, 12-dehydrolupanine, 17-oxolupanine, ammodendrine and 11,12-dehydro-oxosparteine. The lupanine and 3 β -hydroxylupanine were the principal alkaloids in the extract (21.2 and 2.6 mg g⁻¹), the former contributed with 76.2% and the latter with 9.4% of the total content, whereas the minority alkaloids represented approximately 4.5% (Table 2).

According to Przybylak *et al.* (2005), lupanine is the major alkaloid in seeds of *L. rotundiflorus* and *L. exaltatus* of Mexico, with 62.2 and 47.3%, but these values are inferior to those determined in the present study with *L. mexicanus* (76.2%). In other wild species

of Mexico, *L. hintonii* and *L. campestris*, the majority alkaloids are 13 α -hydroxylupanine and hydroxyafillidine (Bermúdez *et al.* 2002; Martínez *et al.* 2001). These variations in the profile and composition of alkaloids in the seeds of the different species of the genus *Lupinus* have been related with the species, place and date of collection.

ANTIFUNGAL ACTIVITY

There were significant differences ($p \leq 0.05$) in the average growth of the mycelia of *S. rolfsii* and *R. solani* from the effect of the extract. The lowest concentrations of the extract did not show important effects on the growth of the mycelia with respect to the control; however, when the concentrations were increased, there was a reduction of mycelia growth with a different response of susceptibility with respect to the pathogen. Thus, when the concentrations of the extract were increased, the growth of the mycelia of *R. solani* had an average inhibition of 81.6, 87.7 and 100% with 3, 4, and 5 mg mL⁻¹ (Table 3).

This behavior was similar in *S. rolfsii*, but the inhibition of mycelia growth was lower when the same concentrations of the extract were applied: with 3, 4 and 5 mg mL⁻¹, the growth of the mycelia was only inhibited by 31, 35 and 72.5%. The above indicates that *S. rolfsii* was less susceptible than *R. solani* to the alkaloid extract of *L. mexicanus*. The mycelia growth of *F. oxysporum* was not different ($p > 0.05$) among treatments, that is, the concentrations of the extract did not inhibit the growth of this fungus. The resistance of *F. oxysporum* to the alkaloids of *L. mexicanus* has been reported when using extracts of *L. montanus* and *L. rotundiflorus* (Zamora *et al.* 2002); furthermore, the mycelia growth of *F. moniliforme* was not inhibited when ground seeds of *L. campestris* and *L. mutabilis* were incorporated to the culture medium (Bravo *et al.* 2000).

Table 3. Effect of the extract of alkaloids on the growth and percentage of inhibition of the mycelia of phytopathogenic fungi.

Alkaloid concentration in the culture medium (mg mL ⁻¹)	<i>Sclerotium rolfsii</i>		<i>Rhizoctonia solani</i>		<i>Fusarium oxysporum</i>	
	MGR [§] (cm)	PI [¶] (%)	MGR [§] (cm)	PI [¶] (%)	MGR [§] (cm)	PI [¶] (%)
0	8.0 ± 0.0 a	0	8.0 ± 0.0 a	0	8.0 a	0
1	8.0 ± 0.0 a	0	8.0 ± 0.0 a	0	8.0 a	0
2	7.5 ± 0.24 a	12.4	5.7 ± 0.42 b	32.4	8.0 a	0
3	5.5 ± 0.46 b	31.2	1.5 ± 0.17 c	81.6	8.0 a	0
4	5.2 ± 0.53 b	35.1	1.0 ± 0.11c	87.7	8.0 a	0
5	2.2 ± 0.13 c	72.5	0.0 ± 0.0 d	100	8.0 a	0

[§]MGR= Mycelia Growth Radial, [¶]PI= Percentage of Inhibition. Within columns, means followed by the same letter are not significantly different at the 0.05 probability level according to Tukey test.

Sas-Piotrowska *et al.* (1996) reported inhibition percentages lower than those found in the present study against *R. solani* and other species of fungi when using different fractions obtained from an ethanol extract of *L. angustifolius*. The differences observed in other studies and those reported in the present investigation are probably due to the variation in the profile and concentration of alkaloids in the extracts of seeds of the different species of *Lupinus*, as well as the possible variations in the methodology of extraction. The results are interesting because there are few references of the fungicidal activity of quinolizidine alkaloids of Mexican species of *Lupinus*.

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