SEASONAL STUDY OF THE ALKALOID PATTERN OF *HUPERZIA SAURURUS* WITH HABITAT IN CÓRDOBA PROVINCE (ARGENTINA)

Ortega M. G. [1,2]; Agnese A. M. [1,2]; Barboza G. E. [2,3] ; Cabrera J. L. [1,2]

[1]Farmacognosia, [3]Botánica, Departamento de Farmacia, Fac. de Ciencias Químicas, Universidad Nacional de Córdoba, Edificio de Ciencias 2, Ciudad Universitaria, 5000 Córdoba, Argentina

[2]IMBIV (Instituto Multidisciplinario de Biología Vegetal, UNC- CONICET).C.C. 495, 5000 Córdoba, Argentina.

E-mail: jcabrera@mail.fcq.unc.edu.ar

Received July 21, 2007. In final form November12, 2007.

Abstract

Previous chemical studies on specimens of *Huperzia saururus* (Lam.) Trevis. collected in different parts of the world showed significant differences in the alkaloid content. In Córdoba (Argentina) the chemical pattern was characterized by the presence of three main alkaloids: sauroine, sauroxine, and 6-hydroxylycopodine. This fact remarkably differs from the patterns informed for specimens collected in Quito (Ecuador), Kahuzi and Ruwenzori (Zaire), and Sabyinyo (Rwanda) where their marker alkaloids are clavolonine and lycopodine that in time differ from those mentioned by other authors of unknown specimen provenance. As a consequence, we undertook a seasonal study by analyzing the qualitative-quantitative content of alkaloids by using GLC-MS and GLC-FID respectively, of specimens collected in Cordoba (Argentina) at the same site during the four seasons of the year in order to investigate if the discrepancies were a result of biosynthetic modifications due to seasonal reasons. Obtained results did not confirm the proposed hypothesis since the chemical pattern showed no significant variations among the different seasons of the year. Thus, the chemical differences among own results and the studies in other parts of the world could complement the systematic antecedents existent to help to resolve on the possibility that more than one species has been involved in the different investigations.
Keywords: *huperzia saururus*, *Lycopodium* alkaloids, alkaloid pattern, seasonal study.

Resumen

Estudios químicos previos en especimenes de *Huperzia saururus* (Lam.) Trevis. recolectados en distintas partes del mundo, mostraron diferencias significativas en su

contenido alcaloídico. En Córdoba (Argentina) el patrón químico fue caracterizado por la presencia de tres alcaloides mayoritarios: sauroína, sauroxina y 6-OH licopodina. Este hecho difiere en forma sustancial a lo informado para especímenes recolectados en Quito (Ecuador), Kahuzi y Rwenzori (Zaire) y Sabyinyo (Rwanda) donde sus alcaloides marcadores son clavolonina y licopodina, que a su vez difieren de los mencionados por otros autores donde el origen del espécimen es desconocido. Como consecuencia, abordamos el estudio estacional de especímenes recolectados en Córdoba en el mismo hábitat pero en diferentes estaciones del año, analizando el contenido cuali-cuantitativo de sus alcaloides por CGL-EM y CGL-FID respectivamente, con el objeto de determinar si dichas discrepancias eran motivadas por modificaciones biosintéticas debido a razones estacionales. Los resultados obtenidos no confirmaron la hipótesis propuesta, ya que el patrón químico no mostró variaciones significativas en las distintas estaciones del año. De este modo, las diferencias químicas entre nuestros resultados y los estudios en otras partes del mundo podrían complementar los antecedentes sistemáticos existentes para ayudar a resolver acerca de la posibilidad de que más de una especie haya estado involucrada en las diferentes investigaciones.
Palabras claves: *huperzia saururus*, alcaloides del *Lycopodium* alkaloids, patrón de alcaloides, estudio estacional.

Introduction

Lycopodiaceae is a family that is comprised of four genera: *Huperzia* Bernh., *Phylloglossum* Kunze, *Lycopodium* L., and *Lycopodiella* Holub [1-3] and has a wide distribution throughout the world. *Huperzia* is a virtually cosmopolitan genus with ca. 300 species in the world and nearly 150 in the Neotropics [4]. Among the twelve groups of species represented in the Neotropics proposed by Øllgaard [1, 4], the group named "*Huperzia saururus* group" is one of the largest (ca. 40 species), including species closely related and highly problematic to delimit.

