UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO
POSGRADO EN CIENCIAS BIOLÓGICAS
INSTITUTO DE GEOLOGÍA

ALCALOIDES DEL TIPO IBOGANO EN CUATRO ESPECIES MEXICANAS DE

***Tabernaemontana* (APOCYNACEAE): SU TRASCENDENCIA QUIMIOTAXONÓMICA,**

ETNOBOTÁNICA, FARMACOLÓGICA Y PRODUCCIÓN *IN VIVO* E *IN VITRO*.

TESIS

QUE PARA OPTAR POR EL GRADO DE:

DOCTOR EN CIENCIAS

PRESENTA:

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CIUDAD UNIVERSITARIA, CD. MX., AGOSTO 2020

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ASUNTO: Oficio de Jurado

M. en C. Ivonne Ramirez Wence
Directora General de Administración Escolar, UNAM
Presente

Me permito Informar a usted, que en la reunión ordinaria del Subcomité de Biología Evolutiva, Ecología, Manejo Integral de Ecosistemas y Sistemática, del Posgrado en Ciencias Biológicas, celebrada el día 04 de noviembre de 2019, se aprobó el siguiente jurado para el examen de grado de DOCTOR EN CIENCIAS del estudiante KRENGEL FELIX con número de cuenta 409490097 con la tesis titulada "ALCALOIDES DEL TIPO IBOGANO EN CUATRO ESPECIES MEXICANAS DE *Tabernaemontana* (APOCYNACEAE): SU TRASCENDENCIA QUIMIOTAXONÓMICA, ETNOBOTÁNICA, FARMACOLÓGICA Y PRODUCCIÓN *IN VIVO* E *IN VITRO*", realizada bajo la dirección del DR RICARDO REYES CHILPA, quedando integrado de la siguiente manera:

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Suplente:	DR. VÍCTOR MANUEL CHÁVEZ ÁVILA

Sin otro particular, me es grato enviarle un cordial saludo.

ATENTAMENTE
"POR MI RAZA HABLARA EL ESPÍRITU"
Ciudad Universitaria, Cd. Mx., a 18 de febrero de 2020

COORDINADOR DEL PROGRAMA

DR. ADOLFO GERARDO NAVARRO SIGÜENZA

COORDINACIÓN

C. c p. Expediente del alumno

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A los miembros del Comité Tutor:

Dr. Ricardo Reyes Chilpa

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AGRADECIMIENTOS A TÍTULO PERSONAL

A los miembros del Comité Tutor:

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Dr. Alexandre Toshirrico Cardoso Taketa

Por sus invaluables comentarios, críticas y consejos que permitieron dirigir mi proyecto de investigación al éxito.

A mi hija Inda Jani

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RESUMEN

México es uno de los países con mayor número de especies de *Tabernaemontana* L. (Apocynaceae), varias endémicas. El género biosintetiza una amplia gama de alcaloides indólicos monoterpenoides (MIAs, por sus siglas en inglés), especialmente del tipo ibogano, algunos de los cuales presentan propiedades antiadictivas contra varias drogas de abuso. La presente tesis recopila una serie de publicaciones realizadas durante el programa doctoral del autor, donde se reporta el desarrollo y la optimización de un protocolo simple y eficiente para el análisis cualitativo y cuantitativo de los perfiles alcaloideos de plantas productoras de alcaloides del tipo ibogano. El procedimiento se apoya en la microextracción de pequeñas cantidades de material vegetal con metanol bajo sonicación por periodos cortos. Los extractos obtenidos se analizan mediante cromatografía de gases acoplada a espectrometría de masas (GC-MS), y los datos cuantitativos resultantes pueden ser sometidos a análisis multivariante para la conveniente visualización de las similitudes y diferencias entre los contenidos alcaloideos de distintas especies y/o grupos experimentales. La comparación fitoquímica de las especies mexicanas *Tabernaemontana alba*, *T. amygdalifolia*, *T. arborea* y *T. donnell-smithii* con las también apocináceas *Tabernanthe iboga* y *Voacanga africana*, las fuentes naturales principales para la producción comercial del alcaloide antiadictivo ibogaína, confirmó la cercana relación quimiotaxonómica entre los tres géneros. Este hecho reveló por un lado el gran potencial de las primeras cuatro especies como fuentes alternativas de ibogaína y los compuestos estructuralmente relacionados coronaridina, ibogamina y voacangina. Por otro lado, a diferencia de las especies de África Central que se emplean en ritos como enteógenos, para las especies mexicanas no se encontraron evidencias documentales de ese tipo de aplicaciones etnobiológicas. Esto probablemente se debe a razones socioculturales e históricos, más que fitoquímicos. Además, se determinó la actividad contra *Mycobacterium tuberculosis* y la citotoxicidad en células Vero de extractos

alcaloideos de *T. alba* y *T. arborea*, en comparación con los compuestos puros ibogaína, voacangina y voacamina. Los resultados obtenidos apoyan los principales usos del género en la medicina tradicional mexicana. Se comprobó por primera vez que la coronaridina y la voacangina se pueden convertir en ibogamina e ibogaína, respectivamente, mediante su desmetoxicarbonilación directa en los extractos metanólicos crudos y en un solo paso. Finalmente, se revisaron las técnicas biotecnológicas y de cultivo *in vitro* útiles para establecer la producción sustentable y competitiva de alcaloides del tipo ibogano utilizando especies apocináceas, con particular énfasis en la ruta biosintética de dichos compuestos.

ABSTRACT

Mexico is one of the countries with the highest number of *Tabernaemontana* L. (Apocynaceae) species, several of them being endemic. The genus biosynthesizes a wide range of monoterpenoid indole alkaloids (MIAs), especially of the ibogan type, some of which exhibit antiaddictive properties against various drugs of abuse. As a result of the author's doctoral project, this thesis compiles a series of publications that report the development and optimization of a simple and efficient protocol for the qualitative and quantitative analysis of the alkaloid profiles of ibogan type alkaloid-producing plants. The procedure relies on the microextraction of small amounts of plant material with methanol under sonication for short periods of time. The extracts obtained are analyzed by gas chromatography-mass spectrometry (GC-MS), and the resulting quantitative data can be subjected to multivariate analysis for the convenient visualization of the similarities and differences between the alkaloid contents of different species and/or experimental groups. The phytochemical comparison of the Mexican species *Tabernaemontana alba*, *T. amygdalifolia*, *T. arborea*, and *T. donnell-smithii* with the also apocynaceous African plants *Tabernanthe iboga* and *Voacanga africana*, the main natural sources for commercial production of the antiadictive alkaloid ibogaine, confirmed the close chemotaxonomic relationship between the three genera. This fact revealed the first four species' great potential as alternative sources of ibogaine and the structurally related compounds coronaridine, ibogamine, and voacangine. Moreover, it also suggested that sociocultural and historical factors are better suited than phytochemical reasons to explain why only the Central African but not the Mexican species could be associated with ritualistic entheogenic uses. *Tabernaemontana alba* and *T. arborea* alkaloid extracts, as well as the pure compounds ibogaine, voacangine, and voacamine, were then evaluated for their activity against *Mycobacterium tuberculosis* and cytotoxicity on Vero cell lines. The results support the main applications of the genus in Mexican traditional

medicine. It was shown for the first time that coronaridine and voacangine can be converted into ibogamine and ibogaine, respectively, by a single-step demethoxycarbonylation process applied directly to the crude methanolic extracts. Finally, biotechnological and *in vitro* culture techniques that may prove useful in establishing sustainable and competitive production of ibogan type alkaloids from Apocynaceae species were reviewed, with particular emphasis on the biosynthetic pathway of these compounds.

INTRODUCCIÓN GENERAL

El género *Tabernaemontana* en México

Tabernaemontana L. (Apocynaceae, subfamilia Rauvolfioideae, tribu Tabernaemontaneae; Sennblad & Bremer, 2002) es un género que comprende más de 100 especies de árboles y arbustos perennifolios tropicales en todo el mundo (Van Beek et al., 1984). En México se distribuye a lo largo del territorio nacional, sobre todo en zonas asociadas con selvas altas perennifolias y selvas bajas caducifolias (Juárez Jaimes et al., 2007). Con 18 especies (casi el 20% de las especies conocidas a nivel mundial) de las cuales 8 son endémicas (marcadas con *), el país es un *hotspot* para el género: *Tabernaemontana alba* Mill., *Tabernaemontana amygdalifolia* Jacq., *Tabernaemontana arborea* Rose ex J.D.Sm., *Tabernaemontana chamelensis* L.O. Alvarado & Lozada-Pérez*, *Tabernaemontana citrifolia* L., *Tabernaemontana divaricata* (L.) R. Br. ex Roem. & Schult., *Tabernaemontana donnell-smithii* Rose ex J.D.Sm., *Tabernaemontana eubracteata* (Woodson) A.O. Simões & M.E. Endress, *Tabernaemontana glabra* (Benth.) A.O. Simões & M.E. Endress, *Tabernaemontana hannae* (M. Méndez & J.F. Morales) A.O. Simões & M.E. Endress, *Tabernaemontana litoralis* Kunth, *Tabernaemontana mixtecana* L. O. Alvarado et Juárez-Jaimes*, *Tabernaemontana oaxacana* (L.O. Alvarado-Cárdenas) A.O. Simões & M.E. Endress*, *Tabernaemontana ochoterenae**, *Tabernaemontana riverae* L.O. Alvarado & V. Saynes*, *Tabernaemontana stenoptera* (Leeuwenb.) A.O. Simões & M.E. Endress*, *Tabernaemontana tomentosa* (Greenm.) A.O. Simões & M.E. Endress*, *Tabernaemontana venusta* (J.F. Morales) A.O. Simões & M.E. Endress*. El estado de Oaxaca presenta la mayor cantidad de especies (13), seguido por Chiapas y Veracruz (8 a 10 cada uno; Alvarado Cárdenas et al., 2019).

Fitoquímica de especies mexicanas de *Tabernaemontana*

Tabernaemontana biosintetiza una gran variedad de metabolitos secundarios,

particularmente alcaloides, triterpenos, flavonoides y glicósidos cardiotónicos (Van Beek et al., 1984). No obstante, el grupo de metabolitos secundarios producidos por el género que ha generado el mayor interés científico debido a su amplia actividad biológica y farmacológica son los alcaloides indólicos monoterpenoides (MIAs, por sus siglas en inglés), un amplio y muy diverso grupo de sustancias compuestas por una unidad indólica y otra monoterpenoide (derivadas del triptofano y la secologanina, respectivamente) (Danieli & Palmisano, 1986). Los escasos antecedentes fitoquímicos respecto de las especies mexicanas se limitan a *T. alba*, *T. amygdalifolia*, *T. arborea*, así como *T. donnell-smithii*, y datan de 1958 a 1980 (Achenbach, 1967a, 1967b, 1966; Chaverri Chaverri & Cicció Alberti, 1980; Collera et al., 1962; Kingston, 1978; Walls et al., 1958). Durante los estudios de licenciatura y maestría del autor de la presente tesis, se detectaron varios MIAs en diferentes órganos de la primera y la tercera especie, algunos por primera vez. De éstos destacaron particularmente la coronaridina, ibogamina, voacangina e ibogaína, compuestos pertenecientes al tipo ibogano, una de 11 clases estructurales en las que se dividen los MIAs, y con mayor distribución en el género *Tabernaemontana* (Danieli & Palmisano, 1986; Van Beek et al., 1984). Dentro del tipo ibogano, los cuatro alcaloides mencionados forman un complejo estructural cuyas únicas diferencias residen en la presencia o ausencia de un grupo metoxicarbonilo en posición 16 y/o un grupo metoxilo en posición 10 (para una representación gráfica, véase las figuras de los siguientes capítulos).

Etnobotánica de especies apocináceas productoras de alcaloides del tipo ibogano

La medicina tradicional mexicana se ha servido de distintas especies de *Tabernaemontana* (*T. alba*, *T. amygdalifolia* y *T. donnell-smithii*, *T. citrifolia*, *T. glabra*, *T. litoralis* y *T. tomentosa*) para tratar infecciones dermatológicas, heridas, piquetes y tumores externos, así como para aliviar distintos tipos de dolor (Biblioteca Digital de la Medicina

Tradicional Mexicana (BDMTM), 2009). Curiosamente, en otras regiones del mundo, varias especies de la famila Apocynaceae que biosintetizan alcaloides del tipo ibogano muestran usos etnomedicinales similares, pero solamente de manera secundaria; primordialmente se utilizan por sus efectos sobre el sistema nervioso central. Así, el arbusto *Tabernanthe iboga* Baill. tiene gran importancia como estimulante y enteógeno en África Central. Lo mismo es cierto, en menor medida, para el árbol *Voacanga africana* Stapf y algunas especies africanas, asiáticas y sudamericanas de *Tabernaemontana* (Dickinson, 2016; Pope, 1969; Rätsch, 2007; Schultes et al., 2001; Van Beek et al., 1984). Por el contrario, en México existe un solo registro de aplicación etnobotánica con fines sicoactivos: El látex de *T. donnell-smithii* ha sido utilizado como estimulante (Caballero et al., 1978).

Propiedades antiadictivas de los alcaloides del tipo ibogano

A nivel farmacológico y en concordancia con los usos etnobotánicos descritos para las especies africanas mencionadas, los alcaloides del tipo ibogano se caracterizan por presentar efectos significativos sobre el sistema nervioso central (Van Beek et al., 1984). La ibogaína, por ejemplo, tiene propiedades tanto estimulantes como oneirogénicas (con aspectos alucinógenos y disociativos), no obstante, ha sido asociada sobre todo con actividad antiadictiva en animales (incluyendo al humano) dependientes de varias drogas de abuso como cocaína, morfina, anfetaminas, nicotina y alcohol. Esta actividad parece deberse a la capacidad de la ibogaína de modular varios sistemas de neurotransmisores, interactuando con receptores acetilcolinérgicos (nicotínicos y muscarínicos), de N-metil-D-aspartato (NMDA), opioides (kappa, mu y delta), sigma (1 y 2) y serotoninérgicos (5-HT$_{2A}$ y 5-HT$_{2C}$), así como transportadores de serotonina y dopamina en el sistema nervioso central. En consecuencia, los efectos placenteros y/o los síndromes de abstinencia causados por las drogas de abuso pueden ser reducidos significativamente (Alper, 2001; Alper et al., 2008;

Brown & Alper, 2017; Dickinson, 2016). Otros alcaloides del tipo ibogano como la coronaridina y sobre todo la ibogamina podrían mostrar efectos antiadictivos incluso mayores a los de la ibogaína, a la vez de presentar menor toxicidad (Glick et al., 1994).

Fuentes naturales de ibogaína de importancia comercial actual y potencial

Debido a la complejidad de la síntesis total de la ibogaína (y otros MIAs del tipo ibogano) (Jana et al., 2011; Jana & Sinha, 2012a, 2012b), sus principales fuentes siguen siendo vegetales. El alcaloide es particularmente abundante en la corteza radical de *T. iboga*, especie cuyas poblaciones silvestres están expuestas a severos grados de sobreexplotación a causa de la demanda terapéutica y recreativa de dicho MIA (Dickinson, 2016; Tonye Mahop et al., 2000). Alternativamente, la ibogaína se puede obtener semisintéticamente a partir de la voacangina, compuesto que ocurre en cantidades considerables en las cortezas de tronco y de raíz de *V. africana* (Janot & Goutarel, 1957; Jenks, 2002). De hecho, la mayoría de la ibogaína pura disponible en el mercado global se deriva de esta fuente, ya que en contraste con *T. iboga*, existen plantaciones comerciales de *V. africana* en distintos países de África Occidental (Brako Danquah, 2012; Dickinson, 2016). Aunque al menos diez especies de *Tabernaemontana* biosintetizan ibogaína y 35 voacangina (Van Beek et al., 1984), ninguna ha sido utilizada para la producción comercial de estas sustancias, lo que posiblemente se deba en parte a la falta de información fitoquímica cuantitativa al respecto.

Cabe destacar que hoy día, la producción de ibogaína depende del aprovechamiento de poblaciones silvestres o cultivos de plantas completas (Dickinson, 2016). Las cada vez más sofisticadas herramientas biotecnológicas no se han aplicado a la fabricación masiva del compuesto, a pesar de la existencia de investigación básica referente al cultivo de tejidos vegetales (CTV) de especies de los tres géneros arriba señalados (Basile et al., 1999; Pawelka & Stöckigt, 1983; Sierra et al., 1991; Stöckigt et al., 1982; Van der Heijden et al.,

1988, 1986). Incluso, estudios recientes han contribuido a elucidar detalladamente la ruta biosintética de los MIAs del tipo ibogano en *T. iboga* (Farrow et al., 2019, 2018). Por ende, la producción *in vitro* de dichas sustancias mediante diferentes enfoques biotecnológicos como el CTV, la expresión de enzimas relacionadas con su biosíntesis en sistemas heterólogos y/o la ingeniería genética podrían cobrar importancia a nivel comercial en un futuro cercano.

Presentación de tesis

Por todo lo anterior, la presente tesis consiste de una serie de artículos publicados, enviados y por enviar que ofrecen respuestas a algunas preguntas surgidas del panorama arriba descrito: Los Antecedentes y el Capítulo 1 comparan cuantitativamente los perfiles alcaloideos de las especies mexicanas *T. alba*, *T. amygdalifolia*, *T. arborea* y *T. donnell-smithii* con los de *T. iboga* y *V. africana*, con el objetivo de examinar su relación quimiotaxonómica y evaluar el potencial de las primeras cuatro como fuentes alternativas de alcaloides del tipo ibogano antiadictivos. El Capítulo 2 revisa la etnobotánica de las primeras cinco especies, y explica los diferentes patrones de uso con base en factores ambientales, socioculturales e históricas. Ensayos de efectos farmacológicos diferentes a los antiadictivos, realizados con extractos alcaloideos de *T. alba* y *T. arborea*, así como los compuestos puros ibogaína, voacangine y voacamina en modelos de actividad contra *Mycobacterium tuberculosis* y citotoxicidad en células Vero, se presentan en el Capítulo 3. El Capítulo 4 se enfoca en aspectos asociados con la producción *in vivo* e *in vitro* de MIAs del tipo ibogano. Por un lado, se describe un protocolo optimizado para la extracción y conversión de coronaridina, ibogamina, voacangina e ibogaína, y por otro, se esbozan estrategias para la producción sustentable de estas sustancias mediante el cultivo de tejidos vegetales de especies apocináceaes, con énfasis en las rutas biosintéticas involucradas.

ANTECEDENTES

Quantification of Anti-Addictive Alkaloids Ibogaine and Voacangine in *In Vivo*- and *In Vitro*-Grown Plants of Two Mexican *Tabernaemontana* Species

Felix Krengel, Josefina Herrera Santoyo, Teresa de Jesús Olivera Flores, Víctor M. Chávez Ávila, Francisco J. Pérez Flores, Ricardo Reyes Chilpa

Artículo de investigación derivado del proyecto de investigación de maestría del primer autor, publicado en *Chemistry and Biodiversity, 13*(12):1730-1737 (2016)

Quantification of Anti-Addictive Alkaloids Ibogaine and Voacangine in *In Vivo*- and *In Vitro*-Grown Plants of Two Mexican *Tabernaemontana* Species

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Tabernaemontana alba and *Tabernaemontana arborea* are Apocynaceae species used in Mexican traditional medicine for which little phytochemical information exists. In this study, preliminary gas chromatography/mass spectrometry analyses of different organs obtained from wild plants of both species identified a total of 10 monoterpenoid indole alkaloids (MIAs) and one simple indole alkaloid, nine of which were reported for the first time in these species. Furthermore, callus cultures were established from *T. alba* leaf explants and regeneration of whole plants was accomplished *via* somatic embryogenesis. The anti-addictive MIAs ibogaine and voacangine were then quantified by gas chromatography with flame ionization detection in wild plants of both species, as well as greenhouse-grown plants, *in vitro*-grown plantlets and embryogenic callus of *T. alba*. Ibogaine and voacangine were present in most samples taken from the whole plants of both species, with stem and root barks showing the highest concentrations. No alkaloids were detected in callus samples. It was concluded that *T. alba* and *T. arborea* are potentially viable sources of ibogaine and voacangine, and that these MIAs can be produced through somatic embryogenesis and whole plant regeneration of *T. alba*. Approaches to increase MIA yields in whole plants and to achieve alkaloid production directly in cell cultures are discussed.

Keywords: *Tabernaemontana alba*, *Tabernaemontana arborea*, Ibogaine, Voacangine, Monoterpenoid indole alkaloid, Plant tissue culture, Somatic embryogenesis.

Introduction

Ibogaine is a monoterpenoid indole alkaloid (MIA) which has been investigated for decades, particularly because of its anti-addictive effects in humans and other mammals. Ibogaine and its metabolite noribogaine have been shown to modulate several neurotransmitter systems by interacting with acetylcholine, N-methyl-$_D$-aspartate, opioid, sigma and serotonin receptors, as well as serotonin and dopamine transporters. These complex interaction effects could explain ibogaine's efficacy in reducing self-administration of opiates, amphetamines, cocaine, alcohol and nicotine in animal addiction models.[1][2] Much information regarding the alkaloid's anti-addictive activity in humans comes from the so-called 'global ibogaine medical subculture', a loosely interconnected community of individuals and private clinics that use ibogaine to reduce drug withdrawal symptoms and to induce positive, often long-lasting neurophysiological and psychological changes enabling pharmacodependent persons to break the chain of substance abuse.[2]

The most common source of pure ibogaine is *Tabernanthe iboga*, a shrub native to West Africa.[3] Due to the growing demand for this plant by traditional healers and followers of the Bwiti religion in Gabon and Cameroon, and also international researchers, pharmaceutical companies and the above-mentioned 'ibogaine medical subculture', natural populations of *T. iboga* may have already been

subjected to severe pressure caused by overexploitation.[4] An alternative way to obtain ibogaine consists of extraction of the structurally related MIA voacangine from *Voacanga africana*, and converting the latter into the former by a simple semisynthetic procedure.[3] Although ibogaine and voacangine are principally associated with the *Tabernanthe* and *Voacanga* genera, they have been found in other members of the Apocynaceae family, particularly in at least 10 species of the closely related *Tabernaemontana* genus.[5]

Thus, the research presented in this article was aimed at determining the alkaloid profiles of different organs of the Mexican species *Tabernaemontana alba* and *T. arborea*, with special attention to ibogaine and voacangine. *T. alba* is used in Mexican traditional medicine to treat dermatological infections and to relieve pain.[6] However, little information exists regarding the phytochemical constituents of these species. The MIAs coronaridine and tabersonine were detected in the seeds of *T. alba*,[7] while *T. arborea* is known to contain epivoacorine, voacamine and voacangine in its sap,[8] and voacangine, isovoacangine and tabersonine in its seeds.[9]

In addition to detecting ibogaine and voacangine in wild plants of both species by gas chromatography/mass spectrometry (GC/MS), plant tissue culture techniques were applied in order to induce callus formation and whole plant regeneration from *T. alba* leaf explants. Content of both MIAs was then quantified in wild *T. alba* and *T. arborea* plants, as well as greenhouse- and *in vitro*-grown plants of the former species by gas chromatography with flame ionization detection (GC-FID), contributing in this way to the development of new sustainable options for production of these pharmaceutically important compounds.

Results and Discussion

Qualitative Analysis of Alkaloid Extracts by GC/MS

GC/MS Analyses of the alkaloid extracts obtained from different organs of wild mature *T. alba* and *T. arborea* plants led to the identification of 10 MIAs and one simple indole alkaloid (*N,N*-dimethyltryptamine) (*Table 1*). Only coronaridine in the case of *T. alba* and voacangine in the case of *T. arborea* had previously been reported for these species.[7–9] The substances of

Table 1. Alkaloids identified by gas chromatography/mass spectrometry in *Tabernaemontana alba* and *T. arborea* extracts

Compound	M^+	Base peak	Identification	Extract
10-Hydroxycoronaridine	354	354	[10][a]	T1, T9 ND in *T. arborea*
Apparicine	264	264	NIST[b]	T1, T3, T5, T9, T11 ND in *T. arborea*
Coronaridine	338	338	[11][a]	T5, T7, T9, T11 T8
Ibogaine	310	136	Standard[c], NIST[b]	T1, T3, T5, T7, T9, T11 T2, T4, T6, T8, T12
Ibogamine	280	136	NIST[b]	T5, T7, T9, T11 T2
N,N-Dimethyltryptamine	188	58	NIST[b]	T3 ND in *T. arborea*
Norseredamine	324	324	NIST[b]	T5 T2
Pericyclivine	322	322	[12][a]	ND in *T. alba* T10
Voacangine	368	136	Standard[c], NIST[b]	T1, T3, T5, T7, T9, T11 T2, T4, T6, T8, T12
Vobasine	352	180	NIST[b]	T11 T2, T4, T6, T8, T10, T12
Vobasinol	354	182	NIST[b]	ND in *T. alba* T4, T10

T1 and T2 = leaves; T3 and T4 = twigs; T5 and T6 = stem bark; T7 and T8 = seeds; T9 and T10 = fruits; T11 and T12 = root bark; odd numbers = *T. alba*; even numbers = *T. arborea*. ND = not detected. [a] Compound identification based on comparison of experimentally obtained mass spectra with information reported in the scientific literature. [b] Compound identification based on comparison of experimentally obtained mass spectra with reference mass spectra of NIST Mass Spectral Search Program (Version 2.0). [c] Compound identification based on comparison of experimentally obtained mass spectra with those of authentic samples of pure chemical standards.

interest of this study, ibogaine and voacangine, were detected in all plant organs of *T. alba* and most of *T. arborea*, with the exception of the fruits. Although apparent contamination of the respective mass spectrograms with peaks of coeluted substances made identification of the two alkaloids doubtful in three other cases (extracts T3, T4 and T6; see the corresponding rows in *Table 1*), their presence was later confirmed by GC-FID (see below). Since many MIAs have analgesic (like the detected compounds coronaridine and vobasine), antimicrobial and antiviral (*e.g.*, the likewise detected substance apparicine) properties,[5] use of *T. alba* in Mexican traditional medicine to treat pain and dermatological infections may thus be partially attributed to the presence of these alkaloids in the respective herbal preparations.

Somatic Embryogenesis and Whole Plant Regeneration of T. alba

The growth medium containing (2,4-dichlorophenoxy)-acetic acid (2,4-D) and kinetin proved to be effective in the induction of callus in *T. alba* leaf explants from all five donor plants, resulting in a callus formation rate of virtually 100% between the first and fourth subculture. The only explants that did not develop any callus showed signs of bacterial and/or fungal contamination and had to be disposed of. Nevertheless, treating the greenhouse-grown donor plants with agrochemicals and disinfecting the explants accordingly, as well as adding antibiotics and antimycotics to the growth medium basically eliminated the occurrence of contamination of *in vitro* cultures. Oxidation of explants and callus was very common and increased with the number of subcultures, but could be controlled by supplementing the growth media with ascorbic and citric acid. Failure to prevent oxidation completely might be due to the decomposition of these acids in the media, as a result of their exposure to autoclaving, high light intensity and increasing pH.[13] Nonetheless, it should be noted that even highly oxidized explants and calli continued to produce healthy, non-oxidized callus for at least 15 subcultures. Photoperiod was not a key factor in callus induction, as explants developed this type of undifferentiated cell mass in both light conditions tested.

The same was true for somatic embryogenesis, a phenomenon which appeared in only a few batches of callus originated from one donor plant after at least seven subcultures, most probably due to the effects of 2,4-D, a potent inducer of this specific embryogenic route of development in plant cells.[14] Overall, the embryogenic response was observed in less than 3%

of the total number of jars containing callus, but was consistent in these batches even after one year of subcultures and advanced degrees of oxidation. When the resultant embryos were transferred to MS medium without growth regulators, *ca.* 60% differentiated into whole plants with at least one leaf pair. These could be established in commercial potting soil and acclimatized to non-axenic greenhouse conditions with a 90% survival rate. *Fig. 1* shows different stages of whole plant regeneration from *T. alba* leaf explants. Alternatively, *in vitro*-grown plantlets derived from somatic embryos could be used as a source of axenic root, stem and leaf explants highly susceptible to callus induction. This desirable trait can hypothetically be associated with a high abundancy of actively dividing meristematic cells in somatic embryos and plantlets.[14] Regarding *T. arborea*, attempts to germinate seeds were not successful, and consequently, plant tissue cultures of this species could not be established.

Quantification of Ibogaine and Voacangine in Alkaloid Extracts by GC-FID

Ibogaine and voacangine content of the alkaloid extracts derived from wild mature plants of both species, as well as from several organs taken from young greenhouse-grown *T. alba* plants, from embryogenic callus induced in explants of the latter and from *in vitro*-grown plantlets regenerated from that callus was quantified by GC-FID (*Table 2*). Both MIAs were found in all samples obtained from whole plants of the two species, being the fruits of *T. arborea* the only exception. The highest yields were detected in the root bark of *T. arborea*, followed by the root and stem bark of *T. alba*. Regarding the latter species, similarly high combined ibogaine and voacangine contents were observed in the stem bark of wild mature plants, the roots of young greenhouse-grown plants and *in vitro*-grown plantlets. Nevertheless, alkaloid concentrations fluctuated considerably between samples of the same organ but taken from different donor plants, suggesting the influence of genetic, developmental and/or environmental factors on MIA production. For example, the combined ibogaine and voacangine content of root segments from one greenhouse-grown plant was between 11 and 17 times greater than that of field-collected root bark or that of stem and root segments derived from another plant of a similar age grown under the same greenhouse conditions.

The highest quantities of ibogaine and voacangine detected in *T. alba* (almost 0.04% of ibogaine in root bark and 0.08% of voacangine in stem bark) and *T. arborea* (almost 0.1% of ibogaine and 0.14% of

a)

b)

c)

d)
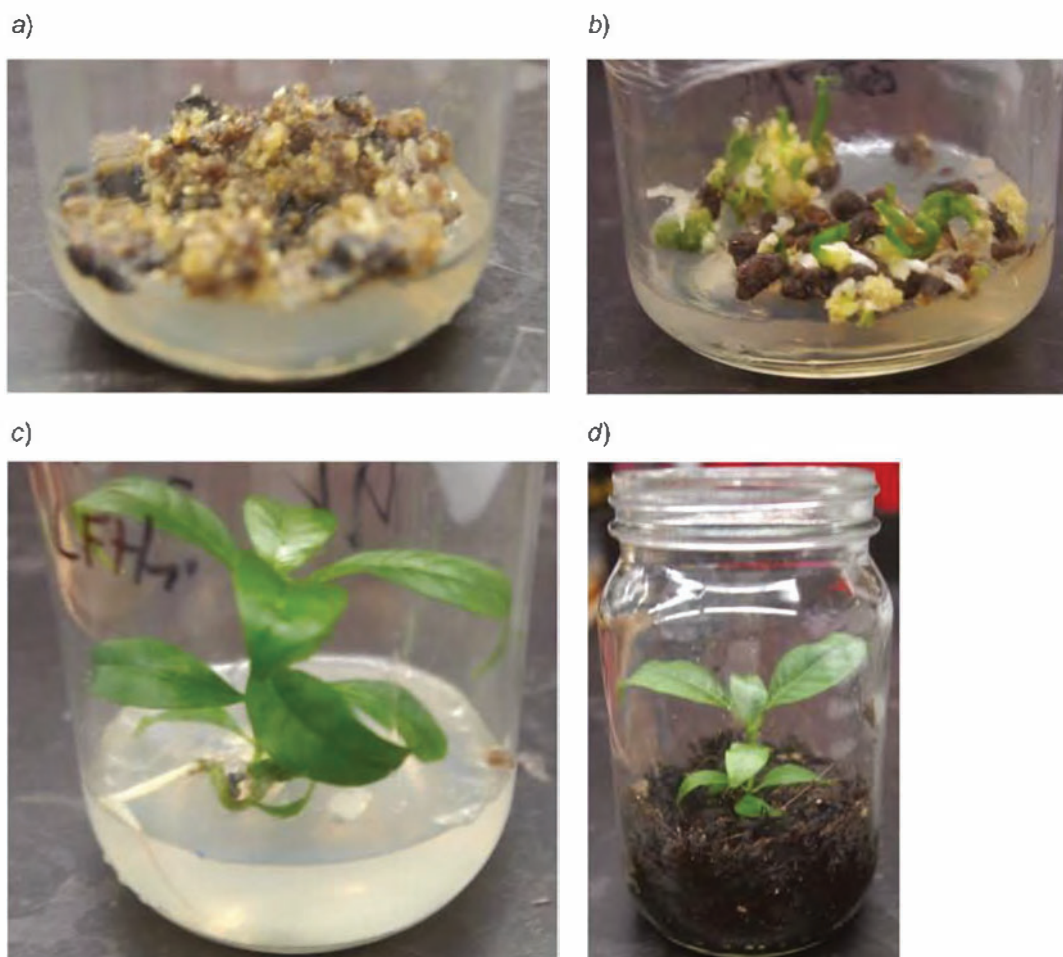
Figure 1. Different stages of *Tabernaemontana alba* whole plant regeneration *via* somatic embryogenesis: *a)* oxidized leaf explants with embryogenic callus; *b)* callus with somatic embryos and plantlets; *c)* whole plant in *Murashige* and *Skoog* medium; *d)* whole plant acclimatized in soil.

voacangine in root bark) were lower but still compara-ble to those reported for *T. iboga* (0.3% of ibogaine in root bark) and *V. africana* (between 0.14% and 0.5% of voacangine in stem bark), the current main sources of these compounds.[3] Thus, research efforts should be undertaken to identify high-yielding genotypes of *T. alba* and *T. arborea* which could then be propa-gated by somatic embryogenesis, and finally be grown *in vitro*, in greenhouse, or in field conditions. In particular, the *in vitro* production of ibogaine and voa-cangine through plant tissue culture offers an exciting alternative to the exploitation of wild populations or the establishment of plantations, as the latter approaches frequently imply environmental risks like overexploitation or loss of biodiversity. Additionally,

plant tissue culture techniques can significantly improve production efficiency by generating biomass in controlled conditions, independently of seasonal factors and natural hazards.[14]

Interestingly, no alkaloids were detected in callus samples of *T. alba*, whereas the plantlets that had been regenerated from the very same calli showed significant ibogaine and voacangine levels. As men-tioned above, these plants cultured *in vitro* under axe-nic and controlled nutritional and environmental conditions produced relatively high concentrations of the two MIAs, comparable to the highest-yielding organs of greenhouse-grown and wild *T. alba* whole plants exposed to a moderate (in the first case) or great (in the second case) variety of herbivores and

Table 2. Ibogaine and voacangine content determined by GC-FID in alkaloid extracts and original plant material obtained from *Tabernaemontana alba* and *T. arborea* whole plants, as well as *T. alba* callus

Alkaloid extract	Ibogaine content in percent of dry weight		Voacangine content in percent of dry weight	
	Alkaloid extract	Plant material	Alkaloid extract	Plant material
T. arborea plant material collected in the field				
T2 (leaves)	5.4532	0.0166	0.3002	0.0009
T4 (twigs)	0.6438	0.0031	0.3922	0.0019
T6 (stem bark)	1.3815	0.0345	1.1192	0.0279
T8 (seeds)	1.8682	0.0011	13.9810	0.0079
T10 (fruits)	ND	ND	ND	ND
T12 (root bark)	3.7196	0.0970	5.3247	0.1388
T. alba plant material collected in the field				
T1 (leaves)	0.6757	0.0008	1.5601	0.0019
T3 (twigs)	1.6185	0.0026	2.0614	0.0033
T5 (stem bark)	1.8127	0.0148	10.2750	0.0838
T7 (seeds)	0.1491	0.0003	0.4507	0.0009
T9 (fruits)	2.1376	0.0036	1.3769	0.0023
T11 (root bark)	0.2975	0.0028	0.4453	0.0042
Plant material taken from greenhouse-grown *T. alba* plants obtained *via* seed germination				
Leaves	0.5333	0.0037	0.3200	0.0022
Stem and root	0.4459	0.0021	0.4992	0.0024
Root	1.4875	0.0397	1.4555	0.0388
Plant material taken from embryogenic callus and *in vitro*-grown *T. alba* plantlets obtained *via* somatic embryogenesis from that callus				
Callus	ND	ND	ND	ND
Whole plant	1.4961	0.0244	4.0332	0.0659

ND, not detected.

microorganisms, as well as other environmental fluctuations. It can therefore be concluded that MIA production was not significantly affected by environmental factors. Taking into account the same genetic origin of the calli and the *in vitro*-grown plantlets analyzed, it rather seems that differentiation plays a critical role in the biosynthesis of ibogaine and voacangine (and probably other MIAs) in *T. alba* (and likely *T. arborea*). In *Catharanthus roseus*, belonging like *Tabernaemontana* to the Apocynaceae family, at least three cell types and five subcellular compartments have to be present in order to produce the enzyme complexes required to biosynthesise the whole range of MIAs that occur in the species.[15] However, the key enzymes of the iboga-type MIA pathway are probably all present in the epidermal cells of aerial organs of *C. roseus*,[16] although restricted to different subcellular compartments.[15] As ibogaine and voacangine are ibogan alkaloids, their biosynthesis in *T. alba* and *T. arborea* may depend highly on subcellular differentiation. Consequently, it is quite probable that the callus propagated in this study lacked the levels of cellular and/or subcellular differentiation required for alkaloid production, but this limitation was overcome by the callus differentiating into plantlets *via* somatic

embryogenesis. Similar examples can be found in the scientific literature for different alkaloids and plant species, *e.g.*, the MIA umbellatine in *Psychotria umbellata*[17] or the protoberberine isoquinoline alkaloids corydaline and cavidine in *Corydalis ambigua*.[18]

Whether or not the growth regulators added to the culture medium had an impact on alkaloid production in the callus remains unclear. On the one hand, 2,4-D has been proposed to reduce alkaloid synthesis in *in vitro* cultures.[19] MIA Production increased when cell suspension cultures of *Tabernaemontana divaricata* were supplemented with naphthalen-1-yl-acetic acid instead of 2,4-D,[20] having been demonstrated that the latter growth regulator can reduce availability of iridoid and indole precursors of the MIA pathway in cell suspension cultures of *C. roseus*.[21] Addition of exogenous tryptamine and loganin to the growth medium can increase MIA production under certain circumstances, with the proportion between both precursors being particularly important.[22] Thus, it cannot be excluded that the lack of MIA production in callus of *T. alba* was caused by insufficient precursor availability and/or the presence of 2,4-D, a compound absent in the media of *in vitro*-grown whole plants derived from the callus. On the other hand, the

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general capability of callus and cell suspension cultures of *T. divaricata* and *Tabernaemontana elegans* to biosynthesise MIAs in the presence of 2,4-D has been documented on several occasions.[20][23][24]

Since direct production of ibogaine and voacangine in callus or cell suspension cultures of *T. alba* would be desirable, but was not observed in this study, research should be done to overcome this constraint. The comparison of growth media with different chemical composition (including nutrients, plant growth regulators and chemical precursors), as well as the isolation of fast-growing and high-yielding cell lines may prove useful. However, if cell differentiation plays an essential role in ibogaine and voacangine biosynthesis, the establishment of hairy root cultures would seem more plausible. Utilizing a common technique to augment MIA yields in *in vitro* cultures of Apocynaceae species, *T. alba* callus, cell suspension or hairy root cultures could also be elicited with signalling compounds involved in the biosynthetic pathway of ibogaine and voacangine, *e.g.*, methyl jasmonate.[25] Eventually, the identification of enzymes engaged in the production of these two MIAs in *T. alba* would open the door to genetic engineering of the species, most notably by making it possible to overexpress key enzymes. This approach has been successfully applied to several Apocynaceae species, focusing on important enzymes of the MIA pathway, like strictosidine synthase and tryptophan decarboxylase.[26][27]

Conclusions

This study reports for the first time the occurrence of ibogaine and eight other alkaloids in different organs of *T. alba* and *T. arborea*, as well as the successful regeneration of *T. alba* whole plants from callus cultures *via* somatic embryogenesis. Ibogaine and voacangine were found in wild, greenhouse-grown and *in vitro*-grown plants of the last species, indicating that *T. alba* (and probably *T. arborea*) is a potentially viable and sustainable source of these two anti-addictive compounds.

