LC-ESI-HRMS-BASED CHEMICAL CHARACTERIZATION OF *Lupinus bogotensis* ROOTS

CARACTERIZACIÓN QUÍMICA POR LC-ESI-HRMS DE LAS RAÍCES DE *Lupinus bogotensis*

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ABSTRACT

Plants of the genus *Lupinus* (Fabaceae) have been studied due to the occurrence of different compounds, especially those possessing quinolizidine and isoflavone-like structures. These kinds of compounds are characterized by both medical and industrial applications, providing various benefits to human being. However, organs such as roots have not been equally studied and there is a lack of such records for native species. Therefore, in the present study, the chemical composition of nodulated roots from greenhouse-established *L. bogotensis* plants was determined. The resulting crude ethanolic extract was then analyzed by LC/HRMS and chemical nature of most compounds was determined by analyzing the high resolution mass spectra. Recorded profile showed adequate separation allowing tentative identification of detected compounds. 47 secondary metabolites (mainly isoflavones and quinolizidine-type compounds) were thus identified. Most phenolic compounds were found to be conjugated flavonoids (e.g., genistin and genistein malonylglucoside) and lupanine, sparteine and hydroxylupanine were noticed as the main alkaloids. Among alkaloid-like compounds, dehydromitomycin C, a compound produced by *Streptomyces caespitosus* was identified. Lupadienediol (a lupane-type triterpene recognized for being involved in rhizobacteria:legumes symbiosis) was the only terpene-related component identified in the extract. The present work corresponds to the first report on the chemical composition of *L. bogotensis* root and constitutes an adequate basis for phytoconstituents finding from nature to support the use of native species.

Keywords: Secondary metabolites, Native species, *Lupinus bogotensis*, Root, LC/MS.

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RESUMEN

Las plantas del género *Lupinus* (Fabaceae) han sido estudiadas debido a la ocurrencia de diferentes compuestos, principalmente aquellos con núcleo quinolizidínicos e isoflavónico, los cuáles se caracterizan por presentar aplicaciones tanto medicinales como industriales, proporcionando diversos beneficios. Sin embargo, órganos como las raíces no han sido igualmente estudiados y, mucho menos, se tiene tales registros para especies nativas. Por tanto, en el presente trabajo se determinó la composición química de raíces noduladas de *L. bogotensis* de plantas establecidas en invernadero. El extracto etanólico crudo resultante se analizó por LC/HRMS y la naturaleza química de los compuestos mayoritarios se determinó mediante el análisis de los espectros de masas de alta resolución. El perfil obtenido evidenció una adecuada separación que permitió por consiguiente la identificación tentativa de los compuestos detectados. De esta manera, se identificaron 47 metabolitos secundarios correspondientes principalmente a isoflavonas y alcaloides quinolizidínicos. La mayor cantidad de compuestos fenólicos se identificaron como flavonoides conjugados (genistína y genisteína malonilglucósido). Los alcaloides mayoritarios fueron isómeros de lupanina, esparteína e hidroxilupanina. Entre los compuestos alcaloidales, se identificó a la mitomicina, un compuesto producido por *Streptomyces caespitosus*. En cuanto a los terpenoides, se identificó como único componente al lupadienediol, un triterpeno de tipo lupano reconocido por estar involucrado en la simbiosis con rizobacterias en leguminosas. Este trabajo corresponde al primer reporte sobre la composición química de la raíz de *L. bogotensis*. Asimismo, se constituye como una adecuada base para la búsqueda de fitoconstituyentes que respalde el aprovechamiento de especies nativas.

Palabras clave: Metabolitos secundarios, Especies nativas, *Lupinus bogotensis*, Raíz, LC/MS.