Huperzia saururus (Lam.) Trevis. (= *Lycopodium saururus* Lam., *H. axillaris* (Roxb.) Rothm., *H. sanctae-barbarae* (Rolleri) Rolleri & Deferrari; *L. elongatum* Sw.) grows in South America from northern Peru to Argentina, and also in Africa, Madagascar and the Mascarenes [4]. In Argentina, it occurs from the north-west (Jujuy, Salta, Catamarca, San Luis) to the central part of this country (Córdoba and Buenos Aires) at high altitudes [5]. Although this species is most easily recognised by its green, somewhat glossy leaves, and its erect shoots densely aggregated and pressed together so that leaves at the stem bases are squeezed and appear etiolated, it is often confused with some other South American allied species such as *H. crassa* (Willd.) Rothm., *H. badiana* B. Øllg. & Wind., and *H. andina* (Rosenst.) Holub among others [4] (Øllgaard pers. com.).

From a chemical point of view, the first study about *H. saururus* on specimens collected in Argentina was by Arata and Canzoneri [6] who isolated a base named pillijanine. Subsequently, Deulofeu and De Langhe [7] isolated two bases: saururine, and sauroxine. However, structural elucidation was not achieved in either of these publications. Later, Ayer et al. [8] elucidated the structure of sauroxine (isomer of α-obscurine) for the first time from Argentinean specimens.

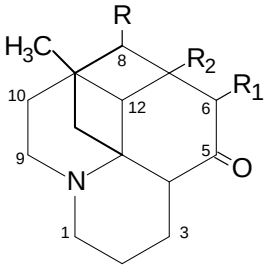
In addition, Braekman et al. [9] reported other alkaloids: lycopodine, clavolonine, fawcettiine, acetylfawcettiine and selagine (=huperzine A) being isolated from specimens of *H. saururus* from Ecuador (South America), Rwanda and Zaire (Africa).

In a revision of Lycopodium alkaloids in the eighties, MacLean [10] added another five more alkaloids: anhydrolycodoline, lycodoline, dihydrolycopodine, saururidine and LS14. However, the two latter structures were not elucidated.

In Argentina, *H. saururus* is commonly known as "cola de quirquincho" and has an extensive ethnomedical use, mainly because of its aphrodisiac properties [11]. It is important to point out that in Argentina, but not in other countries, toxic effects have been reported to be caused by ingestion of this species [12]. For this reason, we undertook both chemical and activity studies which demonstrated that the purified alkaloid extract exhibits an important inhibitory activity on acetylcholinesterase (LD_{50}= 0.58 μg/ml) [13]. From a chemical analysis, we determined that the bioactive extract obtained from specimens collected in spring contained: sauroine [14], sauroxine, 6-hydroxylycopodine, *N*-acetyllycodine, lycopodine, lycodine, *N*-methyllycodine, and clavolonine [13] (Fig 1).

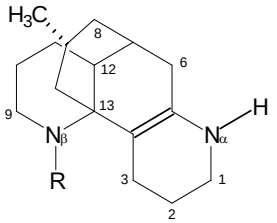
Although *H. saururus* has a wide distribution, it is both ecologically and morphologically specialized and also uniform [4]. However, taking into account that previous analysis of *H. saururus* found significant chemical differences [6-10, 13,14] and that these discrepancies could be attributed to different factors, among them biosynthetic modifications by seasonal causes, we report here on a quali-quantitative content study of the alkaloids present in *H. saururus* collected during all the four seasons of the year, with the aim of verifying if the above mentioned factor has any influence on the observed chemical discrepancies.

Fig. 1. Lycopodium alkaloids isolated from Argentinean specimens of Huperzia saururus

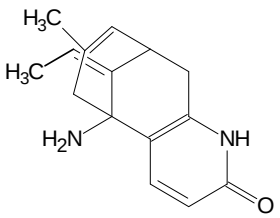


$R = R_1 = R_2 = H$	Lycopodine
$R = OH; R_1 = R_2 = H$	Clavolonine
$R_1 = H; R = R_2 = OH$	Sauroine
$R = R_2 = H; R_1 = OH$	6-hydroxylycopodine

Lycopodane group



$1, C=O; R = CH_3$	Sauroxine
$\Delta^{N\alpha,1}; \Delta^{2,3}; R = H$	Lycodine
$\Delta^{N\alpha,1}; \Delta^{2,3}; R = CH_3$	N-methyllycodine
$\Delta^{N\alpha,1}; \Delta^{2,3}; R = COCH_3$	N-acetyllycodine



Huperzine A

Flabellidane group

Experimental

Plant Material

Plant material was collected in Pampa de Achala, Dpto. San Alberto, province of Córdoba, in different seasons of the year, and was identified by Dr. Gloria Barboza, Instituto Multidisciplinario de Biología Vegetal, Universidad Nacional de Córdoba. Four voucher specimens are deposited at the herbarium of the Museo Botánico de Córdoba as CORD 573 (Summer sample), 395 (Autumn), 572 (Winter) and 684 (Spring).