Experimental Section

Plant Material

Tabernaemontana alba and *T. arborea* leaves, twigs and fruits, as well as stem and root bark were collected in the vicinity of the Estación de Biología Los Tuxtlas (UNAM) at 30 km of the Catemaco-Montepío road. The material was taken from several mature plants of each species and dried at r.t. or in an oven at 35 °C. Voucher specimens of *T. alba* and *T. arborea* were deposited with the Herbarium of the Facultad de Ciencias (UNAM) (voucher numbers 132793 and 133359, resp.).

Recently collected seeds of both species were submerged in dist. H_2O for 24 h and germinated in commercial potting soil (Nutrigarden Tierra Negra) at r.t. Upon reaching a height of *ca.* 10 cm, plantlets were transferred into individual pots (diameter 15 cm, height 14 cm) in a greenhouse (25 ± 3 °C, sunlight intensity oscillating between 111 and 1110 $\mu Em^{-2} s^{-1}$ during the day), irrigating them twice a week and fertilizing the soil with commercial fertilizer (17-17-17 NPK) every 2 to 3 months. Additionally, plants of at least 30 cm in height were pruned and irrigated with a soln. of benzyl adenine (BA; 1 mg/l) in two-week intervals during 5 months, alternating this treatment with the application of a soln. containing benomyl and *Agri-Mycin*® (2 g/l each). This was done in order to break apical dominance and to control systemic contamination of the plants, resp.

Alkaloid Extraction

Alkaloid extracts were obtained from different organs of wild mature plants of *T. alba* and *T. arborea*, from leaves, stem and root segments of five seed-germinated and greenhouse-grown *T. alba* plants ranging in age from 1 to 2 years, from embryogenic callus derived from explants of the latter, and from five *in vitro*-grown plantlets (having between two and four leaf pairs and a height of 6–10 cm) regenerated *via* somatic embryogenesis from that callus (see below).

Extraction of the plant material was carried out as follows: The dried tissue was ground to a fine powder using a mortar and pestle or a *Retsch SM 2000* cutting mill. The material obtained from wild plants was defatted thrice with hexane and then extracted the same number of times with MeOH for 3 days under constant agitation. The smaller amounts of tissue taken from greenhouse- and *in vitro*-grown plants, as well as callus, were defatted and extracted with the same solvents, resp., but making use of an ultrasonic bath and three extraction cycles of 30 min. After filtration, the MeOH extracts were taken to dryness under reduced pressure, dissolved in 0.001M HCl and filtered again. The filtrate was adjusted to a pH of 10 by adding dropwise a soln. of 28% $NH_3 \cdot H_2O$ with constant agitation. The alkaline soln. was then extracted thrice with CH_2Cl_2 in a separation funnel. The org. phase was separated, dried (Na_2SO_4) and evaporated to dryness under reduced pressure. The resulting alkaloid extracts were stored at −20 °C. For a list of the dry

weights and organic extract yields of the extracted plant material, see *Table S1* in the *Supporting Information*.

Qualitative Analysis of Alkaloid Extracts by GC/MS

The alkaloid extracts derived from the plant material collected in the field were qualitatively analyzed by GC/MS using an *Agilent GC 6890* equipped with a fused silica gel *HP-5* (30 m) cap. column directly coupled to a *JEOL GCMate II* mass spectrometer. The method was based on,[28] recording EI-mass spectra at 70 eV, with the injector in split mode (1:50) at 300 °C and the ramp temp. program raising the oven temp. from 150 to 300 °C at 4 °C/min. He was used as carrier gas at 1 ml/min. Samples were dissolved in $CHCl_3$ (5 mg/ml) and manually injected in volumes of 1 µl. Peaks were identified by comparing their mass spectra with spectra contained in NIST Mass Spectral Search Program (Version 2.0), reported in the scientific literature or experimentally obtained from authentic samples of two standard compounds, ibogaine and voacangine, analyzed under the same conditions as the alkaloid extracts. The standards were kindly donated by *Phytostan Enterprises, Inc.* (Montreal, Quebec) and their identity and purity confirmed by ^{1}H- and ^{13}C-NMR using a *Varian Unity 300* MHz spectrometer. Samples were dissolved in $CDCl_3$ and the spectra recorded at 75 MHz in the case of ^{13}C-NMR or 300 MHz in the case of ^{1}H-NMR.

Somatic Embryogenesis and Whole Plant Regeneration of T. alba

Leaves were taken from greenhouse-grown *T. alba* plants using a sterile scalpel and tweezers, placing them immediately in a jar containing a sterile soln. of ascorbic and citric acid (100 mg/l each) to prevent oxidation. The leaves were then submerged in EtOH (70% *v/v*) for 1 min, followed by exposure to a soln. of NaClO (5% *v/v*), *Microdyn*® and *Triton*™ *X-100* (15 and 3 drops per 250 ml, resp.) for 10 min under constant agitation. Further disinfection of the plant material was achieved by exposing the leaves consecutively to sterile solns. of benomyl (4 g/l) for 15 min, *Agri-Mycin*® (4 g/l) for 15 min and a mixture of cefotaxime and terbinafine (250 mg/l each) for 10 min. The leaves were washed twice in sterile deionized H_2O before and after treatment with each soln. During the last disinfection step, they were cut into explants (0.5–1 cm^2 in size).

Altogether, 180 explants were obtained from five genetically different donor plants, and tissue cultures were initiated independently on five occasions within 1 year. Only explants from the same donor plant were placed in groups of three to five in baby food jars containing solid *Murashige* and *Skoog* (MS) medium supplemented with sucrose (30 g/l), ascorbic acid and citric acid (100 mg/l each), (2,4-dichlorophenoxy)acetic acid (2,4-D) (2 mg/l) and kinetin (0.2 mg/l). The pH value of the medium was adjusted to 5.7 before adding *Agargel*™ (7 g/l) and autoclaving it in an *Erlenmeyer* flask at 120 °C and a pressure of 1.2 kg/cm^2 for 18 min. The medium was allowed to cool to *ca.* 40 °C, supplemented with cefotaxime and terbinafine (250 mg/l each) and dispensed into baby food jars in volumes of 20 ml in a laminar flow cabinet. The cultures were maintained at 25 ± 2 °C in a photoperiod of 16 h of fluorescent white light (intensity of 29 µEm^{-2} s^{-1}) and 8 h of darkness or, alternatively, in continuous darkness. Subcultures were carried out every 3 weeks, omitting the addition of cefotaxime and terbinafine to the medium after the first two subcultures. Callus formed on the explants was separated and transferred to individual jars containing the same medium. Embryogenic callus, embryos and plantlets derived from these cultures were placed onto solid MS medium free of plant growth regulators but supplemented with the concentrations of sucrose, ascorbic acid, and citric acid described above. They were then maintained in the photoperiod condition, regardless of their derivation from cultures grown in presence or absence of light. When plantlets had developed two to three leaf pairs, they were transplanted to jars containing sterile commercial potting soil, acclimatizing them to non-axenic conditions and lower air humidity by removing the lid of the jars during increasing periods of time. Eventually, the acclimatized plantlets could be grown in individual pots containing commercial potting soil under greenhouse conditions.

Quantification of Ibogaine and Voacangine in Alkaloid Extracts by GC-FID

Ibogaine and voacangine content of plant material collected in the field, obtained from greenhouse- and *in vitro*-grown plants and embryogenic callus was quantified by GC-FID. The analyses were carried out as described above, with the exception that an *Agilent 6890N* gas chromatograph was coupled with a flame ionization detector. The FID was set to a temp. of 300 °C, and the injector operated in split mode (1:1). Ibogaine and voacangine peaks were identified by comparison of their retention times (t_R) to those of the two standard compounds, applying the external

standard calibration method. For both MIAs, five point (0.1, 0.25, 0.5, 0.75 and 1 mg/ml) calibration curves were constructed in triplicate. Linear curve adjustment gave the following formulas which were used to calculate the ibogaine and voacangine content of the alkaloid extracts:

Ibogaine : $y = 2.10589x + 6.40926$ $(r^2 = 0.99442)$,

Voacangine : $y = 2.96426x + 4.07363$ $(r^2 = 0.99525)$.

Supplementary Material

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbdv.201600146.

Acknowledgements

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Table S1. Dry Weights and Organic Extract Yields from T. alba *and* T. arborea *Plant Material*

Organ	Weight (g) [Percent of Dry Weight of Plant Material (%)]			
	Dry Weight	Hexane Extract	Methanol Extract	Alkaloid Extract
T. arborea Plant Material Collected in the Field				
T2 (leaves)	632.36	16.89 [2.67]	79.22 [12.53]	1.92 [0.30]
T4 (twigs)	727.79	9.00 [1.24]	27.17 [3.73]	3.45 [0.47]
T6 (stem bark)	293.54	2.60 [0.88]	20.04 [6.83]	7.32 [2.49]
T8 (seeds)	180.56	52.18 [28.90]	12.39 [6.86]	0.21 [0.12]
T10 (fruits)	35.30	8.07	2.25	0.02

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		[22.86]	[6.37]	[0.06]
T12 (root bark)	211.01	13.30	12.98	5.50
		[6.30]	[6.15]	[2.61]

T. alba Plant Material Collected in the Field

T1 (leaves)	400.85	17.55	50.10	0.50
		[4.38]	[12.50]	[0.12]
T3 (twigs)	282.54	11.28	22.52	0.45
		[3.99]	[7.97]	[0.16]
T5 (stem bark)	251.36	11.68	10.80	2.05
		[4.65]	[4.30]	[0.81]
T7 (seeds)	43.89	6.80	3.76	0.09
		[15.49]	[8.57]	[0.20]
T9 (fruits)	78.21	22.57	5.35	0.13
		[28.86]	[6.84]	[0.17]
T11 (root bark)	35.35	2.50	1.22	0.33
		[7.07]	[3.45]	[0.93]

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Plant Material Taken from Greenhouse-Grown *T. alba* Plants Obtained via Seed Germination

Leaves	3.27	0.13	0.36	0.0227
		[4.07]	[11.2]	[0.69]
Stem and root	9.69	0.56	1.05	0.0461
		[5.85]	[10.87]	[0.47]
Root	1.26	0.09	0.28	0.0337
		[7.53]	[22.89]	[2.66]

Plant Material Taken from Embryogenic Callus and *in vitro*-Grown *T. alba* Plantlets Obtained via Somatic Embryogenesis from that Callus

Callus	1.21	0.13	0.42	0.0022
		[11.34]	[34.86]	[0.18]
Whole plant	0.36	0.09	0.07	0.006
		[26.1]	[19.92]	[1.63]

ND = not determined

CAPÍTULO 1: FITOQUÍMICA Y QUIMIOTAXONOMÍA

1.1. Metabolite Profiling of Anti-Addictive Alkaloids from Four Mexican *Tabernaemontana* Species and the Entheogenic African Shrub *Tabernanthe iboga* (Apocynaceae)

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FULL PAPER

CHEMISTRY &
BIODIVERSITY

Metabolite Profiling of Anti-Addictive Alkaloids from Four Mexican *Tabernaemontana* Species and the Entheogenic African Shrub *Tabernanthe iboga* (Apocynaceae)

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Ibogaine and other ibogan type alkaloids present anti-addictive effects against several drugs of abuse and occur in different species of the Apocynaceae family. In this work, we used gas chromatography-mass spectrometry (GC/MS) and principal component analysis (PCA) in order to compare the alkaloid profiles of the root and stem barks of four Mexican *Tabernaemontana* species with the root bark of the entheogenic African shrub *Tabernanthe iboga*. PCA demonstrated that separation between species could be attributed to quantitative differences of the major alkaloids, coronaridine, ibogamine, voacangine, and ibogaine. While *T. iboga* mainly presented high concentrations of ibogaine, *Tabernaemontana* samples either showed a predominance of voacangine and ibogaine, or coronaridine and ibogamine, respectively. The results illustrate the phytochemical proximity between both genera and confirm previous suggestions that Mexican *Tabernaemontana* species are viable sources of anti-addictive compounds.

Keywords: alkaloids, phytochemistry, *Tabernaemontana* (Apocynaceae), *Tabernanthe iboga* (Apocynaceae), ibogaine.

Introduction

Ibogaine and Addiction Treatment

Ibogaine is a monoterpenoid indole alkaloid (MIA) belonging to the ibogan type subclass. The substance has aroused interest among the scientific community since its first isolation in 1901 due to its strong central nervous system (CNS) effects. During the last decades, the substance has found growing use as an aid to

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drug detoxification and alternative psychotherapy through a widespread network of medical practitioners, lay therapists, activists, and some practitioners adapting forms of traditional practice.[1] Despite the fact that supportive evidence of ibogaine's anti-addictive effects is often drawn from a wide sample of anthropological anecdotes, the compound has been successfully tested in animal models of drug abuse.[2] Other ibogan type alkaloids like coronaridine and ibogamine may present an ever higher efficacy while being less toxic than ibogaine, possibly due to the absence of a methoxy group.[3] Notwithstanding, the development of the medical use of ibogaine is limited
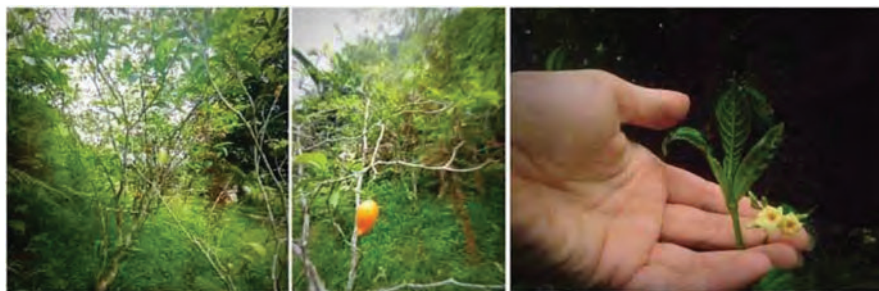
Figure 1. *Tabernanthe iboga.* Whole plant, fruit, and flowers.

by legislation in several countries. In the USA, it was added to Schedule 1 of the US Controlled Substances Act in 1970 because of its psychoactive effects.[4] It is similarly restricted in a number of other countries, while New Zealand, South Africa, and the city of Sao Paolo, Brazil, all have versions of policies that allow for its use.[5] However, the majority of ibogaine treatment occurs outside of prescription programs, through private providers, some of which are licensed treatment centers.[6] Although the total synthesis of ibogaine and structurally related compounds was achieved in the mid-1960s and more efficient chemical procedures were presented a few years ago,[7,8] virtually all the ibogaine currently in existence originates from natural sources.[6]

Tabernanthe iboga *and the* Bwiti *Practice*

Tabernanthe iboga BAILL. (Apocynaceae; *Figure 1*) is a shrub endemic to Central African rainforests where it is commonly known as *iboga*, although the term may apply as well to any of the other eight species of the genus.[9] The plant has a long history of cultural use as a ritual entheogen and medicine, which is predominantly rooted and concentrated in Gabon, especially in the context of *Bwiti*, a spiritual practice of great local importance.[6,10] Although the whole plant of *T. iboga* contains up to 17 MIAs of the ibogan type,[11–15] the ritually most valued material is the bitter inner layer of the root bark which tends to accumulate high concentrations of ibogaine and cause strong stimulant and oneirogenic effects.[16] Being the best known ibogaine-containing plant species, natural populations of *T. iboga* have been exposed to unregulated exploitation in order to meet the expanding demand for this substance from mostly western therapeutic communities. In consequence, *iboga*'s market price has increased at least tenfold in Gabon since the mid-

1990s, creating potential challenges and barriers to access for local traditional practitioners, as well as leading to concerns about sustainability, since traditionally, there has been no significant agricultural *iboga* production.[6,9,17–19]

Mexican Tabernaemontana *Species*

Apart from *T. iboga*, ibogaine has been found in a variety of species of the Apocynaceae family, particularly within the *Tabernaemontana* and *Voacanga* genera.[20] Actually, a significant share of the commercial ibogaine preparations available on the market are now produced via semisynthesis from voacangine isolated from the stem bark of *Voacanga africana* STAPF ex SCOTT-ELLIOT.[6] Being phytochemically more diverse than *Tabernanthe* and *Voacanga*, the pantropical *Tabernaemontana* genus produces 11 classes of MIAs and can be characterized by the presence of compounds belonging to the aspidospermatan, corynanthean, plumeran, and above all ibogan types.[21] Mexico is a diversity hotspot for the genus, with so far 16 identified species, six of which are endemic to the country.[22–24] *Tabernaemontana alba* MILL., *Tabernaemontana amygdalifolia* JACQ., *Tabernaemontana arborea* ROSE ex J.D.SM., and *Tabernaemontana donnell-smithii* ROSE ex J.D.SM. (*Figures 2* and *3*) are known to produce ibogan type alkaloids as major compounds.[21,25–33] These species frequently thrive in disturbed areas associated with tropical deciduous, semi-deciduous, and evergreen forest.[34–36] In the Los Tuxtlas region in the south of the State of Veracruz, *T. alba*, *T. arborea*, and *T. donnell-smithii* are dominant species of the secondary vegetation surrounding the human-induced pasturelands, partially due to the presence of a secondary metabolite-rich latex which acts as a cattle feeding deterrent.[37] The first and the third species are considerably more abundant than the

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Figure 2. *Tabernaemontana amygdalifolia*. Whole plant and flowers.

Figure 3. Fruits of *Tabernaemontana donnell-smithii*, *T. arborea*, and *T. alba* (from left to right).

second.[38] The peculiar morphology of *Tabernaemontana* fruits has earned many species local names alluding to the testicles of mammals, and in the case of the Los Tuxtlas region, the expressions *cojón de gato* (cat balls; *T. alba*), *cojón de venado* (dear balls; *T. arborea*), and *cojón de toro* (bull balls; *T. donnell-smithii*) are common, taking into account the increasing size of the fruits of the three above-mentioned species, respectively (*Figure 3*).

We have previously reported that *T. alba* and *T. arborea* are potentially viable sources of ibogaine and voacangine, which accumulated particularly in the root and stem barks.[26] However, there is a need for quantitative studies that take into account the whole range of alkaloids present in these plants, as some compounds may show anti-addictive effects similar to ibogaine, while others may be toxic. Additionally, these studies should include information regarding the intraspecific variability of alkaloid contents, and the results should be compared with a reference species for these substances. The same procedure could then be applied to other species of the genus.

For these reasons, we followed a metabolite profiling approach based on a microextraction protocol, gas chromatography-mass spectrometry (GC/MS), and principal component analysis (PCA), which allowed for a more comprehensive quantitative analysis of the alkaloid profiles of the barks of four Mexican *Tabernaemontana* species and *T. iboga* than any previous study. The results confirm both the potential of the former as sources of anti-addictive ibogan type alkaloids and the phytochemical proximity between the five species.

Results and Discussion

Interspecific Comparison of the MIA Contents by PCA

Only quantifiable total ion chromatogram (TIC) peaks that could be identified as MIAs were considered relevant to statistical analysis. *Figure 4* illustrates that, generally speaking, the alkaloid profiles of *T. arborea* were more similar to those of *T. donnell-smithii* than to those of *T. amygdalifolia*. *T. alba* root bark samples were divided into two groups, one (2r, 3r, 6r) resembling the mostly ibogamine and coronaridine-containing *T. amygdalifolia* whole root bark, and the other (4r, 5r) being more similar to *T. arborea* root bark which showed a strong association with voacangine and, to a lesser extent, ibogaine. Regarding *T. alba* stem bark, only 3s was associated with *T. amygdalifolia* root bark. The other samples presented greatest similarity to *T. arborea* root bark and/or both types of *T. donnell-smithii* bark, with the latter composing a reasonably homogeneous group defined by its main compound voacangine. *T. amygdalifolia* stem bark formed a quite heterogeneous group, but coronaridine was the major MIA in 12s and 13s. The quantitative predominance of vobasine over voacangine was a unique feature of *T. arborea* stem bark. The *T. iboga* samples 14r and 15r could be distinguished from the different *Tabernaemontana* clusters by their high concentrations of ibogaine.

On an individual scale, the alkaloid profiles of root and stem bark from the same *T. alba* plant could be either similar or considerably different: specimens 3, 4, and 5 showed good coincidence between both types of bark, whereas the opposite was true for specimens 2 and 6. As a general rule for all four *Tabernaemontana* species, the major alkaloid concentrations were always significantly higher in root than in stem bark (*Table S1*).

Two outliers were identified in the scores plot: One *T. amygdalifolia* stem bark sample (11s) that contained
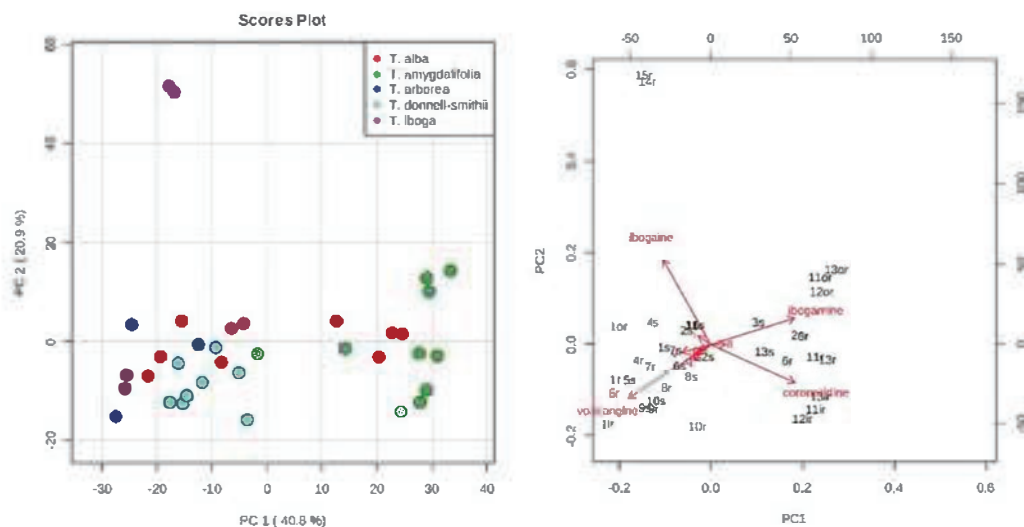
Figure 4. Scores (left) and biplot (right) derived from PCA of the MIA concentrations detected in root and stem bark extracts. 1 = *Tabernaemontana arborea*; 2 – 6 = *Tabernaemontana alba*; 7 – 10 = *Tabernaemontana donnell-smithii*; 11 – 13 = *Tabernaemontana amygdalifolia*; 14 – 16 = *Tabernanthe iboga* (each number represents an individual *Tabernaemontana* spp. plant or *T. iboga* sample). ir = inner root bark; or = outer root bark; r = (whole) root bark; s = stem bark. β= β-hydroxyquebrachamine; a = apparicine; h = 10-hydroxycoronaridine; i = ibogaline; q = quebrachamine; v = vobasine.

only trace amounts of coronaridine, ibogamine, and voacangine, as well as one *T. iboga* root bark sample (16r) with a unique alkaloid profile which was devoid of any of the MIAs detected in the other samples (*Table S1*), but the only one that presented ajmalicine, reserpiline, and yohimbine (data not shown). These three substances were eliminated from the raw data before subjecting it to PCA. Other alkaloids were of minor importance with regard to the PCA outcomes and are discussed in the following paragraphs.

Predominance of the CIVI-Complex Reflects Phytochemical and Taxonomic Proximity between Tabernaemontana *and* Tabernanthe

Ibogamine, coronaridine, ibogaine, and voacangine contributed the most to the separation between samples determined by PCA (*Figure 4*). Interestingly, these alkaloids form a group of structurally related compounds pertaining to the ibogan type of MIAs which hereinafter we will refer to as the 'coronaridine-ibogamine-voacangine-ibogaine (CIVI)-complex': ibogamine represents the basic molecular skeleton, coronaridine its esterified, ibogaine its methoxylated, and voacangine its esterified and methoxylated forms, respectively. However, as the presumed precursor of

the ibogan type alkaloids, stemmadenine presents an ester but not a methoxy group,[39] it seems plausible that, from a biosynthetic point of view, ibogamine is the deesterified-decarboxylated and voacangine the hydroxylated-methoxylated derivative of coronaridine. Ibogaine could then originate either from the hydroxylation-methoxylation of ibogamine or the deesterification-decarboxylation of voacangine (*Figure 5*).

This hypothesis has recently been supported by experimental evidence in a transcriptomic study of *T. iboga* that led to the discovery of two enzymes: ibogamine 10-hydroxylase (I10H) is capable of converting ibogamine to noribogaine and coronaridine to 10-hydroxycoronaridine, while noribogaine-10-*O*-methyltransferase (N10OMT) catalyzes the transformation of noribogaine to ibogaine and 10-hydroxycoronaridine to voacangine.[40] Our results seem to further, albeit indirectly, indicate the validity of this pathway in *Tabernaemontana* species, as the outer root barks of *T. amygdalifolia* (11or, 12or, 13or) and *T. arborea* (1or) accumulated higher concentrations of the non-esterified compounds (ibogamine and ibogaine, respectively) than the inner root barks (11ir, 12ir, 13ir, and 1ir) whose major alkaloids were always present in its esterified form (coronaridine and voacangine, respectively; *Figure 4*, *Table S1*). Taking into account that

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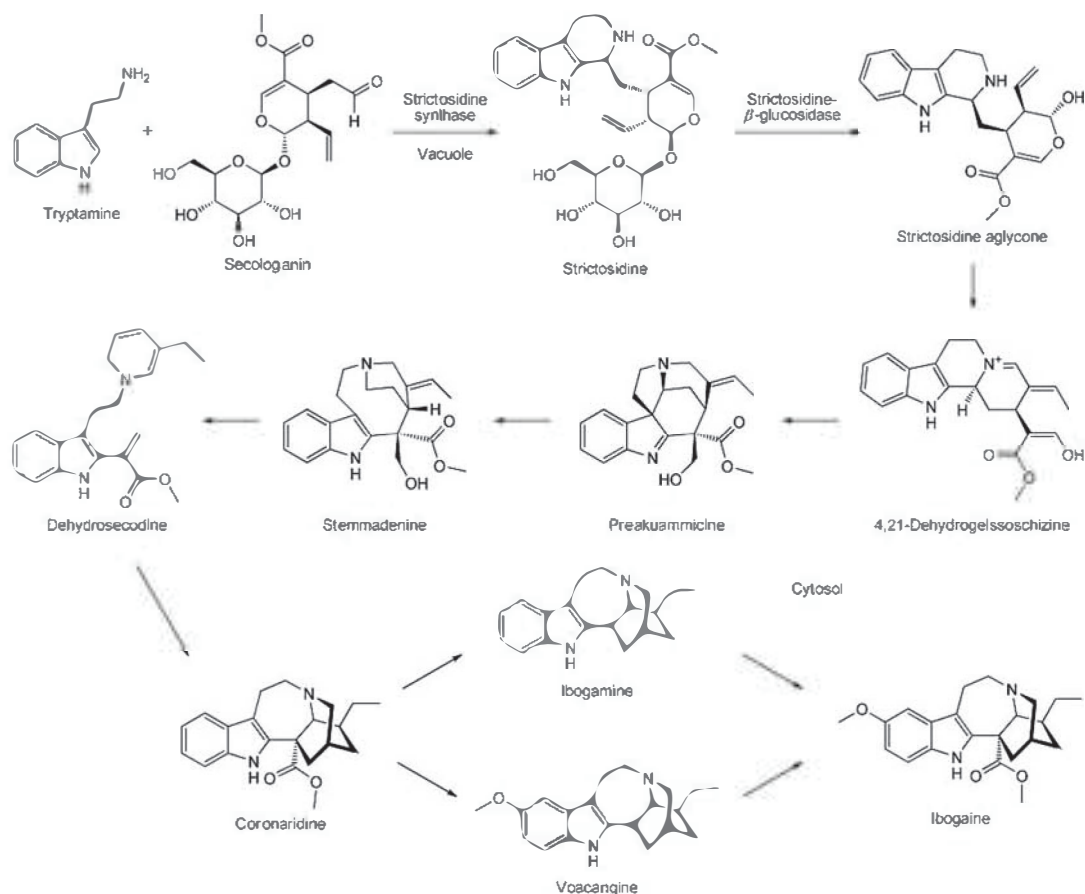
Figure 5. Hypothetical biosynthetic pathway of the CIVI-complex (modified from [39]). Coronaridine and voacangine are the methyl esters of the 16-carbocylic acids of ibogamine and ibogaine, respectively.

outer root bark consists of more mature and supposedly more differentiated tissue than inner root bark, it would make sense that the former accumulated more alkaloids associated with later biosynthetic stages than the latter. In the case of *T. alba* and *T. donnell-smithii*, the root bark was not thick enough to allow separation into inner and outer layers.

From a taxonomic perspective, the predominance of the CIVI-complex in all five species reflects the close relationship between the *Tabernaemontana* and *Tabernanthe* genera. According to *Sennblad* and *Bremer*,[41] the former is a sister group to a clade formed by the latter plus two other African genera (*Carvalhoa* and *Schizozygia*). The fact that we detected only trace amounts of coronaridine in the *T. iboga* root bark samples (*Table S1*) may be due to its occurrence being

restricted to the seeds of the species.[13] The minor alkaloids, vobasine (corynanthean type; *T. alba* and *T. arborea*), apparicine (aspidospermatan type; *T. alba* and *T. amygdalifolia*), 10-hydroxycoronaridine (ibogan type; *T. donnell-smithii*), β-hydroxyquebrachamine, quebrachamine (aspidospermatan type; *T. donnell-smithii*), and ibogaline (ibogan type; *T. iboga*) were of minor importance to the PCA outcomes, but could possibly serve as species-specific chemical markers, either on a whole-plant or organ-specific level: For instance, both quebrachamine-related compounds seem to be exclusive to *T. donnell-smithii*, whereas in the case of 10-hydroxycoronaridine, this may only be true for the barks, since the alkaloid has also been found in the leaves and fruits of *T. alba*.[26] Finally, it should be noted that the alkaloid profiles of *T.*

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amygdalifolia were quite different to previous reports from other researchers, given that we did not detect any compounds of the plumeran type such as cylindrocarpidine and related substances in the barks.[27–29]

Potential of Mexican Tabernaemontana *Species as Alternative Sources of Anti-Addictive Alkaloids*

This study basically confirms the proposal of *Krengel et al.*[26] that *T. alba* and *T. arborea* could be used to produce ibogaine, especially when taking into account the combined ibogaine and voacangine contents, as the latter can be semisynthetically converted to the former.[42] The highest yields of these alkaloids detected in the *Tabernaemontana* samples corresponded to 0.95% of voacangine, as well as 0.22% and 0.27% of ibogaine of root bark dry weight of *T. alba* and *T. arborea*, respectively, hence giving combined concentrations of the two MIAs between 1.17 and 1.22% (*Table S1*). These values are on par with the 1.17% of ibogaine determined in the *T. iboga* root bark sample 14r which originated from a Gabonese farm with commercial goals, and considerably superior (at least 5 times) to what *Krengel et al.*[26] reported for the same species and MIAs. The considerable quantitative differences between the latter and the current study may be explained by the fact that in the first publication, plant material belonging to the same organ and species was pooled together prior to extraction (thus giving average values of high and low yielding individuals), whereas in the present work, samples obtained from individual plants were treated separately. Additionally, the significant variability of ibogaine and voacangine contents between *T. alba* samples suggests that yields could be substantially improved by selecting appropriate chemo- and genotypes, as well as by growing plants in controlled conditions that favor alkaloid production. While in the case of *T. iboga* root bark the inner layer contains most of the ibogaine,[6] in *Tabernaemontana* species there seems to be a tendency to basically restrict the occurrence of non-esterified MIAs to the outer root bark. Consequently, separating inner from outer bark prior to extraction could facilitate isolation of the individual alkaloids. Thorough processing of *Tabernaemontana* bark might also provide a higher-yielding produce and make comparisons with the usually refined *iboga* products more exact.

T. amygdalifolia and *T. donnell-smithii* barks produced only small amounts of ibogaine, but reasonable concentrations of voacangine. Notably, the first species presented considerable concentrations of ibogamine and coronaridine ranging from 0.76 to 0.95%, and from 1.09 to 1.38% of (whole) root bark dry weight, respectively, and even reaching the very high value of 2.05% of ibogamine in outer root bark (*Table S1*).

Considering that *T. alba* presented two clusters of chemotypes, one biosynthesizing mainly the non-methoxylated and the other the methoxylated forms of the (both esterified and non-esterified) ibogamine skeleton in the root bark, this species may actually be a good source of the whole CIVI-complex.

