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Artículo científico

Foliar exudates of *Blakiella bartsiifolia* (S.F. Blake) Cuatrec. (Asteraceae, Astereae). A Preliminary study of the Chemical Composition.

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Resumen

Se presenta el primer análisis fitoquímico de *Blakiella bartsiifolia*, especie endémica del hiperpáramo periglacial andino de Mérida. Del exudado foliar se aislaron e identificaron mediante RMN de alto campo dos flavonoides (5,4´*-dihidroxi*-3,6,7,3´*-tetrametoxiflavona;* 5,7,4´*-trihidroxi*-3,8,3´*-trimetoxiflavona*) y un diterpeno labdánico (*labda-7,14-dien-13(S)-ol*). Se examinó asimismo la capacidad de absorción de la radiación UV del exudado foliar, observándose un coeficiente de absorción específico significativamente mayor en las plantas que, en su estado natural, estaban más expuestas a la radiación solar.

Palabras clave: *Blakiella bartsiifoli*; Asteraceae; exudado; flavonoides; diterpeno labdánico.

Abstract

This is the first phytochemical study of *Blakiella bartsiifolia*, an endemic species of Mérida. From the foliar exudates two flavonoids ((5,4´*-dihydroxy*-3,6,7,3´*-tetramethoxyflavone;* 5,7,4´*-trihydroxy*-3,8,3´*-trimethoxy-flavone*) and a labdane diterpene (*labda-7,14-dien-13(S)-ol*) were identified by high field NMR spectroscopy. Plants naturally exposed to direct sunlight possessed a significantly higher specific absorbance coefficient in the UV region than plants growing in shaded areas.

Keywords: *Blakiella bartsiifolia*; Asteraceae; exudates; flavonoids; labdane diterpene.

Introduction

Blakiella bartsiifolia (S.F. Blake) Cuatrec. (Asteraceae, Astereae), an endemic member of the hiperpáramo plant community in the northern Andes, was first included within the genus *Podocoma* by Sidney Blake (1920)[1]. This genus has a restricted distribution in southeastern South America [2]. Although Cuatrecasas first assigned this plant as *Podocoma bartsiaefolia*, this was changed later to its presently accepted denomination, *Blakiella bartsiifolia*, genus included in Hinterhuberinae subtribe[2,3].

B. bartsiifolia is a perennial sufruticous plant about 30 cm tall. Several unbranched stems develop from a common woody underground pod. Single yellow capitulum crowns each stem and aerial parts wither after fructification during the dry season (December-April). It appears to grow preferentially within rocky outcrops, in crevices and lower openings where small pockets of highly organic soil accumulate. Low density populations are thus formed within a species-rich community of low height plants. *B. bartsiifolia* is an endemic component of high areas as it is found only in a few of such high páramo communities.

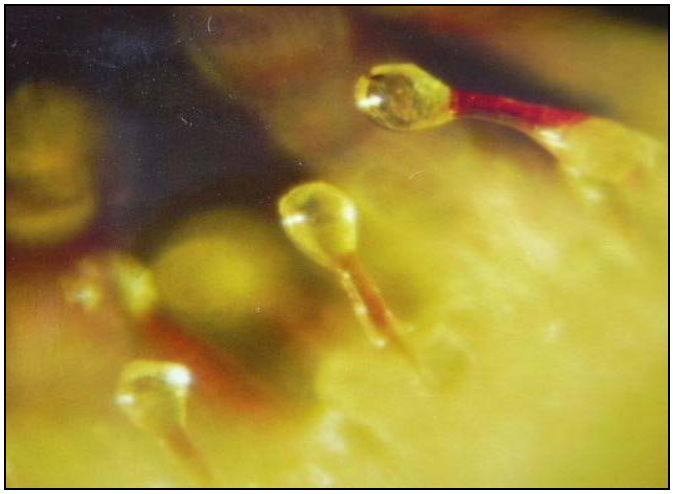

Figure 1: Glandular trichomes of *Blakiella bartsiifolia* (S. F. Blake). (100 X) showing colorless exudate on the tips. (Photograph: M. E. Alonso)

This species has been reported in Páramo de Timotes (3000 - 3500 m asl) (Mérida), Páramo de Piedras Blancas (4000 – 4200 m asl) (Mérida), Laguna Negra (3400 m asl) (Mérida), Páramo de Los Leones (4400 m asl) (Mérida), between Chachopo and Apartaderos (2900-4000 m asl) (Mérida), and Páramo de Cachaco (3700 m asl) (Trujillo)[1]. *B. bartsiifolia* has also been collected in Páramo Almorzadero, Norte de Santader, Colombia (3600 – 3700 m asl)[4].