Preparation and processing of the extracts

Aerial parts of *H. saururus* collected at different seasons of the year were dried at shadow and then crushed. 80 g each were humidified with 0.01 N NaOH solution (50mL) and extracted with $CHCl_3$ by using a Soxhlet extractor. The organic extract was evaporated under reduced pressure until acquiring half of its original volume, partitioned twice with 0.01 N HCl (40 mL each time) and the combined acidic aqueous extract were alkalinized with 0.1 N NaOH to pH 12 and subsequently partitioned with $CHCl_3$ by using a liquid-liquid extractor. The organic phase was evaporated to dryness to yield the crude total alkaloid extract (E_{SU}: 0.30 g; E_A: 0.27 g; E_W: 0.22 g and E_{SP}: 0.25 g). Each extract was submitted to gel filtration on Sephadex LH-20 and MeOH as mobile phase and all the fractions positive to the Dragendorff's reagent were combined and evaporated under reduced pressure. The obtained solid residue represented the total alkaloids. The yield of each one was: E'_{SU}: 0.15 g; E'_A: 0.13 g; E'_W: 0.11 g and E'_{SP}: 0.12 g.

Proccesing and identification of used patterns

In order to obtain the main alkaloids to be used as references, 2700 g of dried and crushed aerial parts of *H. saururus* collected in summer were used. Thus, following the above explained methodology from 10 g of $E_{SP,}$ 2.24 g of E'_{SP} were obtained. This amount was subsequently submitted to different chromatographic techniques (circular, column, and TLC) to yield 20 mg of sauroine (**1**), 7 mg of 6-OH lycopodine (**2**) and 5 mg of sauroxine (**3**) [14, 15]. These three majority alkaloids were identified unmistakably by mass spectrometry and ^1H and 13C NMR, whose data were coincident with the literature [8, 13, 14, 16].

Alkaloid analysis by GC-MS

$E'_{SU,}$ E'_A, E'_W and E'_{SP} were analyzed by using GC-MS in a Perkin Elmer Qmass-910 apparatus, with a capillary column (SE 30) of 30 m in length. The injection volume was 0.5 µl with He as carrier, with the flow rate being 1 ml/min. The temperature program was: 140°C (3 min), 140 to 250°C at 5°C/min, 250°C (5 min), 250-280°C at 5°C/min, 280°C (5 min). The temperatures of the injector, interface, and ion source were 300, 280, and 280°C, respectively. The ionization energy was 70 eV.

Identification

The majority alkaloids present in each different season extract were identified by co-chromatografy in GC-FID using **1, 2,** and **3** as references and by means of their breakdown pattern in GC-EM [8, 14-16].

The minority and traces alkaloids were identified by comparing their breakdown patterns with those found in the literature [8, 13, 14, 17-20].

Quantitative Analyses

The quantitative analyses of the alkaloid extracts $E'_{SU,}$ E'_{A}, E'_{W} and E'_{SP} were carried out by GC/FID in a KONIC KNK 3000 with a capillary column SE 30 of 30 m in length. The analysis conditions (volume of injection, flow rate, temperature program, temperature of injector) were similar to that descript above. The signal was detected with a flame-ionization detector (FID) and the detector temperature was 300 °C.

For the quantitative determination of the alkaloids present in the E' extracts the normalization area method was used. The GC analysis of each extract was developed in triplicate. The percentual average value of each alkaloid that composes the mixture can be observed in Table 1.

Table 1. Quantitative alkaloid content of *Huperzia saururus* seasonal extracts.

