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ALKALOID PROFILE, ANTIBACTERIAL AND ALLELOPATHIC ACTIVITIES OF *LUPINUS JAIMEHINTONIANA* B.L. TURNER (FABACEAE).

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Abstract – Herein we describe some aspects of the ethnobotanical use and the first alkaloid profile of *Lupinus jaimehintoniana*, the 5 to 8 m high arboreous lupine. Five quinolizidine alkaloids identified as sparteine, 5,6-dehydrolupanine, lupanine, nuttalline, and d-thermopsine, were characterized by the respective elution order according to their electronic impact spectra, lupanine being the most abundant in the four different tissues analyzed. Simultaneously, an antibacterial assessment of the four corresponding crude methanolic extracts, as well as the four semi-purified alkaloids was performed on specific *Escherichia coli and Agrobacterium tumefaciens* strains. These experiments resulted in MIC ranges of 37-61 μg mL^{-1} and 130-146 μg mL^{-1}, respectively. for both bacterial species. Finally, the allelopathic activity of these extracts on the germination of *Lactuca sativa* seeds was demonstrated to be in the range of 50-300 μg mL^{-1} for both semi-purified alkaloid and methanolic extracts.

Key words: *Lupinus jaimehintoniana*, alkaloid profile, antibacterial, allelopathic, Mexico

INTRODUCTION

The *Lupinus* genus contains about 200 characterized species distributed in Europe and Africa, but around 90% of the members grow in America (Aïnouche *et al.*, 2004). Although many of these plants have potential use in human activities especially as ornamentals, some examples, such as *L. elegans*, are promising biotools in ecological restoration and for recovering degraded soils (Lara-Cabrera et al., 2009). Due to their high tolerance to hostile environments and their nutritional seed properties, some species such as *L. angustifolious* and *L. exaltatus* are grown in almost any kind of soil and could eventually replace important agronomic plants such as soybean (Bader et al., 2009; Ruiz-López et al., 2006). It is widely known that the *Lupinus* genus contains endogenous concentrations of quinolizidine alkaloids, which are considered as chemotaxonomic markers, but at the same time are toxic compounds for humans, microorganisms,

and even for some plant species (Wink, 1984). The latter property has led to the use of lupine extracts in biological control and in pharmacological trials (Zamora-Nátera et al., 2005, 2008; Ruiz-López et al., 2010). Lupine species are usually herbs or shrubs (*L. arboreus, L. montanus*), 30 cm to 2 m tall. Nevertheless, in the middle of the 1990's, one exception was discovered: the arboreous *Lupinus jaimehintoniana* (Turner, 1995). The plant is commonly known as "chamis de monte" in the southern lowlands of Oaxaca, México. It grows as a subdominant tree of 5 to 8 m height. The autochthonous people from the lands of Ozolotepec, Oaxaca, use this plant as a forage source and a hard wood for various purposes, as well as to fertilize soil prior to sowing. Previously, *L. jaimehintoniana* was observed on the Cerro Quiexobra, the highest mountain in the state (3750 masl; Turner, 1995). In this study we found its presence on the Cerro del Aserradero at 2896 masl. According to phylogenetic studies, *L. jaimehintoniana* is closely related to *L. mexicanus, L. campestris* and *L. elegans* (Aïnouche et al., 2004); however, there is no information about the chemistry and biological activities of this particular plant so far. This work presents the first attempt at obtaining an alkaloid profile of the leaves, seeds, shoots and phloem of *L. jaimehintoniana*, in order to predict its possible biological functions and to identify chemotaxonomic markers in this plant. Simultaneously the antibacterial activity of crude methanolic extracts and semi-purified alkaloids in *Escherichia coli and Agrobacterium tumefaciens* strains were assessed; the allelopathic activity of these preparations in *Lactuca sativa* seeds was demonstrated.

