UNIVERSITY OF SÃO PAULO

SCHOOL OF PHARMACEUTICAL SCIENCES OF RIBEIRÃO PRETO

Metabolomics studies of the subfamily Barnadesioideae (Asteraceae)

Estudos metabolômicos da subfamília Barnadesioideae (Asteraceae)

Gari Vidal Ccana Ccapatinta

Corrected version of the Doctoral Thesis presented to the Post-Graduate Program in Pharmaceutical Sciences on 12/06/2018. The original version is available at the School of Pharmaceutical Sciences of Ribeirao Preto/USP

Ribeirão Preto

2018

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Doctoral Thesis presented to the Graduate Program in Pharmaceutical Sciences of the School of Pharmaceutical Sciences of Ribeirão Preto/USP for the degree of Doctor in Sciences

Area: Natural and Synthetic Products

Supervisor: Dr. Fernando Batista Da Costa

Ribeirão Preto

2018

ABSTRACT

CCANA CCAPATINTA, G. V. **Metabolomics studies of the subfamily Barnadesioideae (Asteraceae)**. 2018. 81 p. Doctoral Thesis. School of Pharmaceutical Sciences of Ribeirão Preto – University of São Paulo, Ribeirão Preto, 2018.

Metabolomics is emerging as an effective approach for the comprehensive evaluation of medicinal plants, classification of raw material, as well as chemotaxonomic studies. This work demonstrates the applicability of metabolomics, using the subfamily Barnadesioideae (Asteraceae) as a study model, for quality assessment and classification purposes of medicinal species (*Chuquiraga* genus) and a chemotaxonomy study of six Barnadesioideae genera (*Arnaldoa*, *Barnadesia*, *Chuquiraga*, *Dasyphyllum*, *Fulcaldea* and *Schlechtendalia*). First, the LC-MS metabolic profiles of Barnadesioideae demonstrated that this subfamily constitutes a chemically underinvestigated taxa with a complex diversity of phenolic compounds, phenylpropanoid derivatives, alkyl glycosides, and triterpenoid glycosides. The intergeneric relationships within Barnadesioideae genera, based on the comparison of their LC-MS metabolic profiles by exploratory and supervised analyses, displayed similarities to those of the intergeneric relationships obtained by the most recent phylogenetic study based on morphological and molecular markers. Second, the LC-MS metabolic profiles of three *Chuquiraga* species (*C. jussieui*, *C. spinosa* and *C. weberbaueri*) lead to the identification of a significant variety of phenolic compounds, phenylpropanoid derivatives, alkyl glycosides, and triterpenoid glycosides, as well as the establishment of prediction models for geographical origin and species classification, as well as the identification of discriminating metabolites by exploratory and supervised multivariate statistical analysis. Third, a classical approach was carried out by acquiring HPLC chromatographic profiles of three *Chuquiraga* species (*C. jussieui*, *C. spinosa* and *C. weberbaueri*) for profiling phenolic compounds and comparison by exploratory and supervised multivariate statistical analysis. Therefore, our results support metabolomics as a valuable tool in the quality control and classification of medicinal plants as well as in chemotaxonomy studies.

Key-words: Asteraceae, Barnadesioideae, liquid chromatography, mass spectrometry, metabolomics, multivariate statistical analysis.

RESUMO

CCANA CCAPATINTA, G. V. **Estudos metabolômicos da subfamília Barnadesioideae (Asteraceae)**. 2018. 81 f. Tese (Doutorado). Faculdade de Ciências Farmacêuticas de Ribeirão Preto – Universidade de São Paulo, Ribeirão Preto, 2018.

A metabolômica vem se tornando uma abordagem eficaz para a avaliação abrangente de plantas medicinais, classificação de matérias-primas, além de estudos quimiotaxonômicos. Este trabalho demonstra a aplicabilidade da metabolômica, utilizando a subfamília Barnadesioideae (Asteraceae) como modelo de estudo, na avaliação da qualidade e classificação de espécies medicinais (espécies de *Chuquiraga*) e no estudo quimiotaxonômico dos principais gêneros de Barnadesioideae (*Arnaldoa*, *Barnadesia*, *Chuquiraga*, *Dasyphyllum*, *Fulcaldea* e *Schlechtendalia*). Em primeiro lugar, a análise dos perfis metabólicos por LC-MS dos membros de Barnadesioideae demonstrou que esta subfamília constitui um grupo quimicamente não explorado com uma diversidade complexa de substancias fenólicas, fenilpropanoides, alquilglicósidos e glicosídeos triterpenoides. As relações intergenéricas dentro da subfamília Barnadesioideae, baseadas na comparação dos seus perfis metabólicos por análises estatísticas multivariadas, mostraram semelhanças com as relações intergenéricas propostas pelo mais recente estudo filogenético com base em marcadores morfológicos e moleculares. Em segundo lugar, a aquisição dos perfis metabólicos de espécies de *Chuquiraga* (*C. jussieui*, *C. spinosa* e *C. weberbaueri*) por analises de LC-MS, levaram à identificação de uma variedade significativa de compostos fenólicos, fenilpropanoides, alquilglicosídeos e glicosídeos triterpenoides, assim como o estabelecimento de modelos de classificação geográfica e de espécies, além da identificação de metabólitos discriminantes por meio de análises estatísticas multivariadas exploratórias e supervisionadas. Terceiro, uma abordagem clássica foi realizada através da aquisição dos perfis cromatográficos por HPLC de espécies de *Chuquiraga* para o perfilhamento de compostos fenólicos e a classificação das espécies por meio de análises estatísticas multivariadas exploratórias e supervisionadas. Logo, os resultados revelam a metabolômica como uma valiosa ferramenta auxiliar no controle de qualidade e classificação de plantas medicinais, bem como em estudos de quimiotaxonômia.

Palavras-chave: Asteraceae, Barnadesioideae, cromatografia liquida, espectrometria de massas, análise estatística multivariada, metabolômica.

1. INTRODUCTION

1.1. The subfamily Barnadesioideae

The subfamily Barnadesioideae (Benth. & Hook. F.) K. Bremer & R.K. Jansen comprises more than 90 species distributed in nine genera entirely restricted to South America. Barnadesioideae members share a number of morphological and molecular features that support their position into a separate subfamily (JANSEN and PALMER 1987; BREMER and JANSEN 1992). The presence of axillary spines and barnadesioid trichomes (pubescences of unbranched three-celled hairs) on floral and vegetative structures constitute unique morphological characteristics within Asteraceae that distinguish Barnadesioideae from the rest of the family (CABRERA 1959; EZCURRA 1985, BREMER and JANSEN 1992; STUESSY et al. 2009). Additionally, another feature of Barnadesioideae is the lack of two DNA inversions in their chloroplast genome, which are present in all other Asteraceae (JANSEN AND PALMER 1987; KIM et al. 2005). Phylogenetically, Barnadesioideae has a well-supported position as the sister group of all other Asteraceae (FUNK et al. 2005; PANERO and FUNK 2008; GRUENSTAEUDL et al. 2009; STUESSY et al. 2009).