The most remarkable feature of *B. bartsiifolia* is the striate cuticle covering abaxial and adaxial leaf surfaces, which probably dampen the strong incident sunlight of the high Páramo. In addition, the leaf surfaces are densely covered by at least two types' trichomes (Figure 1). A through description of these structures will be published elsewhere. Collectively, the trichome assembly impart a sticky sensation upon touching any aerial part of the plant which may protect it from colonization by mites and springtails among other diminutive arthropods, very common in cold areas[5,6]. Also it may protect the plant from herbivory by larger insects such as abundant páramo grasshoppers in *B. bartsiifolia* habitats (*Meridacris subaptera*, *Chibchacris fernandezi*; Cigliano M.M, unpublished obsv.).

The resource allocation to the copious exudate of trichomes must involve considerable metabolic cost which is critical in a resource-limited environment such as the high Páramo. Thus, it was hypothesized that leaf trichome exudates should exert a double-decked protection of exposed plant parts to (1) environmental sources of stress, biological (predation by herbivorous insects, fungal growth, intra-plant competition), and (2) physical (desiccation, excessive solar UV A/B radiation, extreme variations of air temperature), all prevailing in the high Páramo where *B. bartsiifolia* occurs. The aims of the present introductory study were: a) to evaluate the overall UV radiation filtering capacity of cuticular extracts of sun-exposed vs. non-exposed mature individuals of *B. bartsiifolia*, and 2) to study the chemical composition of abundant components of the trichome exudates.

Materials and Methods

General Experimental Procedures

The NMR spectra were measured in $CDCl_3$ or DMSO-d_6 solution, (solvent signal as reference), at 400 MHz (^{1}H NMR) and 100 MHz (^{13}C NMR) using NMR Spectrometer Bruker Avance DRX 400. The MS were recorded with GC/MS Hewlett Packard 5988 (IE 70 eV). Optical rotations were registered with a Rudolph Research Polarimeter, Autopol^{TM} III. Absorption spectra were recorded with a UV-Visible Spectrophotometer Hewlett Packard 8453 using quartz cells. TLC was performed on silica gel 60 F254 0.2 thick plates and for CC silica gel 63-200 mesh.

Plant Material

Aerial parts of *B. bartsiifolia* were collected in Páramo de Piedras Blancas (Mérida, Venezuela), 4100 m asl, in November 2003 (one month apart at the beginning of the dry season) and identified by Dr. Gilberto Morillo (Facultad de Ciencias Forestales y Ambientales, Universidad de los Andes, Mérida, Venezuela**).** A voucher was deposited in the Herbarium MER of this Faculty under nº 54.266.

Extraction and Isolation of compounds

Fresh vegetative aerial parts (175 g) were briefly immersed in dichloromethane (10 mL/g fresh weight; ultrasound, 3 times for 20 s) to dissolve the exudate. Light microscopy observations after this procedure showed that trichomes were free of exudate droplets. The exudates' extract was desiccated with anhydrous sodium sulfate, filtered, and evaporated to dryness in a rotary evaporator at 30 ºC yielding a crude residue (16.0 g, 9.1 % relative to fresh weight). This material was flash-chromatographed through silica gel (180 g) and eluted with a hexane-dichloromethane-ethyl acetate-methanol gradient into 30 fractions. These were regrouped in 11 fractions (A-K) according to their TLC profiles. After elution of cuticular waxes and low polarity material which was not analyzed further, the portion eluted with dichloromethane-ethyl acetate-methanol mixtures gave fractions F-K (15-30; 11.13 g). Fraction G (21-22; 1.58 g) was re-chromatographed on silica gel (40 g) using a similar gradient. After repeated TLC of prominent bands on silica gel employing dichloromethane-ether (9:1) and hexane-ethyl acetate (7:3) and hexane-acetone (6:4), two pure crystalline compounds were obtained. UV spectra and ^{1}H and ^{13}C NMR analysis showed these to be 5,4´-*dihydroxy*-3,6,7,3´-*tetramethoxyflavone*[7] (**1**) (12 mg, 0.08% relative to the crude extract) and 5,7,4´-*trihydroxy*-3,8,3´-*trimethoxyflavone*[8] (**2**) (22 mg, 0.14 %) Fraction F (15-20; 3.85 g) was chromatographed again on the same stationary phase (116 g) using a hexane-dichloromethane-ethyl acetate gradient. The final purification was performed using TLC on silica gel employing hexane-dichloromethane-ether (6:4:0.5), yielding pure *labda-7,14-dien-13(S)-ol* [9,10] (**3**), (203 mg, 1.27 %)