MATERIALS AND METHODS

Metabolite extraction and GC-MS profile

L. jaimehintoniana was collected on Cerro del Aserradero, 16° 09' N 96° 14' W, 2896 masl, in San Juan Ozolotepec, Oaxaca, México, in May 2011 (Spring 2011). The specimen was analyzed and certified by Ramiro Cruz Duran at the FCME-UNAM herbarium, where a voucher specimen (129564) was deposited. Two hundred g of fresh leaves, young shoots, a single phloem (obtained from woody branches) and 50 g of seeds were processed for 10 days in 300 ml of absolute MeOH at 4°C. 150 ml of the alcoholic extracts were processed to obtain semi-purified alkaloids according to García-Mateos et al., 2007. Qualitative alkaloid determination was carried out according to Villa-Ruano et al. (2009), with slight modifications: 2 µl of alkaloid extracts were injected in a Varian CP3800 gas chromatograph equipped with a Factor Four column VF-1ms (30 m X 0.25 mm I.D., covered with a dimethylpolysiloxane plate as the stationary phase) coupled to a Varian quadrupole mass spectrometer 320MS model. The mobile phase was helium at a 1 ml min^{-1} flow rate. The injector temperature was maintained at 200°C, and the oven temperature program was 150°C for 3 min and finally ramped up to 250°C for 20 min. The GC-MS run software was programmed to indentify an *m/z* range of 30-600. The obtained peaks were analyzed according to their mass spectra (Electron Impact) using the NIST Search 2.0 data base and the literature. Total alkaloid content was estimated according to Sreevidya and Mehrotra (2003).

Antibacterial assays

The methanolic and semi-purified alkaloid extracts were concentrated to dryness and resuspended in 20% DMSO to obtain concentrated stocks. *E. coli* DH5α and TOP 10'F (streptomycin resistant) strains were obtained from Sigma-Aldrich Co., and grown respectively in 6 ml of LB and LB-streptomycin 100 µg mL^{-1} starting from an inoculum of OD_{600}= 0.2 (Beckman DU 7400). The liquid media were then adjusted to 10, 20, 50, 80, 100, 150, 200, 250, and 300 µg ml^{-1} for each alkaloid and methanolic extract to perform *in vitro* broth microdilutions (Carson et al., 1995). Subsequently, the cells were immediately incubated with shaking at 37°C for 5 h. In all cases the added 20% DMSO extracts did not exceed 30 µl of final volume and the positive control groups were supplied with 30 µl of the same solution. After incubations the inhibition degree was estimated by end point OD_{600} measurements (Greenwood and O´Grady, 1975) and the minimum inhibitory concentration (MIC) was calculated according to Wiegand et al.,

(2008). *A. tumefaciens* LBA 4404 (rifampicin resistant) was cultured in 6 mL of YEB-rifampicin 100 μg mL^{-1} to be grown at 28°C for 24 h starting from an inoculum of OD_{600}= 0.2. Inhibition assays were done using the same broth dilutions as described above, then the microorganisms were incubated at 28°C for 3 days. The cell density and MIC were calculated as previously described for *E. coli*. All the experiments described were quintuplicated. The MIC of negative controls were obtained by dose response curves using ampicillin (Pentrexyl ®, Tecnofarma Co.) for *E. coli* and cefotaxime sodium (AMSA Laboratories S.A. de C.V.) for *A. tumefaciens* in the range of 1 to 100 μg ml^{-1} for each one.

Allelopathic experiments

Lactuca sativa (cv Great Lakes) seeds were obtained from a local seed market (El Sembrador S.A. de C.V). All undersized and damaged seeds were discarded. Germination was conducted in 100 mm Petri dishes containing a 9.0 cm sheet of Whatman no. 3 filter paper as support. Then, 25 lettuce seeds were placed per dish with 5 ml of a test solution (10, 20, 50, 80, 100, 150, 200, 250, and 300 μg ml^{-1}) or a control solution (0 μg ml^{-1} 20 % DMSO). All tests were quintuplicated. Dishes were covered with Parafilm to reduce evaporation and incubated in the dark at 25°C, in a controlled-environment growth chamber for 7 days. After this, the number of germinated seeds were counted (a seed was considered to be germinated when the radicle was at least 0.2 mm long). During the measurement process, the dishes were kept at 4°C to avoid subsequent growth. An analysis of variance and a multiple range test with the least significant difference (LSD) of Fisher was performed to validate the results at a significance level of 0.05 using the StatAdvisor® software.