1.1.1. Distribution

Despite the small number of species, Barnadesioideae genera display a broad range of habits and distinct geographic distributions. The **Table 1** summarizes the current recognized taxa of Barnadesioideae and their correspondent geographic distribution.

The monotypic genera *Duseniella* K.Schum., *Huarpea* Cabrera, and *Schlechtendalia* Less. are herbaceous/subshrubby plants distributed in isolated areas of Argentina, Brazil and Uruguay (STUESSY et al. 2009). The shrubby genus *Fulcaldea* Poir. was considered monotypic until 2011, when a second species was described in the “Chapada Diamantina”, Bahia, Brazil (FUNK and ROQUE 2011). The genus *Doniophyton* Wedd. includes two herbaceous species, which are found in xeric areas of Chile and Argentina (KATINAS and STUESSY 1997). The three shrubby species of *Arnaldoa* Cabrera have a narrow distribution in southern Ecuador and northern Peru and grow in more or less xerophytic habitats (STUESSY and SAGÁSTEGUI 1993; ULLOA ULLOA et al. 2002).

Table 1. Species of Barnadesioideae and their geographic distribution.

Genus	Species	Distribution*
Arnaldoa Cabrera	*A. argentea* C.Ulloa, P.Jørg. & M.O.Dillon	EC
	A. macbrideana Ferreyra	PE
	A. weberbaueri (Muschl.) Ferreyra	PE
Barnadesia Mutis ex L.f	*B. aculeata* (Benth.) I.C.Chung	EC
	B. arborea Kunth	EC, PE
	B. blakeana Ferreyra	PE
	B. caryophylla (Vell.) S.F.Blake	BO, BR, PE
	B. corymbosa (Ruiz & Pav.) D.Don	BO, PE
	B. dombeyana Less.	PE
	B. glomerata var. *glomerata* Kuntze	BO
	B. glomerata var. *mucronata* I.C.Chung	BO
	B. horrida Muschl.	BO, PE
	B. jelskii Hieron.	EC, PE
	B. lehmannii var. *lehmannii* Hieron.	EC, PE
	B. lehmannii var. *angustifolia* I.C.Chung	PE
	B. lehmannii var. *ciliata* I.C.Chung	EC
	B. lehmannii var. *villosa* (I.C.Chung) Urtubey	EC, PE
	B. macbridei Ferreyra	PE
	B. macrocephala Kuntze	BO
	B. odorata Griseb.	AR, BO
	B. parviflora Spruce ex Benth. & Hook. f.	CO, EC, PE
	B. polyacantha Wedd.	BO, EC, PE
	B. pycnophylla Muschl.	BO, PE
	B. reticulata D.Don	PE
	B. spinosa L.f.	CO, EC
	B. woodii D.J.N.Hind	BO
Chuquiraga Juss	*C. acanthophylla* Wedd.	AR, BO
	C. atacamensis Kuntze	AR, BO, CH
	C. arcuata Harling	EC
	C. aurea Skottsb.	AR
	C. avellanedae Lorentz	AR
	C. calchaquina Cabrera	AR
	C. echegarayi Hieron.	AR
	C. erinacea subsp. *erinacea* D.Don	AR
	C. erinacea subsp. *hystrix* (D.Don) C.Ezcurra	AR
	C. jussieui J.F.Gmel.	BO, CO, EC, PE
	C. kuschelii Acevedo	CH
	C. longiflora (Griseb.) Hieron.	AR, BO
	C. oblongifolia Sagást. & Sánchez Vega	PE
	C. raimondiana A.Granda	PE
	C. morenonis (Kuntze) C.Ezcurra	AR
	C. oppositifolia D.Don	AR, BO, CH
	C. parviflora (Griseb.) Hieron.	AR, BO
	C. rosulata Gaspar	AR
	C. ruscifolia D.Don	AR
	C. spinosa subsp. *spinosa* Less.	PE
	C. spinosa subsp. *australis* C.Ezcurra	AR, BO, CH
	C. spinosa subsp. *huamanpinta* C.Ezcurra	PE
	C. spinosa subsp. *rotundifolia* (Wedd.) C.Ezcurra	CH, PE
	C. straminea Sandwith	AR
	C. ulicina subsp. *ulicina* Hook.	CH
	C. ulicina subsp. *acicularis* (D.Don) C.Ezcurra	CH
	C. weberbaueri Tovar	PE
Dasyphyllum Kunth	*D. argenteum* Kunth	EC
	D. armatum (J.Kost.) Cabrera	AR, BO
	D. brasiliense var. *brasiliense* (Spreng.) Cabrera	AR, BR, PA
	D. brasiliense var. *barnadesioides* (Tovar) Cabrera	BO, PE
	D. brasiliense var. *divaricatum* (Griseb.) Cabrera	AR, BO
	D. brasiliense var. *latifolium* (Don.) Cabrera	BR
	D. brevispinum Sagást. & M.O.Dillon	PE
	D. cabrerae Sagást.	PE
	D. candolleanum (Gardner) Cabrera	BO, BR, PA
	D. colombianum (Cuatrec.) Cabrera	CO

	D. cryptocephalum (Baker) Cabrera	BR
	D. diacanthoides (Less.) Cabrera	CH, AR
	D. diamantinense Saavedra & M.Monge	BR
	D. donianum (Gardner) Cabrera	BR
	D. excelsum (D.Don) Cabrera	CH
	D. flagellare (Casar.) Cabrera	BR
	D. ferox (Wedd.) Cabrera	BO, PE
	D. floribundum (Gardner) Cabrera	BR, PR
	D. fodinarum (Gardner) Cabrera	BR
	D. horridum (Muschl.) Cabrera	PE
	D. hystrix var. *hystrix* (Wedd.) Cabrera	BO
	D. hystrix var. *peruvianum* (Wedd.) Cabrera	PE
	D. inerme (Rusby) Cabrera	AR, BO, PA
	D. infundibulare (Baker) Cabrera	BR
	D. lanosum Cabrera	BR
	D. lanceolatum (Less.) Cabrera	BR
	D. latifolium (Gardner) Cabrera	BO, BR, PA
	D. lehmannii (Hieron.) Cabrera	EC
	D. leiocephalum (Wedd.) Cabrera	BO, PE
	D. leptacanthum (Gardner) Cabrera	BR
	D. maria-lianae Zardini & Soria	PA
	D. orthacanthum (DC.) Cabrera	BR, PA
	D. popayanense (Hieron.) Cabrera	EC
	D. reticulatum var. *reticulatum* (DC.) Cabrera	BR
	D. reticulatum var. *robustum* Domke ex Cabrera	BR
	D. retinens (S.Moore) Cabrera	BR
	D. spinescens (Less.) Cabrera	BR
	D. sprengelianum var. *sprengelianum* (Gardner) Cabrera	BR
	D. sprengelianum var. *inerme* (Gardner) Cabrera	BR
	D. synacanthum (Baker) Cabrera	BR
	D. tomentosum var. *tomentosum* (Spreng.) Cabrera	AR, BO, BR
	D. tomentosum var. *multiflorum* (Baker) Cabrera	BR
	D. trichophyllum (Baker) Cabrera	BR
	D. vagans (Gardner) Cabrera	BR
	D. varians (Gardner) Cabrera	BR, PR
	D. velutinum (Baker) Cabrera	BR, BO
	D. vepreculatum (D.Don) Cabrera	VE
	D. weberbaueri (Tobar) Cabrera	EC, PE
Doniophyton Wedd.	*D. anomalum* (D.Don) Kurtz	AR, CH
	D. weddellii Katinas & Stuessy	AR, CH
Duseniella K.Schum.	*D. patagonica* (O.Hoffm.) K.Schum.	AR
Fulcaldea Poir.	*F. laurifolia* (Bonpl.) Poir.	EC, PE
	F. stuessyi Roque & V.A.Funk	BR
Huarpea Cabrera	*H. andina* Cabrera	AR
Schlechtendalia Less.	*S. luzulaefolia* Less.	AR, BR, UR