5,4´-*Dihydroxy*-3,6,7,3´- *tetramethoxyflavone* (chrysosplenetin) (1). Yellow solid, mp 178-180 ºC. (acetone, lit[11] mp 177-179 ºC). IR ν_{max} (KBr) cm^{-1}: 3398, 3000, 2936, 2850, 1656, 1602, 1570, 1514, 1476, 1166, 804. UV (λ_{max}) (MeOH) nm: 351, 257; (+ NaOMe): 407 ↑, 269; (+ $AlCl_3$): 379, 269; (+$AlCl_3$ + HCl): 375,267; (+NaOAc): 413, 256; (+NaOAc + H_3BO_3): 350, 257. EIMS *m/z* (% rel. int.): 374

$[M]^+$ (100), 373 $[M-H]^+$ (41), 359 $[M - Me]^+$ (77), 356 $[M – H_2O]^+$ (24), 344 $[M – CH_2O]^+$ (7), 151 $[B_2]^+$ (15). ^{1}H NMR ($CDCl_3$): δ 12.61 (s, OH-5), 7.71 (*d*, J = 2.0 Hz, H-2'), 7.67 (*dd*, J = 8.0, 2.0 Hz, H-6'), 7.05 (*d*, J = 8.0 Hz, H-5'), 6.50 (s, H-8), 3.98 (3H, s, 3'-OMe), 3.96 (3H, s, 7-OMe), 3.92 (3H, s, 6-OMe), 3.86 (3H, s, 3-OMe). ^{13}C NMR ($CDCl_3$): δ 178.9 (C-4), 158.8 (C-7), 156.0 (C-2), 152.8 (C-5), 152.3 (C-9), 148.4 (C-4'), 146.4 (C-3'), 138.7 (C-3), 132.3 (C-6), 122.6 (C-6'), 122.5 (C-1'), 114.6 (C-5'), 106.6 (C-10), 110.9 (C-2'), 90.3 (C-8), 60.9 (6-OMe), 60.2 (3'-OMe), 56.1 (3-OMe), 50.3 (7-OMe).

5,7,4´-*Trihydroxy*-3,8,3´-*trimethoxyflavone* (gossypetin 3,8,3'-trimethyl ether) (2). Yellow solid, mp 218-220 ºC. (acetone, lit[8] mp 217-218 ºC). IR ν_{max} (KBr) cm^{-1}: 3478, 3000, 2937, 2847, 1651, 1606, 1567, 1557, 1469, 1172, 802. UV (λ_{max}) (MeOH) nm 362, 275; (+ NaOMe): 419, 283; (+ $AlCl_3$): 415, 366, 283; (+$AlCl_3$ + HCl): 415, 361, 283; (+NaOAc): 362, 275; (+NaOAc + H_3BO_3): 362, 275. EIMS *m/z* (% rel. int.): 360 $[M]^+$ (52), 345 $[M - Me]^+$ (100), 151 $[B_2]^+$ (11). ^{1}H NMR ($CDCl_3$): δ 12.34 (s, OH-5), 7.67 (*d*, J = 2.0 Hz, H-2'), 7.62 (*dd*, J = 8.0, 2.0 Hz, H-6'), 6.99 (*d*, J = 8.0 Hz, H-5'), 6.28 (s, H-6), 3.86 (3H, s, 3'-OMe), 3.83 (3H, s, 8-OMe), 3.81 (3H, s, 3-OMe). ^{13}C NMR ($CDCl_3$): δ 178.2 (C-4), 157.1 (C-2), 156.1 (C-7), 155.3 (C-5), 150.0 (C-4'), 148.7 (C-9), 147.7 (C-3'), 137.9 (C-3), 127.7 (C-8), 122.3 (C-6'), 121.1 (C-1'), 116.0 (C-5'), 111.9 (C-2'), 104.2 (C-10), 99.0 (C-6), 61.1 (8-OMe), 59.8 (3-OMe), 55.7 (3'-OMe).