RESULTS AND DISCUSSION

The alkaloid profile of leaves, seeds, young shoots and isolated phloem obtained from woody branches of *L. jaimehintoniana* is shown in Fig. 1. Five quinolizidine alkaloids identified as sparteine 5,6-dehydrolupanine, lupanine, nuttalline (traces), and d-thermopsine were eluted and characterized in respective order according to the NIST Search 2.0 data base, and their identity was corroborated in the literature (Vulfson and Zaikin, 1976; Tei and Wink, 1999; al-Azizi et al., 1994 and García-Mateos et al., 2007) (Table 1). These results assigned a particular alkaloid GC-MS profile to *L. jaimehintoniana* at this phenological stage. In terms of relative ion abundance, lupanine was the most plentiful compound in all the tissues. This property confers bitter characteristics to *L. jaimehintoniana* but at the same time it represents a source of Lupanine for distinct uses. Chemical profiles of Mexican lupines as *L mexicanus*, *L. exaltatus* and *L. campestris* report Lupanine as the main alkaloid and sparteine derivatives as the less abundant compounds (Martínez-Herrera et al., 2001; Ruiz and Sotelo, 2001; Zamora-Nátera et al., 2005, 2008). Sparteine was observed only in small quantities in leaves and seeds (Figs. 1LE and 1SE), whereas in the rest of the tissues there was no sign of this compound. On the other hand, a particular 5,6-dehydrolupanine increase in the young shoots and phloem with respect to the other tissues was observed (Figs. 1SH and 1PH). Considering the 5,6-dehydrolupanine increase in the phloem and its participation as an important precursor in the quinolizidine alkaloid pathways, its active transport in the vascular tissues in order to participate in the biosynthesis of more lupine alkaloids and/or contribute to chemical defense mechanisms in the studied species could be predicted (Saito et al., 1989). Despite *L. exaltatus* sharing 5,6-dehydrolupanine with *L. jaimehintoniana*, d-thermopsine has not been reported in the Mexican lupine species here mentioned. Nuttalline was detected in the shoots including phloem, but its presence was not observed in the other tissues (Figs. 1SH and 1PH). Lupine seeds regularly contain more alkaloids than other plant tissues (Ruiz–López et al., 2010); our results showed a homogeneous alkaloid pattern among the seeds and leaves of *L. jaimehintoniana* (Figs. 1LE and 1SE), at least at this phenological stage. Further phytochemical analyses are required to determine the alkaloid content in the other stages. According to Sreevidya and Mehrotra (2003), the estimated alkaloid concentration in the studied tissues was as follows: 32.5 mg g^{-1} seeds, 21.2

Table 1. Relative abundance and mass determination of quinolizidine alkaloids from *L. jaimehintoniana.*

Alkaloid	Tissue	% of minimum and maximum abundance*	M^+	Characteristic ions (% abundance)
Lupanine	+LE, SE, SH, PH	100	248	136(100) 149(51) 98(29) 248(31) 110(23)
5,6-dehydrolupanine	LE, SE, SH, PH	20-75	246	98(100) 96(32) 40(20) 246 (15) 55 (11)
d-thermopsine	LE, SE, SH, PH	30-75	244	98(100) 244 (15) 146(11) 136(8) 160 (6)
Sparteine	LE, SE	5-25	234	98(100) 137(95) 41(35) 193(23) 234 (14)
Nuttalline	SH, PH	0.5-1	264	32(100) 135(86) 43(54) 264(46) 150(40)

* Abundance respect to lupanine. +LE, leaves; SE, seeds; SH, shoots; PH, phloem.

Table 2.Effects of semi-purified alkaloid and methanolic extracts from *L. jaimehintoniana* and conventional antibiotics against *E. coli* and *A. tumefaciens* strains.

Microorganism	Alkaloid extract	Methanolic extract	Negative controls	Positive controls (DMSO effect)
	MIC (µg/mL)	MIC (µg/mL)	Ampicillin (µg/mL)	OD_{600}
Escherichia coli TOP 10	53.4 (LE)*, 50.2 (SE), 45.1 (SH), 43.3 (PH)	166.6 (LE), 153.4 (SE), 103.5 (SH), 115.3 (PH)	10.5	0.85±0.005
Escherichia coli DHα5	56.5 (LE), 61.1 (SE), 49.2 (SH), 37.8 (PH)	125.5 (LE), 111.4 (SE), 109.7 (SH), 103.6 (PH)	8.2	0.87±0.005
			Cefotaxime (µg/mL)	OD_{600}
Agrobacterium tumefaciens LBA 4404	130.3 (LE), 135.5 (SE), 132.7 (SH), 145.1 (PH)	395.5 (LE), 374.2 (SE), 385.9 (SH), 375.3 (PH)	5.3	0.9±0.005

*LE leaves; SE, Seeds; SH, shoots; PH, phloem

mg g^{-1} leaves, 9.3 mg g^{-1} young shoots, and 7.4 mg g^{-1} phloem. These data demonstrated that seeds are the best alkaloid source, followed by leaves. A slight difference between the phloem and young shoots was observed, but according to the obtained results, phloem is the main alkaloid provider in the shoots of *L. jaimehintoniana*.