Taxonomy according to STUESSY and SAGÁSTEGUI 1993, and ULLOA ULLOA et al. 2002 for *Arnaldoa*; URTUBEY 1999 and HIND 2001 for *Barnadesia*, EZCURRA 1985, HARLING 1991, SAGÁSTEGUI AND SÁNCHEZ 1991, and GRANADA 1997 for *Chuquiraga*; CABRERA 1959 and 1997, SAGÁSTEGUI 1980, SAGÁSTEGUI and DILLON 1985, ZARDINI and SORIA 1994 and SAAVEDRA et al. 2014 for *Dasyphyllum*; KATINAS and STUESSY 1997 for *Doniophyton*; FUNK and ROQUE 2011 for *Fulcaldea*, CABRERA 1951 for *Huarpea*, and STUESSY et al. 2009 for *Duseniella* and *Schlechtendalia*. Abbreviations: AR = Argentina, BO = Bolivia, BR = Brazil, CH = Chile, CO = Colombia, EC = Ecuador, PA = Paraguay, PE = Peru, UR = Uruguay, VE = Venezuela. *Updated distribution data were consulted on TROPICOS database (www.tropicos.org).

The genera *Barnadesia* Mutis ex L.f, *Chuquiraga* Juss, and *Dasyphyllum* Kunth constitute the largest and most representative taxa of Barnadesioideae; pictures of representative species are displayed in **Figure 1**. *Barnadesia* comprises 19 species of shrubs and trees, mainly distributed in the Andes from Colombia to Argentina, and one species is found in Brazil, mostly restricted to elevations of 1800-3400 m (URTUBEY 1999; HIND 2001). *Chuquiraga* is a genus of 22 spiny evergreen shrubs that grow along the Andes and the Patagonia at high altitude habitats; however, some species are found at sea level areas in central Chile and Argentina (EZCURRA 1985; HARLING 1991; SAGÁSTEGUI and SÁNCHEZ 1991; GRANADA 1997). *Dasyphyllum* is a genus of shrubs or trees, which comprises 41 species distributed throughout South America, with two centers of diversity, one in western South America, in Andean mountains from Venezuela to north-western Argentina, occupying arid regions such as the Puna, and the other in eastern South America, in Brazil, Bolivia, and Paraguay in Atlantic forest and savanna (CABRERA 1959, 1997; SAGÁSTEGUI 1980; SAGÁSTEGUI and DILLON 1985; ZARDINI and SORIA 1994; SAAVEDRA et al. 2014).

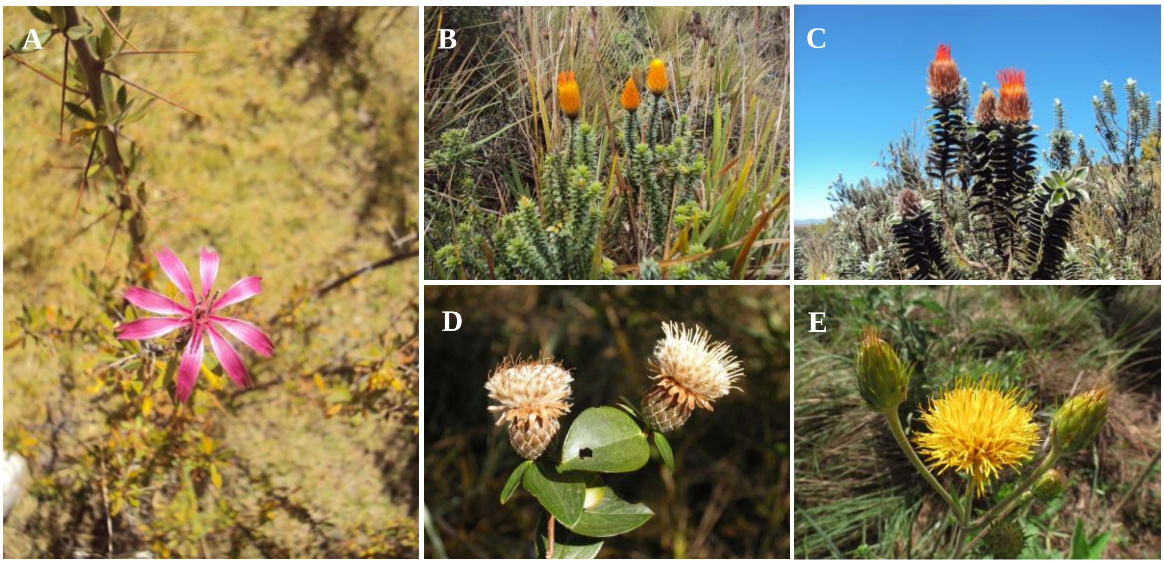

Figure 1. Pictures of representative species of Barnadesioideae: **A**) *Barnadesia horrida* (Q'orimarka, Cusco, Peru), **B**) *Chuquiraga jussieui* (Huancabamba, Piura, Peru), **C**) *Chuquiraga weberbaueri* (Celendin, Cajamarca, Peru), **D**) *Dasyphyllum sprengelianum* (Serra do Cipó, Minas Gerais, Brazil), **E**) *Schlechtendalia luzulaefolia* (Cerro do Tigre, Manoel Viana, Brazil). Photos by: G.V. Ccana-Ccapatinta, G. Shimizu and G. Heiden.