Quantification of Ibogaine in Tabernanthe iboga *Root Bark*

Two *T. iboga* samples (14r and 15r) showed alkaloid profiles characteristic of the species, ibogaine being by far the most abundant compound.[20,43] With the exception of *Jenks*[42] who isolated 3.666 g of ibogaine hydrochloride from 1000 g of *T. iboga* root bark, no published quantitative data on the species' MIA content could be found (the total alkaloid content of *T. iboga* root bark can be as high as 6%[43]). Our results suggest a significant intraspecific variability in ibogaine concentration with one sample (15r) showing a 4-times higher value than the other (14r; *Table S1*), although it is worth noting that we do not know whether the *T. iboga* samples were each obtained from only one plant or rather from a pool of several plants. The very high concentration of 4.75% of ibogaine per dry weight of plant material was actually determined in the root bark produced by a Gabonese Pygmy community. This ethnic group has probably the longest history of ethnobotanical use of *T. iboga*,[6] which could imply that the high alkaloid content in sample 15r is the result of sophisticated plant selection or specific local growing conditions. In contrast, the 14r sample originated from Otong Mekok, a farm in Northern Gabon that aims to produce *iboga* sustainably and economically for both the local and international market. In any case, our results underline the difficulty of correct dosing when using *T. iboga* root bark directly or in the form of extracts (instead of pure ibogaine) in an ethnobotanical or therapeutic context, as depending on the administered dose, ibogaine can act as a stimulant or entheogen, but can also have cardiotoxic effects, particularly in combination with risk factors involving preexisting cardiovascular diseases or adverse drug interactions.[44] Even in traditional settings with a long history of usage and knowledge of *iboga* such as *Bwiti* rituals, incidental

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fatalities related to medical complications do occur.[20,43]

Actually, there is a controversy concerning the degree to which these fatalities are caused by the consumption of 'false *iboga*'. In fact, the plant's long harvest cycle, its rapid rise in value, and its unregulated trade[6] have created conditions that incentivize the adulteration or counterfeit of *T. iboga* root bark with other plant materials, sometimes dangerous. Reports of 'false *iboga*' leading to adverse physical reactions and even to death have been heard within user communities and previously confirmed by *Guignon*[19] in the context of Gabonese traditional medicine, although it is important to point out that the phenomenon is not limited to a local or regional scale, but has also been associated with international mail order and internet sales.[45] It consequently fits the picture that the sample purchased on a local market in Cameroon (16r) did not contain any ibogan type alkaloids, but reserpiline, yohimbine, and ajmalicine (all corynanthean type). The sample was probably obtained from another species of Apocynaceae, most likely belonging to the *Rauvolfia* genus,[46] and mislabeled as *T. iboga* root bark.

Conclusions

Our research offers rare quantitative data on the MIA profiles of the Mexican *Tabernaemontana* species, *T. alba, T. amygdalifolia, T. arborea,* and *T. donnell-smithii,* as well as the African shrub *Tabernanthe iboga.* The predominance of a complex of four structurally related ibogan type alkaloids – coronaridine, ibogamine, voacangine, and ibogaine – in root and/or stem bark proved to be a characteristic chemical feature of the five species. This illustrates the phytochemical and taxonomic proximity between the two genera, and strongly suggests that *Tabernaemontana* species are promising candidates for the production of anti-addictive alkaloids from alternative plant sources. Furthermore, we hope to contribute to the risk reduction regarding both the traditional and therapeutic use of ibogan type alkaloid-containing plant material, particularly with reference to *T. iboga,* the sacred plant that is so essential to Central African spirituality and innovative addiction treatment.

Experimental Section

Plant Material

Root and stem bark was taken from young and mature *Tabernaemontana alba* MILL. (*cojón de gato*), *Tabernaemontana arborea* ROSE ex J.D.SM. (*cojón de venado*), and *Tabernaemontana donnell-smithii* ROSE ex J.D.SM. (*cojón de toro*) plants in the vicinity of the Estación de Biología Los Tuxtlas (UNAM) in Veracruz, Mexico (18°33' to 18°36' N 95°04' to 95°09' W), in February 2017. *Tabernaemontana amygdalifolia* JACQ. (*jasmín de perro*) bark was collected in San Antonio Mulix (20°40'6" N, 89°45'24" W) and Valladolid (20°41'39.872" N, 88°13'38.964" W), Yucatán, Mexico, in April 2017. Taxonomic identification of the species was carried out by Delfino Álvaro Campos Villanueva (Estación de Biología Los Tuxtlas, UNAM) and Leonardo Osvaldo Alvarado Cárdenas (Facultad de Ciencias, UNAM). Voucher specimens were deposited with the Herbarium of the Facultad de Ciencias (FCME), UNAM (voucher numbers 161424 to 161427), and plant names were checked with http://www.theplantlist.org. Root bark samples of *Tabernanthe iboga* BAILL. (*iboga*) were acquired from a distributor in Western Cameroon, a traditional Gabonese Pygmy village, and from Otong Mekok, a commercial farm in Northern Gabon.

Extraction

The dried bark was pulverized using a cutting mill. The extraction procedure was loosely based on the protocol published by *Kim et al.*[47]: 100 mg samples, each associated with an individual plant (trunk bark as well as inner, outer, and whole root bark from five *Tabernaemontana alba,* three *T. amygdalifolia,* one *T. arborea,* and four *T. donnell-smithii* plants, plus the already processed *Tabernanthe iboga* samples), were placed in 2 ml Eppendorf tubes, to which a small piece of degreased cotton wool (approximately 10 mg) and a mixture of dichloromethane (1.5 ml) and ammonium hydroxide (50 μl) was added. The tubes were vortexed and put into an ultrasonic bath for 30 min. After centrifuging at 14500 rpm for 2 min, the supernatant was sucked up into a Pasteur pipette through the cotton wool and evaporated to dryness at room temperature in darkness. This procedure was repeated four more times for each sample. The alkaloid extracts were stored at −20 °C.

Analysis of Alkaloid Extracts by GC/MS

The extracts were dissolved in 2 ml of methanol and analyzed using an Agilent 7890B/5977A GC/MSD with a HP-5ms (30 m) capillary column following the method described by *Krengel et al.*[26], with minor modifications: EI-mass spectra recording at 70 eV after 8 min of solvent delay; injector in split mode (1:5) at 300 °C with automatic injection of 1 µl aliquots; ramp temperature program from 150 to 300 °C at 4 °C/min; Helium carrier gas at 1 ml/min. Peak identification was carried out by the NIST Mass Spectral Search Program for the NIST/EPA/NIH Mass Spectral Library (Version 2.2, built on June 10, 2014) and by comparison of the respective mass spectra with published data or experimentally obtained spectra of standard compounds (representative chromatograms and mass spectra can be found in *Figures S1* and *S2* in the *Supporting Information*). These compounds, ibogaine and voacangine, were kindly donated by Phytostan Enterprises, Inc. (Montreal, Quebec) and used as external standards to construct calibration curves consisting of six points (25, 50, 100, 200, 300, and 400 µg/ml) with three repetitions each. The ibogaine and voacangine contents of the samples were calculated by the respective formula, while other MIAs were quantified based on the calibration curve of the structurally more similar alkaloid.

Ibogaine (appARICine, β-hydroxyquebrachamine, ibogamine, ibogaline, quebrachamine):

$$y = 212807.90171x + 2760970.43093 \ (r^2 = 0.99766)$$

Voacangine (coronaridine, 10-hydroxycoronaridine, vobasine):

$$y = 194161.98834x + 2381117.06883 \ (r^2 = 0.99789)$$

Statistical Analyses

Statistical analyses of the MIA concentrations were performed with the free R-based online software MetaboAnalyst 3.0 (http://www.metaboanalyst.ca/#). Data was normalized by sum and Pareto-scaled before subjecting it to PCA.

Acknowledgements

Author Contribution Statement

F. K., Q. C., and R. R. C. collected the *Tabernaemontana* plant material. J. D. provided the *iboga* samples. F. K. and Q. C. extracted the samples and conducted the GC/MS and statistical analyses. F. K., J. D., and J. H. S. wrote the draft. Based on the comments of all authors, F. K. and R. R. C. wrote the final version of the manuscript.

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CORRIGENDUM

CORRIGENDUM

Felix Krengel, Quentin Chevalier, Jonathan Dickinson, Josefina Herrera Santoyo, and Ricardo Reyes-Chilpa*

Metabolite Profiling of Antiaddictive Alkaloids from Four Mexican *Tabernaemontana* Species and the Entheogenic African Shrub *Tabernanthe iboga* (Apocynaceae)

On page 5, the structures of voacangine and ibogaine were confused in *Figure 5*. The correct structures are:

Voacangine

Ibogaine
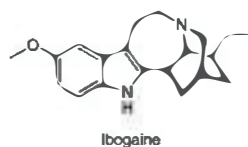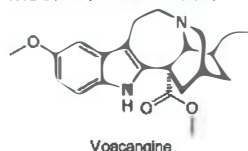
Consequently, *Figure 5* should be as follows:

CHEMISTRY & BIODIVERSITY

Supporting Information

Metabolite Profiling of Anti-Addictive Alkaloids from Four Mexican *Tabernaemontana* Species and the Entheogenic African Shrub *Tabernanthe iboga* (Apocynaceae)

Felix Krengel, Quentin Chevalier, Jonathan Dickinson, Josefina Herrera Santoyo, and Ricardo Reyes Chilpa*

Metabolite Profiling of Antiaddictive Alkaloids from Four Mexican *Tabernaemontana* Species and the Entheogenic African Shrub *Tabernanthe iboga* (Apocynaceae).

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Ibogaine and other ibogan type alkaloids present antiaddictive effects against several drugs of abuse and occur in different species of the Apocynaceae family. In this work, we used chromatography-mass spectrometry (GC-MS) and principal component analysis (PCA) in order to compare the alkaloid profiles of the root and stem barks of four Mexican *Tabernaemontana* species with the root bark of the entheogenic African shrub *Tabernanthe iboga*. PCA demonstrated that separation between species could be attributed to quantitative differences of the majority alkaloids coronaridine, ibogamine, voacangine, and ibogaine. While *T. iboga* mainly presented high concentrations of ibogaine, *Tabernaemontana* samples either showed a predominance of voacangine and ibogaine, or coronaridine and ibogamine, respectively. The results illustrate the phytochemical proximity between both genera, and confirm previous suggestions that Mexican *Tabernaemontana* species are viable sources of antiaddictive compounds.

Keywords: Alkaloids · Phytochemistry · *Tabernaemontana* (Apocynaceae) · *Tabernanthe iboga* (Apocynaceae) · Ibogaine

Supporting Information

a)

b)

c)

d)

e)

2
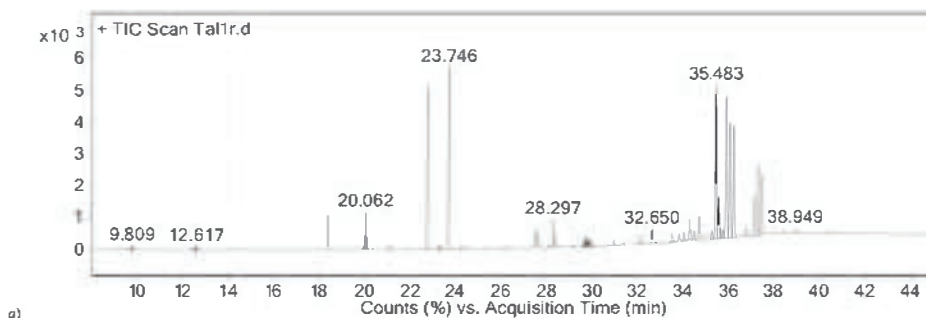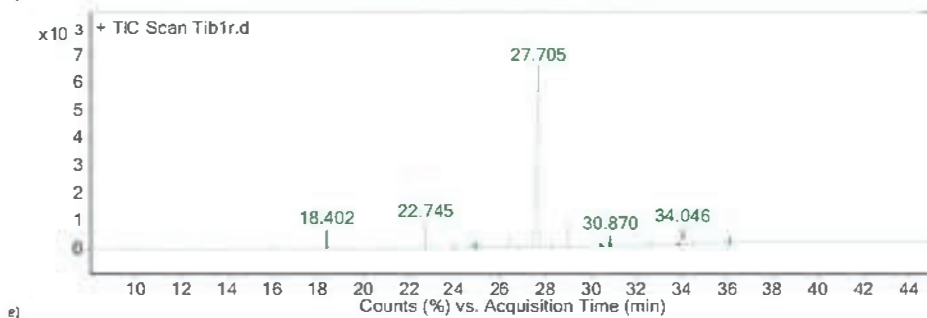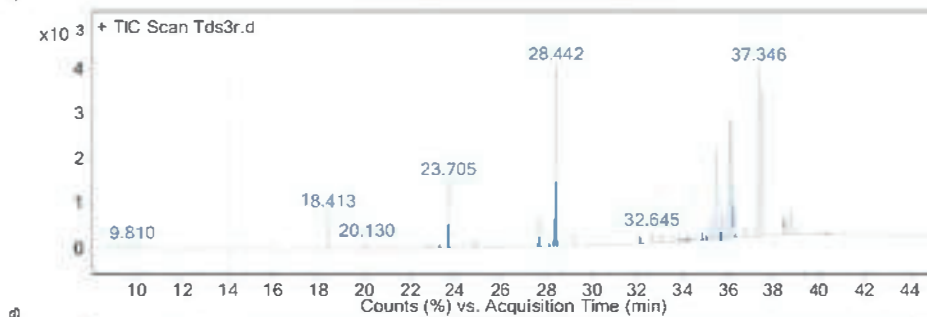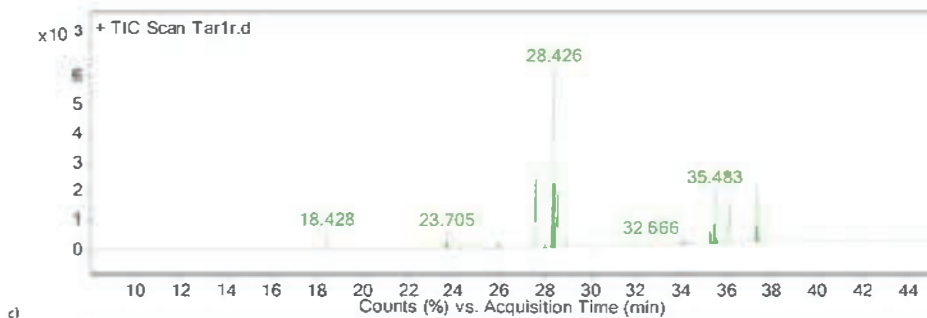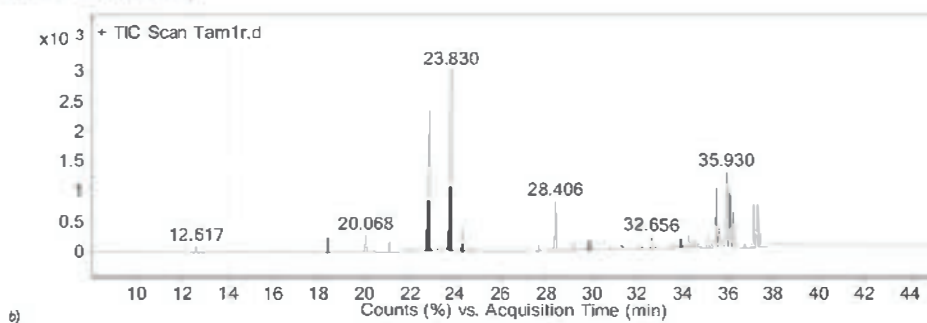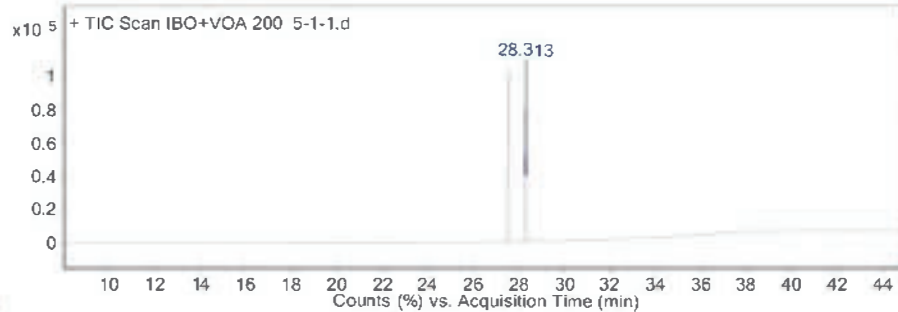
Figure S1. Chromatograms from the root barks of a) *Tabernaemontana alba* (sample 2r); b) *T. amygdalifolia* (sample 11r); c) *T. arborea* (sample 1r); d) *T. donnell-smithii* (sample 9r); e) *Tabernanthe iboga* (sample 14r); and f) chemically pure ibogaine and voacangine.

Retention times (RT) for ibogamine, coronaridine, ibogaine, and voacangine are 22.8±0.1, 23.8±0.1, 27.6±0.2, and 28.3±0.2 min, respectively. Compounds with RT > 34 min are phytosterols and terpenoids.

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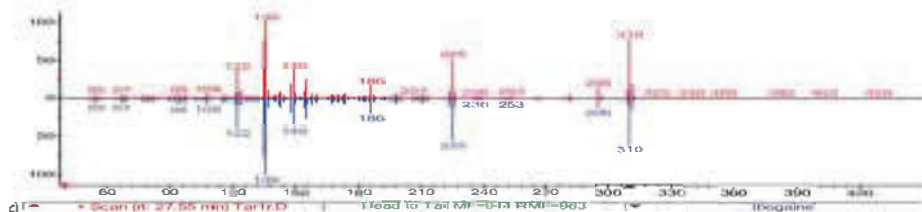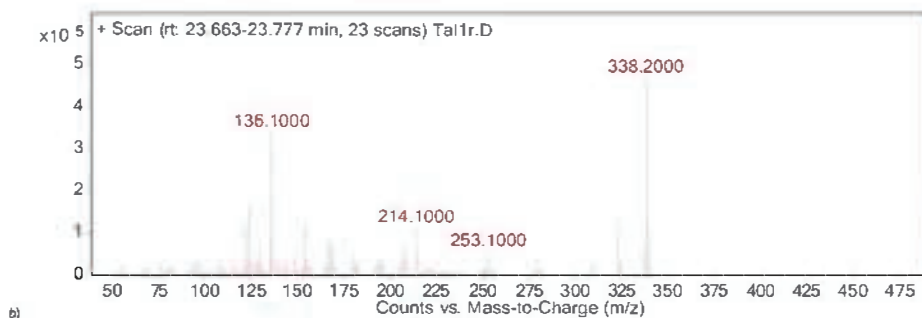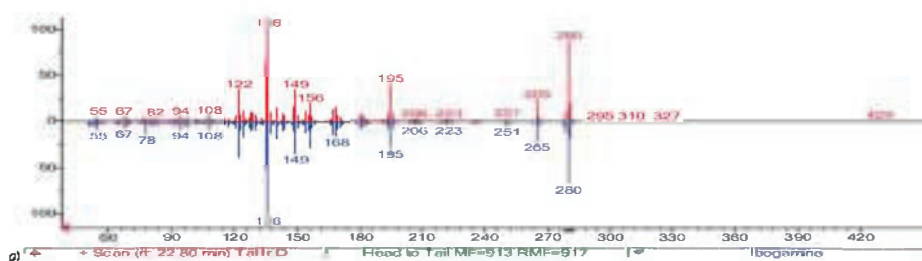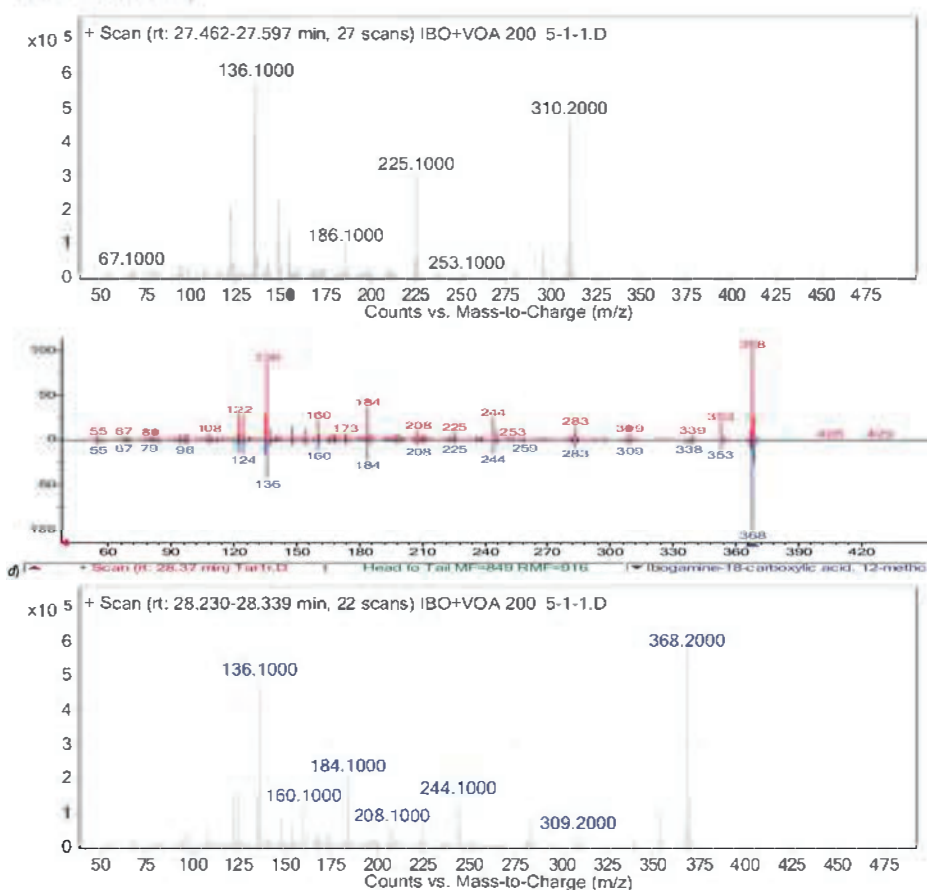
Figure S2. Mass spectra of a) Ibogamine (experimental spectrum obtained from sample 2r (red) vs spectrum from NIST/EPA/NIH Mass Spectral Library (blue)); b) coronaridine (experimental spectrum obtained from sample 2r); c) Ibogaine (experimental spectrum obtained from sample 1r (red) vs spectrum from NIST/EPA/NIH Mass Spectral Library (blue) vs experimental spectrum obtained from chemically pure ibogaine); and d) voacangine (experimental spectrum obtained from sample 1r (red) vs spectrum from NIST/EPA/NIH Mass Spectral Library (blue) vs experimental spectrum obtained from chemically pure voacangine).

Coronaridine was identified by comparison of the experimental mass spectra obtained from the samples with data reported in the scientific literature as described by *Krengel et al.* [26] .

Table S1. MIA concentrations detected in root and stem bark extracts (in percent of dry weight of plant material).

Sample	ß-HO-quebrachamine	Apparicine	Quebrachamine	Ibogamine	Coronaridine	Ibogaine	Voacangine	Vobasine	10-HO-coronaridine	Ibogaline
1r	0.0000	0.0000	0.0000	0.0362	0.0728	0.2728	0.9545	0.1827	0.0000	0.0000
2r	0.0000	0.0787	0.0000	0.2953	0.3483	0.0460	0.0643	0.0431	0.0000	0.0000
3r	0.0000	0.0391	0.0000	0.2014	0.2037	TR	0.0330	TR	0.0000	0.0000
4r	0.0000	0.0000	0.0000	0.0501	0.0888	0.1840	0.4882	0.0651	0.0000	0.0000
5r	0.0000	TR	0.0000	0.0415	0.0749	0.2235	0.9588	0.0779	0.0000	0.0000

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6r	0.0000	0.0497	0.0000	0.2149	0.5181	0.0779	0.1300	TR	0.0000	0.0000
7r	0.0000	0.0000	0.0370	0.0286	0.0458	0.0697	0.2068	0.0000	0.0000	0.0000
8r	0.0000	0.0000	0.0404	TR	0.1239	0.0742	0.2448	0.0000	0.0499	0.0000
9r	0.0000	0.0000	0.0354	0.0322	0.1416	0.0739	0.4400	0.0000	0.0463	0.0000
10r	TR	0.0000	0.0849	0.0311	0.2289	0.0000	0.2742	0.0000	0.0363	0.0000
11r	0.0000	0.0814	0.0000	0.7643	1.0921	0.0470	0.2175	0.0000	0.0000	0.0000
13r	0.0000	0.0785	0.0000	0.9545	1.3887	TR	0.1868	0.0000	0.0000	0.0000
14r	0.0000	0.0000	0.0000	0.0974	0.0000	1.1751	0.0435	0.0000	0.0000	0.0736
15r	0.0000	0.0000	0.0000	0.3993	TR	4.7570	0.2825	0.0000	0.0000	0.2483
16r	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
1s	0.0000	0.0000	0.0000	0.0000	0.0000	TR	0.0295	0.1161	0.0000	0.0000
2s	0.0000	0.0309	0.0000	0.0000	0.0302	0.0330	0.0428	0.0340	0.0000	0.0000
3s	0.0000	0.0425	0.0000	0.1134	0.1373	0.0510	0.0458	0.0371	0.0000	0.0000
4s	0.0000	0.0000	0.0000	TR	TR	0.0495	0.0700	0.0426	0.0000	0.0000
5s	0.0000	0.0000	0.0000	TR	0.0279	0.0607	0.2386	0.0611	0.0000	0.0000
6s	0.0000	0.0000	0.0000	0.0000	0.0470	0.0292	0.0810	0.0000	0.0000	0.0000
7s	TR	0.0000	0.0327	0.0000	0.0316	0.0307	0.0588	0.0000	0.0000	0.0000
8s	TR	0.0000	0.0292	TR	0.0437	TR	0.0543	0.0000	TR	0.0000
9s	TR	0.0000	0.0344	TR	0.0538	0.0335	0.2304	0.0000	0.0335	0.0000
10s	0.0463	0.0000	0.0848	TR	0.0341	0.0000	0.1281	0.0000	0.0270	0.0000
11s	0.0000	0.0000	0.0000	TR	TR	0.0000	TR	0.0000	0.0000	0.0000
12s	0.0000	0.0000	0.0000	TR	0.0371	0.0000	0.0307	0.0000	0.0000	0.0000
13s	0.0000	0.0371	0.0000	0.0581	0.1026	0.0000	0.0295	0.0000	0.0000	0.0000
1or	0.0000	0.0000	0.0000	0.0528	0.0934	0.6422	1.1159	0.1843	0.0000	0.0000
1lr	0.0000	0.0000	0.0000	TR	0.0810	0.1323	1.2503	0.3373	0.0000	0.0000
11or	0.0000	0.0972	0.0000	1.2159	0.2993	0.0681	0.1080	0.0000	0.0000	0.0000
11lr	0.0000	0.0664	0.0000	0.2063	1.2482	TR	0.1694	0.0000	0.0000	0.0000
12or	0.0000	0.1896	0.0000	2.0591	0.6518	0.0674	0.2468	0.0000	0.0000	0.0000
12lr	0.0000	0.0584	0.0000	0.1941	1.4169	TR	0.3087	0.0000	0.0000	0.0000
13or	0.0000	0.1185	0.0000	1.6140	0.2917	TR	0.0629	0.0000	0.0000	0.0000
13lr	0.0000	0.0423	0.0000	0.4284	1.4645	TR	0.2050	0.0000	0.0000	0.0000

1 = *Tabernaemontana arborea*; 2-6 = *Tabernaemontana alba*; 7-10 = *Tabernaemontana donnell-smithii*; 11-13 = *Tabernaemontana amygdalifolia*; 14-16 = *Tabernanthe iboga*

(each number represents an individual plant)

ir = inner root bark; or = outer root bark; r = (whole) root bark; s = stem bark

TR = trace amounts

CAPÍTULO 1: FITOQUÍMICA Y QUIMIOTAXONOMÍA

1.2. Quantitative Evaluation of a Mexican and a Ghanaian *Tabernaemontana* Species as Alternatives to *Voacanga africana* for the Production of Antiaddictive Ibogan Type Alkaloids

Felix Krengel, Jonathan Dickinson, Christopher Jenks, Ricardo Reyes Chilpa

FULL PAPER

Quantitative Evaluation of a Mexican and a Ghanaian *Tabernaemontana* Species as Alternatives to *Voacanga africana* for the Production of Antiaddictive Iboga Type Alkaloids

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In continuation of our efforts to provide quantitative information on antiaddictive ibogan type alkaloid-producing *Tabernaemontana* species, we used gas chromatography-mass spectrometry (GC/MS) to compare the alkaloid profiles of the barks and/or leaves of one Mexican and one African species – *T. arborea* and *T. crassa*, respectively, with the primary sources of commercially available semisynthetic ibogaine, *Voacanga africana* root and stem bark. The qualitative and quantitative similarities between *T. arborea* and *V. africana* barks consolidate previous reports regarding the potential of the former as a promising alternative source of voacangine and ibogaine. The results also suggest that *T. crassa* could be used to produce conopharyngine and ibogaline, two compounds with the same basic skeletal structure and possibly similar antiaddictive properties as ibogaine.

Introduction

Monoterpenoid indole alkaloids (MIAs) of the ibogan type could be of great value in treating drug addiction, with particular utility in the context of the current opioid and stimulant epidemics in the USA. Representative compounds of this type, such as ibogaine, act on a broad range of neurotransmitter systems, resensitize the dopaminergic system, and at the same time, cause introspective, 'oneirogenic' visions and cognitive processing. In consequence, patients report dramatic mitigation of withdrawal symptoms from opioids, as well as sustained reduction of cravings for opioids, stimulants, alcohol, and nicotine.[1–4] Observational research suggests that part of the treatment effect can include personal insight regarding underlying reasons for addiction.[5–8]

Ibogaine is tightly associated with two African Apocynaceae species: While the substance occurs in uniquely high concentrations in the root bark of *Tabernanthe iboga* BAILL., its commercial production mainly relies on *Voacanga africana* STAPF ex SCOTT-ELLIOT, as only the latter has been systematically cultivated throughout West Africa.[4,9] In detail, the alkaloid voacangine is extracted from the barks of *V. africana* and semisynthetically transformed into ibogaine, by cleavage of a methyl ester group.[4,10–12] This procedure can be applied to both pure voacangine, as well as alkaloid-containing crude extracts.[13] The pantropical *Tabernaemontana* L. (Apocynaceae) genus is rich in ibogan type alkaloids and other MIAs.[14] It therefore may provide many new natural sources of antiaddictive compounds. Especially some Mexican species yield considerable concentrations of the civil-

complex, which comprises the structurally and pharmacologically related ibogan type alkaloids coronaridine, ibogamine, voacangine, and ibogaine (*Figure 1*).[10,13,15,16] In order to put these relative recent findings in a context of commercial viability, we compared the alkaloid contents of the barks and/or leaves of *Tabernaemontana arborea* ROSE ex J.D.SM. (from Mexico) and *Tabernaemontana crassa* BENTH. to those of *V. africana* (both from Africa). As a complement to our above-cited publications, the present study contributes to closing the gap between the already existing qualitative information and the rare or non-existing quantitative data concerning ibogan type alkaloids-producing Apocynaceae species.
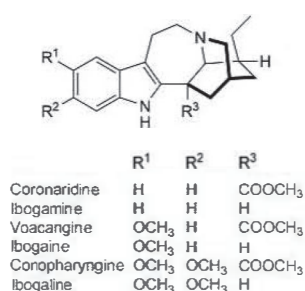
Figure 1. Basic skeletal structures of the ibogan type alkaloids and substituents of the CIVI-complex.

Results and Discussion

In both qualitative and quantitative terms, the alkaloid profiles of *T. arborea* and *V. africana* proved to be astonishingly similar and could be clearly differentiated from the one determined for *T. crassa* (*Table 1*). This result is quite notable, taking into consideration that on the one hand, the first and the third species belong to the same genus and are taxonomically more closely related to each other than to the second species. On the other hand, *V. africana* and *T. crassa* occur in the same geographic region in tropical Africa, while *T. arborea*'s evolutionary history developed in the New World. The yields of the majority alkaloid voacangine were in accordance with recently published information in the case of *T. arborea* root bark,[10] but significantly superior to the available semiquantitative data in the case of the *V. africana* barks.[11,17,18] Coronaridine, ibogamine, ibogaine, and vobasine (the latter being the only non-ibogan type alkaloid) were also present in similar amounts in the respective samples of the two species. Concerning other unidentified minority alkaloids, four out of ten were detected in both *T. arborea* and *V. africana* barks, one in the latter and *T. crassa* leaves, two only in *T. crassa* barks, and three were exclusively associated with either *T. arborea* or *V. africana* barks, or *T. crassa* leaves, respectively (data not shown).

Table 1. Alkaloid contents of different organs of *V. africana*, *T. arborea*, and *T. crassa* (in dry weight percentage of plant material).

Species and organ		Coronaridine	Ibogamine	(Iso)voacangine[a]	Ibogaine	Conopharyngine	Vobasine	Appáricine
V. africana stem bark		TR[b]	TR[b]	0.83	0.07	ND[c]	0.13	ND[c]
V. africana root bark		TR[b]	TR[b]	1.67	0.25	ND[c]	0.09	ND[c]
T. arborea root bark	AVG[d]	0.06	0.02	1.24	0.44	ND[c]	0.24	ND[c]
	CV [%][e]	87.09	173.21	15.71	105.69	ND[c]	49.15	ND[c]
	IPS[f]	TR[b]	TR[b]	1.03	0.16	ND[c]	0.12	ND[c]
	(N=3)	0.09	TR[b]	1.27	0.97	ND[c]	0.24	ND[c]
		0.10	0.07	1.42	0.18	ND[c]	0.36	ND[c]
T. crassa root bark		ND[c]	ND[c]	0.09	ND[c]	0.07	ND[c]	0.16
T. crassa stem bark		ND[c]	ND[c]	TR[b]	ND[c]	0.19	ND[c]	0.08
T. crassa leaves	AVG[d]	ND[c]	ND[c]	0.02	ND[c]	0.21	ND[c]	0.01
	CV [%][e]	ND[c]	ND[c]	83.87	ND[c]	29.36	ND[c]	138.13
	IPS[f]	ND[c]	ND[c]	TR[b]	ND[c]	0.12	ND[c]	TR[b]
	(N=8)	ND[c]	ND[c]	TR[b]	ND[c]	0.13	ND[c]	0.03
		ND[c]	ND[c]	TR[b]	ND[c]	0.17	ND[c]	0.03
		ND[c]	ND[c]	0.03	ND[c]	0.21	ND[c]	TR[b]
		ND[c]	ND[c]	0.03	ND[c]	0.23	ND[c]	TR[b]
		ND[c]	ND[c]	0.03	ND[c]	0.25	ND[c]	TR[b]
		ND[c]	ND[c]	0.03	ND[c]	0.28	ND[c]	TR[b]
		ND[c]	ND[c]	0.04	ND[c]	0.29	ND[c]	0.03

[a] In the case of *T. crassa*, the compound detected may be isovoacangine instead of voacangine. [b] TR = traces. [c] ND = not detected. [d] AVG = average. [e] CV [%] = coefficient of variation. [f] IPS = individual plant samples. All values have been rounded to two decimal places.

T. crassa presented relatively small amounts of a compound with a retention time (t_R) and fragmentation patterns characteristic of voacangine (*Figures S1* and *S2*). This alkaloid has not been previously reported for the species, however, we cannot rule out that we actually detected its constitutional isomer isovoacangine, which has been found before in *T. crassa*.[14,19] In agreement with Cava et al.[20] and Mairura and Schmelzer,[19] the majority alkaloids were conopharyngine, a dimethoxylated derivative of voacangine (predominant in stem bark and leaves), and apparicine, a MIA of the aspidospermatan class (predominant in root bark). Just as coronaridine and voacangine can be semisynthetically converted into ibogamine and ibogaine, respectively, conopharyngine could be transformed into ibogaline. Particularly the last substance may show antiaddictive properties, given that animal studies have revealed its strong central-stimulating effects, similar to those caused by ibogaine. On the contrary, the methoxycarbonyl group of conopharyngine and voacangine seems to be responsible for the compounds being comparatively weak central nervous system stimulants.[14] However, the presence of methoxy groups might implicate higher toxicity, as suggested by Glick et al.[21] who observed that ibogaine, but neither ibogamine nor coronaridine, induced tremors in rats, while antiaddictive effects were similar in all three cases. In any case, the LD_{50} values of conopharyngine and ibogaline (both having two methoxy groups) in mice (i.v.) are higher than those of ibogaine (145, 46, and 42 mg/kg, respectively).[14] Surprisingly, conopharyngine contents were comparable or even higher in the leaves than in the barks of *T. crassa*. This finding is very interesting from both commercial and chemotaxonomic points of view, as leaves are generally the material that can be harvested easiest from most plants, but in the case of *T. arborea*, have been found to present considerably lower alkaloid contents than the barks.[15]

Conclusions

In this study, we continue to provide quantitative information on ibogan type alkaloid-producing Apocynaceae species. By direct comparison with the stem and root barks of *V. africana*, the currently most important source of commercially available semisynthetic ibogaine, we consolidate our previous findings that *T. arborea* root bark is a promising alternative source for this antiaddictive substance. *T. crassa* may be a good source of less explored, yet possibly similarly effective, compounds with the same basic skeletal structure, namely conopharyngine and ibogaline.