Labda-7,14-dien-13(S)-ol **(3)**. Colorless oil; $[\alpha]^{21}_D$ +10,1º ($CHCl_3$); IR ν_{max} cm^{-1} 3406, 3084, 2960, 2920, 2864, 2846, 1640, 1458, 1384, 1138, 994, 918 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 5.91 (*dd*, *J* = 11.0 ,17.0 Hz, H-14), 5.37 ($w_{1/2}$= 11.0 Hz, H-7), 5.20 (*dd*, *J* = 17.0, 1.0 Hz, H-15'), 5.06 (*dd*, *J* = 11.0,1.0 Hz, H-15), 1.97 (m, H-6'), 1.85 (m, H-1'), 1.81 (m, H-6), 1.75 (m, H-12′), 1.65 (3 H, s, Me-17), 1.56 (m, H-9), 1.54 (m, H-2′), 1.50 (m, H-11′), 1.49 (m, H-12), 1.41 (m, H-3′), 1.40 (m, H-2), 1.28 (3H, s, Me-16), 1.16 (m, H-5), 1.14 (m, H-11), 1.13 (m, H-3), 0.95 (*dt*, *J* = 3.8, 13.1 Hz, H-1), 0.86 (3H, s, Me-19), 0.84 (3H, s, Me-18), 0.74 (3H, s, Me-20). ^{13}C NMR ($CDCl_3$): δ 144.9 (C-14), 135.3 (C-8), 122.2 (C-7), 111.8 (C-15), 73.6 (C-13), 55.1 (C-9), 50.2 (C-5), 44.9 (C-12), 42.3 (C-3), 39.2 (C-1), 37.0 (C-10), 33.1 (C-18), 32.9 (C-4), 27.7 (C-16), 23.8 (C-6), 22.2 (C-17), 21.8 (C-19), 21.2 (C-11), 18.8 (C-2), 13.5 (C-20).

UV spectra of foliar exudates.

Aerial parts of *B. bartsiifolia* were collected in Páramo de Piedras Blancas at 4100 m asl in December 2004 and January 2005 at the beginning of the dry season, when sun radiation reaches maximum annual values due to reduced cloud cover. Five samples each of direct sunlight exposed (E) and shaded (NE) individuals were collected. For each individual the middle part of 5 non-senescent stems were excised. The samples were extracted as described above and the dried exudate weight after water removal and solvent evaporation in vacuum was obtained. Solutions 0.01% w/v in dichloromethane (10 mL) of each extract of the samples were prepared and the UV spectrum determined (200 – 450 nm) in quartz cells. After extraction the residual leaf material was dried in a ventilated oven at 50 ºC for 48 h for moisture content analysis.

Statistical analysis

Significant differences of spectral absorbance between plant type and month were tested using two-way ANOVA. Post-hoc comparisons were made by Tukey test. Both tests were done using Statistix package v 4.5 (Analytical Software, St. Paul, Minnesota).

Result and Discussion

Plants of high elevation Andean páramos and other Alpine environments are exposed to an inordinate solar UV-A/B radiation[12]. This is seasonally dependent not only because of sun ray inclination but because of reduced cloud cover in the dry season of tropical areas[13]. This abiotic stress has a particularly strong impact on the phytochemistry of exposed plants[14]. Filtration of this radiation by compounds contained in well exposed plants, such as highly substituted polyaromatic compounds[15,16], is one of the various adaptive strategies to high irradiance[17]. Accumulation of UV-B filtration compounds may converge towards thermal protection against low temperatures as well[18]. Flavonoids are well known for their high molar absorption coefficients in the UV-B region of the EM spectrum. Thus, their accumulation in exposed parts of many heliophyle plants suggests a protective role against environmental irradiance. *B. bartsiifolia* glandular trichomes produced a large quantity of a complex mixture of secondary metabolites in addition to cuticular waxes protecting the epidermis, which represent to 9% of the leaf weight on a FW basis. Accumulation of cuticular exudate in such quantity has scarcely been described is only rarely recorded. Therefore, cuticular investment in *B. bartsiifolia* must be adaptive. While the amount of trichome exudate did not differ appreciably between E and NE plants, there was a significant increase in the cuticular extracts UV radiation absorbance (275 and 340 nm) of E relative to NE plants sampled during the high sun irradiance season (Table 1).

Table 1: Spectral absorbance at 275 and 340 nm of glandular trichome exudate (0.01% w/v) in dichloromethane of exposed (E) or shaded (NE) *Blakiella bartsiifolia* plants in Páramo de Piedras Blancas, 4100 m asl, Mérida, Venezuela.