1.1.2. Chemistry

The secondary metabolite chemistry of Barnadesioideae has been sometimes described as following a simple profile (BOHM and STUESSY 1995; ZDERO et al. 1987). This possible simple chemistry profile was proposed and hypothesized as further evidence of the basal position of Barnadesioideae in the Asteraceae family (BOHM and STUESSY 1995; BOHM and STUESSY 2001; CALABRIA et al. 2007). In total, two acetophenones (**1** and **2**) (SENATORE 1996; SENATORE et al. 1999), vanillin (**3**) (HOENEISEN et al. 2000), gallic acid (**4**) (CASTELUCCI et al. 2007), umbelliferone (**5**) (HOENEISEN et al. 2000), 13 flavonoids (kaempferol, quercetin, isorhammetin, and their 3-*O*-glycosides, **6**-**18**) (BOHM and STUESSY 1995; MENDIONDO et al. 1997, 2000; SENATORE et al. 1999; MENDIONDO and JUÁREZ 2001; JUAREZ and MENDIONDO 2002a, 2002b, 2007; LANDA et al. 2009), and 21 triterpenoids (taraxastane-, lupane-, ursane- and oleanane-type pentacyclic triterpenoids, **19**-**39**) (ZDERO et al. 1987; FLAGG et al. 1999; HOENEISEN et al. 2000; GUROVIC et al. 2010) have been described to date in Barnadesioideae. The correspondent trivial names and chemical structures are presented in **Figure 2** and **Table 2**.

Table 2. Chemical constituents reported in members of Barnadesioideae.

No	Chemical class	Trivial name
1	Phenols	*p*-Hydroxyacetophenone
2		*p*-Methoxyacetophenone
3		Vanillin
4		Gallic acid
5		Umbelliferone
6	Flavonoids	Eriodictyol
7		Kaempferol
8		Kaempferol-3-*O*-glucoside
9		Kaempferol-3-*O*-glucuronide
10		Kaempferol-3-*O*-rutinoside
11		Quercetin
12		Quercetin-3-*O*-glucoside
13		Quercetin-3-*O*-glucuronide
14		Quercetin 3-*O*-rhamnoside
15		Quercetin-3-*O*-rutinoside
16		Isorhamnetin-3-*O*-glucoside
17		Isorhamnetin-3-*O*-glucuronide
18		Isorhamnetin-3-*O*-rutinoside
19	Triterpenoids	α-Amyrin
20		α-Amyrin acetate
21		β-Amyrin
22		β-Amyrin acetate
23		Erythrodiol
24		Friedelinol
25		Taraxasterol
26		Pseudotaraxasterol
27		Faradiol
28		3β,6β-Dihydroxytaraxasta-20-ene
29		3β-Acetoxy-6β-hydroxytaraxasta-20-ene
30		6β-Dydroxytaraxasta-20-ene 3β-palmitate
31		6β-Dydroxytaraxasta-20-en-3-one
32		Lupeol
33		Lupeyl acetate
34		Calenduladiol
35		Betulin
36		Heliantriol B2
37		Lupenone
38		30-Nor-lupan-3β-ol-20-one
39		3β-Acetoxy-30-nor-lupan-20-one

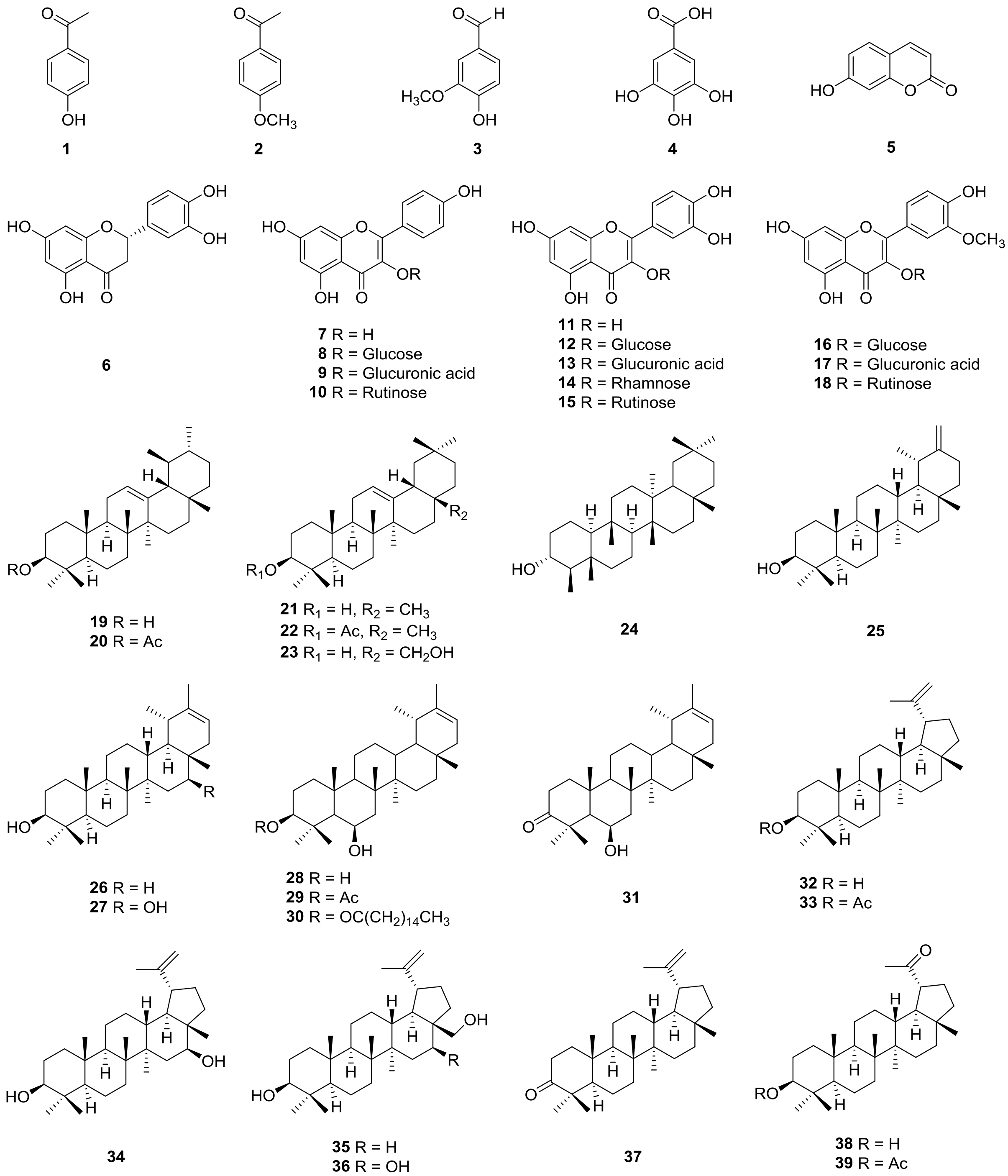

Figure 2. Chemical constituents reported in members of Barnadesioideae.

1.1.3. Medicinal uses

The infusion of the leaves of *Barnadesia arborea* Kunth, distributed among localities of Ecuador and Peru (HIND and HALL 2003), is applied externally for the relief of spasms in children (URTUBEY 1999), while the topical application of its flowers by rubbing is used in the treatment of dermatitis and influenza (TENE et al. 2007). Similarly, the infusion of the flowers of *B. horrida* Muschl., distributed among highlands of Bolivia and Peru (HERRERA 1933), is used for the treatment of common cold, bronchopneumonia, bronchitis, cough, headache, fever, and stomach ache (YAKOVLEFF and HERRERA 1934; HERRERA 1938; ROERSCH 1994).