Experimental Section

Plant Material

Root bark from *T. arborea* was obtained in the Los Tuxtlas region in Veracruz, Mexico, near the Los Tuxtlas Biology Station (National Autonomous University of Mexico (UNAM))[10] and the Sontecomapan lagoon shore in February 2017 and April 2019. Delfino Álvaro Campos Villanueva (Los Tuxtlas Biology Station, UNAM) and Leonardo Osvaldo Alvarado Cárdenas (Faculty of Sciences, UNAM) identified the species, and voucher specimens were deposited with the Herbarium of the Faculty of Sciences (FCME), UNAM (voucher numbers 161424 to 161427). *T. crassa* leaves, root and stem bark, as well as *V. africana* root and stem bark were harvested from mature wild trees near Akim Oda (Birim Central Municipal District, Eastern Region, Ghana) between November 2018 (*V. africana*) and May 2019 (*T. crassa*), respectively, and provided by Bulk African Trade Ltd. (P.O. Box 439, Akim Oda, Eastern Region, Ghana; Tax ID: C0007289111; e-mail: madoe@bulkafricantrade.de). Species were identified by Rahinatu Salam and Edwin Dadzie. All plant names were checked with http://www.theplantlist.org.

Alkaloid Extraction and GC/MS Analysis

The dried and powdered barks were extracted thrice with methanol and analyzed by GC/MS as reported by Krengel et al.[13] EI-mass spectra recording in full scan mode (*m/z* 50 to 500) at 70 eV after 8 min of solvent delay; injector in split mode (1:5) at 300°C with automatic injection of 1 µl aliquots of the methanol-dissolved samples; ramp temperature program from 150 to 300°C at 4°C/min; helium carrier gas at 1 ml/min. Peaks in the resulting total ion current (TIC) chromatograms were identified with the NIST Mass Spectral Search Program for the NIST/EPA/NIH Mass Spectral Library (Version 2.2, build June 10, 2014) and by comparison with previously published data. Quantification of the detected alkaloids was based on six point calibration curves (25, 50, 100, 200, 300, and 400 µg/ml; three repetitions each) of two authentic chemical standards, voacangine and ibogaine, kindly donated by Phytostan Enterprises, Inc. (Montreal, Quebec). For details, see Krengel et al.[10]

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Acknowledgements

Author Contribution Statement

F. K., J. D., and R. R. C. collected the Mexican plant material. C. J. provided the samples of the two African species. F. K. conducted the experimental work. F. K., J. D., and C. J. wrote the draft. Based on the comments of all authors, F. K. and R. R. C. wrote the final version of the manuscript.

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Supporting Information

Quantitative Evaluation of a Mexican and a Ghanaian *Tabernaemontana* Species as Alternatives to *Voacanga africana* for the Production of Antiaddictive Ibogan Type Alkaloids

Felix Krengel, Jonathan Dickinson, Christopher Jenks, and Ricardo Reyes-Chilpa*

Quantitative Evaluation of a Mexican and a Ghanaian *Tabernaemontana* Species as Alternatives to *Voacanga africana* for the Production of Antiaddictive Ibogan Type Alkaloids

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In continuation of our efforts to provide quantitative information on antiaddictive ibogan type alkaloid-producing *Tabernaemontana* species, we used gas chromatography-mass spectrometry (GC-MS) to compare the alkaloid profiles of the barks and/or leaves of one Mexican and one African species – *T. arborea* and *T. crassa*, respectively – with the primary sources of commercially available semisynthetic ibogaine, *Voacanga africana* root and stem bark. The qualitative and quantitative similarities between *T. arborea* and *V. africana* barks consolidate previous reports regarding the potential of the former as a promising alternative source of voacangine and ibogaine. The results also suggest that *T. crassa* could be used to produce conopharyngine and ibogaline, two compounds with the same basic skeletal structure and possibly similar antiaddictive properties as ibogaine.

Keywords: Alkaloids · Phytochemistry · Ibogan type alkaloids · *Tabernaemontana* (Apocynaceae) · *Voacanga* (Apocynaceae)

Supporting Information

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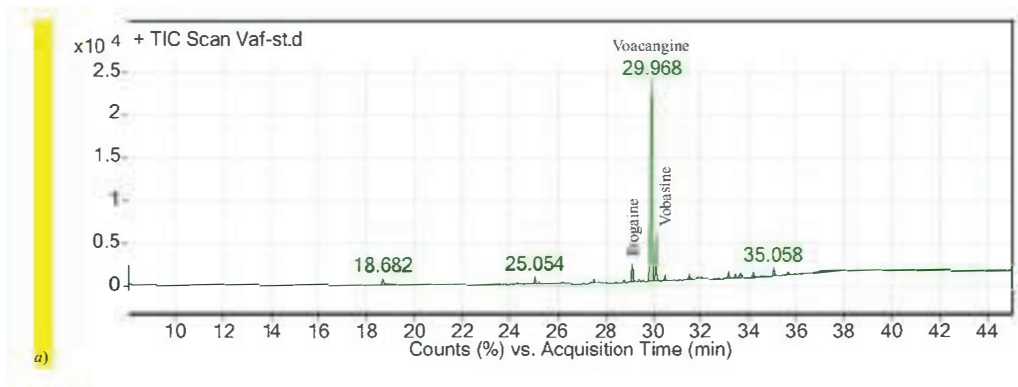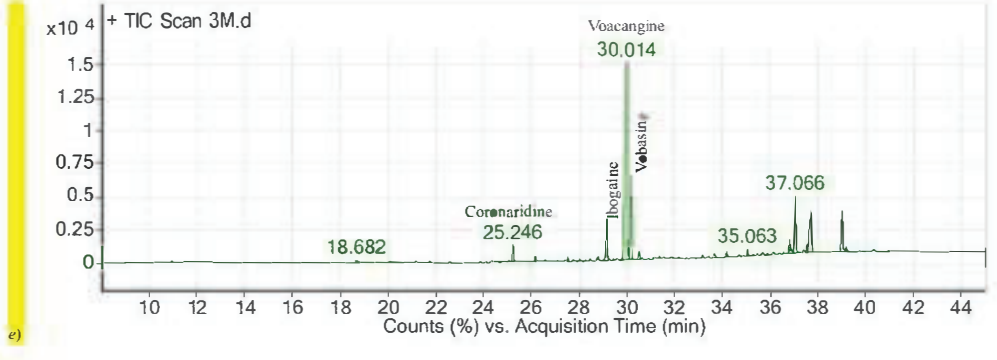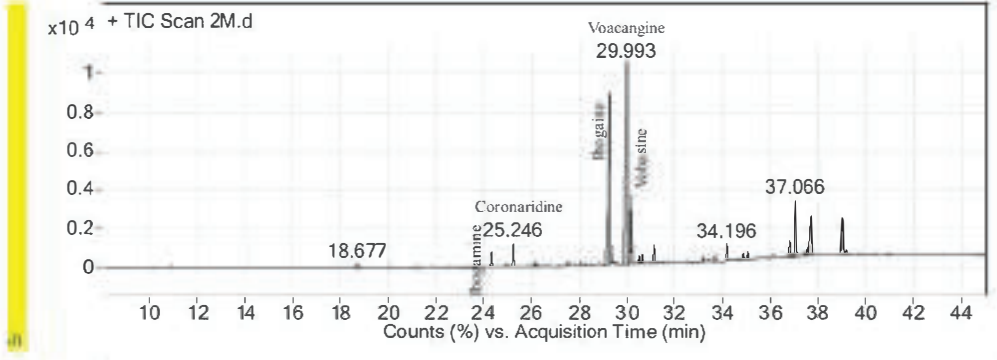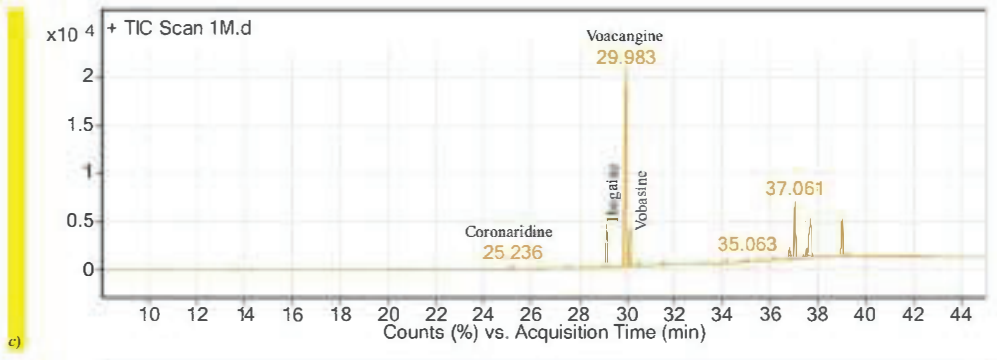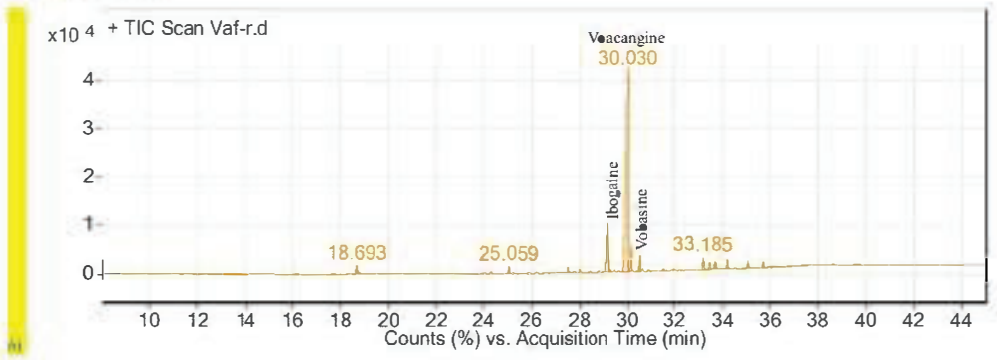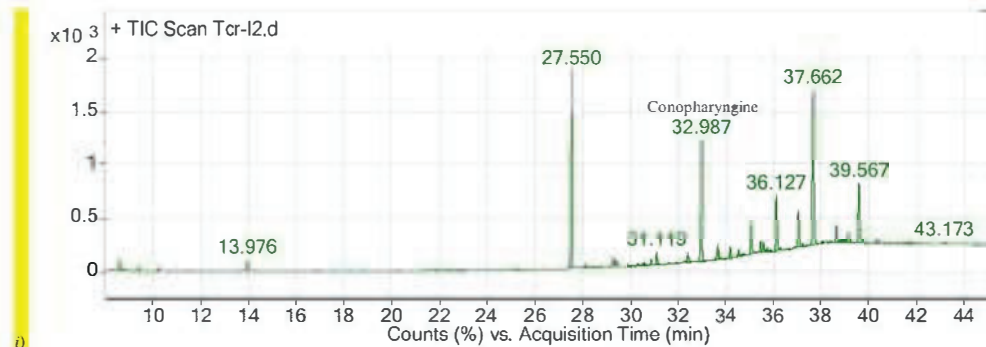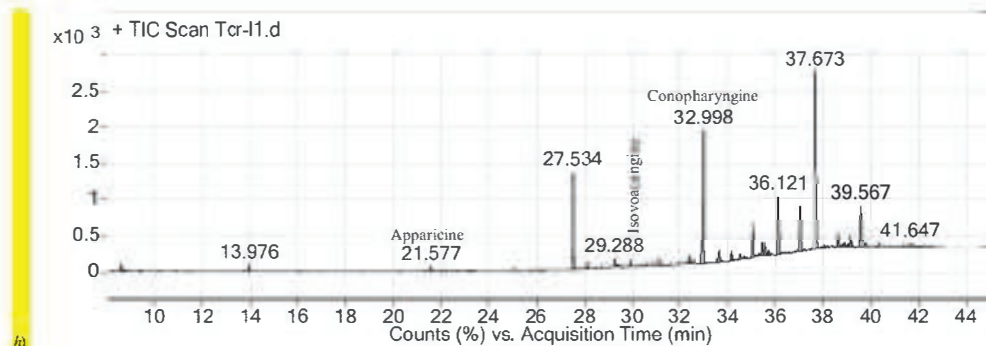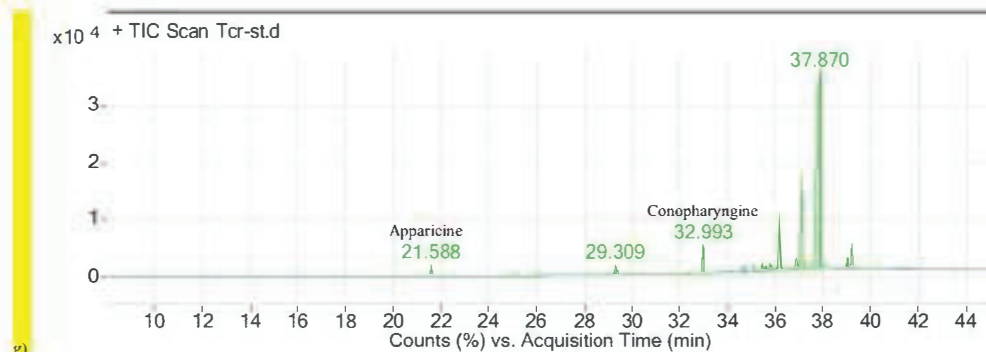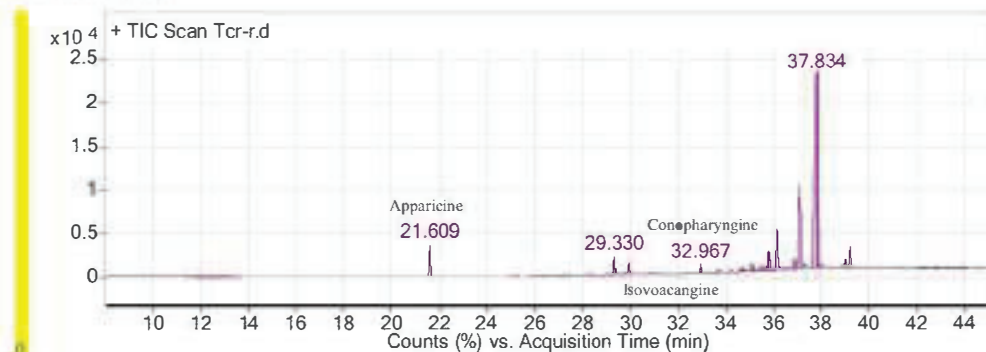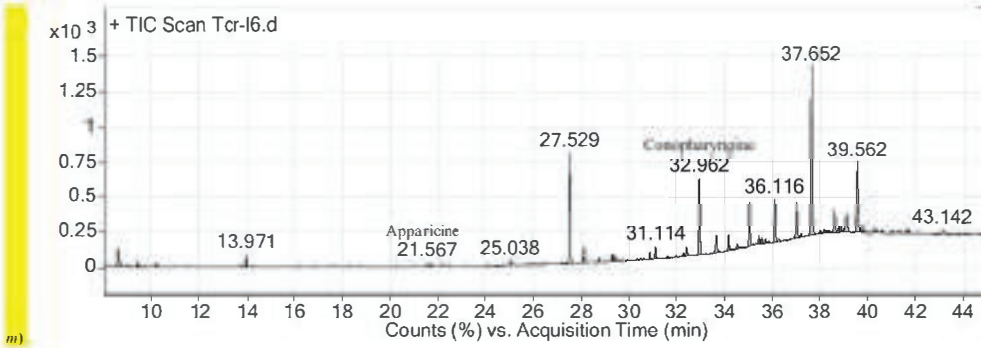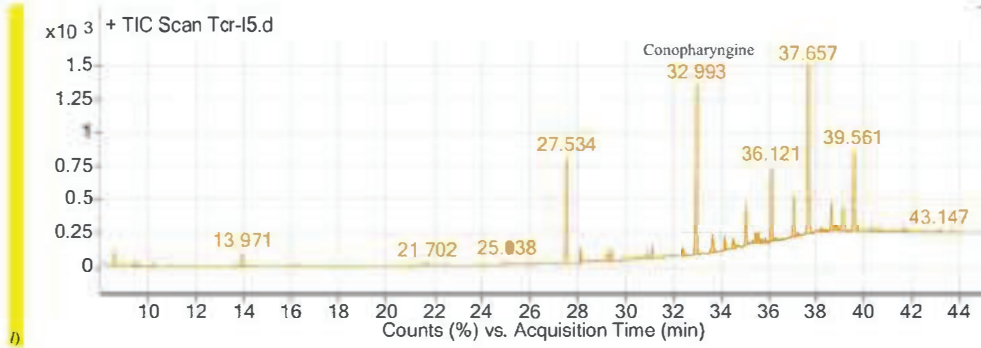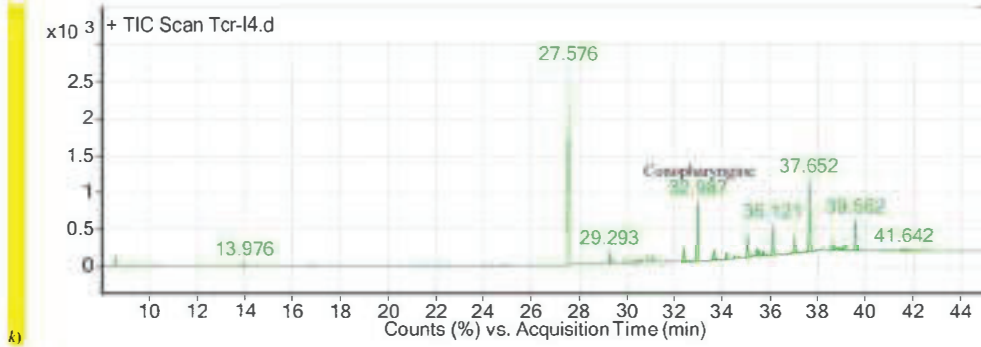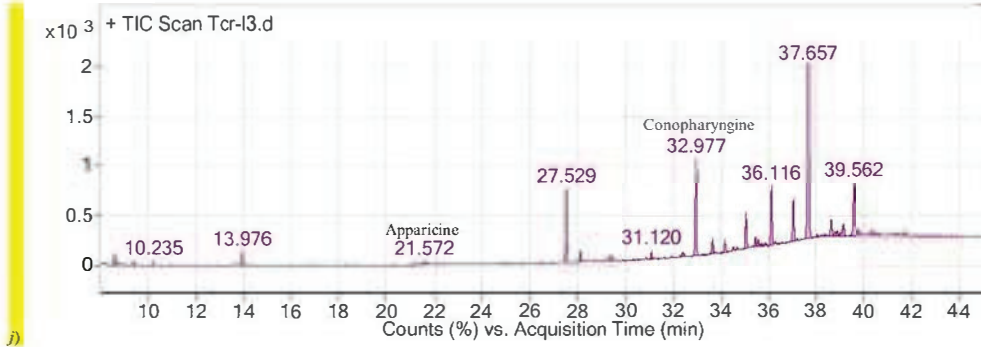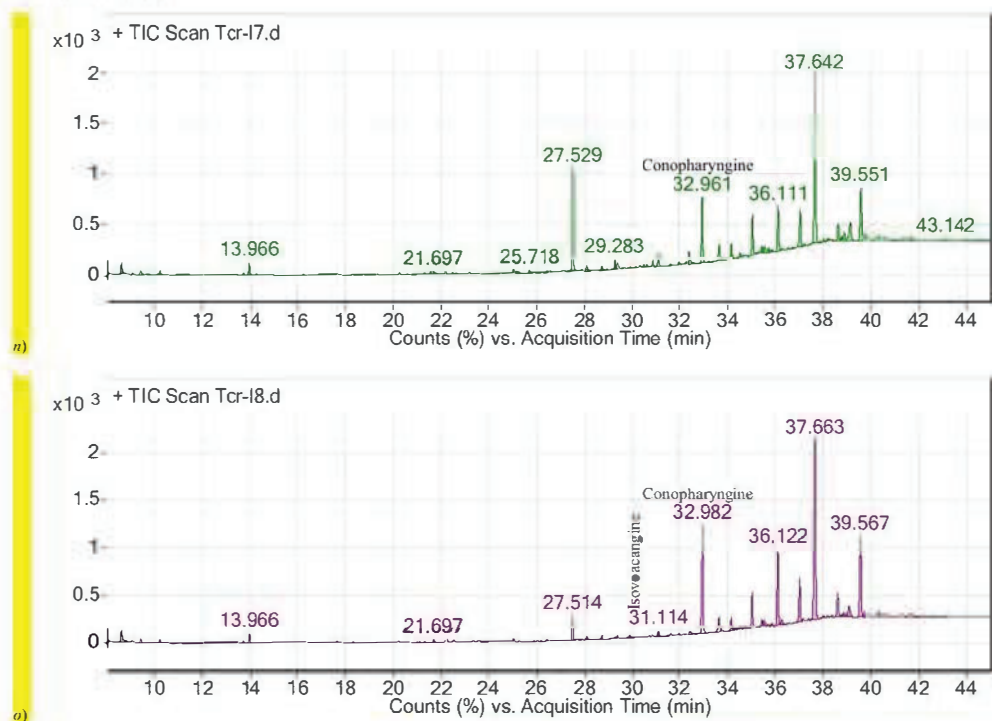
Figure S1. Experimental chromatograms of the extracts obtained from *a) V. africana* stem bark, *b) V. africana* root bark, *c)* to *e) T. arborea* root bark, *f) T. crassa* root bark *g) T. crassa* stem bark, and *h)* to *o) T. crassa* leaves.

Retention times (RT) for apparicine, ibogamine, coronaridine, ibogaine, isovoacangine, vobasine, and conopharyngine are 21.5±0.1, 24.3±0.1, 25.2±0.1, 29.2±0.1, 29.9±0.1, 30.1±0.1, and 32.9±0.1 min respectively. The prominent peak with RT 25.5±0.1 in chromatograms *h)* to *o)* represents a phthalate. Compounds with RTs higher than 33 min correspond to non-alkaloidal compounds like phytosterols and terpenoids.
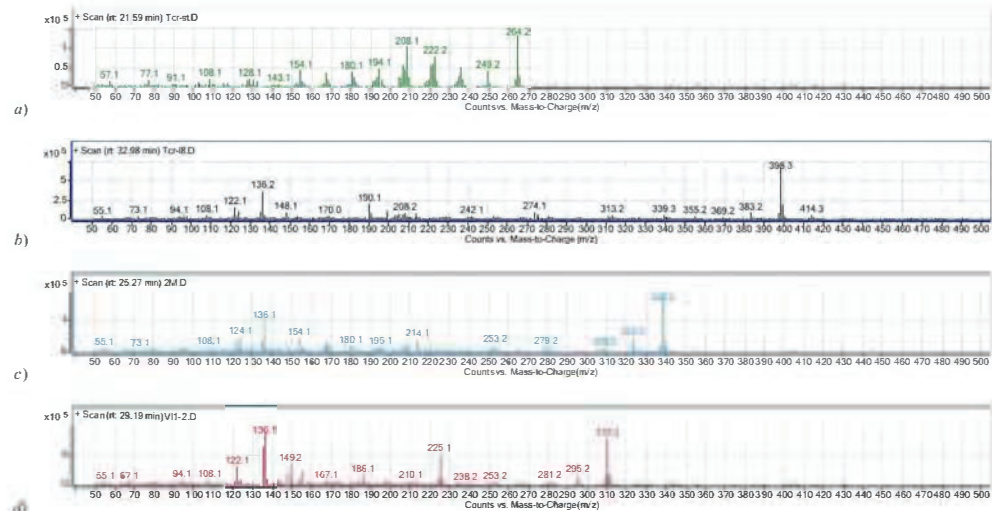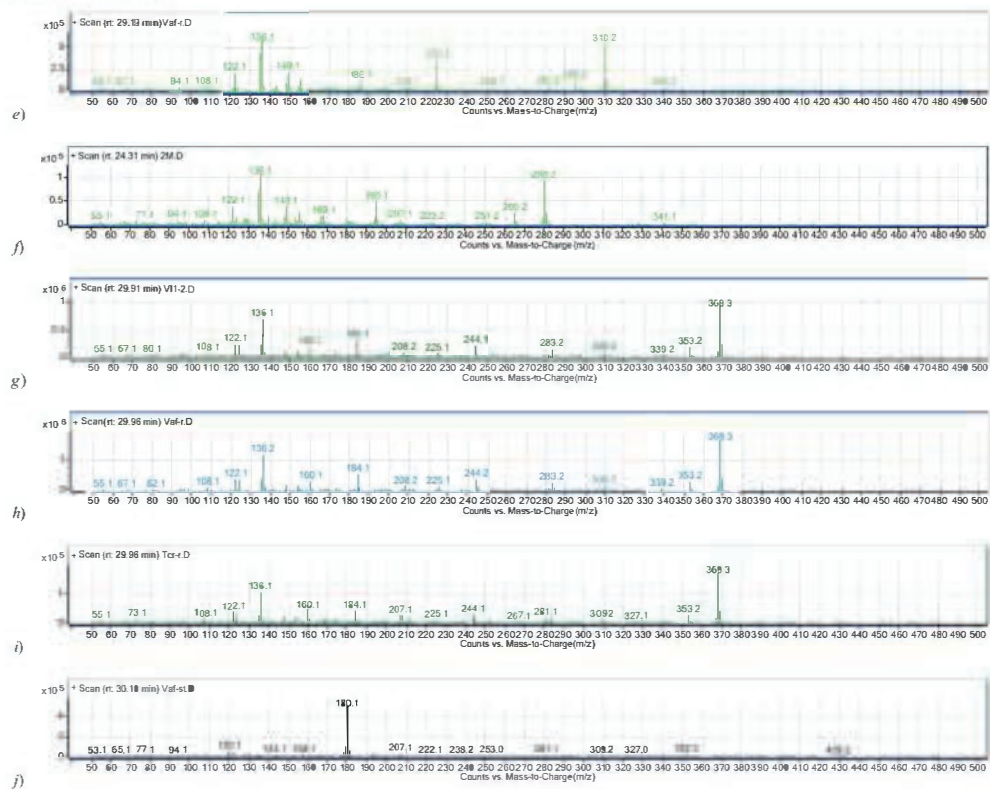
Figure S2. Experimental mass spectra of *a*) apparicine (*T. crassa* stem bark), *b*) conopharyngine (*T. crassa* leaves), *c*) coronaridine (*T. arborea* root bark), *d*) pure ibogaine, *e*) ibogaine (*V. africana* root bark), *f*) ibogamine (*T. arborea* root bark), *g*) pure voacangine, *h*) voacangine (*V. africana* root bark), *i*) (iso)voacangine (*T. crassa* root bark), and *j*) vobasine (*V. africana* stem bark).

CAPÍTULO 2: ETNOBOTÁNICA

Beyond Phytochemistry: Explaining Different Ethnobotanical Use Patterns of the Entheogenic African Shrub *Tabernanthe iboga* and Four Mexican *Tabernaemontana* Species (Apocynaceae) Despite Similar Psychoactive Alkaloid Profiles.

Felix Krengel, Ricardo Reyes Chilpa, Jonathan Dickinson, Laura Cortés Zárraga, Javier Caballero Nieto

1 **Beyond Phytochemistry: Explaining Different Ethnobotanical Use Patterns of the**

2 **Entheogenic African Shrub *Tabernanthe iboga* and Four Mexican *Tabernaemontana* Species**

3 **(Apocynaceae) Despite Similar Psychoactive Alkaloid Profiles.**

4

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20 **Abstract**

21 *Ethnopharmacological relevance: Tabernanthe iboga* (Apocynaceae) has great ethnomedical and

22 spiritual importance in Central Africa due to its stimulant and oneirogenic properties which can

23 be attributed to the presence of ibogan type alkaloids, particularly ibogaine. Several Mexican

24 *Tabernaemontana* species also produce ibogan type alkaloids, and are likewise used in traditional

25 medicine. However and in contrast to *T. iboga*, they are not known as entheogenic plants.

26 *Aim of the study:* To interpret the ethnobotanical uses of the Old and New World species in

27 Central African and Mexican traditional medicine, respectively, in consideration of chemical,

28 historical, and sociocultural factors.

29 *Materials and methods:* An ethnobotanical and phytochemical literature survey of *T. iboga* and

30 four Mexican *Tabernaemontana* species was conducted.

31 *Results:* Some ethnomedical applications such as analgesic and antipyretic treatment could be

32 found for both genera, but entheogenic uses were effectively limited to *T. iboga*, notwithstanding

33 that the alkaloid profiles proved to be quite similar in all cases.

34 *Conclusions:* The lack of registered entheogenic uses of *Tabernaemontana* species by currently

35 existing Mexican ethnic groups or pre-Hispanic civilizations is probably due to unique historical,

36 sociocultural, and environmental circumstances, rather than to the absence of psychoactive

37 properties akin to those of *T. iboga*.

38 **Keywords:** *Tabernaemontana*, *Tabernanthe iboga*, iboga alkaloid, ibogaine, voacangine, CIVI-

39 complex

1. Introduction

40

41   Phytochemistry is currently recognized as a powerful tool for understanding the

42   ethnobotanical use patterns that humanity has assigned to certain plant species. "Plants of the

43   Gods" (Schultes et al., 2001) is a classic and representative work of this research area. The book

44   explains the medical and, above all, entheogenic uses of numerous species on a chemical basis.

45   While many ethnobotanical applications may be unique to a certain territory and/or culture, there

46   are also striking examples of phylogenetically and/or chemotaxonomically related plant species

47   being utilized for similar purposes in distinct world regions that are separated by thousands of

48   kilometers of land or sea (Buenz et al., 2018; Saslis-Lagoudakis et al., 2012). The reason for

49   these analog yet independent discoveries often reside in the presence of the same groups of

50   secondary metabolites in the respective plant species. E. g., different taxa of the Papilionoideae

51   subfamily (Fabaceae) that all contain rotenoids have been used during centuries as fish poisons

52   and/or topical insecticides in many parts of the world. In Mexico, native peoples have used

53   *Pachyrhizus erosus* (L.) Urb., as well as several species of *Brongniartia* Kunth and *Tephrosia*

54   **P**ers. for these purposes, whereas in the Amazon Basin and Maritime Southeast Asia, the

55   corresponding herbal preparations have been obtained from *Lonchocarpus* Kunth and *Derris*

56   Lour., respectively (Béjar et al., 2000; Estrella-Parra et al., 2016; Heizer, 1953; Reyes-Chilpa et

57   al., 1994; Rickard and Cox, 1986; Tobler et al., 2011; Van Andel, 2000). Therefore, it can

58   reasonably be assumed that the peoples inhabiting these areas discovered the piscicide and

59   insecticidal properties independently by observation, trial and error, which are indeed elements of

60   the scientific method. However, does this process of acquiring knowledge always occur

61   throughout time and space? Or can the knowledge be ignored, lost, or simply not be documented?

62   To what degree do historical, sociocultural, and cosmovision-related factors influence the

63   preservation of this knowledge?

64   In this contribution, we apply these questions to five chemotaxonomically related species,

65   namely the entheogenic African shrub *Tabernanthe iboga* Baill. and the four Mexican

66   *Tabernaemontana* L. species *T. alba* Mill., *T. amygdalifolia* Jacq., *T. arborea* Rose ex J.D.Sm.,

67   and *T. donnell-smithii* Rose ex J.D.Sm. (Apocynaceae). In a recent publication (Krengel et al.,

68   2019a), we found that the alkaloid profiles of root and stem barks from the five species consisted

69   mainly of monoterpenoid indole alkaloids (MIAs) of the ibogan type, particularly coronaridine,

70   ibogamine, voacangine, and/or ibogaine. These compounds, which in conjunction we termed the

71   CIVI-complex, share the same basic structure (Figure 1) and presumably similar bioactive

72   properties. Hence, it would seem likely that *Tabernaemontana* species and *T. iboga* presented

73   similar uses in Mexican and African traditional medicine, respectively, especially with regard to

74   the powerful psychoactive effects of the CIVI-complex. However, whereas *T. iboga* is a well-

75   known entheogen in Central Africa, Mexican *Tabernaemontana* species are certainly not

76   representative of the considerable number of plants that have been ritualisticly utilized by the

77   country's indigenous peoples. In this review, we therefore revise the ethnobotanical information

78   available for the New and Old World plants and interpret the registered uses in the light of the

79   reported alkaloid contents of the five species.

80

	R₁	R₂
Coronaridine	H	COOCH₃
Ibogamine	H	H
Voacangine	OCH₃	COOCH₃
Ibogaine	OCH₃	H
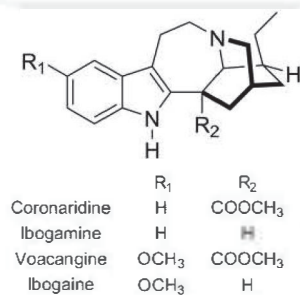
Figure 1. Chemical structures of the CIVI-complex alkaloids.

81

82 **2. Materials and methods**

83   Ethnobotanical and phytochemical information on *T. iboga* and four Mexican

84 *Tabernaemontana* species was collected by using general (Google Scholar, PubMed, Scopus,

85 Web of Science, etc) and specific (Base de Datos Etnobotánica de Plantas Útiles de México –

86 BADEPLAM, UNAM) scientific search engines and databases. Plant names were checked with

87 http://www.theplantlist.org and https://www.tropicos.org. Both the accepted names and

88 synonyms were used to search for published reports and studies. Images of herbarium specimens

89 were retrieved from the herbarium catalogue of the Royal Botanic Garden Edinburgh

90 (https://data.rbge.org.uk/search/herbarium/) and the Herbario Nacional de México (MEXU),

91 accessed via the Portal de Datos Abiertos UNAM (https://datosabiertos.unam.mx/). Chemical

92 structures were drawn with ChemBioDraw Ultra 13.0 (vector).

93 **3. Results and discussion**

94 3.1. Taxonomic classification and distribution of the *Tabernanthe* and *Tabernaemontana* genera

95   The genera *Tabernanthe* Baill. and *Tabernaemontana* L. (Figure 2) are sister taxa within

96 the tribe Tabernaemontaneae (subfamily Rauvolfioideae, family Apocynaceae; Sennblad and

97 Bremer, 2002). With eight species native to Central Africa (*Tabernanthe albiflora* Stapf,

98 *Tabernanthe bocca* Stapf, *Tabernanthe elliptica* (Stapf) Leeuwenb., *Tabernanthe iboga* Baill.,

99 *Tabernanthe mannii* Stapf, *Tabernanthe pubescens* Pichon, *Tabernanthe subsessilis* Stapf,

100 *Tabernanthe tenuiflora* Stapf; The Plant List, 2012), the former is much less extensive than the

101 latter, which comprises about 100 pantropical shrub and tree species (Alvarado-Cárdenas and

102 Saynes-Santillán, 2018). Mexico presents a particularly high number of *Tabernaemontana*

103 species, the fourth and the last seven of the following list being endemic: *Tabernaemontana alba*

104 Mill., *Tabernaemontana amygdalifolia* Jacq., *Tabernaemontana arborea* Rose ex J.D.Sm.,

105 *Tabernaemontana chamelensis* L.O. Alvarado & Lozada-Pérez, *Tabernaemontana citrifolia* L.,

106 *Tabernaemontana divaricata* (L.) R. Br. ex Roem. & Schult., *Tabernaemontana donnell-smithii*

107 Rose ex J.D.Sm., *Tabernaemontana eubracteata* (Woodson) A.O. Simões & M.E. Endress,

108 *Tabernaemontana glabra* (Benth.) A.O. Simões & M.E. Endress, *Tabernaemontana hannae* (M.

109 Méndez & J.F. Morales) A.O. Simões & M.E. Endress, *Tabernaemontana litoralis* Kunth,

110 *Tabernaemontana mixtecana* L. O. Alvarado et Juárez-Jaimes, *Tabernaemontana oaxacana* (L.O.

111 Alvarado-Cárdenas) A.O. Simões & M.E. Endress, *Tabernaemontana ochoterenae*,

112 *Tabernaemontana riverae* L.O. Alvarado & V. Saynes, *Tabernaemontana stenoptera* (Leeuwenb.)

113 A.O. Simões & M.E. Endress, *Tabernaemontana tomentosa* (Greenm.) A.O. Simões & M.E.

114 Endress, *Tabernaemontana venusta* (J.F. Morales) A.O. Simões & M.E. Endress (Alvarado-

115   Cárdenas et al., 2019).

116

Figure 2. Herbarium specimens of *Tabernanthe iboga*[1], *Tabernaemontana alba*[2], *T. amygdalifolia*[3], *T. arborea*[4], and *T. donnell-smithii*[5] (top to bottom, left to right).