Lycopodium alkaloids (MW)	**Season**			
	Summer (% ± SD)	**Autumn (%± SD)**	**Winter (%± SD)**	**Spring (%± SD)**
sauroine (279)	65.2 ± 0.20	57.6 ± 0.15	67.9 ± 0.17	60.9 ± 0.23
6-OH lycopodine (263)	16.7 ± 0.10	13.9 ± 0.10	13 ± 0.15	12.2 ± 0.15
sauroxine (274)	9.3 ± 0.08	19.6 ± 0.05	12.2 ± 0.10	19 ± 0.10
N-acetyllycodine (284)	2.07 ± 0.02	0.70 ± 0.001	3.95 ± 0.02	0.66 ± 0.006
HS1 (261)	3.65 ± 0.01	1.19 ± 0.01	1.07 ± 0.012	4.04 ± 0.015
clavolonine (263)	0.63 ± 0.006	3.63 ± 0.02	0.33 ± 0.015	0.53 ± 0.008
HS3 (260)	1.4 ± 0.03	1.01 ± 0.015	0.56 ± 0.003	1.72 ± 0.01
HS4 (276)	0.23 ± 0.003	0.19 ± 0.002	0.52 ± 0.006	0.63 ± 0.02
HS2 (261)	0.59 ± 0.005	0.27 ± 0.006	0.22 ± 0.004	-
lycodine (242)	0.10 ± 0.006	0.03 ± 0.0006	0.06 ± 0.0008	0.07 ± 0.0012
N-methyllycodine (256)	0.08 ± 0.001	0.02 ± 0.0001	0.07 ± 0.001	0.07 ± 0.001
lycopodine (247)	0.04 ± 0.0006	0.06 ± 0.0002	0.12 ± 0.005	0.18 ± 0.0015
huperzine A (242)	0.01 ± 0.0005	1.80 ± 0.004	-	-

Data are expressed as mean (%) ± S.D.

Results and discussions

In the present study of seasonal monitoring on *H. saururus´* Lycopodium alkaloids by GC-MS, nine *Lycopodium* alkaloids were identified in Summer extract

(E'_{SU}). Four of them belonged to the Lycopodane group (lycopodine, clavolonine, sauroine, 6-hydroxylycopodine) and five, to the flabellidane group (sauroxine, lycodine, *N*-methyllycodine, *N*-acetyllycodine, huperzine A) (Table 1 and Fig. 1). On the other hand, four alkaloids were only characterized as *Lycopodium* alkaloids by their typical fragmentation pattern [17]: being from the first group HS1 ($C_{16}H_{23}NO_2$, MW 261), and HS2 ($C_{16}H_{23}NO_2$, MW 261), and from the second group HS3 ($C_{16}H_{24}N_2O$, MW 260), and HS4 ($C_{16}H_{24}N_2O_2$, MW 276) (Table 1). Autumn extract (E'_A) showed the same pattern as E'_{SU}. Winter extract (E'_W) and Spring extract (E'_{SP}) were very similar but huperzine A was absent from both, and HS2 was not found in E'_{SP}.
It is important to point out that no alkaloid responding to the molecular weight of pillijanine (244) or sauririne (153) was obtained (Table 2).

The relative amounts of the alkaloids were classified into three categories: majority (>9%), minority (<9% and >1%) and traces (< 1%). Three alkaloids occurred as majority throughout the year: sauroine, 6-OH-lycopodine and sauroxine. Substantial quali-quantitative differences in the chemical pattern were not observed during the period studied, as can be seen in Table 1.

Qualitative variations were minor and related to two alkaloids, huperzine A was absent in winter and spring and HS2 did not occur during spring. In the quantitative analysis, it is easy to observe that sauroine, 6-OH lycopodine and sauroxine were the alkaloids present at the highest concentrations throughout all the year, especially the former that in winter and summer exceeded 65 % of the total alkaloids present in the extract (Table 1). The remaining alkaloids were present at minor concentrations (< 9 %) or at trace levels (< 1 %). Thus, sauroine, 6-OH lycopodine and sauroxine chemically characterize *H. saururus* with habitat in the mountainous region of Córdoba (Argentina).

Using these data it was possible to contrast our chemical pattern with that obtained by other authors who studied specimens collected in other geographic regions [9].

With respect to studies on specimens also collected in Argentina, it is important to point out that the structures of pillijanine [6] and saururine [7] were never elucidated. In the present study no structures with the molecular weight assigned to these alkaloids occurred in any of the four seasons. Probably they could have been derived from artifacts or mixtures of alkaloids, considering the time when the studies were developed. On contrary we did confirm sauroxine precense which was already reported by Ayer et al. (Table 2).