The infusion of leaves and thorns of *Dasyphyllum brasiliense* is used for the treatment of inflammatory diseases in São Paulo and Minas Gerais states, Brazil (CASTELUCCI et al. 2007). The cortex decoction of *D. diacanthoides* (Less.) Cabrera is used for the treatment of contusions and rheumatism in Mapuche traditional medicine in Chile (de MÖSBACH 1991).

Several species of the genus *Chuquiraga* are described as being used in the traditional medicine of Argentina, Bolivia, Chile, Colombia, Ecuador and Peru. The medicinal uses of *Chuquiraga* can be traced to times of pre-Columbian South American cultures such as the Incas (GIBERTI 1983; ROERSCH 1994; BRACK 1999; DE-LA-CRUZ et al. 2007), Aymaras (VILLAGRÁN et al. 1998, 2003), and Tehuelches (RAMÍREZ and BELOSO 2002). As a general trend, *Chuquiraga* medicinal species are used as infusions, alone or in mixture with other plants (BUSSMAN et al. 2010; BUSSMAN et al. 2015), for the treatment of respiratory, gastrointestinal, genitourinary and reproductive disorders. The common names and medicinal uses of species of the genus Chuquiraga are displayed in **Table 3**.

Chuquiraga jussieui and *C. spinosa* are frequently used in the treatment of prostatitis and prostate cancer. In this context, ARROYO-ACEVEDO et al. (2017, 2018) described for the first time the protective effect of the administration of *C. spinosa* alcoholic extract on N-methyl nitrosourea (NMU)-induced prostate cancer and gastric cancer in rats. The same extract displayed cytotoxicity in the DU-145 (prostate carcinoma) cell line with a IC_{50} of 2.98 µg/ml (ARROYO-ACEVEDO et al. 2017). Additionally, HERRERA-CALDERON et al. (2017) investigated the cytotoxicity of *C. spinosa* ethanolic extract on the MCF-7 (breast adenocarcinoma), K-562 (chronic myelogenous leukemia), HT-29 (colon adenocarcinoma), H-460 (lung large cell carcinoma), M-14 (amelanotic melanoma), HUTU-80 (duodenum adenocarcinoma), and DU-145 cell lines, obtaining IC_{50} values of 5.32-9.25 µg/ml. Interestingly, the lipophilic fractions (hexane, petrol, chloroform and ethyl acetate) obtained from the initial extract displayed IC_{50} values of 24.19-54.12 µg/ml, suggesting that the active constituents may remain in the polar fractions. The up-to-date reported biological activities of *Chuquiraga* species are summarized in **Table 4**.

Table 3. Common names and medicinal uses of species of the genus *Chuquiraga*.

Species	Country	Common name	Indications*	Reference
C. acanthophylla	AR	Espina amarilla	Cold, cough and fever. Stomachache. Urinary tract infections.	BARBARÁN 2008
C. atacamensis	AR	Hierba de san Pedro, san Pedro, kishka tola	Conjunctivitis, for which the plant is used to make a medicinal smoke. Rheumatic pain, where the plant infusion is used to wash rheumatic legs to relieve pain.	GIBERTI 1983
	BO	San Gerónimo, fundición, kutu kutu, chajllampa	Cold, cough, fever. Urinary tract infections, cystitis, prostatitis. Relief of postpartum symptoms. Not recommended in pregnant women.	ZAMORA 2008
	CH	Lengua de gallo, tastará, quebrolla, killokisca, chana chaklamba	The infusion is used as hot baths against colds. Productive and non-productive cough, fever. Genitourinary and reproductive disorders in women.	VILLAGRÁN et al. 1998, 2003
C. avellanedae	AR	Quilimbay-trayao, tratrakcha, trayau	Cough. Headache and fever, boiled leaves are chewed in a mixture with sugar.	RICHERI et al. 2013
C. erinacea	AR	Romerillo, falsa uña de gato, trifrif mamull	Stomachache and liver disease. Kidney disease. Strengthens the brain and nerves.	LADIO and LOZADA 2009
C. jussieui	CO	Chuquiragua, vela de páramo	Febrifuge, diuretic, kidney stones.	DÍAZ-PIEDRAHITA and VÉLEZ-NAUER 1993
	EC	Chuquira, chuquiragua	Liver disease, diabetes. Allergy and skin disorders. Pain of the bones, rheumatism and other inflammations. Toothache, stomachache and gastrointestinal disorders. Cold, fever, cough and respiratory disorders. Malaria, malarial fever, smallpox, internal infections. Urogenital disorders, diuretic. Relief of postpartum symptoms.	MARTÍNEZ 2006; TENE et al. 2007; ANSALONI et al. 2010
	PE	Chiquiragua (northern Peru). Inca llaulli, kentayllaulli, quishuara, kiswara, kiswara tiutumpi, qharisirviy (southern Peru)	Stomachache and liver disease. Musculoskeletal pain. Skin eruptions, inflammations. Common cold, cough, sore throat, fever, respiratory disorders. Vaginitis and vaginal infections, as external washing. Urinary tract infections, kidney disease, stones, prostatitis. Postpartum symptoms. Endoparasiticide (intestinal worms), and ectoparasiticide (lice). Rheumatic pain, an infusion is used to wash the legs.	TORRES et al. 1992; ROERSCH 1994; DE FEO 2003; VÁSQUEZ et al. 2010
C. longiflora	AR	Azafrán de la puna	The plant is added to water for personal washing.	GIBERTI 1983
C. oppositifolia	AR	Azafrán del campo	Hypoglycaemic, hypocholesterolaemic. Antifungal.	RAAD 2012
C. parviflora	BO	Chiñi michi michi	Against curse.	VANDEBROEK et al. 2003
C. spinosa	AR	Charkoma	Regulation of the menstrual cycle.	GIBERTI 1983
	BO	Huamanpinta	Kidney stones and cystitis.	CEUTERICK et al. 2011
	EC	Chuquiragua	Cold, cough and fever. Pain of the bones. Malaria.	BUSSMANN and SHARON 2006b
	PE	Huamanpinta, huancapita, huancaspita, laulinco, pucacasha, pazpapamaquin, qharisirviy, cjari sirvi	Respiratory affections. Antiblenorrhaegic and vermifuge. Conjunctivitis. Gonorrhoea. Urinary system disorders in women and men. Vaginitis and vaginal infection, the infusion of the plant is uses for external washing. Kidney and prostate inflammations. Prostate cancer. Diuretic. Sexual impotence.	BRACK 1999; MADALENO 2007; REHECHO et al. 2011
C. weberbaueri	PE	Amaro amaro	Cough, bronchitis, asthma. Liver disease. Diuretic and depurative.	BRACK 1999

*Commonly, aerial parts are used to make infusions or decoctions in water; other modes of use are detailed in the table text. Abbreviations: AR = Argentina, BO = Bolivia, CH = Chile, CO = Colombia, EC = Ecuador, PE = Peru.