[1]Adapted from Herbarium catalogue, by Royal Botanic Garden Edinburgh, 2008, Retrieved from

http://data.rbge.org.uk/herb/E00486107. Copyright by Royal Botanic Garden Edinburgh.

[2-5]Adapted from Portal de Datos Abiertos UNAM, by Herbario Nacional de México (MEXU), Departamento de Botánica,

Instituto de Biología (IBUNAM), Universidad Nacional Autónoma de México (UNAM), 2013. Copyright by IBUNAM.

[2]Retrieved from http://datosabiertos.unam.mx/IBUNAM:MEXU:1219268.

[3]Retrieved from http://datosabiertos.unam.mx/IBUNAM:MEXU:1228171.

[4]Retrieved from http://datosabiertos.unam.mx/IBUNAM:MEXU:850624.

[5]Retrieved from http://datosabiertos.unam.mx/IBUNAM:MEXU:671168.

117

118   3.2. Ethnobotany of *Tabernanthe iboga* and Mexican *Tabernaemontana* species

119   When comparing the reported ethnobotanical uses of *T. iboga* in African traditional

120   medicine with those of *T. alba*, *T. amygdalifolia*, *T. arborea*, and *T. donnell-smithii* in Latin

121   America (Table 1), it becomes evident that, first, information is scarce in all the cases revised.

122   Regarding *T. arborea*, there are no reports on ethnobotanical applications at all. Second, some

123   uses occur in both genera, notably those associated with analgesic, antimicrobial, antipyretic, and

124   tonic properties. Third, only the African species appears to be utilized internally for central

125   nervous system (CNS) activity-related reasons on a regular basis, in order to cause stimulant or

126   entheogenic effects. The *Tabernaemontana* species are mostly applied externally for the

127   treatment of dermatological conditions, as well as for their analgesic or antiinflammatory

128   activities. Fourth, the vast majority of herbal preparations of *T. iboga* are obtained from the roots

129   (or root bark), and only three minor uses are based on the plant's latex. In Mexican traditional

130   medicine, in contrast, the latex is the most widely used plant material from *Tabernaemontana*

131   species, followed by the leaves, seeds, and twigs. Root or stem bark preparations have only been

132   reported for these species in Central and South America. With respect to Mexican

133   *Tabernaemontana* species other than the three listed in Table 1, only *T. citrifolia*, *T. glabra*, *T.*

134   *litoralis*, and *T. tomentosa* presented ethnomedical records. These confirmed the above-

135   mentioned use patterns for the genus (Barrera-Marín et al., 1976; Batis, 1994; Burgos-Hernández

136   et al., 2014; Bye, 1985; Caballero et al., 1978; Cuevas, 1991; Kelly and Palerm, 1952; Marquez-

137   Salazar, 1997; Mendoza-Marquez, 2000; Monroy-Ortiz and Castillo-España, 2000; Pennington,

138   1969, 1963; Rodríguez-López, 2003; Yetman, 2002), adding only the treatment of respiratory

139   (latex and leaves of *T. glabra*) and eye diseases, such as conjunctivitis (latex of *T. tomentosa*), to

140 the list (Alonso-Castro et al., 2011; Frei-Haller, 1997; Yetman and Van Devender, 2002).

141

142 **Table 1.** Ethnobotanical uses reported in the scientific literature from three Mexican

143 *Tabernaemontana* species and *Tabernanthe iboga*.

Species	Reported ethnobotanical uses						References
	Analgesic	Antiinflammatory	Antimicrobial	CNS-related	Dermatological	Others	
Tabernaemontana alba	Mexico: Toothache (external application of crushed seeds or latex), body aches (leaves), headache	ND	Mexico: *Dermatobia hominis* (Linnaeus Jr. in Pallas, 1781) myiasis, filariasis (external application of latex) Brazil: Anthelmintic (*Taenia* Linnaeus, 1758)	ND	Mexico: Pimples (external application of latex or leaves), burns, furuncles, skin infections, skin patches (Pityriasis alba), ulcers, warts, wound healing (external application of latex)	Mexico: Dog bites, mumps (external application of latex) Brazil: Antipyretic (leaves and bark), purgative (leaves), tonic (bark)	(Alcorn, 1983) (Aparicio-Alegría and García, 1995) (Batis, 1994) (BDMTM. 2009a) (Caballero et al., 1978) (Cifuentes and Ortega, 1990) (Geck et al, 2016) (Leonti, 2002; Leonti et al., 2001) (Martínez-Murillo, 1992) (Mendoza-Marquez, 2000) (Van Beek et al., 1984)
Tabernaemontana amygdalifolia	Mexico: Pain relief	ND	Puerto Rico and Central America: Syphilis (bark infusions)	ND	Mexico: Warts (external application of latex or leaf infusions), skin infections like pellagra (external application of twig infusions) or exanthemas (external application of latex infusions), blepharitis, Herpes labialis (external application of latex), wound cleansing and healing, treatment of wounds caused by	Mexico: Antidiarrhoeal, antipyretic, purgative (internal application of latex) Puerto Rico and Central America: Antipyretic (bark infusions)	(Anderson et al. 2005) (Ankli et al, 2002) (Barrera-Marín et al., 1976) (BDMTM. 2009b) (Estrada-Lugo et al., 2011) (Nieves, 2003) (Sanabria. 1986) (Van Beek et al., 1984)

					flies (external application of latex) or poisonous animals (leaves) Colombia: Warts (latex), cataplasm for tumor treatment and wound healing (leaves) Puerto Rico and Central America: Ulcers (external application), warts (latex)	
Tabernaemontana donnell-smithii	ND	Mexico: Swellings, sprains (external application of leaves)	Mexico: *D. hominis* myiasis (external application of latex), *Lutzomyia* França, 1924 bites (Leishmaniasis)	Mexico: Stimulant (latex)	Mexico: Wound healing (external application of leaf infusions), external tumors	ND	(BDMTM. 2009c) (Caballero et al.. 1978) (Flores-Guido et al., 2010) (Martínez-Alfaro et al.. 1995) (Mendoza-Marquez. 2000) (Rodríguez-Acosta et al.. 2010)
Tabernanthe iboga	West Africa. Congo, and Gabon: Toothache	ND	West Africa. Congo, and Gabon: Sleeping sickness (African trypanosomiasis?; root)	West Africa. Congo, and Gabon: Aphrodisiac, neurasthenia, stimulant, (divination, magicoreligious, ritualistic) entheogenic (all root)	ND	West Africa. Congo, and Gabon: Antihypertensive, antipyretic, arrow poison ingredient (latex), cough medicine, infertility treatment (antimicrobial?), ophthalmic, tonic (all root)	(Alper et al.. 2008) (Neuwinger, 1996) (Pope, 1969) (Rätsch, 2007) (Schultes et al., 2001)

144   ND = not detected

145

146   In Central Africa, *T. iboga* (known under the common name *iboga*) is traditionally used

147   on a regional scale tightly associated with the Gabonese *Bwiti* practice (Fernández, 1982; Pope,

148   1969). Although the species has occasionally been employed in treatments that do not rely on

149   CNS activity, its main ethnobotanical uses are related to its aphrodisiac, stimulant, and above all,

150   oneirogenic effects which enable the *Bwiti* practitioner to communicate with the ancestors and

151   gain insight into the nature of life and death (Fernández, 1982; Pope, 1969; Ravelec et al., 2007).

152   The spiritual practice and initiatory society of *Bwiti* arose in the mid 19th century due to the

153   exposure of the coastal Bantu population to the Pygmy peoples inhabiting the interior of the

154   country. Similar to other traditional practices connected with *iboga*, it focuses variably on

155   mysticism, warriorship, and healing. Treatments and initiations can serve different and

156   overlapping purposes, such as rites of passage, grieving, and physical, emotional, and spiritual

157   healing. Today, *Bwiti* is recognized as one of Gabon's official religions. Its various branches

158   (*Dissoumba*, *Mitsogo*, *Fang*, etc.) are practiced in numerous villages throughout Gabon,

159   extending into Cameroon and Equatorial Guinea (Fernández, 1982). In 2005, Gabon's former

160   President Omar Bongo declared *iboga* to be a "national cultural heritage" and a "strategic

161   reserve." This classification placed it under the general protection of Gabon's culture ministry

162   and international treaties like the Nagoya Protocol, a segment of the UN Convention on

163   Biological Diversity that focuses on access and benefit sharing for traditional knowledge holders

164   in relation to biological or genetic resources (Dickinson, 2016).

165   The reported ethnobotanical uses of *Tabernaemontana* species are much less prevalent in

166   Mexico than those of *T. iboga* in Central Africa. For instance, *T. alba*, *T. arborea*, and *T. donnell-*

167   *smithii* grow abundantly in the tropical Los Tuxtlas region in the eastern Mexican state of

168 Veracruz (Krengel et al., 2020, 2019a, 2019b, 2016), but despite several field trips to this region,

169 we have not been able to obtain testimonies from the inhabitants that confirm the information

170 displayed in Table 1. On the contrary, people would state that the respective *Tabernaemontana*

171 species had no medicinal application and were mainly used for the construction of (living) fences

172 and agricultural tools, while the latex served sporadically as adhesive or as a chewing gum

173 substitute. Effectively, all four practices have been registered in the literature (Avendaño-Reyes

174 and Acosta-Rosado, 2000; Caballero et al., 1978; Flores-Guido et al., 2010; Martínez-Alfaro et

175 al., 1982; Rodríguez-Acosta et al., 2010). In the case of *T. amygdalifolia*, the barks analyzed by

176 Krengel et al. (2019) were collected in the southern Mexican state of Yucatán, but again was there

177 no evidence of this plant being utilized for medicinal purposes by the local population. To the

178 best of our knowledge, neither is any of the four *Tabernaemontana* species sold on the important

179 markets for traditional medicine in Mexico City, like the *Mercado de Sonora*. Taken together, the

180 previous observations suggest that the ethnomedicinal use of Mexican *Tabernaemontana* species

181 is limited to a local, and not like in the case of *T. iboga*, to a regional scale.

182

183 3.3. Alkaloid profiles of *Tabernanthe iboga* and Mexican *Tabernaemontana* species

184   The psychoactive properties of *T. iboga* root bark are essentially due to the majority

185 compound ibogaine, but other ibogan type alkaloids such as coronaridine, ibogamine, ibogaline,

186 tabernanthine, and voacangine have also shown central-stimulating CNS activity (Van Beek et al.,

187 1984), and probably contribute to the overall effects (Rätsch, 2007). Table 2 summarizes the

188 MIAs that have been found in *T. iboga* and the Mexican *Tabernaemontana* species *T. alba*, *T.*

189 *amygdalifolia*, *T. arborea*, and *T. donnell-smithii*. The CIVI-complex occurs in all five species

190   and is highlighted in bold.

191

192   **Table 2.** MIAs reported in the scientific literature from the five species.

Species	MIAs	References
Tabernaemontana alba	Apparicine, **coronaridine**, 10-hydroxycoronaridine, **ibogaine**, **ibogamine**, norseredamine, tabersonine, **voacangine**, vobasine	(Collera et al., 1962) (Guzmán-Gutiérrez et al., 2020) (Krengel et al., 2019a, 2019b, 2016) (Van Beek et al., 1984)
Tabernaemontana amygdalifolia	Apparicine, demethylaspidospermine, **coronaridine**, N-acetyl-12-demethoxycylindrocarine, cylindrocarpidine, 12-demethoxycylindrocarpidine, 17-demethoxycylindrocarpidine, homocylindrocarpidine, 5-oxocylindrocarpidine, 10-oxocylindrocarpidine, **ibogaine**, **ibogamine**, O-demethylpalosine, **voacangine**, voacangine hydroxyindolenine	(Achenbach, 1967a, 1967b, 1966) (Krengel et al., 2019a) (Van Beek et al., 1984) (Zhu et al., 1990)
Tabernaemontana arborea	**Coronaridine**, **ibogaine**, **ibogamine**, norseredamine, pericyclivine, tabersonine, voacamine, **voacangine**, isovoacangine, 19-epivoacorine, vobasine, vobasinol	(Chaverri-Chaverri and Cicció-Alberti, 1980) (Guzmán-Gutiérrez et al., 2020) (Kingston, 1978) (Krengel et al., 2020, 2019a, 2019b, 2016) (Van Beek et al., 1984)
Tabernaemontana donnell-smithii	**Coronaridine**, 10-hydroxycoronaridine, **ibogaine**, **ibogamine**, quebrachamine, ß-hydroxyquebrachamine stemmadenine, tabernanthine, tabersonine, voacamine, **voacangine**, isovoacangine	(Collera et al., 1962) (Krengel et al., 2019a) (Walls et al., 1958)
Tabernanthe iboga	**Coronaridine**, desmethoxyiboluteine, gabonine, **ibogaine**, ibogaine hydroxyindolenine, ibogaline, ibogamine, **ibogamine** hydroxyindolenine, iboluteine, ibophylline, iboquine, iboxygaine (kimvuline), iboxyphylline, kisantine, tabernanthine, **voacangine**, voaphylline	(Dickel et al., 1958) (Goutarel et al., 1974, 1958) (Khuong-Huu et al., 1976) (Krengel et al., 2019a) (Neuss, 1959)

193

70

194   3.4. Beyond phytochemistry: Explaining the different patterns of ethnobotanical use of

195   *Tabernanthe iboga* and Mexican *Tabernaemontana* species

196   A direct ethnobotanical comparison of the four Mexican *Tabernaemontana* species with

197   the African *T. iboga* may prove problematic due to the geographic, historical, and cultural

198   differences implied. Nevertheless, with this in mind, the available phytochemical information can

199   be interpreted in a manner that sheds light on why plants with related secondary metabolisms

200   show quite distinct patterns of ethnobotanical use, especially if geographic and cultural

201   boundaries are involved. It seems that although many times explanations can be based on

202   concrete chemical findings, aspects of a more fortuitous nature are at least equally important. The

203   following hypothesis is meant to offer a reasonable approach to explaining the distinct patterns of

204   ethnobotanical use between the Mexican *Tabernaemontana* species and *T. iboga*:

205   1.   The presence of several MIAs with analgesic, antiinflammatory, dermatological, and

206   antimicrobial properties confers a certain efficacy to herbal preparations of the five species in

207   terms of treating related diseases and medical conditions. Nonetheless, this efficacy is inferior to

208   that of other locally available plant species, and consequently, neither *Tabernaemontana* species

209   nor *T. iboga* are extraordinarily valued in traditional Mexican or African medicine, respectively,

210   for these purposes.

211   2.   Ibogaine causes unique stimulant and oneirogenic CNS effects that convert *T. iboga* into

212   an authentic "plant of the gods" of the greatest ethnobotanical value in Central African cultures.

213   The same cannot be said for Mexican *Tabernaemontana* species, either due to their inferior or

214   undesirable CNS activity, lack or loss of knowledge of this activity in traditional medicine, or

215   preferences intrinsic to indigenous civilizations.

216   3.   In consequence, the ethnobotanical significance of a plant species that contains mostly

217   MIAs of the ibogan type depends to a large degree on whether or not the respective human

218   cultures discover and appreciate the CNS effects of these alkaloids.

219   So why would Mexican indigenous civilizations not include *Tabernaemontana* species in

220   their otherwise wide range of psychoactive plants with ritualistic applications? One possible

221   answer is that Mexican *Tabernaemontana* species have alkaloid profiles whose constituents act

222   synergistically in a distinct manner compared to the MIAs associated with *T. iboga*, due to

223   qualitative (chemical structure) and/or quantitative (ratio between individual MIAs) differences.

224   Hence, herbal preparations of *Tabernaemontana* species could exhibit CNS effects quite

225   dissimilar to *iboga*. However, Krengel et al. (2019) showed that ibogamine, ibogaine, and

226   voacangine were majority alkaloids in the barks of *T. alba*, *T. amygdalifolia*, *T. arborea*, and *T.*

227   *donnell-smithii*, as well as *T. iboga*. And although the authors detected only trace amounts of

228   coronaridine in the latter, this compound has been reported for the species in previous studies

229   (Table 2). Leaving aside the minority alkaloids, the main difference between the alkaloid profiles

230   of the two genera consists in the predominance of ibogaine in *iboga* but not Mexican

231   *Tabernaemontana* barks. The lower contents of ibogaine in the last ones may not be sufficient to

232   produce considerable CNS effects, and the other majority alkaloids of the ibogan type, namely

233   ibogamine, coronaridine, and voacangine, might not share comparable stimulant and entheogenic

234   properties with the first compound. Taking into account the similarities of chemical structures

235   and pharmacological activities observed in animal models (Glick et al., 1994; Van Beek et al.,

236   1984) between the four MIAs, this explanation seems unlikely. Additionally, there is at least one

237   report of *T. donnell-smithii* latex being used as a stimulant in Veracruz, Mexico (Table 1;

238 Caballero et al., 1978), and South-American *Tabernaemontana* species have provided brain

239 tonics (voacangine-containing *Tabernaemontana heterophylla* Vahl), stimulants

240 (*Tabernaemontana tetrastachya* Kunth), hypnotics (*Tabernaemontana rimulosa* Woodson), or

241 additives to entheogenic herbal preparations like *Ayahuasca* (coronaridine, ibogamine, and

242 voacangine-containing *Tabernaemontana sananho* Ruiz & Pav.). The bark and seeds of

243 *Tabernaemontana dichotoma* Roxb. ex Wall. are the sole or main ingredients of narcotic and

244 hallucinogenic preparations in India and Sri Lanka, containing coronaridine, ibogamine, and

245 vobasine, among other MIAs. In Madagascar, *Tabernaemontana coffeoides* Bojer ex A.DC.

246 which presents coronaridine, ibogamine, voacangine, and vobasine, serves as a stimulant (Rätsch,

247 2007; Schultes et al., 2001; Van Beek et al., 1984). All of these uses imply significant CNS

248 activity in the absence of ibogaine but the presence of coronaridine, ibogamine, and/or

249 voacangine.

250   An elevated toxicity of *Tabernaemontana* preparations due to other MIAs is likewise

251 improbable. *T. alba* and *T. arborea* root bark's minority alkaloid vobasine does not belong to the

252 ibogan but to the corynanthean class (Van Beek et al., 1984), and it is therefore possible that its

253 biological activities are quite different from the first group. Nevertheless, the above-mentioned

254 psychoactive uses of vobasine-containing *T. dichotoma* and *T. coffeoides* suggest that this

255 compound does not easily cause life-threatening or otherwise deterrent effects. Toxicological data

256 summarized by Van Beek et al. (1984) supports this claim, with orally (p.o.) administered

257 vobasine showing non-fatal signs of toxicity at 100 mg/kg in rats and between 200 and 300 mg/kg

258 in mice. The median lethal dose (LD_{50}) of intravenously (i.v.) administered vobasine in mice was

259 higher than the respective values for voacangine, ibogaline, and ibogaine (58, 54, 46, and 42

260   mg/kg, respectively). Similarly, *T. arborea* and *T. donnell-smithii* are known to contain the

261   dimeric bis-indole alkaloid (consisting of two MIAs coupled together) voacamine (Kingston,

262   1978; Walls et al., 1958), whose toxicity is fairly low (LD_{50} 360 mg/kg). Much on the contrary,

263   the compound seems to have neuroprotective properties (Currais et al., 2014). However, it

264   remains unclear whether or not possibly toxic cardiac glycosides occur in the genus (Van Beek et

265   al., 1984).

266   An example that substantiates nicely the arguments made in the last two paragraphs are

267   the root and stem barks of *Voacanga africana* Stapf (Apocynaceae) whose alkaloid profiles

268   revealed astonishing similarities to those of *T. arborea* root bark: In both cases, the

269   concentrations of the predominant monomeric MIAs voacangine (high), ibogaine, and vobasine

270   (low to intermediate) are in the same range (Krengel et al., 2020), and as far as bis-indole

271   alkaloids are concerned, voacamine can be found in either species, too (Chen et al., 2016;

272   Guzmán-Gutiérrez et al., 2020; Kingston, 1978; Wang et al., 2019). Yet only *V. africana* has been

273   associated with psychoactive ethnobotanic uses in its respective native range, which is basically

274   limited to West Africa, including Gabon, and thus overlaps with the geographic distribution of *T.*

275   *iboga* (Rätsch, 2007; Schultes et al., 2001). Hence, sociocultural and historical factors may better

276   explain why only the African but not the Mexican species have been documented as traditional

277   stimulants and entheogens.

278   It is, of course, perfectly feasible that the indigenous peoples of Mexico simply never

279   discovered the CNS properties of *Tabernaemontana* species, that the corresponding knowledge

280   was lost during or after the Spanish colonization of the Americas, or that it was never revealed to

281   outsiders. The use of pre-Columbian psychoactive herbal preparations like *Ayahuasca* and

282 *Jurema*, for example, has been widely reported in large parts of South America (De Souza et al.,

283 2008; Dos Santos et al., 2016), but not in Mexico, in spite of the fact that the same or

284 phytochemically similar plant species occur throughout the tropical Americas. The N,N-

285 dimethyltryptamine (N,N-DMT)-containing root and stem barks of *Mimosa tenuiflora* (Willd.)

286 Poir. (Fabaceae) are the main ingredients for the psychoactive brew *Jurema* in northeastern

287 Brazil, whereas in Mexico, they are used to treat dermatological problems and burns, and to

288 construct living fences (BDMTM, 2009d; De Souza et al., 2008). The same bark in combination

289 with β-carboline alkaloid-containing plant material from, e. g., *Banisteriopsis* C.B. Rob.

290 (Malpighiaceae) species, capable of inhibiting the monoamine oxidase A (MAO-A), and thus, the

291 degradation of N,N-DMT, would provide an *Ayahuasca*-like mixture (Dos Santos et al., 2016). In

292 analogy with Mexican *Tabernaemontana* species, the non-existence of reports about the

293 psychoactive use of *Ayahuasca*- or *Jurema*-like preparations in pre-Hispanic Mexico may be

294 attributed to either of the three reasons mentioned at the beginning of this paragraph. It is

295 certainly remarkable that the stem and root barks of *T. alba*, *T. amygdalifolia*, *T. arborea*, and *T.*

296 *donnell-smithii* do not present registered ethnobotanical applications. As these are the organs

297 with the supposedly highest alkaloid contents, both generally speaking and with respect to the

298 CIVI-complex (Krengel et al., 2019a, 2016), it is plausible that if the barks were, for whatever

299 reason, simply never ethnomedicinally used, the discovery of the four species' psychoactive

300 effects would consequently be less likely. Nonetheless, the fact that the already mentioned

301 stimulant use of the latex of *T. donnell-smithii* was registered in an indigenous *Chinanteco*

302 community (Caballero et al., 1978) can be interpreted to imply that at least some pre-Columbian

303 Mexican cultures knew about the CNS-related activities of this and probably other

304 *Tabernaemontana* species. It could also indicate that this knowledge has been conserved over the

305 centuries, albeit in rudimentary form. Ergo, the most evident reasons for the lack of reported

306 psychoactive uses of the genus in Mexican traditional medicine are concealment and partial loss

307 of the original knowledge.

308 Parts of the los Tuxtlas region have been inhabited by two native peoples, the *Popoluca*

309 and the *Nahua*, since 500 and 800 A. D., respectively (Velázquez-Hernández, 2015), but there is

310 no evidence of *Tabernaemontana* species being used for stimulant or entheogenic purposes.

311 Indeed, two ethnobotanical studies of *Popoluca* communities documented only non-psychoactive

312 applications of *T. alba* (Leonti, 2002; Leonti et al., 2001). It should be mentioned, though, that

313 both local *Popoluca* and *Nahua* communities possess sacred spiritual and ritualistic knowledge

314 that has been conserved and transmitted by a small circle of highly respected leaders only

315 (Velázquez-Hernández, 2015). During the colonial period of Mexico, spiritual practices involving

316 entheogenic plants or mushrooms were discouraged, suppressed, and often persecuted with

317 particular eagerness by the religious and secular authorities of the Viceroyalty of New Spain.

318 Most written primary sources of the pre-Hispanic era, the so-called *códices*, were destroyed.

319 Nowadays, at least some clerical institutions like the Catholic Church continue to look upon such

320 "pagan" customs with distrust (Béjar et al., 2000; Guzmán, 2008; Schultes et al., 2001).

321 Accordingly, it sounds reasonable that the historical experience of many indigenous communities

322 would have led them to conceal the use of entheogenic plants from outsiders.

323 Furthermore, the region once covered by tropical rainforests has suffered severe

324 ecological and cultural devastation during the last decades, largely due to governmental policies

325 designed in the 1950s and aimed to convert "uninhabited" land into private property for

326 immigrant *mestizo* cattle breeders (Durand, 2005; Velázquez-Hernández, 2015). As a result,

327 knowledge of the psychoactive properties of the local *Tabernaemontana* species might have been

328 lost, just as according to our experience mentioned earlier, the ethnomedicinal uses of these

329 plants listed in Table 1 appear not to have been conserved by several *mestizo* communities of the

330 Los Tuxtlas region.

331 Nonetheless, there is another explanation that plausibly combines chemical-biological and

332 cultural aspects: Pre-Hispanic Mexican civilizations possibly had a certain preference for the

333 "classical hallucinogens" which according to the Hollister-definition causes "changes in thought,

334 perception and mood," but only minimal "intellectual or memory impairment," "stupor, narcosis

335 or excessive stimulation," and "autonomic nervous system side effects," in the absence of

336 "addictive craving" (Glennon, 1994). Several representatives of this group, specifically the

337 ergoline derivatives-containing *ololiuqui* (*Turbina corymbosa* (L.) Raf. [Convolvulaceae]) and

338 *tlitliltzin* (*Ipomoea violacea* L. [Convolvulaceae]), mescaline-containing *peyotl* (*Lophophora*

339 *williamsii* (Lem. ex Salm-Dyck) J.M. Coult. [Cactaceae]), as well as psilocybin and psilocin-

340 containing *teonanácatl* (*Psilocybe* (Fr.) P. Kumm. [Strophariaceae]) were widely available in pre-

341 Columbian Mexico and continue to be an essential part of the cosmogony and rituals of many

342 indigenous civilizations (Rätsch, 2007; Schultes et al., 2001). Plants presenting other types of

343 psychoactive compounds are often used to a lesser degree and generally considered "less sacred"

344 than the aforementioned species. For example, in Mazatec culture, the Lamiaceae *hierba de la*

345 *pastora* (*Salvia divinorum* Epling & Játiva) is mainly used in rituals when *Psilocybe* mushrooms

346 are scarce. Similarly, the Huichol people regard *peyotl* as part of their "holy trinity," together with

347 the deer and the maize (Rätsch, 2007; Schultes et al., 2001). The Solanaceae *kieri* (*Datura*

348   *innoxia* Mill. and *Datura stramonium* L.), on the other hand, is considered a malignant and

349   dangerous force in opposition to the benevolent *peyotl* (BDMTM, 2009e). Both *S. divinorum* and

350   *Datura* species contain psychoactive compounds which induce dissociative or deliriant CNS

351   effects very different to those of the "classical hallucinogens," namely the neoclerodane

352   diterpenoid salvinorin A in the former, and the tropane alkaloids hyoscyamine and scopolamine

353   in the latter case (Rätsch, 2007; Schultes et al., 2001). Following the same logic in this particular

354   cultural context, it should be noted that the effects of ibogaine are also qualitatively dissimilar to

355   other entheogenic drugs. At lower doses, both *T. iboga* root bark and its active principle act as a

356   stimulant much appreciated by hunters and warriors in order to suppress fatique. High to

357   excessive doses are usually taken at *Bwiti* ceremonies, and cause an introversive self-reflective

358   state accompanied by closed-eye visions that resemble more of a dream than the

359   pseudohallucinations and "psychedelic" mindset associated with the "classical hallucinogens",

360   which is why the term "oneirogenic" is often used in connection with ibogaine. Furthermore,

361   although the substance presents a moderate toxicity, it is certainly more toxic than the "classical

362   hallucinogens", and high amounts tend to severly impair motor activity, while overdoses can

363   paralyze and even kill humans (Alper, 2001; Nichols, 2004; Ott, 1996; Schultes et al., 2001),

364   especially those suffering from cardiovascular diseases (Koenig and Hilber, 2015). Pope

365   (1969) states that "The hallucinogenic dose is several times the normal stimulant dose, so that the

366   user must endure intense and unpleasant central stimulation in order to experience the

367   hallucinogenic effects". From a pharmacodynamic point of view, the entheogenic properties of

368   the "classical hallucinogens" are a consequence of them being agonists of the 5-HT$_{2A}$ serotonin

369   receptor. Ibogaine, on the contrary, interacts with a much wider range of receptors and

370   transporters known to modulate psychoactive effects. Actually, the compound has been found to

371   both activate 5-HT$_{2A}$ (like mescaline and psilocin) and kappa opioid receptors (like salvinorin A),

372   while inhibiting muscarinic (like hyoscyamine and scopolamine) and nicotinic acetylcholine and

373   N-methyl-D-aspartate (NMDA) receptors (like the synthetic dissociative ketamine). It also binds

374   to sigma receptors, as well as to serotonin and dopamine transporters (Alper, 2001; Glick and

375   Maisonneuve, 1998; Johnson et al., 2011; Lochner and Thompson, 2016; Nichols, 2004;

376   Sweetnam et al., 1995; Zorumski et al., 2016). Thus, it is reasonable to assume that the

377   indigenous Mexican civilizations simply preferred the "classical hallucinogens" to ibogan type

378   alkaloid-containing plants, due to a cultural bias towards the former and a natural environment

379   offering many varieties of this type of entheogen. As a matter of fact, nowhere else in the world

380   are as many indigenous uses of psychoactive plants registered as in Mexico (Schultes et al.,

381   2001). Whether or not *Tabernaemontana* species were utilized ritualistically on a minor scale and

382   this knowledge then forgotten, cannot be answered at this time.

383   In Central Africa, in contrast, the only "classical hallucinogenics" available may be

384   represented by *Psilocybe* species, whereas *iboga* is by far the most important natural source of

385   entheogenic preparations (Schultes et al., 2001). Clearly, the regional cultures perceived the

386   oneirogenic properties of the latter as something highly desirable and sacred, not minding, or

387   perhaps even appreciating, the strong stimulating effects. The absence or negligible use of

388   "classical hallucinogenics" may be explained by the relatively low abundance and variety of the

389   respective natural sources compared to Mexico, and/or a cultural bias towards *iboga*, rendering

390   other entheogenic preparations unimportant. It would thus seem that the demand for psychoactive

391   natural sources belonging to the "classical hallucinogenics" or ibogan type alkaloid-containing

392   plants was substantially created by the local natural environment and, above all, sociocultural and

393   historical developments intrinsic to two very distinct culture groups with similar spiritual needs.

394

395   **Glossary**

396   CIVI-complex: coronaridine-ibogamine-voacangine-ibogaine-complex

397   CNS: central nervous system

398   GC-MS: gas chromatography-mass spectrometry

399   Ibogaine HCl: ibogaine hydrochloride

400   I.v.: intravenous

401   LD_{50}: median lethal dose

402   MAO-A: monoamine oxidase A

403   MIA: monoterpenoid indole alkaloid

404   NMR: nuclear magnetic resonance

405   N,N-DMT: N,N-dimethyltryptamine

406   PCA: principal component analysis

407   P.o.: per os

408   PTA: purified total alkaloid

409   TA: total alkaloid

410   TIC: total ion chromatogram

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Declarations of interest

None to declare.

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CAPÍTULO 3: FARMACOLOGÍA

Antimycobacterial activity of alkaloids and extracts from *Tabernaemontana alba* and *T. arborea*

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Antimycobacterial Activity of Alkaloids and Extracts from *Tabernaemontana alba* and *T. arborea*

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ABSTRACT

Tuberculosis is the main cause of death from a single infectious agent. Globally, according to the World Health Organization, in 2018, there were an estimated 1.2 million tuberculosis deaths. Moreover, there is a continuous appearance of drug-resistant strains. Thus, development of new antituberculosis medicines should receive high priority. Plant-derived natural products are promising candidates for this purpose. We therefore screened alkaloid extracts obtained from the root and stem barks of the Mexican Apocynaceae species *Tabernaemontana alba* and *Tabernaemontana arborea*, as well as the pure alkaloids ibogaine, voacangine, and voacamine, tested for activity against *Mycobacterium tuberculosis* H37Rv and cytotoxicity to mammalian Vero cells using the resazurin microtiter and the MTT assays, respectively. The extracts were analyzed by GC-MS and HPLC-UV. *T. arborea* root bark alkaloid extract showed the highest activity against *M. tuberculosis* ($MIC_{100} = 7.8$ µg/mL) of the four extracts tested. HPLC suggested that voacangine and voacamine were the major components. The latter was isolated by column chromatography, and its chemical structure was elucidated by ^1H and ^{13}C NMR, and MS. Unambiguous assignation was performed by HSQC, HMBC, and NOESY experiments. Voacamine is a dimeric bis-indole-type alkaloid and is 15 times more potent than the monomeric ibogan-type alkaloids ibogaine and voacangine ($MIC_{100} = 15.6$, 250.0, and 250.0 µg/mL, respectively). However, all of these compounds showed cytotoxicity to Vero cells, with a poor selectivity index of 1.00, 0.16, and 1.42, respectively. This is the first report of voacamine activity against *M. tuberculosis*.

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Introduction

Continuous development of bacterial resistance to antituberculous medicines has reached an alarming magnitude. Ethambutol, isoniazid, rifampicin, and pyrazinamide are the most important first-line drugs due to their good cost-effectiveness ratios, but their long history of medical use has led to the appearance of several rifampicin- and multidrug-resistant tuberculosis (RR/MDR-TB) strains. Even worse, extensively drug-resistant TB (XDR-TB) has become relatively common, rendering several second-line drugs useless [1,2]. According to the World Health Organization (WHO), in 2018, there were an estimated 1.2 million TB deaths globally, about half a million new cases of rifampicin-resistant TB, with 78% of them being multidrug-resistant TB [3]. Natural products from plants are an excellent option to discover new compounds with activity against TB, either on their own, or in synergy with established antituberculous drugs [4].

Mexico's outstanding biodiversity and well-conserved knowledge of traditional medicine makes the country a good starting point for such an endeavor. At least 187 species are ethnobotanically used to treat TB-related symptoms, but only one-third of these plants have been experimentally tested against *Mycobacterium tuberculosis* [5]. The *Los Tuxtlas* Biosphere Reserve in the state of Veracruz harbors the most important remains of the tropical rainforest that once covered a great part of the Gulf Coast of Mexico. It is located near the northern distribution limit of this ecosystem in the American continent. Among the region's 3356 plant species [6], the *Tabernaemontana* L. (Apocynaceae) genus

is represented by the following three: *Tabernaemontana alba* Mill., *Tabernaemontana arborea* Rose ex J. D. Sm., and *Tabernaemontana donnell-smithii* Rose ex J. D. Sm. [7]. The first and the last species have been reported to be used in Mexican traditional medicine due to their analgesic, anti-inflammatory, antiparasitic, and/or dermatological properties [8,9], and all three are known to contain significant amounts of monoterpenoid indole alkaloids (MIAs) of the ibogan-type subclass, particularly, coronaridine, ibogamine, voacangine, and ibogaine [10,11].

Taking into consideration that extracts obtained from Asian and African species of *Tabernamontana* have shown antimycobacterial activity [12,13], we evaluated the alkaloid extracts obtained from the stem and root barks of *T. alba* and *T. arborea* as well as pure voacangine, ibogaine, and voacamine against *M. tuberculosis* strain H37Rv and assessed their cytotoxicity to Vero cells. The *T. arborea* root bark extract and, to a lesser degree, the dimeric bis-indole-type MIA voacamine proved to be worthy candidates for further research directed to develop new antituberculous medicines.

Results

The alkaloid contents were determined in *T. alba* and *T. arborea* extracts from the stem and root bark by GC-MS (▶ Table 1 and Fig. 1). All the samples contained at least one ibogan-type alkaloid (coronaridine, ibogaine, ibogamine, and/or voacangine) and the corynanthean-type MIA vobasine, as previously reported [10, 11, 14]. The aspidospermatan-type alkaloid apparicine was only found in the *T. alba* extracts. The most abundant alkaloids were

▶ **Table 1** Monomeric alkaloid contents of extracts from the barks of *T. alba* and *T. arborea* (μg/mg) determined by GC-MS.