INTRODUCTION

Plants belonging to the genus *Lupinus* are legumes comprising ca. 164 species (www.itis.gov) mainly distributed in the Mediterranean and America (Aïnouche *et al.*, 2004). These plants are characterized by constitutive synthesis of quinolizidine alkaloids involving defense functions against herbivores and pathogens as well as competition properties on other plants (Wink, 2003; El-Shazly *et al.*, 2001, Wink *et al.*, 1995). In addition, *Lupinus* spcies are also characterized by the synthesis of flavonoids presenting symbiotic associations with Rhizobiaceae bacteria (Barsch *et al.*, 2006) since flavonoid excretion is required for the development of specialized structures (e.g., nodules) in plant roots to use atmospheric nitrogen (Long, 1996; Fernández-Pascual *et al.*, 2007). Reduction of ammonium-derived nitrogen is accompanied by changes in the plant metabolism which may modify the production of such compounds. In Fabaceae, it has been demonstrated that the chemical composition of nodulated individuals is differential to those of plants without such interaction (Paul &

Driscoll, 1997; Barsch *et al*., 2006). Despite this fact, so far the relationship on rhizobacteria:*Lupinus* symbiosis regarding its chemical composition is still unknown.

In Colombia, it has been identified 54 *Lupinus* species with some uses at different levels in the fields of food, medicinal, cultural, fodder and transformation (Barney, 2001). Among these plants, *L. bogotensis* (a native legume to the Colombian Andes) is characterized by its fast growth, abundant seed bank formation, adaptability to different environmental conditions, and as an enabler for secondary vegetation establishing within ecological restoration initiatives (Gomez-Ruiz *et al*., 2013; Vargas-Rios *et al*., 2009). Furthermore, this plant has been recently investigated in Colombia for its potential use as control alternative for insect pests (Molina *et al*., 2014; Molina *et al*., 2010); while aerial part is traditionally consumed through oral administration as medicine in Bolivia (Macía *et al*., 2005). However, ornamental usage is its most known exploitation (Osorio *et al*., 1959).

Presence of *L. bogotensis* in a variety of environments is mostly due to its ability for tolerating frost, drought and nutrients-poor areas (Vargas-Rios *et al*., 2009). These capabilities let it adapting to several zones such as dry and wet forest, moorland and some transformed areas (secondary vegetation-containing areas and grasslands) (Groom, 2012; Barney, 2011). *L. bogotensis* is also able to be settled in places where other plants fail to adapt and for colonizing disturbed areas (Vargas-Rios *et al*., 2009; Meredith, 1988). These features could be rationalized by secondary metabolism variations as adaptive responses into ecosystem changes becoming therefore this plant a suitable model for bioprospecting studies.

As mentioned above, various studies have shown that *Lupinus* plants synthesize a variety of phenolic-type metabolites and, due to its capability for fixing atmospheric nitrogen, also produce *N*-containing compounds such as alkaloids (Wink, 2013; Harborne 1996). However, there is a lack of studies on chemical composition of roots of *Lupinus* plants and they are accordingly limited to flavonoids determination (Wojakowska *et al*., 2013; Kachlicki *et al*., 2005; Stobiecki & Wojtaszek, 1990; Lane & Newman, 1987). In addition, there are no reports on native plants like *L. bogotensis.* According to this context, a LC-ESI-HRMS-based chemical composition of nodulated *L. bogotensis* roots under greenhouse conditions was then investigated in the present study for the first time.

EXPERIMENTAL

Plant Material

L. bogotensis seeds, obtained from wild plants in Samacá, Boyaca (N05°29'06,2' W73°28'37.8''), were germinated into a humid chamber. A voucher specimen from a mother plant was deposited at Herbario Nacional Colombiano (collection number COL576245). A resulting plantlet was planted into a polyethylene bag (1.17 L) with a sand-earth (3:1) substrate with irrigation every two days and without fertilization or fumigation during the experiment. The plants were kept in UMNG's greenhouse, Cajicá, at 2,548 m above sea level and 16 °C average temperature, from June-December 2014. Effective nodules-containing roots were randomly collected to prepare an ethanolic extract.

Preparation of Ethanol Extract

Liquid nitrogen-freezing root materials were macerated and resulting powder was then lyophilized. Dried material (1.5 g) was extracted with 96% ethanol (1:1.5 w/v) for 30 minutes using a three-cycle ultrasound-assisted solid-liquid protocol. Resulting mixture was filtered and concentrated

Tabla 1. Identified compounds in *L. bogotensis* root-derived extracts using LC/HRMS.