Table 4. Biological activities reported for species of the genus *Chuquiraga.*

Plant	Extracting Solvent, standardization	Bioactivity	Results	Reference
C. atacamensis	80% Ethanol (5 g/100 ml)*, 500 µg of GAE/ml	*In vitro* COX-1 inhibition	IC_{50} = 2 µg/ml	ALBERTO et al. 2009
		In vitro COX-2 inhibition	IC_{50} = 4.7 µg/ml	
		Antioxidant, DPPH$^{\cdot}$, ABTS$^{\cdot+}$, $O_2^{\cdot-}$	IC_{50} = 3.5 - 20 µg/ml	
	80% Ethanol (5 g/100 ml)*, 500 µg of GAE/ml	*Staphylococcus aureus* strains	MIC = 80–600 µg/ml	ZAMPINI et al. 2009
		Enterococcus faecalis strains	MIC = 150–300 µg/ml	
		Escherichia coli strain	MIC = 600 µg/ml	
		Other gram-negative bacteria	MIC = 300–600 µg/ml	
	Ethanol (dry extract)	Antioxidant, ABTS$^{\cdot+}$ assay	SC_{50} = 1.5 µg/ml	ZAMPINI et al. 2010
C. erinacea	Ethanolic extract (dry extract)	*In vitro* AChE inhibitory activity	IC_{50} = 7.26 mg/ml	GUROVIC et al. 2010
C. jussieui	Water (2 g/100 ml)*	Antioxidant	IC_{50} = 64.9 mg/L	DUEÑAS et al. 2014
C. spinosa	Water (dry extract), 5.4 mg GAE/mg	Antioxidant, DPPH$^{\cdot}$, ABTS$^{\cdot+}$, $O_2^{\cdot-}$	IC_{50} = 9.6 - 30.5 µg/ml	CASADO et al. 2011
		Candina albicans	MIC = 2.5 µg on TLC plate	
		Cladosporium cucumerinum	MIC = 2.5 µg on TLC plate	
		Rhizopus stolonifer	MIC = 4.6 µg on TLC plate	
	50% Methanol (dry extract), 6.3 mg GAE/mg	Antioxidant: DPPH$^{\cdot}$, ABTS$^{\cdot+}$, $O_2^{\cdot-}$	IC_{50} = 8.5 - 21.7 µg/ml	CASADO et al. 2011
		Antiinflammatory, paw edema in rats	Maximal inhibition = 52.5%	
		Antiinflammatory, ear edema in mice	Inhibition = 88.1%	
		Candina albicans	MIC = 6.3 µg on TLC plate	
		Rhizopus stolonifer	MIC = 13.5 µg on TLC plate	
	Methanol (dry extract), 12.6 mg GAE/mg	Antioxidant, DPPH$^{\cdot}$, ABTS$^{\cdot+}$, $O_2^{\cdot-}$	IC_{50} = 10.5 – 36.5 µg/ml	CASADO et al. 2011
		Rhizopus stolonifer	MIC = 18.5 µg on TLC plate	
	Water (5g/500 ml)*	*Staphylococcus aureus* strain	13 mm, agar diffusion test	BUSSMAN et al. 2008
	96% ethanol (dry extract)	Cytotoxicity in DU-145 cell line	IC_{50} = 2.98 µg/ml	ARROYO-ACEVEDO et al. 2017
	96% ethanol (dry extract)	Cytotoxicity in MCF-7 cell line	IC_{50} = 9.25 µg/ml	HERRERA-CALDERON et al. 2017
		Cytotoxicity in K-562 cell line	IC_{50} = 7.34 µg/ml	
		Cytotoxicity in HT-29 cell line	IC_{50} = 8.52 µg/ml	
		Cytotoxicity in H-460 cell line	IC_{50} = 5.32 µg/ml	
		Cytotoxicity in M-14 cell line	IC_{50} = 8.30 µg/ml	
		Cytotoxicity in HUTU-80 cell line	IC_{50} = 6.20 µg/ml	
		Cytotoxicity in DU-145 cell line	IC_{50} = 7.09 µg/ml	
	Hexane fraction (dry extract)	Cytotoxicity in DU-145 cell line	IC_{50} = 27.03 µg/ml	
	Petroleum ether fraction (dry extract)	Cytotoxicity in DU-145 cell line	IC_{50} = 33.10 µg/ml	
	Chloroform fraction (dry extract)	Cytotoxicity in DU-145 cell line	IC_{50} = 24.19 µg/ml	
	Ethyl acetate fraction (dry extract)	Cytotoxicity in DU-145 cell line	IC_{50} = 54.12 µg/ml	
C. straminea	80% Methanol (dry extract)	Antioxidant, DPPH$^{\cdot}$, ABTS$^{\cdot+}$	SC_{50} = 14.5 - 34.9 µg/ml	MENDIONDO et al. 2011
		Staphylococcus aureus strains	MIC = 200 - 800 µg/ml	

* Plant/solvent ratio. GAE, Gallic acid equivalents

1.1.4. Toxicity

There are few data about the toxicity or side effect of species of the genus *Chuquiraga*. The aqueous extracts of *C. spinosa* and *C. weberbaueri* displayed median lethal doses (LD_{50}) >10,000 µg/mL in the brine shrimp lethality assay, whereas the ethanolic extracts displayed LC_{50} values of 1.1 and 0.25 µg/mL, respectively (BUSSMANN et al. 2011). Even though there is a report discouraging the administration of *C. atacamensis* infusions in pregnant women because it could cause miscarriage (ZAMORA 2008), additional studies are required to reveal the possible toxicity and side effect of *Chuquiraga* species and other representatives of Barnadesioideae subfamily.

1.1.5. Commercialization

The medicinal species of *Chuquiraga* are important and evident elements in medicinal plant markets of traditional cities of Ecuador and Peru but also in modern cities such as Guayaquil and Lima, and at least one species has been introduced in the international market. Differently to markets of Ecuador and Peru, where commercialization of *Chuquiraga* species is frequent, the commercialization of *Chuquiraga* species in Markets of Bolivia seems to be absent (MACÍA et al. 2005; BUSSMANN et al. 2016).

Representative pictures from commercial samples of *Chuquiraga* species are displayed in **Figure 3**. *Chuquiraga jussieui* is one of the most popular medicinal plants in Ecuador and has been noted as a plant with promising industrial potential (BUITRON 1999; MARTÍNEZ 2006; GUPTA 2006). The flowering parts of this species are also found in markets of northern Peru together with *C. weberbaueri* (BUSSMANN et al. 2007). In the markets of southern Peru, the inflorescences of *C. jussieui* are frequently commercialized separately from leaves and steams (**Figure 3A**). The aerial parts of *C. spinosa* are sold along the main cities of Peru (MADALENO 2007; CEUTERICK et al. 2011; HUAMANTUPA et al. 2011; **Figure 3B**). This species is also distributed as a dietary supplement in Europe (Huamanpinta, Esparta GmcH, www.paracelmed.com; **Figure 3C**) and North America (Huamanpinta, Alpha Omega Labs, www.alphaomegalabs.com). Products that contain *C. spinosa*, mixed with other plants, can also be found, for example, Women's Care Blend (Amazon, www.amazon.com), Prostate Care Blend and Kidney Cleanser Blend (Fito Global Inc., www.fitoglobal.com).