Extract	Apparicine	Coronaridine	Ibogamine	Voacangine	Ibogaine	Vobasine
T. alba stem bark	24.07	35.67	27.52	133.65	31.66	35.06
T. arborea stem bark	ND	ND	ND	26.20	ND	58.80
T. alba root bark	34.96	92.40	166.51	28.94	31.56	25.00
T. arborea root bark	ND	ND	ND	53.58	33.74	62.67

ND: not detected
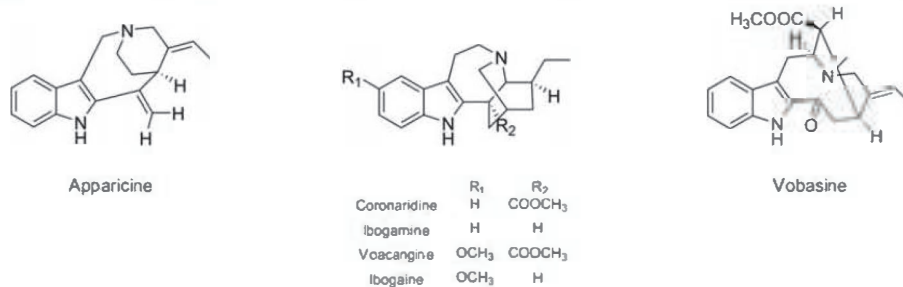
▶ **Fig. 1** Chemical structures of the monomeric MIAs apparicine (aspidospermatan type), coronaridine, ibogamine, voacangine, and ibogaine (ibogan type), as well as vobasine (corynanthe type).
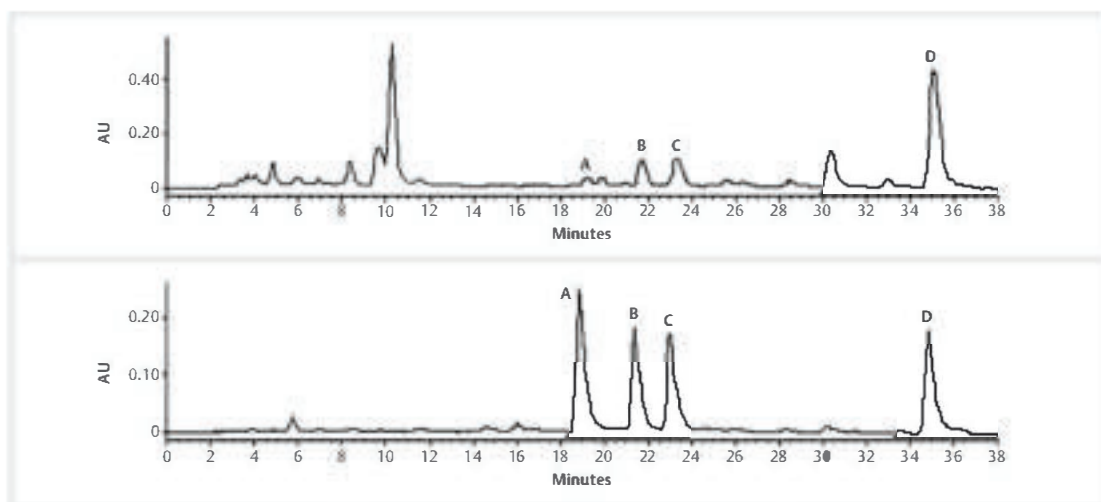
▶ **Fig. 2** HPLC-UV chromatograms (280 nm) of *T. arborea* root bark alkaloid extract (above), as well as pure ibogaine (A), voacamine (B), and voacangine (C) (below).

ibogamine and voacangine in *T. alba* root and stem extracts with 166.51 and 133.65 μg/mg, respectively (▶ **Table 1**).

The GC-MS method described above was only suitable for the determination and quantification of monomeric, but not dimeric, MIAs, which were not detected in our conditions. Since *T. arborea* is known to biosynthesize bis-indole-type alkaloids such as voacamine [15], the root bark extract of this species was also analyzed by HPLC-UV. Voacamine was effectively detected in this sample with a RT (retention time) = 21.5 min compared with an authentic sample isolated by us (▶ **Fig. 2**).

The alkaloid extract from *T. arborea* (2 g) root bark (2 g) was then subjected to column chromatography (CC), affording a light beige solid (28 mg), which was identified as voacamine by NMR and electron ionization (EI) MS. The latter indicated a molecular ion of m/z 704 and the formula $C_{43}H_{52}N_4O_5$. The ^{13}C and HSQC NMR spectra revealed the presence of 43 carbons belonging to 2 methyls, 9 methylenes, 14 methines (6 aromatic), 3 methoxyls, and 1 methylamino group, as well as 14 quaternary carbons (▶ **Table 2**). The ^{13}C and ^1H spectra showed the chemical shifts and proton multiplicities reported for voacamine (▶ **Fig. 3**) [16–18]. The hydrogens attached to C-3, C-5, and C-6 were assigned as follows: H_β = 2.721 and H_α = 2.867, H_β = 3.152 and H_α = 3.364, and H_β = 3.100 and H_α = 2.952 ppm, respectively; these δ values are the opposite of those previously reported [17]. The unambiguous assignation was performed by ^1H-^{13}C HSQCed ^1J(C,H), ^1H-^{13}C HMBC ^nJ(C–H) (n = 2, 3, and 4) (Table 1S, Supporting Information), ^1H-^1H TOCSY (Table 2S, Supporting Information), and ^1H-^1H NOESY (▶ **Fig. 4** and **Table 3**). The most quoted numbering system was used to describe the structure of voacamine [19].

The extracts and pure compounds were tested for their antimycobacterial activity (▶ **Table 4**). *T. arborea* root bark extract showed the highest activity against *M. tuberculosis* (MIC_{100} = 7.8 μg/mL) of the four extracts tested. Voacamine, one of the

most abundant constituents by HPLC, was 15 times more potent than the monomeric ibogan-type alkaloids ibogaine and voacangine (MIC_{100} = 15.6, 250.0, and 250.0 μg/mL, respectively). However, voacamine was also two times lower in potency than the extract of *T. arborea* root bark (▶ **Table 3**), suggesting the presence of other active alkaloids and an additive or synergistic effect. On the other hand, the tested alkaloids showed cytotoxicity to Vero cells, with a poor selectivity index of 1.00, 0.16, and 1.42 for voacamine, voacangine, and ibogaine, respectively.

The *T. arborea* extracts proved to be more potent than their *T. alba* counterparts. The extracts from the latter species contained apparicine, coronaridine, ibogamine, voacangine, ibogaine, and vobasine, as determined by CG-EIMS. Our data suggest some of these alkaloids could be, in part, responsible for their mild antimycobacterial activity (▶ **Table 3**).

Discussion

T. arborea root bark extract was the most potent extract of the four tested extracts (MIC = 7.8 μg/mL) and more active than extracts reported for other species of this genus [12,13]. For instance, *Tabernaemontana divaricata* (L.) R.Br. ex Roem. & Schult. (syn. *Tabernaemontana coronaria* [Jacq.] Willd.) and *Tabernaemontana elegans* Stapf showed MICs of 100.0 and 15.6 μg/mL, respectively [12,13]. The above results sustained the evaluation of pure ibogaine, voacangine, and voacamine present in our best extract against *M. tuberculosis*. Voacamine was the most active alkaloid tested, 15 times more potent than that of the two monomeric alkaloids, but it was two times lower in potency than the alkaloid extract of *T. arborea* root bark.

Voacamine is the condensation product of voacangine with an alkaloid of the vobasine group, probably vobasinol [20] (▶ **Fig. 3**). This bis-indole alkaloid occurred only in *T. arborea*, but not in

▶ **Table 2** ¹H (700 MHz) and ¹³C (175 MHz) NMR for voacamine (▶ **Fig. 3**).

Atom	¹H-¹³C HSQCed[a] ¹J(C,H)	
	δ ¹³C ppm	δ ¹H ppm, J (Hz)
1	-	1H, bb, 7.449
2	137.20	-
3	51.79	2H, bb, 2.867 (¹H$_\alpha$) and bb, 2.721 (¹H$_\beta$)
4	-	-
5	53.08	2H, m, 3.364 (¹H$_\alpha$) and m, 3.152 (¹H$_\beta$)
6	22.22	2H, m, 3.100 (¹H$_\beta$) and m, 2.952 (¹H$_\alpha$)
7	109.97	-
8	127.37	-
9	99.19	1H, brs, 6.922
10	150.84	-
11	129.87	-
12	110.26	1H, brs, 6.747
13	130.22	-
14	27.32	1H, bb, 1.804
15	31.96	2H, bb, 1.676 (¹H$_\alpha$) and dd, 1.081 (¹H$_\beta$) (J= 11.2 y 7.7)
16	54.93	-
17	36.47	2H, bb, 2.470 (¹H$_\alpha$) and 1.748 (¹H$_\beta$) (J= 13.02)
18	11.60	3H, t, 0.874 (J= 7.7)
19	26.73	2H, qd, 1.546 and qd, 1.407 (J = 7 y 7.7)
20	38.99	1H, m, 1.286
21	57.18	1H, bb, 3.501
1'	-	1H, bb, 7.696
2'	-	-
3'	37.27	1H, bd, 5.135 (J = 10 Hz)
4'	-	-
5'	59.94	1H, m, 4.056
6'	19.50	2H, bb, 3.490 (¹H$_\alpha$) and bb 3.255 (¹H$_\beta$)
7'	109.97	-
8'	129.72	-
9'	117.4	1H, m, 7.536
10'	118.93	1H, bb, 7.051
11'	121.57	1H, bb, 7.051
12'	109.82	1H, bb, 7.051
13'	135.77	-
14'	36.26	2H, m, 2.546 (¹H$_\alpha$) and bd, 1.999 (J= 9 Hz)
15'	33.49	1H, m, 3.771
16'	46.94	1H, bb, 2.732
18'	12.33	3H, d, 1.665 (J= 6.37)
19'	118.66**	1H, bb, 5.339
20'	138.00	-
21'	52.43	2H, m, 3.750 and m, 2.94
MeO-10	56.07	3H, s, 4.000
MeO-16' COOCH₃	49.94	3H, s, 2.461
CO-16' COOCH₃	171.67	
MeO-16	52.45	3H, s, 3.652

Atom	¹H-¹³C HSQCed[a] ¹J(C,H)	
	δ ¹³C ppm	δ ¹H ppm, J (Hz)
CO-16	175.22	
MeN-4'	42.29	3H, brs, 2.614

CDCl₃/TMS used as the solvent and internal reference. Chemical shifts (δ, ppm) and coupling constants (J) in Hz. Heteronuclear 2D shift-correlated HSQC [¹] (C,H)]. s = singlet, t = triplet, q = quartet, bb = broad band, m = multiplet, brs = broad singlet, d = doublet, bd = broad doublet, qd = quartet doublet. [a] Overlapped signals deduced by HSQCed, HMBC, TOCSY and NOESY. * Overlapped at 138 ppm. ** Chemical shift deduced by HMBC.

T. alba, which is in agreement with previous results [15, 21–23]. Voacamine was first isolated from *Voacanga africana* Stapf, and subsequently from other species [24, 25]. This species has been traditionally used in West Africa against leprosy, whose causal agent is *Mycobacterium leprae* [26, 27]. Subsequently, voacamine was isolated from *Tabernaemontana* species, also belonging to Apocynaceae. In the decade of the 1970s, a systematic search for novel anticancer drugs from plants was performed. Voacamine isolated from the sap of *T. arborea* (collected in Costa Rica) was cytotoxic against the cell line P-388 (lymphocytic leukemia), showing an ED₅₀ of 2.6 μg/mL [15]. Later, other authors tested voacamine against five other cell cancer lines and found significant inhibitory activity [28].

This bis-indole alkaloid also showed antimicrobial activity against *Plasmodium falciparum in vitro* and *in vivo* [29]. In addition, voacamine is an anti-trypanosomatid agent against Indian strains of *Leshmania donovani*, as well as Brazilian strains of *Leshmania amazonensis* and *Trypanosoma cruzi* [30]. This alkaloid was reported as not toxic in normal fibroblasts [31]. The hydroethanolic extract of *Voacanga africana* and voacamine isolated from this extract exert neuroprotective activity *in vitro* [32].

Regarding the monomeric alkaloids, we determined an MIC of 250 μg/mL for voacangine and ibogaine (▶ Table 2). However, it has previously been reported that ibogaine and voacangine showed an MIC₉₉ of 50 μg/mL against *M. tuberculosis* strain H37Rv [33]. These indole alkaloids were isolated from *Tabernaemontana citrifolia* collected from Guadeloupe Island in the Caribbean. The differences between our data and others previously reported could be due to the experimental methods. We used the REMA assay, and Rastogi et al. [33] used the Bactec method. We adjusted the density of the bacterial suspensions at 600 nm, while the latter authors relied on McFarland standards. All four values determined for ibogaine and voacangine considerably exceed the MICs associated with voacamine and *T. arborea* root bark extract. Another ibogan alkaloid so far tested, coronaridine, has shown an MIC = 82.64 μg/mL [34].

Regarding cytotoxicity, it should be noted that the extract of *T. arborea* root bark also presented the greatest selectivity index (SI) of all samples tested (▶ Table 2), indicating the most favorable relation between antimycobacterial activity and *in vitro* cytotoxicity. The three alkaloids showed *in vitro* cytotoxicity to Vero cells, and a poor selective index of 1.00, 0.16, and 1.42, respectively. However, tests with mice suggest lower *in vivo* toxicity of both ibogan-type alkaloids and voacamine, with intravenously administered median lethal doses ranging from 42 to 360 mg/kg [23].
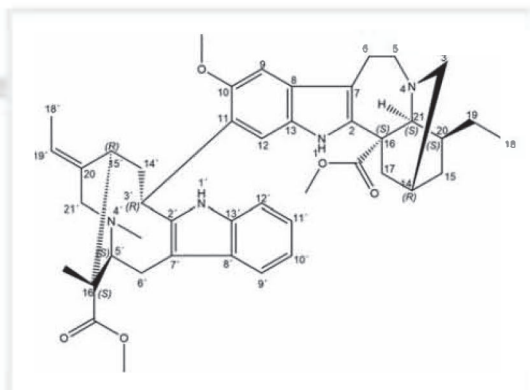
▶ **Fig. 3** Chemical structure of the dimeric MIA voacamine (bis-indole type).

Materials and Methods

Plant material

Root and stem barks from *T. alba* and *T. arborea* were collected in the *Los Tuxtlas* region in Veracruz, Mexico, and identified by Delfino Álvaro Campos Villanueva (Estación de Biología Los Tuxtlas, UNAM) and Leonardo Osvaldo Alvarado Cárdenas (Facultad de Ciencias, UNAM). Voucher specimens were deposited at the herbarium of the Facultad de Ciencias, UNAM (voucher numbers 132793, 133359, 161424 to 161427). For further details, see [10, 11].

General experimental procedures

All alkaloid crude extracts were obtained by extraction and analyzed by GC-MS as described elsewhere [10, 11, 14]. In brief, the dried and powdered barks were defatted with hexane and then extracted thrice with methanol. The dry methanolic extracts were dissolved in dilute hydrochloric acid, filtered, and made basic by the addition of concentrated ammonium hydroxide. The alkaloid extracts were then obtained by liquid-liquid extraction with chloroform. GC-MS analyses were conducted with an Agilent 7890B/5977 A GC/MSD and an HP-5 ms (30 m) capillary column.

HPLC-UV analyses were performed on a Breeze HPLC system (Waters) equipped with a degasser, 1525 binary pump, 2998

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▶ **Fig. 4** Voacamine ^{1}H-^{13}C HSQCed 1 J(C,H), ^{1}H-^{13}C HMBC n J(C–H) (n = 2, 3, and 4), ^{1}H-^{1}H TOCSY, and ^{1}H-^{1}H NOESY.
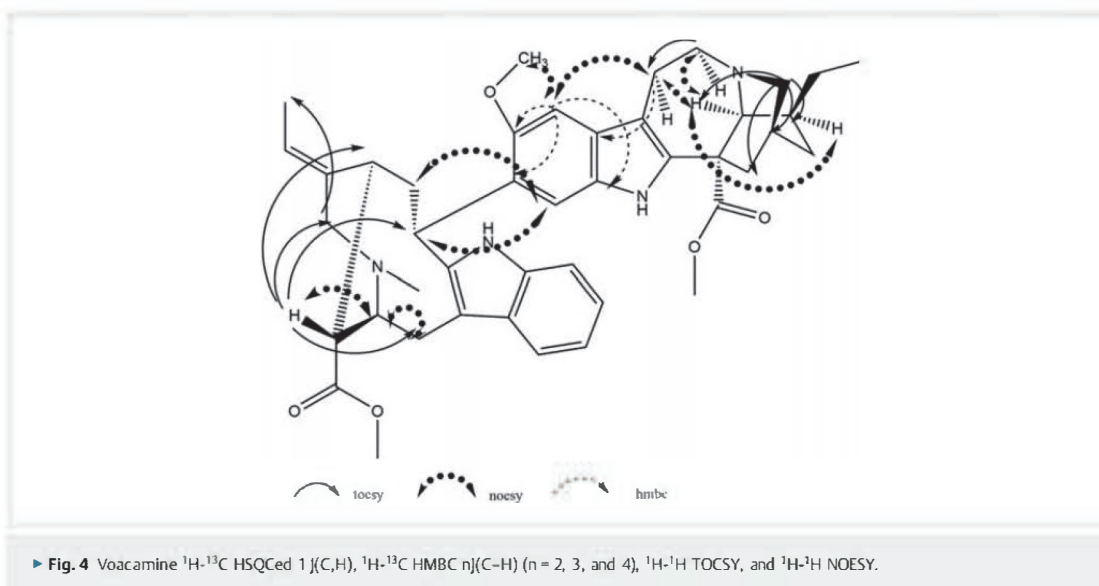
photodiode array detector, and a Phenomenex Luna PFP(2) column (100 Å, 250 × 4.6 mm, 5 μm particle size). Pure ibogaine, voacangine (kindly donated by Phytostan Enterprises, Inc.), and voacamine were used as external standards in GC-MS and HPLC-UV analyses. CC was carried out with silica gel 60 (Macherey-Nagel). Fractions were monitored by TLC using precoated ALUGRAM Xtra SIL G/UV254 aluminum sheets (Macherey-Nagel). EI mass spectra were obtained on a JEOL MStation JMS-700. ^{1}H NMR and ^{13}C NMR spectra were acquired on a Bruker AVANCE III HD spectrometer at 700 and 175 MHz, respectively, using CDCl$_3$ and DMSO as solvents. Chemical shifts are indicated in ppm (δ scale).

GC-MS analysis

The ramp temperature program was raised from 150 to 300 °C at 4 °C/min. The carrier gas was helium (1 mL/min). EI-MS were recorded at 70 eV with the injector set in split mode (1 : 5) at 300 °C and automatic injection of 1 μL aliquots. Peaks were identified using the NIST Mass Spectral Search Program for the NIST/EPA/NIH Mass Spectral Library (Version 2.2) and by comparison with experimentally obtained spectra of voacangine and ibogaine, as well as published data.

HPLC-UV analysis

All analyses were conducted at room temperature. The mobile phase consisted of methanol (A) and phosphate buffer pH 7.4 (B) with the following elution gradient delivered at a flow rate of 1 mL/min: 0–1 min 70% B, 1–30 min 70 to 90% B, 30–35 min 90% B, and 35–38 min 90 to 100% B [35]. The injection volume was 20 μL. The compounds were detected at 280 nm.

Isolation of voacamine

An alkaloid extract obtained from *T. arborea* root bark (2 g) was fractionated by CC. Fractions 42 and 43 were eluted in a dichloro-

methane-methanol (5 : 95) gradient and yielded a light beige solid (28 mg).

M. tuberculosis inocula preparation

M. tuberculosis H37Rv strain was obtained from ATCC (27294) and was cultivated in a 7H9-glycerol-10% ADC-0.01% tyloxapol medium (albumin dextrose catalase and 7H9 are from BBL Middlebrook Enrichment) at 37 °C until an O.D. of 0.4 at a wavelength of 600 nm was reached. Working bacteria solution was obtained by a dilution of 1 : 25 in 7H9-ADC 10%.

Antimicrobial susceptibility test using the resazurin microtiter assay

The assay used here was previously described by Collins and Franzblau. In brief, the outer wells of a 96-well plate were each filled with 200 μL of sterile PBS to prevent dehydration during the incubation (8 days). Rifampicin (Sigma-Aldrich) was used as a reference drug (16.000–0.001 μg/mL serial twofold dilutions) in each plate. Compounds were evaluated at various concentrations from 1.95 to 500.00 μg/mL and controls with DMSO (5%) (Sigma-Aldrich), DMSO + *M. tuberculosis*, medium, and medium + *M. tuberculosis*. Three independent assays were carried out by each experiment. Plates were incubated for 6 days. Next, 30 μL of 0.01% resazurin (weight/volume) (Sigma-Aldrich) were added to each well and the plates were incubated for 2 more days. Visual inspection was used to determine the color of each well, with blue interpreted as no growth, pink as growth, and MIC$_{100}$ as the last concentration in which a blue color was observed [36–38].

Cell culture

The cytotoxicity assays were carried out using a Vero cell line (African green monkey kidney ATCC CCL-81). These cells were cultured in RPMI 1640 medium supplemented with 10% FBS and nonessential amino acids (GIBCO).

Table 3 ^{1}H-^{1}H-NOESY data for voacamine (▶ Fig. 3).

H	δ$_H$	H	δ$_H$
HN-1	7.449	1H-17	1.748 (^{1}H$_\beta$)
3	2.721 (^{1}H$_\beta$)	1H-6	3.100 (^{1}H$_\beta$)
3	2.721 (^{1}H$_\beta$)	1H-5	3.152 (^{1}H$_\beta$)
3	2.721 (^{1}H$_\beta$)	1H-14	1.804
3	2.721 (^{1}H$_\beta$)	1H-17	1.748 (^{1}H$_\beta$)
3	2.721 (^{1}H$_\beta$)	1H-3	2.867 (^{1}H$_\alpha$)
3	2.867 (^{1}H$_\alpha$)	1H-15	1.080 (^{1}H$_\beta$)
5	3.364 (^{1}H$_\alpha$)	1H-6	2.952 (^{1}H$_\alpha$)
5	3.152 (^{1}H$_\beta$)	1H-6	2.952 (^{1}H$_\alpha$)
6	3.100 (^{1}H$_\beta$)	1H-9	6.922
6	2.952 (^{1}H$_\alpha$)	1H-9	6.922
9	6.922	3H-MeO-10	4.000
12	6.747	1H-14'	2.546
12	6.747	1H-3'	5.135
14	1.804	1H-17	2.470 (^{1}H$_\alpha$)
14	1.804	1H-15	1.080 (^{1}H$_\beta$)
15	1.080 (^{1}H$_\beta$)	1H-15	1.676 (^{1}H$_\alpha$)
15	1.676 (^{1}H$_\alpha$)	1H-20	1.280 (^{1}H$_\alpha$)
15	1.080 (^{1}H$_\beta$)	1H-19	1.407
17	2.470 (^{1}H$_\alpha$)	1H-20	1.280 (^{1}H$_\alpha$)
17	2.470 (^{1}H$_\alpha$)	1H-14	1.804
17	1.748 (^{1}H$_\beta$)	1H-3	2.720 (^{1}H$_\beta$)
18	0.874	1H-21	3.501
18	0.874	1H-20	1.280
19	1.546	1H-21	3.501
18	0.870	3H-MeO-16	3.652
21	3.500 (^{1}H$_\alpha$)	1H-5	3.360 (^{1}H$_\alpha$)
21	3.500 (^{1}H$_\alpha$)	1H-6	2.950 (^{1}H$_\alpha$)
1'	7.690	1H-12'	7.051
1'	7.690	1H-3'	5.135
3'	5.135	1H-14'	1.999 (^{1}H$_\beta$)
3'	5.135	1H-12	6.747
MeN-4'	2.614	1H-21'	3.750 (^{1}H$_\beta$)
MeN-4'	2.614	1H-6'	3.255 (^{1}H$_\beta$)
MeN-4'	2.614	1H-5'	4.056
MeN-4'	2.614	1H-6'	3.490 (^{1}H$_\alpha$)
5'	4.056	1H-6'	3.255 (^{1}H$_\beta$)
5'	4.056	1H-16'	2.732
6'	3.255 (^{1}H$_\beta$)	1H-9'	7.536
9'	7.536	1H-10'	7.051
14'	2.546 (^{1}H$_\alpha$)	1H-6'	3.490 (^{1}H$_\alpha$)
15'	3.770	1H-19'	5.339
15'	3.770	1H-18'	1.665
15'	3.770	1H-14'	1.999 (^{1}H$_\beta$)
15'	3.770	1H-3'	5.135
18'	1.665	1H-19'	5.339
18'	1.665	1H-21'	3.750 (^{1}H$_\beta$)
19'	5.339	1H-21'	2.940 (^{1}H$_\alpha$)

▶ **Table 4** Antimycobacterial effects and cytotoxicity of alkaloid extracts and pure compounds.

Sample	MIC$_{100}$ (µg/mL)	IC$_{50}$ (µg/mL)	SI
T. alba stem bark	62.50	70.50	1.10
T. arborea stem bark	31.20	43.30	1.40
T. alba root bark	62.50	87.50	1.40
T. arborea root bark	7.80	35.50	4.50
Voacamine	15.60	16.30	1.00
Voacangine	250.00	40.80	0.16
Ibogaine	250.00	354.00	1.40
Rifampicin	0.06	> 1000.00	> 16.60

MIC: minimum inhibitory concentration as determined by the REMA in the reference strain *M. tuberculosis* H37Rv (ATCC 27294). IC$_{50}$: half-maximal inhibitory concentration as determined by the MTT assay in Vero cells. SI: selectivity index = IC$_{50}$/MIC$_{100}$

Cytotoxicity assay

To assess the cytotoxicity of each tested compound, 10 000 Vero cells were placed in a 96 well-plate per well and incubated for 24 h in 100 µL of RPMI medium. After incubation, the plate was washed and new fresh medium with the compound at a different concentration was added. Each tested compound was incubated for 48 h at 37 °C in a 5% CO_2 atmosphere. Then, a volume of 10 µL of MTT (Sigma) (5 mg/mL in sterile PBS) was added to each well and incubation was allowed to continue for another 4 h. The medium was removed and a volume of 100 µL of DMSO 100% was used to solubilize the formazan. Absorbance was determined at a wavelength of 570 nm and cytotoxicity was calculated as % Toxicity = (1-(ABS problem/ABS control)) *100. Controls were cells without treatment [39]. The maximum concentration of DMSO in each well was 5% and was innocuous to Vero cells. We checked that no change in color occurred in the control wells in the colorimetric assays due only to the compounds or extracts, which could suggest a REDOX reaction.

Supporting information

HMBC and TOCSY data of voacamine, as well as cytotoxicity concentration curves of the alkaloid extracts and pure compounds as determined by MTT in Vero cells are available as Supporting Information.

Contributors' Statement

F.K. and M.L.S. conducted the GC analyses. J.K.D.C. performed the CC. S.L.G.G. ran the HPLC and, together with E.H.S., the NMR analyses. M.S.M. carried out the *in vitro* experiments. S.L.G.G. and F.K. wrote the draft of the manuscript. Based on the comments of all authors, S.L.G.G., C.E., and R.R.C. wrote the final version of the manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Supporting Information

Antimycobacterial Activity of Alkaloids and Extracts from *Tabernaemontana alba* and *T. arborea*

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Table 1S ^{1}H (700 MHz) and ^{13}C (175 MHz) NMR for voacamine (3), heteronuclear 2D HMBC(^{n}J(C,H)) (n = 2, 3, and 4) in CDCl$_3$ as solvent and TMS used as internal reference (δ_H 0.00 TMS and δ_C 77.00 CDCl$_3$) chemical shifts (δ, ppm) and coupling constants (J, Hz in parentheses).

Atom	^{1}H-^{13}C HMBC ^{n}J(C, H)		
	^{2}J(C, H)	^{3}J(C, H)	^{4}J(C, H)
1	-	-	
2	-	H-21, 2H-6	-
3	-	2H-5, H-21	2H-15
4	-	-	-
5	2H-6	H-3, 2.72 (^{1}H$_\beta$)	-
6	2H-5	-	-
7	H-6, 2.952 (^{1}H$_\alpha$)	2H-5, H-9	-
8	-	2H-6	-
9	-	-	-
10	H-9	-	-
11	-	H-9	-
12	-	-	-
13	-	H-9	-
14	H-3, 2.72 (^{1}H$_\beta$)	-	-
15	-	2H-19, H-21, H-3, 2.72 (^{1}H$_\beta$)	3H-18
16	-	H-21	-
17	-	2H-3 H-21	-
18	2H-19	H-20	-
19	3H-18	H-21, H-15, 1.08 (^{1}H$_\beta$)	-
20	H-21, 2H-19, H-15 1.08 (^{1}H$_\beta$)	3H-18	-
21	-	2H-5, 2H-19	-
CO-16	-	H-21, 3H-MeO-16	-
1´	-	-	-
2´	-	-	-
3´	-	-	-
16´	-	-	-
4´	-	-	-
5´	-	-	-
6´	-	-	-
7´	-	H-9´	-
8´	H-9´	H-10´	H-11´
9´	H-10´	H-11´	-
10´	H-11´	-	-
11´	-	H-9´, H-10´, H-12´	-
12´	H-11´	H-10´	H-9´
13´	-	H-9´, H-11´	-
14´	-	H-16´	-
15´	-	-	-
16´	-	-	-
18´	-	-	-
19´	3H-18´	-	-
20´	-	3H-18´	-
21´	-	-	-
MeO-10	H-9	-	-
MeO-16´	-	-	-
COOCH$_3$			
CO-16´ COOCH$_3$	-	3H-MeO-16´, H-15´	-
MeO-16	-	-	-
CO-16	-	H-21, 3H-MeO-16	-
MeN-4´	-		

^{a}Superimposed ^{1}H signals are described without multiplicity and chemical shifts were deduced by ^{1}H-^{13}C HSQCed, ^{1}H-^{13}C HMBC ^{n}J(C,H) (n= 2, 3, and 4), ^{1}H-^{1}H TOCSY, and ^{1}H-^{1}H NOESY. *It was not possible to observe the

signal at ^{13}C, probably due to overlapping at 138.00 ppm. [**]Chemical shift was deduced by HMBC $^nJ(C,H)$ (n= 2, 3, and 4). S = singlet, t = triplet, q = quartet, bb = broad band, m = multiplet, brs = broad singlet, d = doublet, bd = broad doublet, qd = quartet doublet.

Table 2S ^1H-^1H-TOCSY data for voacamine (**Fig. 3**).

H	δ_H	H	δ_H
3	2.721 ($^1H_\beta$)	1H-21	3.501
3	2.721 ($^1H_\beta$)	2H-17	1.748 ($^1H_\beta$), 2.47 ($^1H_\alpha$)
3	2.721 ($^1H_\beta$)	2H-15	1.676 ($^1H_\alpha$), 1.08 ($^1H_\beta$)
3	2.721 ($^1H_\beta$)	1H-20	1.286 ($^1H_\alpha$)
3	2.867 ($^1H_\alpha$)	2H-17	2.470 ($^1H_\alpha$), 1.748 ($^1H_\beta$)
3	2.867 ($^1H_\alpha$)	2H-15	1.676 ($^1H_\bullet$), 1.081 ($^1H_\beta$)
3	2.867 ($^1H_\alpha$)	1H-20	1.286 ($^1H_\alpha$)
3	2.867 ($^1H_\alpha$)	1H-14	1.804
5	3.364 ($^1H_\alpha$)	1H-6	2.952 ($^1H_\alpha$)
5	3.152 ($^1H_\beta$)	1H-6	3.10 ($^1H_\beta$)
14	1.804	2H-15	1.676 ($^1H_\bullet$), 1.08 ($^1H_\beta$)
14	1.804	1H-17	2.470 ($^1H_\bullet$)
14	1.804	1H-20	1.286 ($^1H_\alpha$)
14	1.804	3H-18	0.878
15	1.676 ($^1H_\alpha$)	1H-20	1.286 ($^1H_\alpha$)
15	1.676 ($^1H_\alpha$)	2H-19	1.546, 1.407
15	1.676 ($^1H_\alpha$)	3H-18	0.878
15	1.08 ($^1H_\beta$)	3H-18	0.874
15	1.08 ($^1H_\beta$)	1H-20	1.286 ($^1H_\bullet$)
15	1.08 ($^1H_\beta$)	1H-19	1.407
15	1.08 ($^1H_\beta$)	1H-19	1.541
15	1.08 ($^1H_\beta$)	1H-15	1.676 ($^1H_\alpha$)
15	1.08 ($^1H_\beta$)	1H-14	1.804
18	0.874	2H-19	1.407, 1.541
18	0.874	1H-20	1.286 ($^1H_\alpha$)
18	0.874	1H-15	1.676 ($^1H_\bullet$)
19	1.546	1H-20	1.286 ($^1H_\alpha$)
19	1.546	2H-15	1.676 ($^1H_\bullet$), 1.081 ($^1H_\beta$)
3´	5.135	2H-14´	2.546 ($^1H_\bullet$), 1.999 ($^1H_\beta$)
3´	5.135	1H-15´	3.77
3´	5.135	1H-16´	2.732
5´	4.056	2H-6´	3.490 ($^1H_\bullet$), 3.255 ($^1H_\beta$)
5´	4.056	1H-16´	2.732
9´	7.536	1H-10´	7.051
9´	7.536	1H-11´	7.051
9´	7.536	1H-12´	7.051
15´	3.771	2H-14´	1.99 ($^1H_\beta$), 2.546 ($^1H_\alpha$)
15´	3.771	1H-3´	5.135
15´	3.771	1H-16´	2.732
15´	3.771	1H-18´	1.665
16´	2.732	1H-3´	5.13
16´	2.732	1H-15´	3.771
16´	2.732	1H-5´	4.056
16´	2.732	1H-21	3.75 ($^1H_\beta$)
16´	2.732	2H-6´	3.490 ($^1H_\bullet$), 3.255 ($^1H_\beta$)
18´	1.665	1H-19´	5.339
18´	1.665	2H-21´	2.94 ($^1H_\alpha$), 3.75 ($^1H_\beta$)
19´	5.339	1H-18´	1.665
21´	2.94 ($^1H_\alpha$)	1H-21	3.750 ($^1H_\beta$)
21´	2.94 ($^1H_\alpha$)	1H-18´	1.665
21´	3.750 ($^1H_\beta$)	1H-18´	1.665
21´	3.750 ($^1H_\beta$)	1H-20´	2.94 ($^1H_\alpha$)

Compounds toxicity to Vero Cell Line by MTT
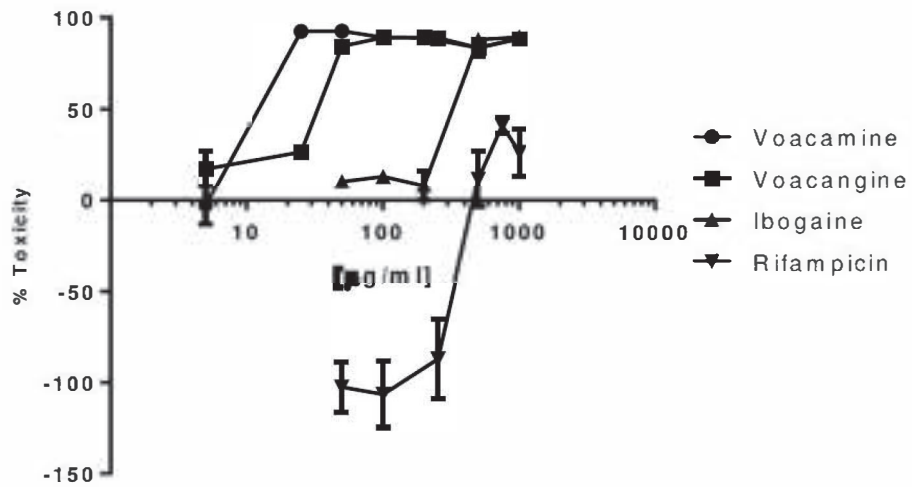
Extracts toxicity to Vero Cell Line by MTT
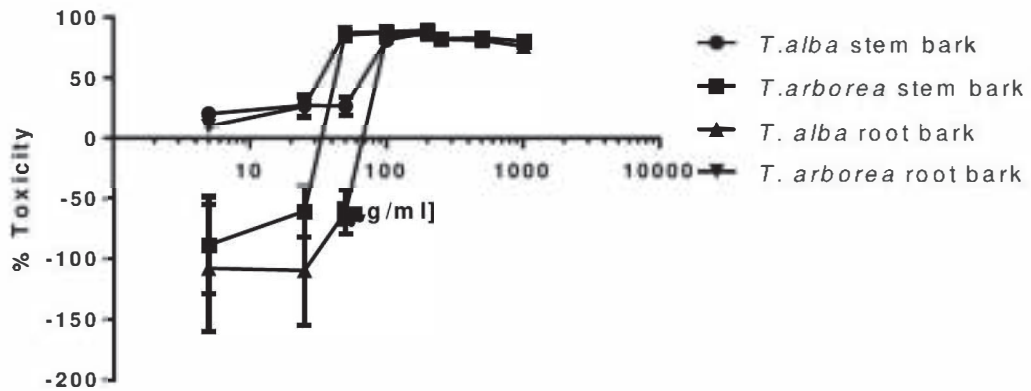
CAPÍTULO 4: PRODUCCIÓN *IN VIVO* E *IN VITRO*

4.1. Extraction and Conversion Studies of the Antiaddictive Alkaloids Coronaridine, Ibogamine, Voacangine, and Ibogaine from Two Mexican *Tabernaemontana* Species (Apocynaceae)

Felix Krengel, Marco V. Mijangos, Marisol Reyes Lezama, Ricardo Reyes Chilpa

FULL PAPER

Extraction and Conversion Studies of the Antiaddictive Alkaloids Coronaridine, Ibogamine, Voacangine, and Ibogaine from Two Mexican *Tabernaemontana* Species (Apocynaceae)

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Several species from the Apocynaceae family, such as *Tabernanthe iboga*, *Voacanga africana*, and many *Tabernaemontana* species, produce ibogan type alkaloids, some of which present antiaddictive properties. In this study, we used gas chromatography/mass spectrometry (GC/MS) to examine the efficiency of methanol, acetone, ethyl acetate, dichloromethane, chloroform, and hydrochloric acid in extracting the antiaddictive compounds coronaridine, ibogamine, voacangine, and ibogaine (altogether the CIVI-complex) from the root barks of *Tabernaemontana alba* and *Tabernaemontana arborea*. These Mexican species have recently shown great potential as alternative natural sources of the aforementioned substances. Methanol proved to be the most suitable solvent. Furthermore, the crude methanolic extracts could be engaged in a one-step demethoxycarbonylation process that converted coronaridine and voacangine directly into its non-carboxylic counterparts ibogamine and ibogaine, respectively, without the intermediacy of their carboxylic acids. The established protocol straightforwardly simplifies the alkaloid mixture from four to two majority compounds. In summary, our findings facilitate and improve both the qualitative and quantitative analysis of CIVI-complex-containing plant material, as well as outlining a viable method for the bulk production of these scientifically and pharmaceutically important substances from Mexican *Tabernaemontana* species.