No	Rt (min)	Metabolite [a]	molecular formula	exact mass	m/z (exp)	error (ppm)	RA[b] (%)	Type[c]
1	1.95	cassigerol B	$C_{21}H_{16}O_6$	365.1025	365.1057	8.765	2.94	E
2a		lupanine isomer 1	$C_{15}H_{24}N_2O$	249.1966	249.1986	8.026		A
2b	2.18*	spartein isomer 1	$C_{15}H_{26}N_2$	235.2174	235.217	-1.701	12.00	A
2c		hydroxylupanine isomer 1	$C_{15}H_{24}N_2O_2$	265.1916	265.1925	3.382		A
3	2.98	hydroxylupanine isomer 2	$C_{15}H_{24}N_2O_2$	265.1916	265.1924	3.005	1.66	A
4	3.24	dehydrolupanine	$C_{15}H_{22}N_2O$	247.1810	247.1814	1.463	0.58	A
5	3.50	lupanine isomer 2	$C_{15}H_{24}N_2O$	249.1966	249.1987	8.427	8.98	A
6	4.80	dehydromitomycin C	$C_{16}H_{18}N_3O_5$	332.1246	332.1230	-4.817	0.56	A
7	6.08	angeloyloxydihydroxylu-panine	$C_{20}H_{30}N_2O_5$	379.2232	379.2233	0.264	1.08	A
8a	7.43*	hydroxytigloyloxylupanine	$C_{20}H_{30}N_2O_4$	363.2292	363.2289	-0.826	0.45	A
8b		dioxopartein	$C_{15}H_{22}N_2O_2$	263.1759	263.176	0.380		A
9	8.48	tetrahydroxyisoflavone glucoside isomer 1	$C_{21}H_{20}O_{11}$	449.1084	449.1105	4.698	1.53	I
10	9.89	lupanine isomer 3	$C_{15}H_{24}N_2O$	249.1966	249.1969	1.204	0.68	A
11	10.38	genistein diglucoside	$C_{27}H_{30}O_{15}$	595.1663	595.1692	4.873	1.62	I
12	13.59	tetrahydroxyisoflavone glucoside isomer 1	$C_{21}H_{20}O_{11}$	449.1084	449.1094	2.249	2.52	I

13	13.94	tetrahydroxyisoflavone glucoside isomer 1	$C_{21}H_{20}O_{11}$	449.1084	449.1094	2.249	0.87	I
14	16.02	hydroxypseudobaptige-nin glucoside	$C_{30}H_{36}O_4$	461.2692	461.2679	-2.218	0.48	I
15	16.55	tetrahydroxyisoflavone isomer 1	$C_{15}H_{10}O_6$	287.0556	287.0555	-0.220	0.96	I
16	17.23	genistin	$C_{21}H_{20}O_{10}$	433.1134	433.1146	2.771	3.90	I
17	17.91	hydroxygenistein isomer 2	$C_{15}H_{10}O_6$	287.0556	287.0555	-0.220	1.41	I
18	18.97	trihydroxyisoflavone isomer 1	$C_{15}H_{10}O_5$	271.0606	271.0603	-1.286	0.65	I
19	21.31	genistein malonylglucoside	$C_{24}H_{22}O_{13}$	519.1138	519.115	2.312	2.83	I
20	23.44	tetrahydroxyisoflavone isomer 2	$C_{15}H_{10}O_6$	287.0556	287.0556	0.129	0.70	I
21	23.83	Tetrahydroxyisoflavone isomer 3	$C_{15}H_{10}O_6$	287.0556	287.0554	-0.568	4.68	I
22	24.26	Tetrahydroxyisoflavone isomer 4	$C_{15}H_{10}O_6$	287.0556	287.0553	-0.916	0.69	I
23a	26.32*	aminohexadecanediol	$C_{16}H_{35}NO_2$	274.2746	274.2747	0.350	0.93	AG
23b		lupinisoflavone A isomer 1	$C_{20}H_{16}O_6$	353.1025	353.1005	-5.702		I
24	26.59	luteone	$C_{20}H_{18}O_6$	355.1181	355.1171	-2.816	0.54	I
25	26.84	trihydroxyisoflavone isomer 2	$C_{15}H_{10}O_5$	271.0606	271.0611	1.666	3.76	I
26	27.15	barpisoflavone A isomer 1	$C_{16}H_{12}O_6$	301.0712	301.0703	-2.989	0.91	I
27	27.64	barpisoflavone A isomer 2	$C_{16}H_{12}O_6$	301.0712	301.0698	-4.650	0.48	I
28	27.78	lupinisoflavone A isomer 2	$C_{20}H_{16}O_6$	353.1025	353.1025	-0.038	0.89	I