The results of antibacterial assays are shown in Table 2. Clear growth inhibition was detected in *E. coli* DH5α and TOP 10 (streptomycin-resistant) strains at relatively low concentrations of both alkaloid and methanolic extracts; nevertheless, alkaloid fractions were more effective than the methanolic ones. Compared to the control groups, these experiments clearly reflected the sensitivity of the *E. coli* strains to the *L. jaimehintoniana* isolates. Despite the fact that the evaluation of methanolic extracts resulted in high MIC values, the numbers revealed a direct correlation with the inhibition patterns of semi-purified alkaloids. This evidence strongly suggests that the endogenous alkaloids of *L. jaimehintoniana* are associated with the anti-*E. coli* effect. Nonetheless, this does not repudiate the synergistic antibacterial activity of other natural products dissolved in methanolic extracts. The MIC range of 37 and 61 µg mL^{-1} for *E. coli* strains was estimated for the different alkaloid preparations of *L. jaimehintoniana*. Antibacterial experiments using leaves and seed solutions resulted in similar MIC values, while the assessment of phloem and young shoots extracts in both DH5α and TOP10 F' strains gave the lowest MIC values. The basic difference in alkaloid content in these tissues is the high abundance of d-thermopsine in the

Table 3. Toxic effects of semi-purified alkaloid extracts from *L. jaimehintoniana* on the germination of *L. sativa* seeds (n=25).

μg mL^{-1}	LE	SE	SH	PH
0	22.4±1.6 a	23.2±1.4 a	23.8±1.6 a	23.6±1.6 a
10	17.4±1.5 b	24.2±0.4 a	22.0±1.5 a	23.2±0.8 a
20	19.6±1.8 b	23.0±1.2 a	22.2±1.6 a	21.8±1.9 a
50	14.0±2.5 c	11.6±1.1 a	21.8±2.1 a	13.0±1.5 b
80	13.4±2.0 c	15.4±2.0 a	15.2±2.1 b	12.4±3.4 b
100	14.0±0.7 c	10.2±1.7 b	12.2±1.4 c	12.0±1.8 b
150	10.8±2.5 d	8.6±1.1 c	8.2±1.9 d	7.0±1.5 c
200	4.6±2.7 e	3.4±2.7 c	7.2±3.0 d	1.6±2.0 d
250	3.6±1.6 e	1.8±1.7 c	3.2±2.3 e	3.8±3.8 d
300	1.4±1.6 e	0.6±0.8 d	1.4±1.6 e	1.2±1.3 d

*LE, leaves; SE, Seeds; SH, shoots; PH, phloem. Means with diverse letter are statically different.

Table 4. Toxic effects of methanolic extracts from *L. jaimehintoniana* on the germination of *L. sativa* seeds (n=25).

μg mL^{-1}	LE	SE	SH	PH
0	23.0±1.2 a	23.6±1.6 a	24.0±1.2 a	22.8±1.9 a
10	24.0±1.7 a	24.4±0.5 a	22.6±1.5 a	22.6±2.0 a
20	12.6±1.5 b	22.4±1.3 a	22.2±1.0 a	23.8±1.3 a
50	13.6±1.3 b	21.6±1.3 a	15.6±2.3 b	22.0±1.0 a
80	12.8±1.6 b	20.6±2.0 a	18.2±0.8 c	14.8±3.7 b
100	5.6±1.9 c	15.0±3.5 b	15.0±2.0 c	15.8±3.0 b
150	3.4±1.5 d	8.8±1.3 c	9.0±1.8 d	8.0±1.5 c
200	1.8±0.8 d	10.6±1.1 c	11.8±1.7 d	2.6±1.5 d
250	1.8±0.8 d	9.2±1.6 c	10.0±2.8 d	3.8±1.3 d
300	1.0±1.2 d	2.0±2.1 d	1.2±1.6 e	2.4±1.5 d

*LE, leaves; SE, Seeds; SH, shoots; PH, phloem. Means with diverse letter are statically different.

phloem (Fig. 1PH). It is probable that d-thermopsine promoted the observed effect, since there is evidence of the induction of cell toxicity by this alkaloid (Geng et al., 2005); or it acted synergistically with other lupine alkaloids to heighten the antimicrobial activity (Wink, 1984). Other approaches using alkaloid fractions from *L. angustifolius* report a weak effect in some *E. coli* strains (not the used in this work) (Erdemoglu et al., 2007). However, the metabolic profile of such extracts did not include the presence of d-thermopsine. Thus the present results demonstrated an efficient anti-*E. coli* activity by both methanolic and semi-purified alkaloids from *L. jaimehintoniana*.