Keywords: alkaloids, phytochemistry, *Tabernaemontana* (Apocynaceae), CIVI-complex, ibogaine.

Introduction

Several ibogan type alkaloids, particularly ibogaine (**4**), have shown antiaddictive effects against different drugs of abuse like alcohol, cocaine, nicotine, and above all, opioids, in animal models.[1] Ibogaine (**4**) itself and addiction treatment involving consumption of the substance is illegal in some countries, e.g. the USA, but legal in others, although restrictions may apply.[2] Most of the ibogaine (**4**) available on the market is obtained from the African species *Tabernanthe iboga* BAILL. and *Voacanga africana* STAPF ex

SCOTT-ELLIOT (Apocynaceae). In the first case, ibogaine (**4**) is extracted directly from the root bark of this shrub, but unfortunately, the practice implies certain ethical and environmental problems. First, *T. iboga* (common name *iboga*) is considered a sacred plant in the Gabonese spiritual discipline Bwiti, due to its entheogenic properties which play a central role in the associated rituals and philosophy.[3,4] Second, the growing demand for ibogaine (**4**) for 'modern' uses not related to Bwiti by Western societies in combination with uncontrolled exploitation of the species has impacted negatively on the conservation status of the latter.[4–6] In view of the above, ibogaine (**4**) can alternatively be produced from the root and stem bark of *V. africana*, a plant which has been cultivated on a much larger scale than *T. iboga*.[4,7] To be precise, the
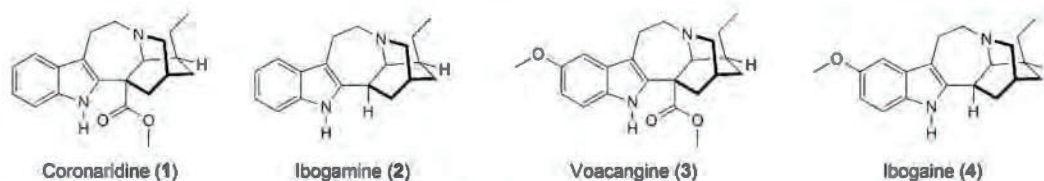
Figure 1. Chemical structure of the alkaloids of the CIVI-complex.

Table 1. Extract, coronaridine (1), and ibogamine (2) yields obtained from *T. alba* root bark using different solvents.

Solvent	Extract DW [%][a]	CV [%][b]	Coronaridine (1) RPA [%][c]	CV [%]	Ibogamine (2) RPA [%]	CV [%]
Methanol	10.03	12.66	**100**	11.29	**100**	15.49
Methanol + ammonia	10.30	**3.88**	93.60	15.48	92.17	23.35
Acetone	**5.80**	12.07	41.97	12.09	72.64	5.48
Acetone + ammonia	6.30	26.51	64.94	17.16	72.92	33.79
Ethyl acetate	6.07	8.46	35.88	19.80	52.95	22.62
Ethyl acetate + ammonia	**5.80**	5.17	64.79	17.53	75.24	13.47
Dichloromethane	6.67	12.12	43.22	9.35	73.47	12.14
Dichloromethane + ammonia	6.53	11.59	67.55	**9.10**	66.41	9.70
Chloroform	7.50	8.33	42.77	22.77	74.35	21.79
Chloroform + ammonia	7.33	7.75	63.52	16.05	76.97	5.63
Acid-base precipitate	8.63	6.98	68.34	12.47	62.10	**2.40**
Acid-base filtrate	4.17	14.06	3.79	74.19	1.68	64.37

Values shown are the average of three repetitions and three extractions each. [a] DW [%] = dry weight percentage of plant material. [b] CV [%] = coefficient of variation. [c] RPA [%] = relative peak area

methyl ester of the compound's 16-carboxylic acid (called voacangine (3)) is isolated, purified, and semi-synthetically converted into ibogaine (4) by a two-step protocol consisting of ester saponification followed by acidification for subsequent decarboxylation.[4,8,9] Based on this information, we recently analyzed four Mexican species from the closely related *Tabernaemontana* L. genus (Apocynaceae) and determined good amounts of the antiaddictive ibogan type alkaloids coronaridine (1), ibogamine (2), voacangine (3), and ibogaine (4), named hereinafter the CIVI-complex.[10,11] We now report a simple and efficient protocol which allows for the extraction and conversion of these compounds – whose structural differences are limited to the presence or absence of a methyl ester and/or a methoxy group (*Figure 1*) – from the root barks of *Tabernaemontana alba* MILL. and *Tabernaemontana arborea* ROSE ex J.D.SM. (Apocynaceae). Both species occur in the tropical zones of Mexico and Central America, often as important elements of the secondary vegetation in disturbed areas. *T. alba* has been used ethnobotanically to treat tooth and headache, as well as dermatological conditions.[12–14]

Results and Discussion

Extraction Efficiency of Different Solvents

Tables 1 and *2* suggest that untreated methanol is the solvent of choice for extraction of the whole CIVI-complex. This trend was most obvious in the case of the nonmethoxylated alkaloids ibogamine (2) and, above all, coronaridine (1). Voacangine (3) and ibogaine (4) were also extracted efficiently with ammonium-containing acetone, ethyl acetate, dichloromethane, and chloroform. Compared to methanol, all the other solvents proved to be more selective (as represented by the respective extracts' dry weight percentage of plant material (DW [%]) which may be of advantage under certain conditions. Addition of ammonia did not improve the extraction efficiency of methanol, but increased alkaloid abundance variability (as represented by the coefficient of variation (CV [%]) between the three repetitions. Concerning the less polar solvents, ammonia treatment improved alkaloid yields in most cases, with the exception of acetone, dichloromethane, and chloroform in association with ibogamine (2). With regard to the acid-base extraction using hydrochloric acid and ammonia, we followed

Table 2. Extract, voacangine (3), and ibogaine (4) yields obtained from *T. arborea* root bark using different solvents.

Solvent	Extract DW [%][a]	CV [%][b]	Voacangine (3) RPA [%][c]	CV [%]	Ibogaine (4) RPA [%]	CV [%]
Methanol	13.13	6.11	95.66	**7.24**	**100**	8.83
Methanol + ammonia	13.77	6.18	88.59	21.38	99.10	17.62
Acetone	8.90	**1.12**	82.59	6.94	75.91	4.03
Acetone + ammonia	11.00	6.36	90.34	18.45	98.18	9.62
Ethyl acetate	**7.53**	12.89	68.40	11.21	52.20	8.63
Ethyl acetate + ammonia	9.50	3.65	**100**	8.89	95.71	**3.77**
Dichloromethane	8.63	14.39	81.80	20.71	63.93	16.31
Dichloromethane + ammonia	9.57	12.64	97.49	11.18	99.02	4.63
Chloroform	10.40	6.00	77.56	10.26	66.84	8.29
Chloroform + ammonia	10.67	10.95	93.83	13.74	99.05	6.95
Acid-base precipitate	13.70	12.06	57.94	9.43	86.37	13.79
Acid-base filtrate	4.23	29.91	0.52	43.42	0.27	33.45

Values shown are the average of three repetitions and three extractions each. [a] DW [%] = dry weight percentage of plant material. [b] CV [%] = coefficient of variation. [c] RPA [%] = relative peak area

the protocol proposed by Jenks.[9] This author intended to develop'a convenient, inexpensive, and efficient procedure to isolate and purify the alkaloids from *T. iboga* root bark and enable affordable processing of the bark in Africa without exportation'. Our findings confirm the efficacy of Jenks' extraction technique in two ways: First, all four alkaloids of the CIVI-complex precipitated easily under alkaline conditions, leaving only traces of these compounds in the filtrate, which made a more laborious water-solvent-partitioning step unnecessary. Second, the protocol yielded similar amounts of *T. iboga*'s majority alkaloid ibogaine (4) as extraction with methanol. Coronaridine (1), ibogamine (2), and voacangine (3), however, were not efficiently extracted with Jenks' method.

In summary, untreated methanol should be used to extract the whole CIVI-complex from Apocynaceae species that contain considerable quantities of these alkaloids, like Mexican *Tabernaemontana* species[10,11] or *V. africana*,[15] particularly when conducting quantitative alkaloid profiling studies. If selective bulk extraction of voacangine (3) and/or ibogaine (4), but not coronaridine (1) and/or ibogamine (2) was the aim, ammonium-supplemented acetone, ethyl acetate, dichloromethane, or chloroform could be an alternative to methanol, particularly when taking into account the higher selectivity of the former compared to the latter. The main benefits of the procedure developed by Jenks[9] are its low cost and environmental acceptability, as only acids and bases in aqueous solutions are required. Nevertheless, its usefulness is limited to the bulk extraction of ibogaine (4).

Table 3. Extract yields obtained from *T. alba* and *T. arborea* root bark macerated or sonicated with methanol six times.

	Methanolic extract (DW [%][a])			
	T. alba root bark		*T. arborea* root bark	
Extraction	Maceration	Sonication	Maceration	Sonication
1	5.85	6.23	8.43	8.75
2	2.24	2.68	2.29	2.73
3	0.81	1.15	0.85	1.16
4	1.11	0.80	0.87	0.77
5	0.44	0.64	0.40	0.58
6	0.21	0.25	0.19	0.19
Total	10.67	11.74	13.03	14.17

[a] DW [%] = dry weight percentage of plant material.

Efficiency of Maceration vs. Ultrasound-Assisted Extraction

Extraction of the barks for 60 min by either maceration or sonication resulted in similar methanolic extract yields (*Table 3*). Furthermore, the longer extraction time did not improve extraction efficiency as can be seen by comparing the dry weight percentage of plant material (DW [%]) of the extracts obtained by sonication with methanol for 20 (*Tables 1* and *2*) or 60 min (*Table 3*, total DW of the first three extractions). It is of course possible that maceration times shorter than 60 min might not be maximally efficient. In any case, three to four extractions were sufficient to obtain well above 80 or 90% of the total methanolic extract yield, respectively.
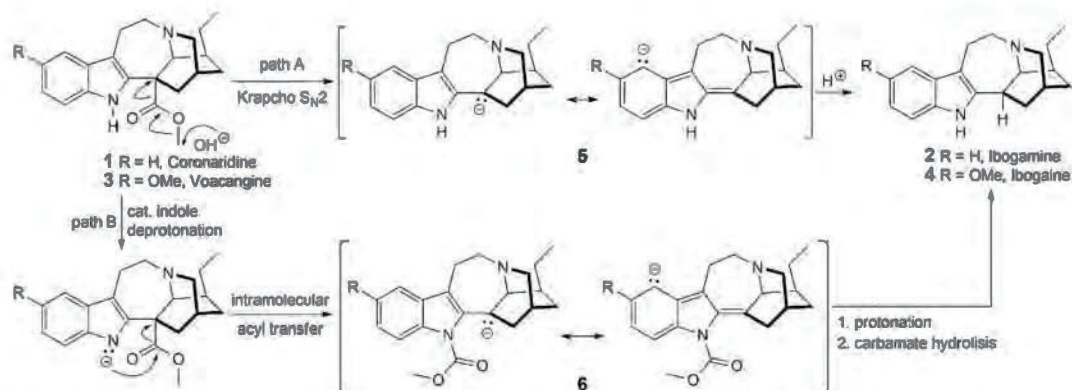
Figure 2. Proposed reaction mechanisms for the conversion of coronaridine (1) and voacangine (3) into ibogamine (2) and ibogaine (4), respectively, by KOH-mediated direct demethoxycarbonylation.

Demethoxycarbonylation of Methanolic Crude Extracts

The methanolic crude extracts of *T. alba* and *T. arborea* root bark described in the previous section were subjected to a saponification and decarboxylation process based on Janot and Goutarel.[8] Gas chromatography/mass spectrometry (GC/MS) analyses of aliquots taken before, during, and after the saponification step, as well as after the decarboxylation process (*Figures S1* and *S2*), revealed three major findings: First, while the aforementioned protocol was developed to convert pure voacangine (3) isolated from *V. africana* into ibogaine (4), we now report for the first time the successful application of this technique to complex crude extracts of ibogan type alkaloid-containing plant material. Previous attempts of Jenks[9] to transform voacangine (3) into ibogaine (4) following the same procedure with alkaloid extracts from *V. africana* did not succeed. Additionally, the present work proves that the process can also be used to convert coronaridine (1) into ibogamine (2).

Second, it was surprising to find that the methoxycarbonyl moiety in coronaridine (1) and voacangine (3) was effectively extruded only with the methanolic KOH treatment, making a posterior acidification step, as reported, not really necessary for the decarboxylation to be accomplished. Before the experiment, it was assumed that the treatment of the ClVI-complex with methanolic KOH would convert coronaridine (1) and voacangine (3) into their respective potassium carboxylates, however our results showed that they were directly transformed into ibogamine (2) and ibogaine (4), respectively. We therefore suggest two alternative

reaction mechanisms that allow for the direct demethoxycarbonylation of coronaridine (1) and voacangine (3) under these seemingly mild conditions: Path A) a Krapcho decarboxylation proceeding through hydroxide S_N2 displacement (due to the high steric hindrance that a carbonyl addition could imply),[16] leading to the resonance-stabilized carbanion 5 which yields the observed products upon protonation. Or, as Krapcho decarboxylations do not usually proceed under such mild conditions, path B) a catalytic indole deprotonation followed by intramolecular acyl transfer to yield the resonance-stabilized anion carbamate 6.[17,18] Subsequent protonation and facile urethane hydrolysis would then account for the observed products (*Figure 2*).

Third, the 6 h saponification period proposed by Janot and Goutarel[8] proved to be suboptimal in terms of product yield. The concentration of the methoxycarbonyl-alkaloids (1 and 3) was indeed lowest in the aliquots taken at the end of the whole process, but their respective demethoxycarbonyl-derivatives (2 and 4) were also present in smaller amounts than in aliquots taken at earlier time points. Furthermore, while the ratio between the former and the latter (1/2 and 3/4) declined exponentially during the 6 h period, the sum of both (1+2 and 3+4) followed a similar pattern, although not exponentially. In consequence, it seems that the saponification process effectively converted coronaridine (1) and voacangine (3) into ibogamine (2) and ibogaine (4), respectively, in a time-dependent manner, but that part of the products (and possibly the substrates) were simultaneously degraded, probably due to their
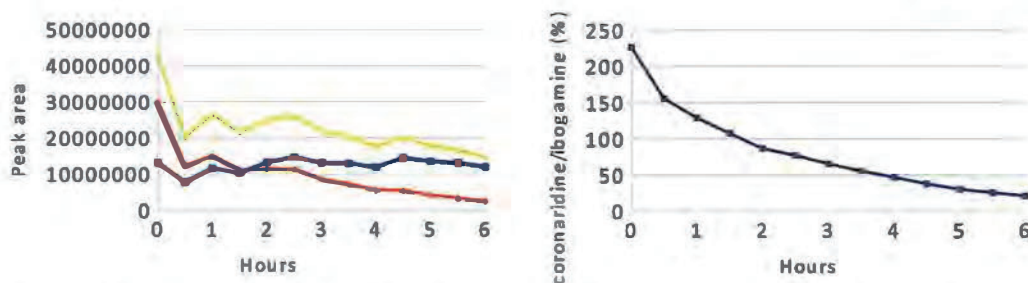
Figure 3. Quantitative changes in the coronaridine (**1**) and ibogamine (**2**) concentrations determined in the *T. alba* root bark methanolic extracts during the 6 h saponification process, expressed as (left) peak area values (orange = coronaridine (**1**); blue = ibogamine (**2**); yellow = coronaridine (**1**) + ibogamine (**2**)) and as (right) the ratio of coronaridine (**1**) to ibogamine (**2**).
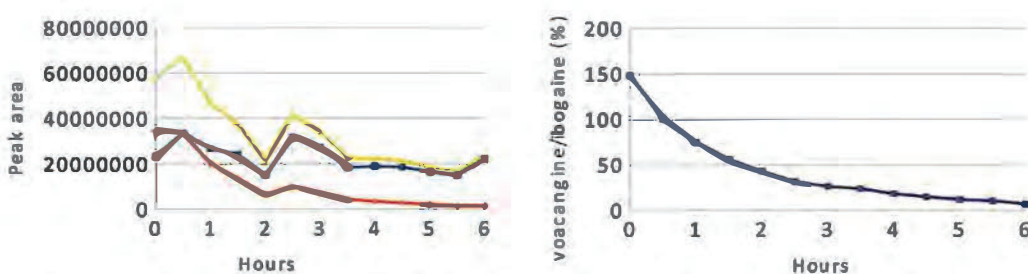

Figure 4. Quantitative changes in the voacangine (**3**) and ibogaine (**4**) concentrations determined in the *T. arborea* root bark methanolic extracts during the 6 h saponification process, expressed as (left) peak area values (orange = voacangine (**3**); blue = ibogaine (**4**); yellow = voacangine (**3**) + ibogaine (**4**)) and as (right) the ratio of voacangine (**3**) to ibogaine (**4**).

prolonged exposure to heat and the alkaline medium. The highest amounts of ibogamine (**2**) and ibogaine (**4**) were actually obtained at 2.5 and 4.5 h, and 0.5 h and 2.5 h, respectively, with the later time points showing significantly smaller concentrations of the methoxycarbonyl-alkaloids (**1** and **3**; *Figures 3* and *4*).

It is striking, though, that the quantitative values of ibogamine (**2**) and ibogaine (**4**) in aliquots 1 to 12 were not far off the amounts determined in the corresponding aliquot 0, which was taken before initiating the saponification process. In other words, during the whole procedure the concentrations of the demethoxycarbonyl-compounds (**2** and **4**) oscillated slightly above and below the concentrations present in the original methanolic crude extracts. Hence, the usefulness of the demethoxycarbonylation protocol described in this study appears to reside more in its capacity to simplify the alkaloid profiles of organic extracts from CIVI-complex-containing plant species than in increasing the ibogamine (**2**) and/or ibogaine (**4**) yields in comparison to the alkaloid contents of the original plant material. On the one hand, the direct

demethoxycarbonylation of crude extracts showing significant amounts of the methoxycarbonyl-alkaloids (**1** and **3**) should greatly facilitate separation and isolation of their demethoxycarbonyl-counterparts (**2** and **4**), which seem to be the more efficient antiaddictive compounds[19] and thus, the commercially more interesting substances. On the other hand, the demethoxycarbonylated crude extracts could be further processed into standardized total alkaloid extracts for addiction treatment. E.g., when the saponified methanolic extract of *T. arborea* root bark was subjected to the decarboxylation procedure under acidic conditions – which basically consisted of Jenks' extraction method plus heat application – the result was a purified ibogaine (**4**)-predominant mixture with smaller but still considerable concentrations of ibogamine (**2**), quite akin to total alkaloid extracts of *T. iboga*, which have already been used in addiction therapy (*Figure S2*).[4,11] Moreover, improvements could be made to the present demethoxycarbonylation protocol, in order to reduce the degradation rate of the CIVI-complex, e.g. by reducing the temperature or

using another solvent or base. If this was not feasible, the highest ibogamine (**2**) and ibogaine (**4**) yield would still be obtained by laboriously isolating pure coronaridine (**1**) or voacangine (**3**) from the plant material for posterior demethoxycarbonylation. However, judging from published reports, even in this case a loss of product is to be expected.[8,20,21]

Conclusions

To our knowledge, this is the first study comparing the extraction efficiency of different solvents with regard to the antiaddictive ibogan type alkaloids coronaridine (**1**), ibogamine (**2**), voacangine (**3**), and ibogaine (**4**) from plant material. We also prove for the first time that the methoxycarbonyl-alkaloids coronaridine (**1**) and voacangine (**3**) can be converted into their non-carboxylic – and seemingly more active – counterparts ibogamine (**2**) and ibogaine (**4**), respectively, in a single synthetic operation. Our results show that coronaridine (**1**) and voacangine (**3**) are very prone to direct demethoxycarbonylation under mild basic conditions (KOH in methanol at 72–75 °C). We propose an unusually mild Krapcho S_N2 mechanism in order to explain the observed results, however, an alternative mechanism may be operating, where a catalytic indole N–H deprotonation triggers an intramolecular acyl-transfer to yield a readily hydrolyzable methylcarbamate, accounting for the mildness of the transformation. Taken together, our findings contribute to improving both quantitative alkaloid profiling studies and bulk production of the CIVI-complex from plant species, with specific regard to Mexican *Tabernaemontana* species as new potential sources of these alkaloids.

Experimental Section

Plant Material

Root bark from *Tabernaemontana alba* Mill. and *Tabernaemontana arborea* Rose ex J.D.Sm was obtained in the Los Tuxtlas region in Veracruz, Mexico, as described by Krengel et al.[11] Delfino Álvaro Campos Villanueva (Estación de Biología Los Tuxtlas, UNAM) and Leonardo Osvaldo Alvarado Cárdenas (Facultad de Ciencias, UNAM) identified the species, and voucher specimens were deposited at the herbarium of the Facultad de Ciencias (FCME), UNAM (voucher numbers 161424 to 161427). Plant names were checked with http://www.theplantlist.org. Root bark from a high-

yielding individual of each species, one showing a predominance of coronaridine (**1**) and ibogamine (**2**), and the other of voacangine (**3**) and ibogaine (**4**), was selected to cover the whole range of the four alkaloids of the CIVI-complex.

Microextraction – Extraction Efficiency of Different Solvents

The dried bark was pulverized using a cutting mill, and 100 mg samples of each species were placed in 33 Eppendorf microcentrifuge tubes (2 ml). 18 tubes of each species were left untreated, while the samples in the remaining 15 tubes were moistened with 0.5 ml of ammonium hydroxide. The tubes were then closed, sonicated for 5 min, and left to stand for 2.5 h. Subsequently, the tubes were opened in order to completely dry the bark under a gentle stream of air. The extraction was carried out as reported by Krengel et al.:[11] A piece of degreased cotton wool (approximately 10 mg) and 1.5 ml of one of six different solvents (0.1 M aqueous hydrochloric acid, methanol, acetone, ethyl acetate, dichloromethane, chloroform) were added to each tube. The basified plant material was extracted with a mixture of any of the last five solvents and concentrated ammonium hydroxide (33 µl/ml). Each of the resulting 11 experimental groups consisted of three repetitions. All tubes were vortexed, sonicated for 20 min, and centrifuged at 14500 rpm for 2 min. The supernatant was sucked up into a Pasteur pipette through the cotton wool and placed in the freezer in 15 ml Falcon tubes (in the case of the hydrochloric acid extracts) or evaporated to dryness at room temperature in darkness (in the case of the other solvents). Each sample was then extracted two more times in the same way and the corresponding fractions pooled together. The hydrochloric acid extracts were thawed and processed according to a protocol inspired by Jenks:[9] The pH was adjusted to 10 by the addition of concentrated ammonium hydroxide, the Falcon tubes were centrifuged at 2400 rpm for 5 min, and the aqueous supernatant gravity filtered through Whatman grade 2 filter paper. The pellets and the paper were dried in the tubes under a stream of air and extracted thrice with a mixture of methanol and ammonium hydroxide (33 µl/ml) for 20 min in an ultrasonic bath. The tubes were centrifuged at 2400 rpm for 5 min and the methanolic supernatant decanted and evaporated. The aqueous supernatant was mixed vigorously with the same volume of dichloromethane in 50 ml Falcon tubes before separating the two phases by centrifugation at

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2400 rpm for 2 min. The dichloromethane fraction was then recovered with a pipette and evaporated to dryness. The aqueous supernatant was extracted twice more and the respective fractions pooled together. All alkaloid extracts were stored at −20 °C.

Macroextraction – Efficiency of Maceration vs. Ultrasound-Assisted Extraction and Preparation of Methanolic Crude Extracts

A greater amount of plant material was extracted with what proved to be the most efficient solvent using a scaled-up version of the microextraction protocol described above: 50 ml Falcon tubes containing 3 g samples of dried and powdered root bark from each species, as well as degreased cotton, were filled with 40 ml of methanol and either macerated or sonicated for 60 min. During this period, the tubes were vortexed four times and finally centrifuged at 2400 rpm for 5 min. The supernatant was sucked up with a pipette through the cotton and evaporated to dryness. The samples were then extracted five more times, storing each fraction separately.

Demethoxycarbonylation of Methanolic Crude Extracts

The saponification and decarboxylation of the methanolic crude extracts from *T. alba* and *T. arborea* root bark were carried out according to Janot and Goutarel:[8] 450 mg of each extract were dissolved separately in 30 ml of a methanol and potassium hydroxide mixture (9 g of the latter were covered with 2 ml of water and diluted with methanol to a final volume of 60 ml) in 50 ml Pyrex tubes which were then submerged in a hot water bath and maintained at an internal temperature of 72–75 °C for 6 h. In both cases, a total of 13×0.5 ml aliquots was taken before, during (every 30 min), and after the heating procedure. These aliquots and the remaining saponified methanolic extracts were evaporated to dryness. The latter were suspended separately in 25 ml of 2 M aqueous hydrochloric acid and gravity filtered through Whatman grade 2 filter papers. The filtrates were heated to 95 °C in 50 ml Pyrex tubes submerged in a hot water bath. After 15 min, the solutions were allowed to cool at room temperature before raising the pH to 10 with ammonium hydroxide and gravity filtering. The resulting filtrates and dried precipitated material were extracted five times with dichloromethane, respectively, which after evaporation yielded the saponified and decarboxylated alkaloid extracts.

Analysis of Alkaloid Extracts by GC/MS

The extracts were dissolved in 2 ml of methanol and analyzed using an Agilent 7890B/5977 A GC/MSD with a HP-5ms (30 m) capillary column following the method described by Krengel et al.:[11] EI-mass spectra recording at 70 eV after 8 min of solvent delay; injector in split mode (1:5) at 300 °C with automatic injection of 1 μl aliquots; ramp temperature program from 150 to 300 °C at 4 °C/min; Helium carrier gas at 1 ml/min. Peak identification was carried out by the NIST Mass Spectral Search Program for the NIST/EPA/NIH Mass Spectral Library (Version 2.2, build Jun 10, 2014) and by comparison of the respective mass spectra with published data and experimental spectra of voacangine (**3**) and ibogaine (**4**). These pure chemical compounds were kindly donated by Phytostan Enterprises, Inc. (Montreal, Quebec).

Author Contribution Statement

F. K. and M. R. L. conducted the experimental work. F. K. and M. V. M. wrote the draft. Based on the comments of all authors, F. K. and R. R. C. wrote the final version of the manuscript.

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Supporting Information

Extraction and Conversion Studies of the Antiaddictive Alkaloids Coronaridine, Ibogamine, Voacangine, and Ibogaine from Two Mexican *Tabernaemontana* Species (Apocynaceae)

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Extraction and Conversion Studies of the Antiaddictive Alkaloids Coronaridine, Ibogamine, Voacangine, and Ibogaine from Two Mexican *Tabernaemontana* Species (Apocynaceae).

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Several Apocynaceae species, such as *Tabernanthe iboga*, *Voacanga africana*, and many *Tabernaemontana* species, produce ibogan type alkaloids, some of which present antiaddictive properties. In this study, we used gas chromatography-mass spectrometry (GC-MS) to examine the efficiency of methanol, acetone, ethyl acetate, dichloromethane, chloroform, and hydrochloric acid in extracting the antiaddictive compounds coronaridine, ibogamine, voacangine, and ibogaine (altogether the CIVI-complex) from the root barks of *Tabernaemontana alba* and *Tabernaemontana arborea*. These Mexican species have recently shown great potential as alternative natural sources of the aforementioned substances. Methanol proved to be the most suitable solvent. Furthermore, the crude methanolic extracts could be engaged in a one-step demethoxycarbonylation process that converted coronaridine and voacangine directly into its non-carboxylic counterparts ibogamine and ibogaine, respectively, without the intermediacy of their carboxylic acids. The established protocol straightforwardly simplifies the alkaloid mixture from four to two majority compounds. In summary, our findings facilitate and improve both the qualitative and quantitative analysis of CIVI-complex-containing plant material, as well as outlining a viable method for the bulk production of these scientifically and pharmaceutically important substances from Mexican *Tabernaemontana* species.

Keywords: Alkaloids · Phytochemistry · *Tabernaemontana* (Apocynaceae) · CIVI-complex · Ibogaine

Supporting Information
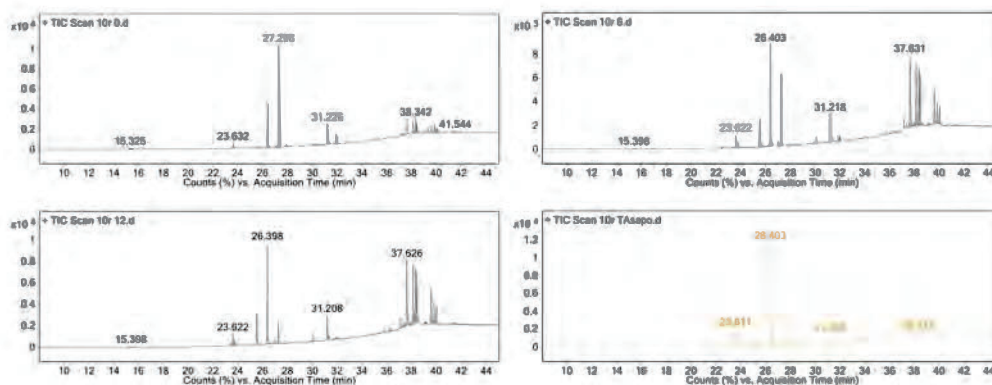
Figure S1. Chromatograms of the methanolic crude extract obtained from *T. alba* root bark before, during (at 3 h), and after the 6 h saponification process, as well as after the decarboxylation process under acidic conditions (from top left to bottom right).

Retention times (RT) for ibogamine (2), coronaridine (1), ibogaine (4) and voacangine (3) are 26.40±0.02, 27.30±0.02, 31.23±0.03, and 31.96±0.02 min, respectively. Peaks with RT > 36 min correspond to non-alkaloidal compounds.

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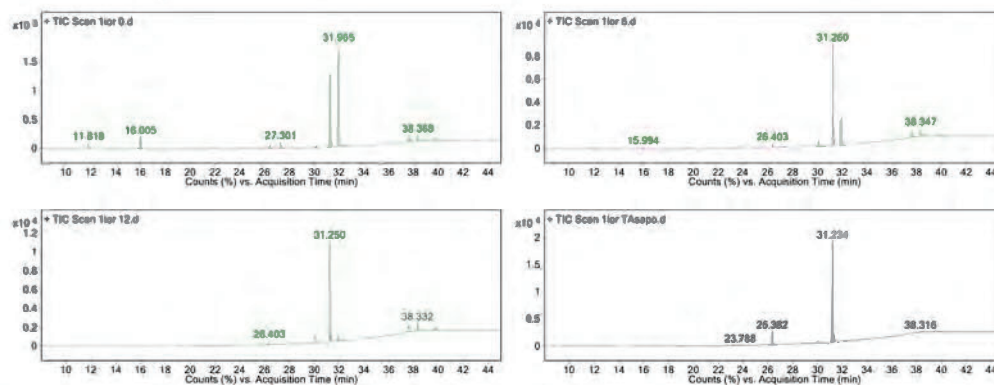
Figure S2. Chromatograms of the methanolic crude extract obtained from *T. arborea* root bark before, during (at 3 h), and after the 6 h saponification process, as well as after the decarboxylation process under acidic conditions (from top left to bottom right).

Retention times (RT) for ibogamine (**2**), coronaridine (**1**), ibogaine (**4**) and voacangine (**3**) are 26.40±0.02, 27.30±0.02, 31.23±0.03, and 31.96±0.02 min, respectively. Peaks with RT > 36 min correspond to non-alkaloidal compounds.

# CAPÍTULO 4: PRODUCCIÓN *IN VIVO* E *IN VITRO*

4.2. Strategies for the *in vitro* production of antiaddictive ibogan type alkaloids from Apocynaceae species

Felix Krengel, Teresa de Jesús Olivera Flores, Josefina Herrera Santoyo, Ricardo Reyes Chilpa

REVIEW

Strategies for the in vitro production of antiaddictive ibogan type alkaloids from Apocynaceae species

Felix Krengel[1,2] · Teresa de Jesús Olivera-Flores[3] · Josefina Herrera-Santoyo[4] · Ricardo Reyes-Chilpa[2]

Abstract

Monoterpenoid indole alkaloids (MIAs) of the ibogan type, such as ibogaine, have shown promising antiaddictive effects against several drugs of abuse in humans and animal models of addiction. Unfortunately, international ibogaine demand has led to the overexploitation of natural populations of the African species *Tabernanthe iboga* (Apocynaceae), the main source of this alkaloid. Therefore, it is necessary to identify alternative ibogan type alkaloid-containing plant species, as well as to develop new sustainable production systems for said group of pharmaceutically important compounds. In this review, we focus on strategies for the in vitro production of the antiaddictive ibogan type MIAs coronaridine, ibogamine, voacangine, and ibogaine (collectively named "CIVI-complex") from Apocynaceae species, with particular emphasis on the *Tabernaemontana* genus. Since plant tissue culture (PTC)-related information on the CIVI-complex is scarce, we also consider reports on the in vitro production of other ibogan type MIAs and where necessary, of compounds belonging to the aspidospermatan, corynanthean, and plumeran type.

Key message

This review aims at giving an overview of potential strategies to produce antiaddictive ibogan type alkaloids from in vitro cultures of Apocynaceae species.

Keywords *Tabernaemontana* · Iboga · Coronaridine · Ibogamine · Voacangine · Ibogaine

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Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
7-DLGT	7-deoxyloganetic acid glucosyltransferase
7-DLH	7-deoxyloganic acid hydroxylase
8-HGO	8-hydroxygeraniol oxidoreductase
ASO	O-acetylstemmadenine oxidase
BA	Benzyladenine
CC	Callus culture
CCC	Compact callus cluster
CIVI	Coronaridine-ibogamine-voacangine-ibogaine
CNS	Central nervous system
CSC	Cell suspension culture
G8O	Geraniol-8-oxidase
GDNF	Glial cell line-derived neurotrophic factor
GES	Geraniol synthase
G(G)PPS	Geranyl(geranyl) diphosphate synthase
GO	Geissoschizine oxidase
GS	Geissoschizine synthase
HL1	Hydrolase 1
HRC	Hairy root culture
I10H	Ibogamine 10-hydroxylase

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IAA	Indoleacetic acid
IBA	Indolebutyric acid
IO	Iridoid oxidase
IPAP	Internal phloem-associated parenchyma
IS	Iridoid synthase
JA	Jasmonic acid
LAMT	Loganic acid-O-methyltransferase
MeJA	Methyl jasmonate
MEP	Methylerythritol phosphate
MIA	Monoterpenoid indole alkaloid
N10OMT	Noribogaine-10-O-methyltransferase
NAA	1-Naphthaleneacetic acid
NMDA	N-methyl-D-aspartate
PGR	Plant growth regulator
PTC	Plant tissue culture
SA	Salicylic acid
SAR	Systemic acquired resistance
SAT	Stemmadenine O-acetyltransferase
SGD	Strictosidine β-glucosidase
SLS	Secologanin synthase
STR	Strictosidine synthase
TDC	Tryptophan decarboxylase

Introduction

The enormous potential that plant tissue culture (PTC) holds for the commercial in vitro production of secondary metabolites has largely remained a promise. However, recent advances in the elucidation of the biosynthetic pathways of several natural products, as well as the emergence of new techniques and technologies, give us a glimpse of a near future in which many compounds of pharmacological importance could be produced in an efficient, economical, and sustainable way by means of in vitro culture techniques. This review aims to provide an overview of realistic approaches to producing antiaddictive monoterpenoid indole alkaloids (MIAs) of the ibogan type, especially coronaridine, ibogamine, voacangine, and ibogaine, from PTCs of Apocynaceae species. Where information on these compounds is incomprehensive, suggested production strategies are derived from the closest examples published in the scientific literature, e.g. preference is given to other ibogan type alkaloids before considering MIAs that belong to distinct structural classes.