29	28.40	lupadienediol	$C_{30}H_{48}O_2$	441.3733	441.3732	-0.126	1.50	T
30	29.82	lupinisoflavone A isomer 3	$C_{20}H_{16}O_6$	353.1025	353.1027	0.529	3.49	I
31a	30.34*	hydroxypseudobapti-genin	$C_{16}H_{10}O_6$	299.0555	299.055	-1.672	2.55	I
31b		tetrahydroxystilbene	$C_{14}H_{12}O_4$	245.0813	245.0796	-6.936		S
32	30.83	hydroxypseudobapti-genin	$C_{16}H_{10}O_6$	299.0555	299.056	1.672	4.05	I
33	31.24	lupinisoflavone A isomer 4	$C_{20}H_{16}O_6$	353.1025	353.1024	-0.321	0.75	I
34	31.44	lupinisoflavone J	$C_{25}H_{26}O_7$	439.1756	439.1755	-0.228	1.54	I
35	31.62	lupinisoflavone A isomer 5	$C_{20}H_{16}O_6$	353.1025	353.102	-1.454	0.67	I
36	31.75	lupinisoflavone A isomer 6	$C_{20}H_{16}O_6$	353.1025	353.1023	-0.604	1.01	I
37	32.32	lupinisoflavone A isomer 7	$C_{20}H_{16}O_6$	353.1025	353.1026	0.246	2.57	I
38	33.50	barpisoflavone C isomer 1	$C_{21}H_{18}O_6$	367.1181	367.1172	-2.452	1.16	I
39	34.55	barpisoflavone C isomer 2	$C_{21}H_{18}O_6$	367.1181	367.1182	0.272	1.07	I
40	35.25	trimethoxyisoflavan	$C_{18}H_{20}O_4$	301.1439	301.1425	-4.649	10.96	Io
41	36.36	lupichromone	$C_{19}H_{22}O_4$	315.1596	315.1577	-6.027	1.48	C
42	46.50	*N*-methylhexadecanoyl-pyrrolidine	$C_{21}H_{41}NO$	324.3266	324.3264	-0.740	5.91	A

[a] correct isomer was not identified; [b] Mean totalized relative abundance; [c] A: Alkaloid; AG: Fatty alcohol; C: Chromone; S: Stilbene; I: Isoflavone; Io: Isoflavan; T: Triterpene.
* Coelution.

under reduced pressure to yield the crude extract (Colegate & Molyneaux, 2008).

LC-ESI-HRMS Analysis

Chemical characterization of ethanol extract was performed on a Shimadzu liquid chromatograph coupled to a Bruker micrOTOFQ II high resolution-mass spectrometer. A Phenomenex Luna® C18 column (150x2.0 mm, 3µm) and mixtures of acetonitrile and trifluoroacetic acid (0.05%) were used as chromatographic system under 254 nm wavelength monitoring. Mass spectrometry was operated in positive ion mode using an ESI source. Final chromatogram was obtained by optimization of separation conditions. Resulting TIC was baseline-corrected, normalized and autoscaled in order to achieve an appropriate determination of the areas under the curve for each signal (i.e., each compound) for calculating the relative abundance percentage.