The most striking differences refer to the alkaloid compounds informed by Braekman et al. [9] and MacLean [10]. In the former case, the authors reported lycopodine and clavolonine as the main constituents, with fawcettiine and acetylfawcettiine at lower amounts, and huperzine A at trace levels from specimens of Quito (Ecuador), Sabyinyo (Rwanda) and Kahuzi and Ruwenzori (Zaire). They also stated that sauroxine, pillijanine and saururine were absent (Table 2). The absence of sauroxine contrasts greatly with our study, where this compound was one of three alkaloids present at the highest concentration.

On the other hand, in his chapter about Lycopodium alkaloids, MacLean [10] mentioned *H. saururus* alkaloids qualitatively without giving details about the collection site, and then added to the list of known compounds other alkaloids taken from a doctoral thesis (Kahindo 1985) whose results, as far as we know, have not yet been published (Table 2).

Table 2. *Huperzia saururus* alkaloid content according to the site of collection

Alkaloids	Provenance			
	MW	**Argentina (present study)**	**Ecuador/ Zaire/Rwanda (1974)**	**Unknown (1985)**
6-OH lycopodine [13]	263	+	-	-
Sauroine [14]	279	+	-	-
lycodine [13]	242	+	-	-
N-methyllycodine [13]	256	+	-	-
N-acetyllycodine [13]	284	+	-	-
HS1	261	+	-	-
HS2	261	+	-	-
HS3	260	+	-	-
HS4	276	+	-	-
sauroxine [8-10, 13]	274	+	-	+
lycopodine [9, 10, 13]	247	+	+	+
clavolonine [9, 10, 13]	263	+	+	+
huperzine A [8, 10, 13]	242	+	+	+
fawcettiine [8, 10]	307	-	+	+
acetylfawcettiine [8, 10]	349	-	+	+
anhidrolycodoline [10]	245	-	-	+
dihidrolycopodine [10]	249	-	-	+
lycodoline [10]	263	-	-	+
saururidine [10]	291	-	-	+
LS14 [10]	467	-	-	+
pillijanine [6,10]	244	-	-	-
saururine [7,10]	153	-	-	-

Considering these data, it is evident that at least two or three compounds (sauroine and 6-OH lycopodine or also sauroxine) isolated from the Argentinean specimens of *H. saururus* are missing from the populations studied by the above-mentioned authors.

Beyond the differences mentioned herein, the most important compound to take into consideration is sauroine (MW 279) present in the greatest concentration among all the alkaloids reported. The remarkable fact is that it was never mentioned in any publication, not even its molecular weight which is not coincident with any of the alkaloids previously informed with structure not elucidated for *H. saururus*: pillijanina (244), saururine (153), saururidine (291) or LS14 (467) (Table 2).

How can all these chemical discrepancies, not due to seasonal variations as we put in evidence, be explained? Are there many taxa involved in this chemical information?

The chemical composition showed in this paper for the specimens collected in Córdoba would probably be the alkaloid pattern to the species growing from Peru to Argentina, named up to present *H. saururus.* Sauroine, 6-OH lycopodine and sauroxine are the most important and majority alkaloids of this species.

In the case of the chemical composition reported by Braekman for specimens from Zaire, Rwanda and Ecuador we can explain the difference based on the absence of sauroine, 6-OH lycopodine and sauroxine.

The different chemical pattern displayed by the African specimens, named also *H. saururus*, would probably due to many reasons. In fact, one can suspect that the chemical composition varies as a consequence of the disjunt geographical distribution of *H. saururus* or that this chemical pattern belongs to other species different to the one growing in America. The question here now is: Do the same plants or species grow in Ecuador as the ones in Africa? It is much probable that Braekman's chemical pattern [9] for Ecuador does not belong to *H. saururus* due to the distribution of this species is restricted to Andean areas in South America southern of Ecuador [21] and, in consequence, excluded from Ecuador [22].

On the other hand, the chemical composition compiled by MacLean, was taken in part from Kahindo´s Doctoral Thesis and, as it was mentioned above, the provenance of the specimens analyzed is unknown. In this latter case, it is curious the chemical pattern obtained: five alkaloids (anhidrolycodoline, dihidrolycopodine, lycodoline, saururidine and LS14) not found neither for Braekman nor for us, sauroxine present, and sauroine and 6-OH lycopodine missing. Again, it is probable that this pattern belongs to other taxa.