Figure 3. Commercial samples of *Chuquiraga* species: **A**) Flowers of *C. jussieui* (Market in Puno City, Peru); **B**) Aerial parts of *C. spinosa* (Market in La Oroya City, Peru); **C**) Capsules containing *C. spinosa* powder (Commercialized in Austria and Germany). Photos by: G.V. Ccana-Ccapatinta.

1.2. Metabolomics

In parallel to the terms "genome" (complete set of genes present in a cell or organism) and "proteome" (entire set of proteins expressed by a cell or organism), the set of metabolites synthesized by a biological system constitute the "metabolome" (OLIVER et al. 1998; FIEHN 2002). Therefore, metabolomics can be defined as the comprehensive, qualitative, and quantitative analysis of all metabolites in a biological system by high-throughput analytical strategies (GOODACRE et al. 2004; KOPKA et al. 2004; GOODACRE 2005; ROCHFORT 2005). Metabolomics is fast becoming the approach of choice across a broad range of sciences including systems biology, drug discovery, molecular and cell biology, and other medical and agricultural sciences because of the continuous analytical and computational developments conducted in this research area.

1.2.1. Metabolomics approaches

Metabolomics, in the strict sense, involves the measurement of all metabolites in a given system; however, this is not yet technically possible because of the lack of a simple automated analytical strategy that can record the metabolome in a reproducible and robust way

(GOODACRE et al. 2004; KOPKA et al. 2004). Accordingly, three technical approaches were initially described that intended to highlight the options available for monitoring the metabolome. However, the practical and conceptual boundaries between "metabolic fingerprinting" and "metabolite/metabolic profiling" could be sometimes unclear. Thus, other researchers subdivided the metabolomics analytical methodologies into only two categories, namely, "untargeted analysis/metabolite profiling" and "target analysis" (VILLAS-BÔAS et al. 2005; VINAYAVEKHIN and SAGHATELIAN 2010; ROBERTS et al. 2012). Although metabolomics is a relatively new research field and the used terminologies are still evolving (ERNST et al. 2014), the **Table 5** presents commonly used terminologies.

Table 5. Terminologies commonly used in metabolomics research.

Term	**Definition**
Metabolomics	Comprehensive, qualitative, and quantitative analysis of all metabolites in a biological system by high-throughput analytical strategies.
Metabolome	Complete set of small-molecule chemicals found within a biological sample.
Metabolic fingerprinting	Rapid high-throughput screening of all detectable analytes in a sample without mandatory identification.
Metabolite/metabolic profiling	The identification and quantification of a number of pre-defined metabolites, which may be associated with the same pathway or belong to the same class of compounds.
Metabolite target analysis	Metabolites are selected prior to analysis, by optimized extraction or specific separation and/or detection.
Untargeted analysis	Rapid analysis of a large number of different metabolites in which quantification is not mandatory.
Targeted analysis	Procedure that must include the identification and absolute quantification of selected metabolites.

Adapted from ERNST et al. (2014).

The analytical techniques used for metabolome data acquisition include spectroscopic (nuclear magnetic resonance, NMR) and spectrometric (mass spectrometry, MS) methods, either directly or in association with chromatography. Among them, the association of high-resolution mass spectrometry with gas or liquid chromatography (GC-MS and LC-MS) are likely the most commonly applied tools because of their high sensitivity and comprehensiveness of the acquired data (CAJKA and FIEHN 2016; FIEHN 2016; GORROCHATEGUI et al. 2016; ROCHAT 2016). Other techniques such as capillary electrophoresis tandem mass spectrometry (CE-MS), high-performance liquid chromatography with diode array detection (HPLC-DAD), infrared spectroscopy (FT-IR), among others, have also been described (ERNST et al. 2014), and are compared in **Table 6**.

Table 6. Some standard techniques for untargeted and targeted metabolomics.

	Sensitivity	Throughput	Comprehensiveness
FT-IR	Low	High	Low
NMR	Low	Low-high	Low-high
LC-NMR	Low	Low	High
LC-MS	High	High	High
GC-MS	High	High	High
CE-MS	High	Medium	High
LC-UV	Medium-high	High	Low

Modified from WECKWERTH and MORGENTHAL (2005).

1.2.2. Multivariate data analysis

Metabolomics is placed at the interface between chemistry, biology, statistics and computer science, thus requiring multidisciplinary skills. The high-dimensional nature of metabolome datasets acquired in a metabolomics study requires multivariate data analyses to turn data into knowledge (GOODACRE et al. 2004, BEISKE et al. 2015). These analyses can be classified as unsupervised and supervised multivariate methods, whose general description are displayed in **Table 7**. Popular unsupervised analyses include hierarchical cluster analysis (HCA) and principal components analysis (PCA) that are also known as exploratory methods. Supervised analyses require adequate validation by establishing a *training set*, used to build a model, a *validation set*, used to validate the model, and a *test set*, used to test the model. Popular algorithms include partial least squares discriminant analysis (PLS-DA), *k*-nearest neighbor classification (*k*NN), and neural networks.

Table 7. Description of unsupervised and supervised multivariate analyses.

Multivariate analysis	Popular algorithms	Description
Unsupervised	HCA, PCA, Kohonen neural networks	The system is shown a set of inputs and then left to cluster the metabolite data into groups. For multivariate analysis this optimization procedure is usually 'simplification' or dimensionality reduction; this means that a large body of metabolite data are summarized by a few parameters with minimal loss of information. After clustering, the ordination plots or dendrograms are interpreted
Supervised	PLS-DA, *k*NN, back-propagation neural networks	The desired responses (Y data or 'traits' or 'classes') associated with each of the inputs (X data, or 'metabolome data') are known. The goal is to find a mathematical transformation (model) that will correctly associate all or some of the inputs with the target traits. Such inductive methods allow one to discover which metabolites (inputs) are key for the separation of the traits to be predicted.

Adapted from GOODACRE et al. (2004).

1.2.3. Metabolite identification in metabolomics

In 2007, the Metabolomics Standards Initiative (MSI) Working Group on Chemical Analysis (CAWG) recommended the minimum standards in chemical analysis reports in metabolomics studies as detailed in **Table 8**. These standards recommend that authors should differentiate and report the level of identification accuracy for all reported metabolites based on a four-level system that varies from *level 1* (identified compound) through *levels 2* and *3* (annotated compounds or compound class identification) to *level 4* (unidentified or unclassified metabolites which, however, can be differentiated based on spectral data). The identification of metabolites is essential for integrating metabolomics data into other information disciplines (SUMNER et al. 2007; CREEK et al. 2014).

Table 8. Proposed minimum metadata relative to metabolite identification in metabolomics.