Compounds Identification

Tentative identity of compounds was performed by determining the molecular formulas using exact mass measurements obtained from high resolution spectra. When possible, chemical nature of compounds was

determined using the databases Knapsack (http://kanaya.naist.jp) and CHEMnetBASE – Dictionary of Natural Products (http://dnp.chemnetbase.com).

RESULTS AND DISCUSSION

LC-HRMS-based analysis of *L. bogotensis* roots-derived extract allowed detection and identification of 47 secondary metabolites. Compound identification was performed using the information provided by mass spectra and determination of molecular formula through exact mass measurements compared to databases Knapsack and CHEMnetBASE. According to signals elution, following types of metabolites were found: alkaloids (12), stilbenes (2), flavonoids (30), fatty alcohols (1), terpenoids (1) and chromones (1). Table 1 shows a list enclosing those identified compounds. Numbers for each type of compound were differentiated by colors: alkaloids in red, phenolics in green, terpenoids in blue and other compounds in black.

L. bogotensis root material mostly presented phenolic metabolites (62.19% totalized relative abundance). These compounds were represented by stilbenes (2) and isoflavone-type flavonoids (29) and an isoflavane
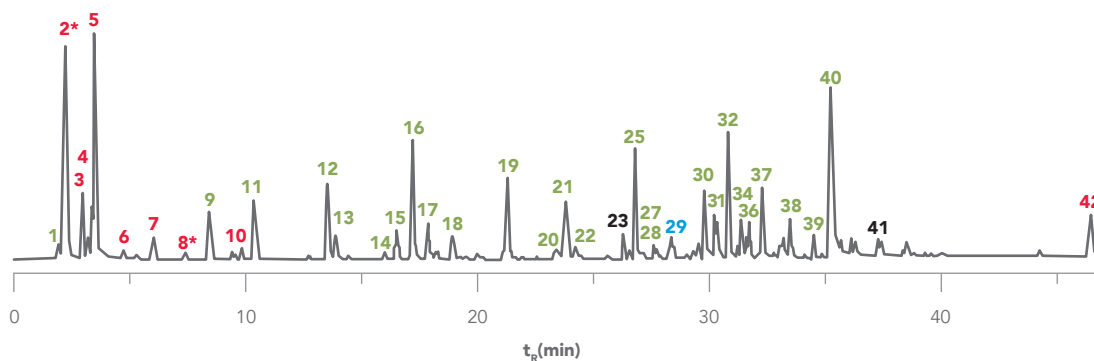

Figure 1. LC/LRMS-derived TIC profiles of *L. bogotensis* root extract.
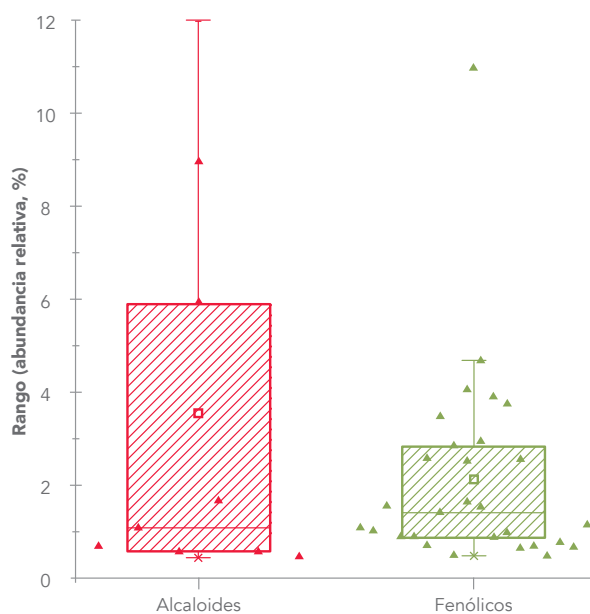
Figure 2. Dispersion on phenolic and alkaloid-type relative abundance. Each point inside the boxes corresponds to a relative abundance value of a metabolite within the group.