A. tumefaciens is a phytopathogen that infects a great variety of dicotyledonous and some gymnospermous plants causing the crown gall disease (López et al., 1989). Due to the colonization methods and virulence of *A. tumefaciens*, there are few and expensive ways to manage it. Biological control using plant extracts can provide an alternative, although there are few reports about this issue (Sahi et al., 1990; Stanojević et al., 2010). Our results indicate that the alkaloid fractions as well as methanolic extracts had an acceptable effect on the growth inhibition of the *A. tumefaciens* 4404 strain (Table 2). A MIC range between 130 and 146 μg mL^{-1} and 375

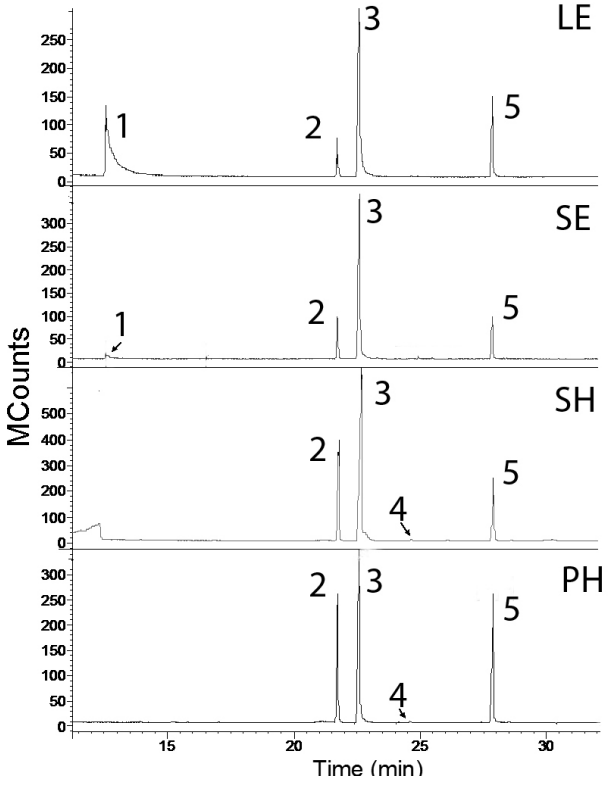

Fig. 1. Quinolizidine alkaloid profile of *L. jaimehintoniana*, obtained GC-MS. LE, leaves; SE, seeds; SH, young shoots; PH, phloem. 1, sparteine (12.58 min); 2, 5,6-dehydrolupanine (21.71 min); 3, lupanine (22.58 min); 4, nuttalline (24.63); 5, d-thermopsine (27.85 min).

and 395 μg mL^{-1} was observed for both alkaloid fractions and methanolic extracts, respectively. However, there was not a remarkable difference between the assayed extracts from the different organs. To our knowledge, this is the first approach to determine the anti-*A. tumefaciens* properties using bitter lupine extracts. Many Leguminosae including bitter lupines are recalcitrant species; contrarily some sweet varieties such as *L. luteus* have been successfully transformed using *A. tumefaciens* strains (Pniewski et al., 2006). The high endogenous levels of quinolizidine alkaloids in bitter lupines could be correlated to the recalcitrant behavior. It is remarkable that these results open the possibility to perform *in vivo* antibacterial evaluations in order to ensure the efficacy of the *L. jaimehintoniana* alcoholic extracts and semi-purified alkaloids.

The allelopathic effect of the methanolic and semi-purified alkaloid extracts of *L. jaimehintoniana* is shown in Tables 1 and 2. The viability of *L. sativa* seeds used in this work was 93.2%. Multiple range tests revealed statistically significant variances between treatments in both group experiments ($P<0.05$). Clearly, both methanolic and semi-purified alkaloids had a clear inhibitory effect starting from 50 μg mL^{-1}. Considering the n=25 seeds, the application of the maximum inhibitory concentration for the studied cases (300 μg mL^{-1}) resulted in 94.4% of inhibition. Previous assays using *L. mexicanus* extracts, seeds from *Amaranthus hybridus* and *Echinochloa crus galli*, as well as different varieties of *L. sativa*, demonstrated an allelopathic effect on the order of milligrams per milliliter, but only achieving 80% of inhibition (Wink, 1983; Zamora-Nátera, et al., 2008). The best efficiency of *L. jaimehintoniana* preparations could be associated with its specific alkaloid profile and concentration.

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