All the statements until here let us to state that the chemical information reported under the same name *H. saururus* deserves more experimental work including samples all along the range of distribution of this species. The data obtained will allow to conclude if the differences are based solely on geobotanical considerations or if different taxa (species or infraspecific taxa) or different races of the same species producing different alkaloids under diverse environmental conditions are the reasons of the discrepancies reported in the literature.

Acknowledgments

The authors thank Dr. B. Øllgaard for the information provided that gave us a better understanding of the issue. This work was carried out thanks to the support of Agencia Córdoba Ciencia, SeCyT-UNC, and FONCYT grants. We also acknowledge native speaker Dr. Paul Hobson for revision of the manuscript.

References

[1] Øllgaard, B., *Opera Botanica* **1987,** *92*, 153.

[2] A. Tryon, B. Lugardon (Eds.), Spores of Pterydophyta. Surface, Wall Structure and Diversity Based on Electron Microspore Studies. Springer Verlag. New York. USA. Inc. **1991,** pp. 589-605.

[3] W. A. Ayer, L. S. Trifonov, In *The Alkaloids*, Brossi, A. (Ed.). Vol. 45, Academic Press, New York (1994), pp. 233-267.

[4] Øllgaard, B. *Ann. Missouri Bot. Gard*. **1992**, *79*, 686.

[5] M. M. Ponce, In *Catálogo de las Plantas Vasculares de la República Argentina*, *I. Pteridophyta, Gymnospermae y Angiospermae (Monocotiledoneae), Monogr. Syst. Bot. Missouri Botanical Garden.* Zuloaga, F., Morrone, O. (Eds.), 1996, 60: 1-79.

[6] Arata, P. N.; Canzoneri, F. *Gazz. Chim. Ital*. **1892**, *22*, 146.

[7] Deulofeu, V.; De Langhe, J. *J. Amer. Chem. Soc*. **1942**, *64*, 968.

[8] Ayer, W.; Habgood, T.; Deulofeu, V.; Juliani, H. *Tetrahedron* **1965,** *21*, 2169.

[9] Braekman, J. C.; Nyembo, L.; Bourdoux, P.; Kahindo, N.; Hootele, C. *Phytochemistry* **1974,** *13*, 2519.

[10] MacLean D.B., In *The Alkaloids*, A Brossi. (Ed.), Academic Press, New York, **1985**, pp. 241-298.

[11] E. R de la Sota, In *Flora de la Provincia de Jujuy*, A L. Cabrera (Ed.). INTA, Bs. As, **1977**, pp. 19-27.

[12] Amorín, J. L. *Farmacobotánica* **1974**, *16*, 3.

[13] Ortega, M.G., Agnese, A.M., Cabrera, J.L. *Phytomedicine* **2004**,*11*, 539.

[14] Ortega, M.G.; Agnese, A.M.; Cabrera, J.L. *Tetrahedron Lett.* **2004**,*45*, 7003.

[15] Ortega, M. G. Estudio de metabolitos secundarios en especies argentinas del género Lycopodium (Lycopodiaceae). Tesis Doctoral, **2002**.

[16] Sun, C.M.; Ho L. K.; Sun, M.L. *Planta Medica* **1993,** *59*, 467.

[17] MacLean, D.B. *Can. J. Chem*. **1963**, *41*, 2654.

[18] Liu, J.S.; Zhu, Y.L.; Yu, C.M.; Zhou, Y.Z.;. Han, Y. Y; Wu, F.W.; Qi, B.F. *Can. J. Chem.* **1986**, *64*, 837.

[19] Alam, S.N.; Adams, A.H.; MacLean, D.B. *Can. J. Chem*. **1964**, *42*, 2456.

[20] Loyola, L.A.; Morales, G.; Castillo, M. *Phytochemistry* **1979,** *18*, 1721.

[21] B. Øllgaard In *Flora of Ecuador*, Harling G. and Andersson L. (Ed.), University of Göteborg, Riksmuseum-Stockholm and Pontificia Universidad Católica del Ecuador, Denmark, **1988**, pp. 1-156.

[22] P.M. Jørgensen, In *Catalogue of the Vascular Plants of Ecuador*. P.M. Jørgensen and S.León-Yánez (Eds.), Missouri Botanical Garden Press, Saint Louis **1999**, pp.148-152.