(1) (Figure 1). The second group of main metabolites was found to be alkaloids (31.89% totalized relative abundance) and triterpene-related lupadienediol (1.50%).

Box-plot (Figure 2) shows alkaloids have higher relative abundance values to than of phenolics (higher mean and median values) involving a greater dispersion. However, phenolics showed the highest amount of metabolites identified to that of alkaloids.

Alkaloid-containing co-elution 2a-c (lupanine, sparteine and hydroxylupanine) (12.00%) was the greatest relative abundant signal, followed by trimethoxyisoflavan 40 (10.95%) and lupanine 5 (8.97%), and other compounds such as flavonoids tetrahydroxyflavone 21 (4.67%), hydroxypseudobaptigenin 32 (4.05%), genistin 16 (3.90%), and genistein 25 (3.75%). Lupinisoflavones A, C and J and barpisoflavone C were also found in smaller amounts (abundances < 3.5%) (Table 1). Seven isomers of

lupinisoflavone A (10.77%) (a prenyl-containing metabolite) were interestingly found as chemical components, suggesting a mixed biosynthetic pathway in *L. bogotensis* roots.

LC/HRMS profiles indicated an adequate separation for chemical characterization of *L. bogotensis* roots-derived extracts. This technique has been successfully used in the chemical study of several *Lupinus* plants (Wojakowska *et al.*, 2013; Stobiecki *et al.*, 2010; Kachlicki *et al.*, 2008; Kachlicki *et al.*, 2005) and for monitoring the composition comprising high-polarity secondary metabolites such as flavonoids (Rijke *et al.*, 2006; Bednarek *et al.*, 2003; Gu & Gu, 2001).

Flavonoids are a group of ubiquitous polycyclic compounds often synthesized by legumes and they tend to occur in conjugate manner (Wojakowska *et al.*, 2013). This kind of metabolites are considered as biologically important agents since they exhibit several functions during the lifecycle of plants (Dixon & Paiva, 1995) such as defense, signaling and chemoattraction involved in mutualistic associations with soil microorganisms [e.g., *Lupinus-Bradyrhizobium* (Jarabo-Lorenzo *et al.*, 2003)]. In the roots of *L. bogotensis* nodulated plants were mostly found conjugated flavonoids which was consistent with those results presented by Kachlicki *et al.* (2005) and Wojakowska *et al.* (2013), who evaluated *Lupinus*-derived radicular materials from North American and Mediterranean plants.

In addition, the high content of alkaloids can be related to the symbiotic relationship with atmospheric nitrogen-fixing rhizobacteria (Gagnon *et al.*, 1995). Alkaloid profiles are generally consistent among species, but their patterns can vary according to the storing organ (Carey & Wink, 1994). In *Lupinus* plants, the most abundant alkaloid content can be found in seeds in comparison to that of other plant parts (Torres *et al.*, 2002). These *N*-containing molecules can be found in *ca.* 20% plant species

and play defense functions against herbivores and pathogens (Zulak *et al.*, 2006). Main alkaloids found in this study (e.g., lupanine, sparteine and hydro-xylupanine) are considered as the principal toxic constituents in various *Lupinus* species and, for that reason, they have been included in several studies for their potential pharmacological uses (Zulak *et al.*, 2006). However, some of these metabolites have a restricted distribution for certain *Lupinus* species such as multiflorine (a dehydrolupanine isomer). This quinolizidine alkaloid has been reported only in *Lupinus* plants, and it is biogeographically considered one of the largest components in South American species, but randomly occurred for those plants from North America (Torres *et al.*, 1999). No bicyclic quinolizidine alkaloids were identified (e.g., lupinine), which usually appear involving a sporadic

and minor presence in South American *Lupinus* plants (Torres *et al.*, 1999).