		Spectral absorbance (AU) and sampling time	
Wavelength (nm)	**Plant type**	**December**	**January**
275	E	1.25 ± 0.23[a]	1.13 ± 0.19[a]
	NE	0.74 ± 0.13[b]	0.90 ± 0.15[a,b]
340	E	0.75 ± 0.19[c]	0.73 ± 0.08[c]
	NE	0.43 ± 0.12[d]	0.53 ± 0.02[d]

[a,b,c,d] Different letters denote significant differences between means ($P < 0.05$) using Tukey test, n = 5 replicates.

Indeed, E plants collected in December were 69% more effective than NE plants to attenuate the 275 nm band at the upper end of the UV-C zone, and 74% more opaque to the 340 nm well within the UV-B band. Similar differences have been recorded for different plant species along altitudinal gradients[16]. This substantial opaqueness does not vary as the dry season advances (December vs. January sampling) when leaf withering has not started yet. This is probably due to plants reaching the end of their growing period in preparation for senescence in the dry months ahead. Further studies will be necessary to test this hypothesis.

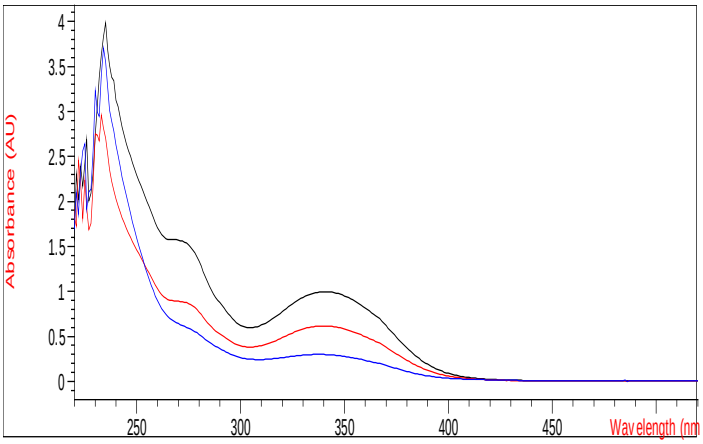

Figure 2: Partial UV-Vis spectra of 0.01 w/v (dichloromethane) solutions of raw *Blakiella bartsiifolia* glandular trichome exudates. Black: sun-exposed plant; Red/Blue: shaded plants

The UV spectrum of the whole exudates (Figure 2) showed the general profile of flavonoids. Therefore, the isolation protocols were designed for this type of medium-high polar material. Indeed, the known 5,4´-*dihydroxy*-3,6,7,3´-*tetramethoxyflavone* (**1**); 5,7,4´-*trihydroxy*-3,8,3´-*trimethoxyflavone* (**2**) were isolated from these fractions. Their UV spectrum was similar to the crude extract, which suggested the significant contribution of these flavonoids to the overall protection against UV-B sun irradiance reaching the high altitude ground. Although the atmosphere at 3,500 – 4,200 m asl filters off radiation below 280 nm, the molecular structure of many flavonoids includes chromophores with important molar extinction coefficients above the cutoff wavelength, thus providing UV-B protection.

The position of hydroxy and methoxy substituents in **1** and **2** was determined by the UV spectrum in various solvents which were confirmed by ^{1}H and ^{13}C NMR and (COSY, HSQC and HMBC) correlations. The UV spectrum of **1** in alkaline medium (MeOH/NaOMe) showed an increment in intensity of band I and a shift of 56 nm relative to **1** in MeOH alone. This result was associated with a OH in position C4' on ring B. The ^{1}H-NMR spectrum showed a AMX coupling pattern with signals at δ: 7.71 (1H, *d*, J=2 Hz, H-2'), δ: 7.67 (1H, *dd*, J=2 and 8 Hz, H-6'), δ: 7.05 (1H, *d*, J=8 Hz, H-5') typical of a 1,3,4 tri-substituted aromatic ring (B ring). This result is compatible with a 4´-OH and 3´-OMe substitution.

A bathochromic shift of 28 nm in the UV spectrum of **1** in acidic medium ($AlCl_3$ + HCl) suggested a C5-OH on ring A which is responsible of the signal at δ 12.61 in the ^{1}H-NMR spectrum. The data also suggests the presence of two other CH_3O- groups in positions C3 and C6. The peak at δ 6.50 was assigned to the C8 proton in ring A and thus the fourth CH_3O- unit was placed at C7 on this same ring. The spectral data are compatible with 5,4´-*dihydroxy*-3,6,7,3´-*tetramethoxyflavone* (**1**, Figure 3), previously reported as chrysosplenetin, isolated from *Artemisia hispanica* Lam. non Weber ex Stechm.[7].