No	Level of identification	Details
1	*Identified compounds*	**Non-novel metabolite**: a minimum of two independent and orthogonal data relative to an authentic compound analyzed under identical experimental conditions to validate non-novel metabolite identifications (e.g. retention time/index and mass spectrum, retention time and NMR spectrum, accurate mass and tandem MS, accurate mass and isotope pattern, full ^{1}H and/or ^{13}C NMR, 2D NMR spectra). **Novel metabolite**: metabolites identified for the first time and which represent novel identifications should include sufficient evidence for full structural identification. Traditionally, it involves extraction, isolation, and purification followed by accurate mass measurement, ion mass fragmentation patterns, NMR (^{1}H, ^{13}C, 2D), and other spectral data or chemical derivatization.
2	*Putatively annotated compounds*	Without chemical reference standards, based upon physicochemical properties and/or spectral similarity with public/commercial spectral libraries. The use of literature values reported for authentic samples by other laboratories.
3	*Putatively characterized compound classes*	Based upon characteristic physicochemical properties of a chemical class of compounds, or by spectral similarity to known compounds of a chemical class.
4	*Unknown compounds*	Although unidentified or unclassified these metabolites can still be differentiated and quantified based upon spectral data.

Levels of identification following SUMNER et al. (2007).

1.2.4. Metabolomics for chemotaxonomy

According to ERDTMAN (1963), chemotaxonomy developed as “very early in the development of natural products chemistry it occurred to many botanists and chemists that it should be possible to characterize and classify plants based on their chemical constituents”.

Then, chemotaxonomy is the attempt to classify and identify organisms based on differences and similarities in their biochemical compositions (ERDTMAN 1963; WINK and WATERMAN 1999). Chemotaxonomy accompanied the technical advances in phytochemical analysis from paper chromatography (PC), throughout thin layer chromatography (TLC), high-performance liquid chromatography (HPLC) to finally rely on the isolation and structure elucidation (principally by NMR and MS spectrometry) of plant constituents (WINK and WATERMAN 1999; WINK et al. 2010).

In this regard, the recent analytical improvements established by metabolomics platforms offer the possibility of scanning the metabolome of a numerous set of plants to compare them and evaluate taxonomic hypothesis (WATERMAN 2007, REYNOLDS 2007). The use of metabolomics in chemotaxonomy studies contributes to the classification of plants when uncertainty exists using classical botanical methods. For example, MESSINA et al. (2014) used LC-MS-based metabolomics to test taxonomic boundaries in the *Olearia phlogopappa* (Asteraceae) complex, confirming the limits of closely related taxa where DNA sequence data has been uninformative. Similar studies have been conducted with Brazilian members of the genus *Vernonia* and tribe *Vernonieae* (Asteraceae) (MARTUCCI et al. 2014; GALLON et al. 2018).

1.2.5. Metabolomics for quality control of medicinal plants

The quality control of botanical drugs begins with the authentication of the botanical raw material, continues through the preparation of the botanical drug extract and culminates in the botanical drug product. These products (e.g. crude plant extracts) sold as nutraceuticals or phytopharmaceuticals require that their composition is assessed with precision and kept constant. For this purpose, a usual way to standardize an extract is to quantify its active(s) principle(s). Often, however, the active(s) principle(s) are not clearly defined, and the standardization can be made on a characteristic compound of a given plant, which serves as a marker but may not be directly linked to the biological activity of the extract. Chromatographic (TLC, HPLC) and spectroscopic (NMR, IR, UV) fingerprinting have been used as tools for the quality control of medicinal plants with few phytochemical information, however, representing a challenging analytical task since these mixtures are usually composed of hundreds of different compounds (ULRICH-MERZENICH et al. 2007; WOLFENDER et al. 2010).

In this context, metabolomics enables to obtain a global idea of the compositions of a crude extract, and consequently evaluation of its quality. One advantage of applying metabolomics for quality control is that medicinal plants are evaluated based not only on a limited number of metabolites that may be (or not) pharmacologically important, but on the overall metabolome that includes known/unknow, minor/major metabolites. Then, metabolomics is now established as an approach for the comprehensive evaluation and quality control of medicinal plants, classification of raw material, definition of the degree of similarity between extracts (*phytoequivalence*), and identification of adulterations (ULRICH-MERZENICH et al. 2007; HEINRICH 2008; OKADA et al. 2010; WOLFENDER et al. 2010).

1.3. Metabolomic studies of the subfamily Barnadesioideae

Compared to other tribes or genera of, the subfamily Barnadesioideae constitutes a phytochemically underinvestigated group of Asteraceae, therefore, in this project a LC-MS approach was used to establish a metabolomics-based chemotaxonomic classification and to explore its phytochemical composition. On the other hand, species of the genus *Chuquiraga* are frequently used in traditional medicine and some products are even distributed in the international market, demanding adequate phytochemical characterization and quality control procedures that are not available in the current literature. For this purpose, two analytical setups, HPLC-DAD and LC-MS, were used to establish a species classification of medicinal species of the genus *Chuquiraga*. Therefore, the present project aimed to establish:

- A metabolomics-based chemotaxonomic classification of the subfamily Barnadesioideae.
- A metabolomics-based species classification of medicinal species of the genus *Chuquiraga*.
- A chromatographic profile-based species classification of medicinal species of the genus *Chuquiraga*.

2. OBJECTIVE

To carry out metabolomic studies and develop strategies for species classification and chemotaxonomy on members of the subfamily Barnadesioideae (Asteraceae).

CONCLUSIONS

This work demonstrates the applicability of metabolomics for quality assessment of medicinal species of the genus *Chuquiraga* as well as a chemotaxonomy study of the subfamily Barnadesioideae.

The subfamily Barnadesioideae constitutes a chemically underinvestigated taxa with a complex diversity of phenolic compounds, alkyl glucosides, and triterpenoid glycosides. The intergeneric relationships of the subfamily Barnadesioideae based on their LC-MS metabolome data displayed similarities to those intergeneric relations proposed by the most recent phylogenetic study based on morphological and molecular markers, therefore showing metabolomics as a valuable auxiliary tool in chemotaxonomy studies.

Liquid chromatography associated to high-resolution mass spectrometry (LC-MS) and ultraviolet detection, constitutes a high-throughput platform in metabolomics studies for the quality assessment of medicinal plants. In addition to the phenolic compounds, the occurrence of phenylpropanoid derivatives of malic, tartaric and tartronic acids was recognized. Several flavonoid glycosides were also identified along with alkyl glycosides and triterpene glycosides. Exploratory and supervised multivariate analyses enabled geographical discrimination, species classification and identification of discriminating metabolites.

The HPLC chromatographic profiles of *Chuquiraga* species showed the occurrence of *p*-hydroxyacetophenone glycosides, flavonoids glycosides, and caffeic acid ester derivatives. Although the HPLC-DAD method is limited to the detection of chromophoric compounds, the multivariate analysis of HPLC chromatographic fingerprints enabled the establishment of a species classification model for *Chuquiraga* species.

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