Similarly, in those phytochemical studies on plants extracts have been reported the presence of compounds produced by microorganisms. In this study, it was identified dehydromitomycin C, an aziridine alkaloid produced by the actinobacteria *Streptomyces caespitosus* (Danshiitsoodol *et al.*, 2006). This microorganism, usually found on soil (Crocker & Jones, 1983), could be considered as responsible for the presence of this metabolite in the root extract. Finally, lupadienediol 29 was the only identified terpenoid-related compound. This metabolite is a lupane-type triterpene, which is known to be involved in the formation of hydrophobic and antibiotic barriers during root nodules development within rizobacterias:legumes symbiosis (Hayashi
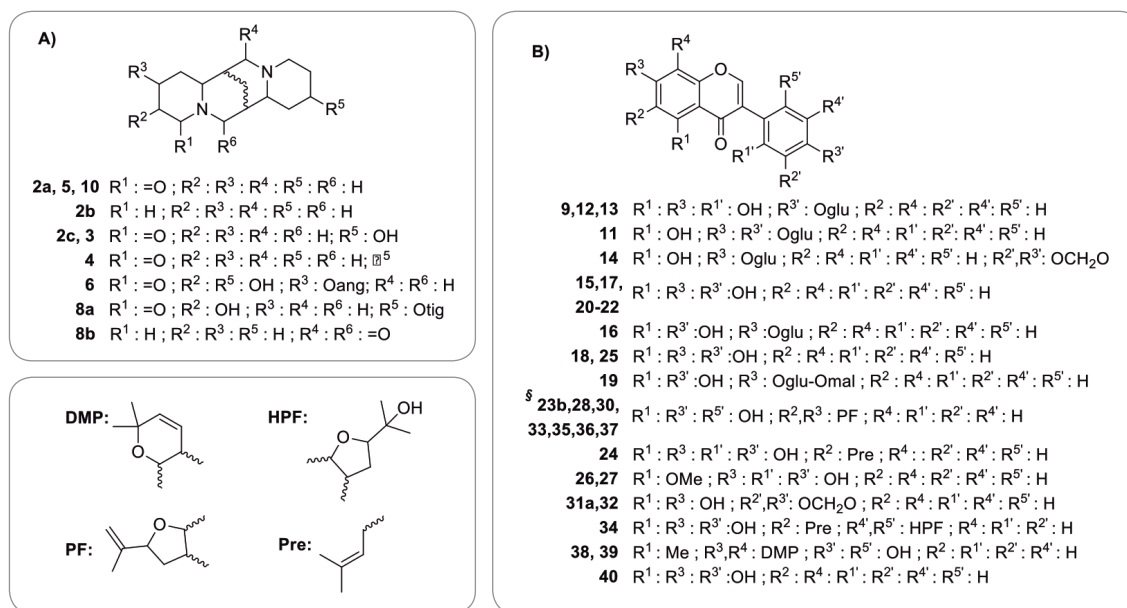

Figure 3. Planar chemical structures of most-probable isomers of A) quinolizidine alkaloids and B) isoflavones identified in *L. bogotensis* roots (glu: glucose; ang: angeloyl; tig: tigloyl; mal: malonate); §Most-probable isomer no differenced due to several possibilities of positions for a free/cycled prenyl and hydroxyls groups.

et al., 2004). Studies such as those by Barsch *et al.* (2006) on the symbiotic interaction between *Sinorhizobium meliloti* and *Medicago sativa* (alfalfa) show that there are changes in metabolic composition according to the symbiotic association in the host. When alfalfa plants were unable to establish symbiosis that effectively fixes nitrogen, they had low levels of glutamine and glutamate and, when plants exhibit nodulation, ketoglutaric acid amount was found to be increased implying a diminished nitrogen load in the plant.

In conclusion, isoflavones and alkaloids were mostly identified among 47 secondary metabolites detected in *L. bogotensis* root ethanol extract. Most phenolic compounds were found to be conjugated flavonoids. Alkaloids were found to be related to quinolizidine-like compounds, but, dehydromitomycin C was also detected. Although there is a lack of studies on chemical composition of roots from *Lupinus* plants, identified compounds were consistent to that of previous studies. The present work accordingly corresponds to the first report on the chemical composition of *L. bogotensis* root and constitutes an adequate basis for phytoconstituents finding from nature to support the use of native species.

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