By contrast, the base peak in the mass spectrum [M^{+}-15] of compound **2** suggests a C8-OMe substituent[8]. The substitution pattern of the B ring was identical to chrysosplenetin. The signal at δ 12.34 (C5-OH) in the ^{1}H-NMR spectrum was compatible with the bathochromic shift on UV band I in $AlCl_3$ and $AlCl_3$/HCl in MeOH. The HMBC spectrum shows correlations of the proton at δ 6.28 with carbons C10, C8, and C7, thus placing this proton on ring A. In comparing the ^{13}C-NMR signal of the C-H unit in this ring (δ 99.0) with the unsubstituted carbon on ring A of chrysosplenetin (C8; δ 90.3), it is possible to assign the aromatic proton of **2** to C6. This position is less hindered than C8 owing to the neighboring OH on C5 which is hydrogen-bonded to the C4 carbonyl. These features are compatible with 5,7,4´-*trihydroxy*-3,8,3´-*trimethoxyflavone* which has been isolated previously from *Gutierrezia microcephala* A. Gray[8].

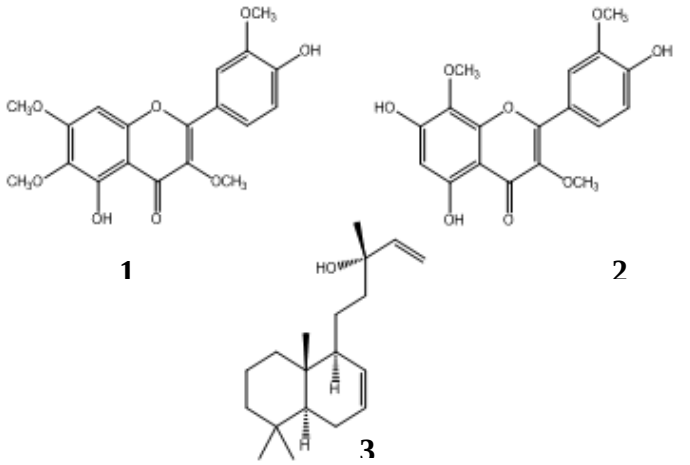

Figure 3: Molecular structures of compounds from *Blakiella bartsiifolia* glandular trichome exudates reported in the present work.

A third compound was also obtained from the lower polar fraction F in high yield which was identified as *labda*-7,14-*dien*-13(*S*)-*ol* (**3**), a labdane diterpene. The IR spectrum showed the presence of hydroxyl (3399, 1138 cm^{-1}), and terminal olefinic groups (1694, 994 and 919 cm^{-1}). The ^{1}H NMR spectrum at low field included a AMX coupling pattern with signals at δ: 5.91 (1H, *dd*, J=17 and 11 Hz, H-14), δ: 5.20 (1H, *dd*, J=17 and 1 Hz H-15´), δ: 5.06 (1H, *dd*, J=11 and 1 Hz H-15) typical of a terminal, disubstituted olefinic group. The HMBC spectrum showed correlations of the protons H-14, H-15 and H-15´ with the quaternary C-OH (δ 73.6, C-13) carbon. A thorough spectral analysis using COSY, HSQC and HMBC allowed to assign completely all the ^{1}H NMR signals leading to *labda-7,14-dien-13(S)-ol* as the molecular structure of compound **3**[9,10]. This compound has been isolated previously from *Salvia broussonetii* Benth[9] and *Aster oharai* Nakai[10]. The absolute configuration (*S*) of C-13 was obtained from the optical rotation data[19]. The absence of chromophores with absorption above 210 nm rejected its function as sun irradiation filter. There are scant reports on the biological activity of this compound with impact on the chemical ecology of plants and further discussion on its role in *B. bartsiifolia* will be postponed until this function is better determined.

Conclusions

B. bartsiifolia trichomes produce a large quantity of exudates to protect exposed plant parts from excessive solar UV A/B radiation typical of the high Andean Páramo. From the leaf exudates *chrysosplenetin*, *gossypetin 3,8,3'-trimethyl ether* (flavones) and *labda-7,14-dien-13(S)-ol*) (a labdane diterpene) were isolated and characterized. These compounds have been described earlier in other species of Astereae. The UV spectrum of isolated flavonoids was similar to the whole extract suggesting the involvement of these flavonoids in the protection of leaf surfaces against excess solar UV-B radiation common in high mountain tropical ecosystems.

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