The monoterpenoid indole alkaloids (MIAs) are a structurally diverse group of compounds

The condensation of tryptamine with the iridoid secologanin originates strictosidine, the structural prototype from which all monoterpenoid indole alkaloids (MIAs) derive. Based on its skeleton, the MIAs can be divided into 11 classes, namely the vincosan, corynanthean, vallesiachotaman, strychnan, aspidospermatan (all with intact secologanin skeleton), plumeran, eburnan (both with restructured secologanin skeleton), ibogan, and tacaman (both with the highest degree of secologanin skeleton restructuring) types, in addition to the alkaloids whose biosynthetic routes are ignored and the bis-indole alkaloids (dimers generally composed of MIAs of the corynanthean, plumeran, and/or ibogan types) (van Beek et al. 1984).

Several ibogan type alkaloids present antiaddictive properties for different drugs of abuse

MIAs have a wide range of biological activity. Some examples of pharmaceutically valuable compounds are the antihypertensive drugs ajmalicine and reserpine (corynanthean type) (Kumari et al. 2013), as well as the antineoplastic bis-indole alkaloids vinblastine and vincristine (Almagro et al. 2013, 2015). The ibogan type MIAs are particularly interesting due to their central nervous system (CNS) activity (van Beek et al. 1984). Within this structural class, ibogaine has attracted most of the research, partly in view of its potentially antiaddictive properties for several drugs of abuse, such as alcohol, amphetamines, cocaine, nicotine, and above all, opiates. Regarding the pharmacological effects of ibogaine, it is known that this substance modulates different neurotransmitter systems simultaneously. For example, it interacts with acetylcholine (nicotinic and muscarinic), N-methyl-D-aspartate (NMDA), opioid (kappa, mu, and delta), sigma (1 and 2), and serotonin (5-HT2A and 5-HT2C) receptors. In addition, ibogaine interacts with serotonin and dopamine transporters in the CNS (Alper 2001; Alper et al. 2008), and induces the expression of the glial cell line-derived neurotrophic factor (GDNF) (He and Ron 2006). Taken together, these actions can result in a significant reduction of the pleasant effects and/or abstinence syndromes that drugs of abuse cause in the drug-dependent person (Alper 2001; Alper et al. 2008). Brown and Alper (2008) concluded in an observational study that "ibogaine was associated with substantive effects on opioid withdrawal

symptoms and drug use in subjects for whom other treatments had been unsuccessful, and may provide a useful prototype for discovery and development of innovative pharmacotherapy of addiction."

Other MIAs structurally similar to ibogaine, such as coronaridine, desethylcoronaridine, ibogamine, and tabernanthine, attenuated the self-administration of cocaine and morphine in rats with similar or even higher efficiency than the former (Glick et al. 1994).

Several Apocynaceae species are natural sources of ibogan type alkaloids

Traditionally, ibogaine is obtained from the root bark of *Tabernanthe iboga* Baill. (Apocynaceae), a Central African shrub of great spiritual importance in some local cultures. The probably most conspicuous example is the *Bwiti* practice in Gabon which makes use of the oneirogenic properties of *T. iboga*'s root bark to communicate with the ancestors and learn about the mysteries of life and death in a sophisticated ritualistic context. Unfortunately, the interest that ibogaine has aroused in non-traditional consumers throughout the world during the last decades, be it for recreational, spiritual, medicinal or scientific purposes, has resulted in the overexploitation of the natural populations of *T. iboga*. In order to counteract this development, as well as biopiracy, the Gabonese government banned the export of the species unless a license from the Ministry of Culture is granted (Dickinson 2016). The total synthesis of ibogaine was reported for the first time in 1966 and improved in recent years (Jana and Sinha 2012a, b), but like in the case of most MIAs, the compound's complex chemical structure has greatly complicated commercial application of this approach (Scossa et al. 2018). Hence, the search for alternative sources and sustainable production methods of ibogaine and other ibogan type MIAs represents the most obvious solution to the aforementioned supply problem. And indeed, a considerable part of the ibogaine sold on the global market is produced semi-synthetically from voacangine isolated from the trunk bark of *Voacanga africana* Stapf (Apocynaceae). In contrast to *T. iboga* whose systematic and controlled cultivation is limited to sporadic and incipient projects (Dickinson 2016), there are commercial plantations of *V. africana* in several West African countries, thus allowing for the sustainable use of this natural resource (Brako-Danquah 2012; Dickinson 2016). Among the plants of the New World, the *Tabernaemontana* genus stands out because of its taxonomic and phytochemical closeness to *T. iboga*, reflected by its capacity to biosynthesize a great variety of MIAs of the plumeran, corynanthean, and ibogan types (Danieli and Palmisano 1986). Regarding the Mexican flora, Krengel et al. (2016, 2019) detected several alkaloids


	R₁	R₂
1	H	COOCH₃
2	H	H
3	OCH₃	COOCH₃
4	OCH₃	H
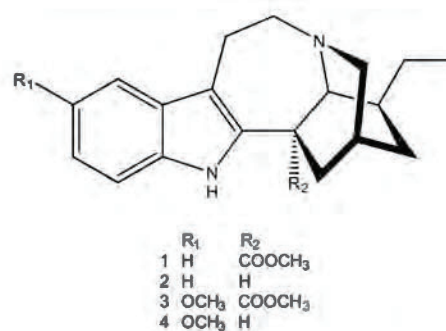
Fig. 1 Chemical structures of the alkaloids of the CIVI-complex

of the last structural class in *Tabernaemontana alba* Mill., *Tabernaemontana amygdalifolia* Jacq., *Tabernaemontana arborea* Rose ex J.D.Sm., and *Tabernaemontana donnellsmithii* Rose ex J.D.Sm. (Apocynaceae). The root barks of the four species contained coronaridine (1), ibogamine (2), voacangine (3), and ibogaine (4), all of which share the same structural skeleton and only differ in the presence or absence of a methyl ester and/or methoxy group in fixed positions (Fig. 1). Therefore, we proposed the denomination "CIVI-complex" for this characteristic group of compounds. The combined voacangine and ibogaine contents of *T. alba* or *T. arborea* root bark were quantitatively comparable to the amounts of voacangine in *V. africana* or ibogaine in *T. iboga*. *T. amygdalifolia* root bark showed high concentrations of coronaridine and ibogamine. Consequently, Mexican *Tabernaemontana* species hold great potential as alternative sources of ibogan type MIAs.

In vitro production of ibogan type alkaloids: culture techniques

Generally speaking, the in vitro production of ibogan type MIAs by PTC is viable. Nonetheless, there are few publications that have reported the biosynthesis of the CIVI-complex—or structurally closely related alkaloids—in different types of cell cultures, as shown in Table 1.

Furthermore, a variety of aspidospermatan, corynanthean, and/or plumeran type MIAs were detected in PTCs of the above-mentioned species, as well as in CSCs of two other *Tabernaemontana* species (*Tabernaemontana africana* A. DC., *Tabernaemontana orientalis* R. Br.) and *V. africana* (Dagnino et al. 1991, 1993a, b, 1994, 1995; Lucumi et al. 2001, 2002; Schripsema and Verpoorte 1992; Schripsema et al. 1991, 1994; Sierra et al. 1992; Stevens et al. 1993; Stöckigt et al. 1983; van der Heijden et al. 1988b, 1989a). Krengel et al. (2016) did not find alkaloids in CCs of *T. alba*, however, when indirect somatic embryogenesis was induced

Table 1 In vitro production of the CIVI-complex and related ibogan type alkaloids from Apocynaceae species (in chronological order)

Species	Culture type	Alkaloids	References
Tabernaemontana tomentosa (Greenm.) A.O. Simões & M.E. Endress	Cell suspension cultures (CSCs)	Coronaridine	Stöckigt et al. (1982)
Tabernaemontana divaricata (L.) R. Br. ex Roem. & Schult.	CSCs	Coronaridine, catharanthine, 19-S-heyneanine	Pawelka and Stöckigt (1983) van der Heijden et al. (1988a)
Tabernaemontana elegans Stapf	Callus cultures (CCs)	Isovoacangine, 3-oxo-isovoacangine, 3-R/S-hydroxyisovoacangine, 3-R/S-hydroxycoronaridine	van der Heijden et al. (1986)
Tabernaemontana pandacaqui Poir.	CSCs, CCs, shoot cultures	3-hydroxyvoacangine	Sierra et al. (1991)
Tabernanthe iboga Baill.	CSCs	Ibogaine, dihydroxyibogamine, ibogamine, voacangine, ibogaline	Basile et al. (1999)
Tabernaemontana alba Mill.	Somatic embryos derived from CCs	Ibogaine, voacangine	Krengel et al. (2016)

in the callus, the resulting shoots showed ibogaine and voacangine contents similar to the trunk and root bark of mature whole plants of the same species. Hence, cellular and/or subcellular differentiation seems to play a key role in MIA biosynthesis. Sierra et al. (1991) observed an increase in alkaloid yield proportional to the degree of differentiation in suspension cells, callus, morphogenetic callus, proliferated shoots, and functional buds of *T. pandacaqui*. Plumeran type alkaloids predominated in the poorly differentiated CSCs, whereas experimental groups with higher levels of differentiation presented the ibogan type MIA 3-hydroxyvoacangine. Similar results were obtained in the case of *T. elegans*: Alkaloids of the ibogan type were detected in CCs, but not in CSCs. In whole plants, this structural class occurred in combination with alkaloids of the corynanthean type forming bis-indole MIAs (van der Heijden et al. 1989a).

Catharanthus roseus (L.) G. Don (Apocynaceae) is the most exhaustively investigated MIA-producing species. In order to biosynthesize the entire range of its more than 130 MIAs, including the aforementioned antineoplastic drugs vinblastine and vincristine (Almagro et al. 2013, 2015; Scossa et al. 2018), different cell types and compartments have to be present in the whole plant. At the cellular level, epidermal, mesophyll, and internal phloem-associated parenchyma (IPAP) cells, idioblasts and laticifers are required, as well as vacuoles, endoplasmic reticula, thylakoids, nuclei, and cytosol at the subcellular level (de Luca et al. 2014; Guirimand et al. 2011; Scossa et al. 2018). As many enzymes of the MIA pathway are exclusively located in certain cell types and compartments, the inter- and intracellular transport of intermediate compounds is essential. For example, the precursors of vindoline (plumeran type) are transported from the epidermal to the mesophyll cells, and subsequently to the idioblasts and laticifers, where the final product is biosynthesized (Salim and de Luca 2013; Scossa et al. 2018). In addition, a part of the biosynthetic pathway is carried out in the chloroplasts. In consequence,

the occurrence of structurally complex compounds like vindoline is scarce or non-existing in poorly differentiated in vitro cultures of *C. roseus*. The same is true for bis-indole MIAs, which is why the leaves or whole plants of the species remain the most important commercial sources of vinblastine and vincristine, the condensation products of catharanthine with vindoline (Almagro et al. 2013; Scossa et al. 2018; Zhao et al. 2013).

Based on published research studies conducted on *C. roseus* and *T. iboga*, Fig. 2 illustrates a plausible metabolic pathway of the CIVI-complex in Mexican *Tabernaemontana* species. Being structurally less complex than vindoline and bis-indole MIAs, ibogan type alkaloids and its direct precursors are entirely biosynthesized in the cytosol of the epidermal or root apical cells (Almagro et al. 2013; Qu et al. 2019; Salim and de Luca 2013). Hence, the in vitro production of these compounds should be comparatively simple, as only intermediate cellular and subcellular differentiation stages are apparently required. Notwithstanding, the most important limiting factors may be associated with the pathway of the iridoid precursors, which in *C. roseus* are synthesized in the plastids and cytosol of the IPAP cells (Almagro et al. 2013; de Luca et al. 2014; Saiman et al. 2014, 2015, 2018; Salim and de Luca 2013). Accordingly, this means that in order to produce ibogan type alkaloids, in vitro cultures of *Tabernaemontana* species must either present two cell types similar to epidermal and IPAP cells, respectively, or a single cell type capable of producing both the iridoid precursors and the alkaloids. As can be seen in Fig. 2, the presence of vacuoles and plastids would probably be mandatory, too.

As a strong indicator of the general feasibility of the in vitro bulk production of ibogan type alkaloids, it should be mentioned that the commercial production of catharanthine from bioreactor-grown *C. roseus* suspension cell cultures reached economically viable levels in the 1990s. The fact that the example given has not been exploited by any company (Zhao et al. 2013), can perhaps be explained

by the molecule's minor pharmaceutical importance. The antiaddictive compounds of the CIVI-complex do certainly not present this limitation, and suspension cell cultures of appropriate Apocynaceae species may be the simplest in vitro technique for coronaridine, ibogamine, voacangine, and/or ibogaine production. Nevertheless, the greatest obstacle to producing ibogan type alkaloids in bioreactors on an industrial scale is likely to be of a quantitative rather than a qualitative nature. If the in vitro production process is less profitable than obtaining the natural products by traditional agroforestry techniques, it simply will not attract commercial interest (Almagro et al. 2013). Zhao et al. (2001a, b) reported that compact callus clusters (CCC) of *C. roseus* can be propagated in liquid medium similar to CSCs. Due to a higher degree of cell differentiation, the alkaloid yield of the former tends to be superior to that of the latter, without affecting biomass productivity. Another option to ensure the desired level of differentiation, and thus the occurrence of a greater variety and/or quantity of certain secondary metabolites, consists in the establishment of either non-transformed root cultures or hairy root cultures (HRCs), especially in the case of species that accumulate the highest amounts of MIAs in the root bark, e.g. *T. iboga* and several *Tabernaemontana* species (Dickinson 2016; Krengel et al. 2016). HRCs are obtained by the successful infection and transformation of a plant with *Rhizobium rhizogenes* (Riker et al. 1930) Young et al. 2001, and show faster growth and a higher genetic stability than non-transformed root cultures. Furthermore, only the former grow indefinitely in the absence of exogenous plant growth regulators (PGR) (Zhao et al. 2013). The selection of high yielding cell lines facilitates the production of known quantities of MIAs over time (Almagro et al. 2013). Thakore et al. (2017) were able to obtain 34 ± 2.3 mg/ml of ajmalicine (corynanthean type) from *C. roseus* HRCs in a modified bubble column bioreactor, surpassing all previously reported concentrations at this scale. While the biosynthesis of alkaloids, including catharanthine, has been observed in HRCs of the latter and other Apocynaceae species (Benyammi et al. 2016; Mehrotra et al. 2013a, b), there are no publications regarding the production of the CIVI-complex using root cultures of any species.

Elevated differentiation levels can also be obtained by organogenesis or somatic embryogenesis. Both phenomena can be induced directly in explants or indirectly in callus. Depending on the PGRs added to the growth medium, the formation of roots (rhizogenesis), adventitious shoot buds (caulogenesis), or embryos (somatic embryogenesis) can be favored (Hussain et al. 2012). Unlike CSCs, CCs, and HRCs, shoot and embryo cultures are generally able to biosynthesize the entire range of MIAs of *Tabernaemontana* and other Apocynaceae species (Krengel et al. 2016; Sharma et al. 2019). Unfortunately, the biomass production of the individual batches tends to be low, and therefore, a large number

of the respective plant structures is required to obtain commercially significant amounts of secondary metabolites. For this reason, organogenesis and somatic embryogenesis are usually used for the regeneration of whole plants, particularly clones from desired genotypes (Guan et al. 2016). These plants can be grown in plantations or hydroponic systems, and harvested at the appropriate development stage considering the ratio of biomass to secondary metabolite production (Mall et al. 2019). Protocols for the in vitro multiplication of several *Tabernaemontana* species can be found in the scientific literature (Kodja et al. 1997; Krengel et al. 2016; Oliveira et al. 2003; Sierra et al. 1991).

In vitro production of ibogan type alkaloids: optimization of culture conditions

Once the appropriate type of in vitro culture has been chosen, culture conditions should be optimized in order to improve alkaloid yields. Donor plants with the most suitable genotypes should be selected, e.g. those that accumulate high concentrations of the ibogan type MIAs of interest and facilitate the formation of callus in explants exposed to PGRs. While the explant type may influence the profile of natural products biosynthesized by the resulting CCs, the different cell lines that make up a callus are of greater importance, since they tend to show considerable differences concerning their ability to produce and accumulate secondary metabolites (Ramawat 2007). Thus, the selection of cellular aggregates or individual cells with high ibogan type alkaloid productivity is essential for the establishment of commercial in vitro cultures from Apocynaceae species. Unfortunately, in many cases the selected cell lines have a low genetic and biochemical stability, leading to the loss of their desirable traits, unless selection is carried out continuously (Ramawat 2007). A convenient selection technique consists in exposing CSCs to UV light and then choosing the cellular aggregates with the highest degrees of autofluorescence which can be an indicator of the presence of certain MIAs (Morris et al. 1989).

The environmental conditions should also be finely balanced in PTC. The following variables are key factors for the in vitro production of ibogan type MIAs:

Photoperiod: The induction and activation of several enzymes involved in the MIA pathway, such as tryptophan decarboxylase (TDC), are light-dependent (Molchan et al. 2012; Zhu et al. 2015). Consequently, the presence of light can increase the alkaloid biosynthesis in both in vitro cultures and whole plants of *C. roseus* (Zhu et al. 2015). However, high luminous intensities of 64800 lx or more seem to inhibit the production of ibogan type and other MIAs in plants of *Tabernaemontana pachysiphon* Stapf (Höft et al. 1996, 1998). As a general rule, in vitro cultures
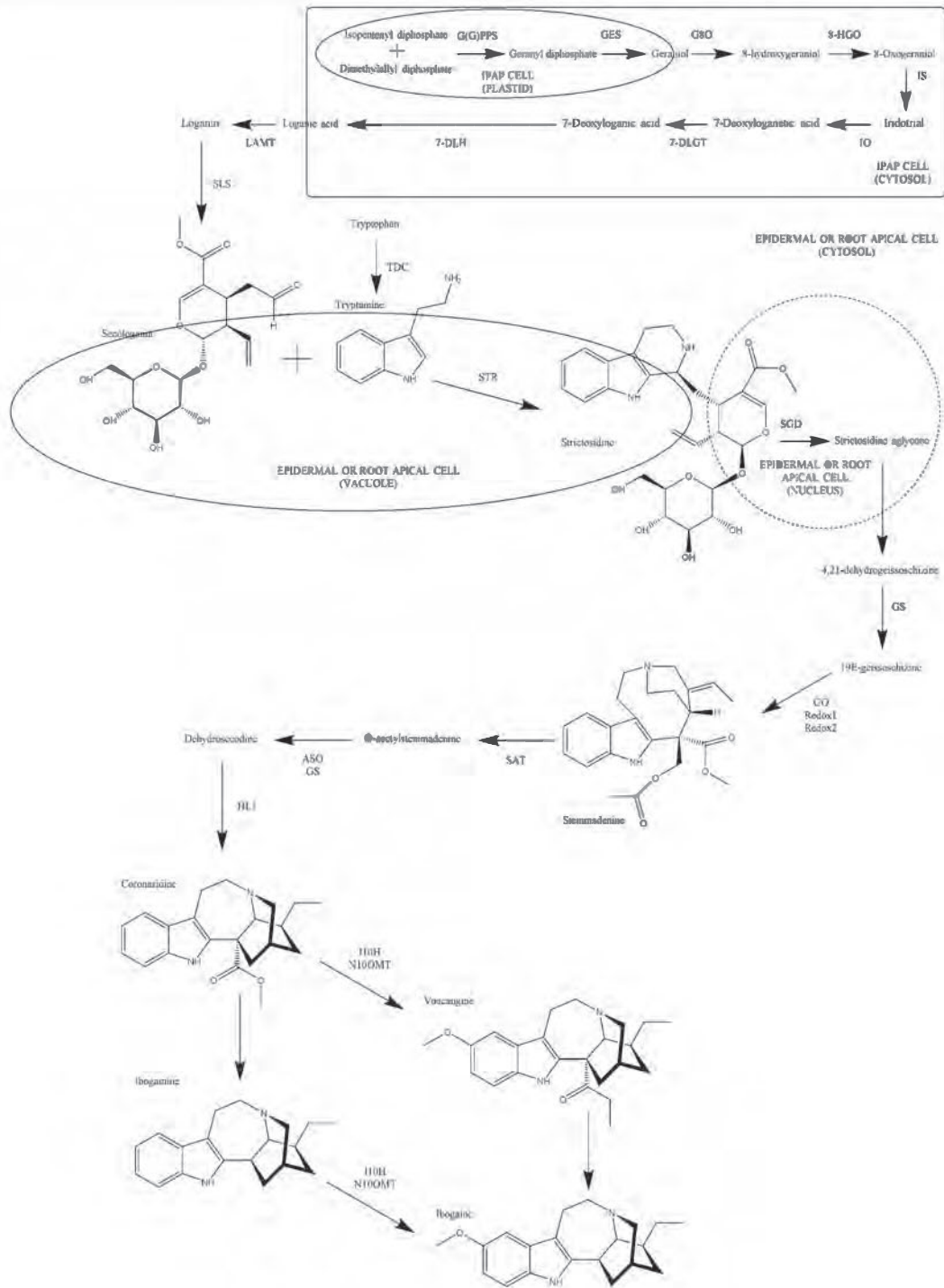
◀Fig. 2 Hypothetical biosynthetic pathway of the alkaloids of the CIVI-complex, with particular focus on the cellular and subcellular localization of enzymes and metabolites. *IPAP* internal phloem-associated parenchyma, *G(G)PPS* geranyl(geranyl) diphosphate synthase, *GES* geraniol synthase, *G8O* geraniol-8-oxidase, *8-HGO* 8-hydroxygeraniol oxidoreductase, *IS* iridold synthase, *IO* iridoid oxidase, *7-DLGT* 7-deoxyloganetic acid glucosyltransferase, *7-DLH* 7-deoxyloganic acid hydroxylase, *LAMT* loganic acid-O-methyltransferase, *SLS* secologanin synthase, *TDC* tryptophan decarboxylase, *STR* strictosidine synthase, *SGD* strictosidine β-glucosidase, *GS* geissoschizine synthase, *GO* geissoschizine oxidase, Redox 1; Redox 2; *SAT* stemmadenine O-acetyltransferase, *ASO* O-acetylstemmadenine oxidase, *HLI* hydrolase 1, *I10H* ibogamine 10-hydroxylase, *N10OMT* noribogaine-10-O-methyltransferase

of Apocynaceae species should be exposed to a photoperiod of 12/12, 16/8 or 24 h of light with an intensity between 150 and 1000 lx during the alkaloid production phase (Basile et al. 1999; Sierra et al. 1991; van der Heijden et al. 1986, 1988a). Nevertheless, it makes sense to keep some batches in continuous darkness for two reasons: First, regardless of alkaloid biosynthesis, in vitro cultures may show superior growth rates and forms when incubated in darkness than under photoperiod conditions (Krengel et al. 2016), ensuring in this way the long-term conservation of batches with desired traits. Secondly, the production of individual MIAs can be more or less light-dependent, meaning that in some cases, continuous darkness may favor the biosynthesis of possibly desired alkaloids while inhibiting the production of possibly undesired compounds (van der Heijden et al. 1988a).

Temperature: The production of ibogan type MIAs from in vitro cultures of *Tabernaemontana* or *Tabernanthe* species is usually carried out at a temperature between 25 and 28 °C (Basile et al. 1999; Pawelka and Stöckigt 1983; Sierra et al. 1991; van der Heijden et al. 1986, 1988a). Toivonen et al. (1992a) did not observe significant differences in the alkaloid yield of *C. roseus* CSCs exposed to 17, 23, or 32 °C, whereas Morris (1986) reported that the ratio between ajmalicine and serpentine (corynanthean type) was temperature-dependent. In HRCs of the same species, MIA accumulation was inversely proportional to the temperature (19.5, 24, or 32 °C). Nonetheless, in terms of volumetric productivity, a temperature of 24 °C resulted in the most convenient ratio between growth rate and alkaloid accumulation (Toivonen et al. 1992b). In *C. roseus* seedlings, catharanthine production was higher at 20 than at 25 °C, but reached the highest values at 35 °C after 16 days of cultivation (Guo et al. 2007). In summary, a temperature of 25 ± 3 °C seems to be adequate for the establishment and propagation of in vitro cultures of most Apocynaceae species, although it may prove worthwhile to elucidate the effects that lower or higher temperatures have on the biosynthesis and accumulation of ibogan type MIAs.

Nutrients: Ibogan type alkaloids can be produced from in vitro cultures of *Tabernaemontana* or *Tabernanthe* species using MS (Murashige and Skoog 1962) or B5 (Gamborg et al. 1968) growth media (Basile et al. 1999; Pawelka and Stöckigt 1983; Sierra et al. 1991; van der Heijden et al. 1986, 1988a). The composition of these complex media may be modified in order to alter alkaloid biosynthesis. The most relevant variables are the sources and levels of carbon, phosphate, and nitrogen (particularly the ammonium to nitrate ratio), in addition to amino acids and vitamins (Ramawat and Mathur 2007). Media containing sucrose concentrations of 20–30 g/l are appropriate for ibogan type alkaloid production (Basile et al. 1999; Sierra et al. 1991; van der Heijden et al. 1988a). However, higher levels of this carbohydrate can increase secondary metabolite biosynthesis (Ramawat and Mathur 2007). For example, a *C. roseus* cell line that had lost the ability to produce ajmalicine in CSCs propagated in B5 medium with 20 g/l sucrose, biosynthesized this MIA again in the presence of 60 g/l sucrose. Serpentine yield was also improved under this condition (Mérillon et al. 1986). In other *C. roseus* CSCs, Zhao et al. (2001b) observed that production of ajmalicine and serpentine was highest in media containing between 50 and 60 g/l sucrose, but in the case of the ibogan type alkaloid catharanthine, the optimal sucrose concentration ranged from 60 to 70 g/l. Furthermore, both concentration intervals increased biomass formation and cell compaction, the latter of which can be interpreted as an indicator of higher differentiation levels. The substitution of sucrose for glucose or fructose enhanced catharanthine production in *C. roseus* HRCs (Jung et al. 1992).

Generally speaking, a lower phosphate and nitrogen availability stimulates alkaloid biosynthesis in *C. roseus* in vitro cultures (van der Heijden et al. 1989c). The same may be true for other macro and micronutrients, as well as vitamins, since Verma et al. (2012) reported favorable results when using half strength instead of standard MS medium. In CSCs of *T. divaricata*, the alkaloid yield increased tenfold when changing the ammonium to nitrate ratio from 1:2 to 1:1 (Schripsema and Verpoorte 1992). The last ratio also caused higher catharanthine production in *C. roseus* plantlets than ratios 0:1 and 3:1 (Guo et al. 2012).

PGRs: Auxins and cytokinins stimulate cell division, and the ratio between them directs plant cell development. High concentrations of auxins (specifically indoleacetic (IAA) and indolebutyric acid (IBA)) favor root formation, while cytokinins usually induce shoot growth. Appropriate ratios between both types of PGRs stimulate callus formation (Smith 2013). It is important to note that these cause-and-effect relationships have only general and in no way universal validity applicable to all plant species and genotypes. In the case of *T. alba*, growth media containing 2 mg/l of the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.2 mg/l of the cytokinin kinetin first

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induced the formation of callus, and subsequently somatic embryogenesis in only a few batches of CCs (Krengel et al. 2016). Although 2,4-D is very efficient in dedifferentiating plant cells and inducing callus formation (Smith 2013), it can also have an inhibitory effect on the MIA production of in vitro cultures (Almagro et al. 2013, 2015). Arvy et al. (1994) proposed that 2,4-D affected the biosynthetic pathway of the iridoid precursors (particularly the conversion of loganic acid to loganin), rather than the MIA pathway itself, in *C. roseus* CSCs. The auxin has indeed be shown to repress the expression of at least three genes associated with the methylerythritol phosphate (MEP) pathway which gives rise to the iridoids, in addition to the gene coding for TDC, the enzyme that catalyzes the reaction that converts tryptophan to tryptamine, the indole precursor of the MIAs (Zhu et al. 2015). However, ibogan type MIAs were detected in CSCs of *T. iboga* and *T. divaricata*, as well as in CCs of *T. elegans* and *T. pandacaqui*, despite the presence of high concentrations of 2,4-D (Basile et al. 1999; Sierra et al. 1991; van der Heijden et al. 1986, 1988a). Verma et al. (2012) even demonstrated that *C. roseus* suspension cells produced significantly higher amounts of MIAs when exposed to 2,4-D than to 1-naphthaleneacetic acid (NAA) and/or IAA. This suggests that the inhibitory effect of 2,4-D on the in vitro production of MIAs is not a general rule, but rather depends on the traits of the specific cell line. Taking into account the high efficiency of 2,4-D in inducing and propagating CCs and CSCs of Apocynaceae species, the search for cell lines that produce ibogan type alkaloids in the presence of this auxin could give better results than replacing the latter with another PGR like NAA or IAA, which by the way are also capable of repressing the TDC gene (Zhu et al. 2015). Alternatively, the biomass could be propagated in medium containing 2,4-D until reaching the exponential phase, and then be transferred to a MIA production medium with no or low auxin, phosphate, and nitrogen concentrations, but with high sucrose content. When reaching the stationary phase, many of the previously produced primary metabolites should be converted to secondary metabolites (Ramawat and Mathur 2007). Cytokinins, on the other hand, tend to increase MIA yield by stimulating the biosynthesis of the iridoid precursors. *C. roseus* CSCs supplemented with benzyladenine (BA) or trans-zeatin showed increased ajmalicine production, especially in combination with ethylene (Papon et al. 2005; Yahia et al. 1998).

A special class of plant hormones are those related to defense mechanisms against herbivores and pathogens. E.g., salicylic acid (SA) triggers plant response to biotrophic pathogens, often in the form of systemic acquired resistance (SAR), whereas jasmonic acid (JA) induces defense mechanisms against necrotrophic pathogens and herbivores (Bari and Jones 2009). Particularly jasmonates have been proved

useful in the induction of MIA biosynthesis in Apocynaceae species, as described in the following paragraphs.

In vitro production of ibogan type alkaloids: strategies for increasing productivity

After selecting high-yielding cell lines and optimizing culture conditions, ibogan type MIA productivity can still be increased by the application of certain techniques, some of which will be briefly described in the following paragraphs.

Elicitation: Any substance that when added to the growth medium stimulates the biosynthesis of certain secondary metabolites in whole plants or in vitro cultures, can be considered an elicitor for the respective natural product and species. There are abiotic and biotic elicitors (Namdeo 2007). For example, CSCs of *T. africana*, *Tabernaemontana catharinensis* A. DC., *T. divaricata*, *T. elegans*, and *T. orientalis* were elicited biotically with mycelial preparations of different ascomycetes, in addition to the enzymes cellulase and pectinase. In consequence, triterpene production increased, but MIA biosynthesis was inhibited (Pereira et al. 2007; van der Heijden et al. 1988b, 1989b). Van der Heijden et al. (1989b) proposed that inhibition of GPP synthase activated the former and blocked the second pathway in *T. divaricata* CSCs elicited with *Candida albicans* (C. P. Robin) Berkhout 1923. *C. roseus* in vitro cultures, on the other hand, have been successfully elicited with abiotic and biotic agents, causing higher MIA yields, including the ibogan type alkaloid catharanthine. Examples are mycelial preparations of *Aspergillus flavus* Link 1809 (Tonk et al. 2016), *Aspergillus niger* van Tieghem 1867, and *Penicillium citrinum* Thom, C. 1910, as well as methyl jasmonate (MeJA), cyclodextrins, and UV light. The last three elicitors have the greatest potential to increase catharanthine production, especially when applied together to CSCs (Almagro et al. 2013, 2014, 2015). In view of the commercial production of ibogan type alkaloids, it also makes sense to consider the most economical elicitors with proven efficacy, such as mannitol or KCl which can induce osmotic and saline stresses, respectively (Sharma et al. 2019; Zhao et al. 2001a). It should be noted that appropriate degrees of osmotic pressure may also have enhancing effects on cell differentiation (Iwase et al. 2005).

Precursor feeding: As already mentioned, the availability of iridoid precursors can be a limiting factor for the in vitro production of ibogan type MIAs due to either the absence of IPAP cells or inhibition of the iridoid (and to a lesser extent, the tryptamine) pathway by auxins like 2,4-D. Both problems can be overcome by the addition of precursors to the culture medium, which often restores or improves the ability to biosynthesize MIAs in *C. roseus* cell lines that normally do not produce alkaloids, be it for intrinsic reasons

or for the composition of the growth medium. In general, the iridoid loganin appears to be more efficient in increasing MIA biosynthesis than its metabolic product secologanin. Tryptophan or tryptamine feeding does not have significant effects, unless applied in combination with the iridoid precursors (Arvy et al. 1994; Mérillon et al. 1986; Moreno et al. 1993; Sharma et al. 2019; Whitmer et al. 2002). On a commercial scale, the addition of great amounts of loganin or secologanin to ibogan type alkaloid producing in vitro cultures is not realistic due to the high costs of these iridoids. However, both substances can be administered in the form of complex plant extracts, either in crude or semipurified form (Geerlings et al. 2001; Hallard et al. 1998; Nam et al. 2007). Several species of the Caprifoliaceae family are good natural sources of loganin and/or secologanin, particularly within the *Lonicera* L., *Symphoricarpos* Duhamel, and *Weigela* Thunb. genera (Hallard et al. 1998).

Genetic engineering: Several genes related to the MIA pathway have been identified in *C. roseus*. Above all, the enzymes associated with the biosynthesis of the iridoid and indole precursors, in addition to those responsible for catalyzing the condensation of both, have been described in detail (de Luca et al. 2014; Miettinen et al. 2014). A considerable number of studies has concentrated on the overexpression of TDC and/or STR (a gene coding for strictosidine synthase, which catalyzes the condensation of tryptamine with secologanin) in the case of enzymes, and on ORCA3 in the case of transcription factors. In several of these experiments, MIA yield, including the ibogan type alkaloid catharanthine, increased significantly, particularly after precursor feeding, in both whole plants and in vitro cultures (Almagro et al. 2013, 2015; Sharma et al. 2018). At present, less research has been devoted to the iridoid precursors, but the results so far are promising. For instance, Kumar et al. (2015, 2018) succeeded in overexpressing geranyl(geranyl) diphosphate synthase (G(G)PPS) and/or geraniol synthase (GES) in *C. roseus* plants, which led to higher levels of secologanin and ultimately catharanthine, as well as other monomeric MIAs. Notwithstanding that both enzymes may be essential for channeling primary metabolites towards the MEP (and therefore the MIA) pathway instead of the mevalonate pathway, these findings were not observed in GES-overexpressed cell suspension cultures of the same species, possibly underlining once again the importance of adequate differentiation levels for MIA biosynthesis (Saiman et al. 2014, 2015, 2018).

Although the last years have seen important advances with respect to the elucidation of the ibogan type alkaloid pathway, many enzymes that give rise to the large number of MIAs downstream from strictosidine remain unknown. Qu et al. (2019) recently identified the last missing enzymes in the pathway leading from strictosidine to vinblastine and vincristine in *C. roseus*, including the biogenesis of

catharanthine. In the case of *Tabernaemontana*, *Tabernanthe*, and *Voacanga* species, the identification of enzymes involved in the interconversion of the four alkaloids of the CIVI-complex should receive special attention. Overexpression of the genes encoding for these enzymes could then provide in vitro cultures or whole plants that produce high concentrations of the desired antiaddictive alkaloid (coronaridine, ibogamine, voacangine, or ibogaine) as the final product, rather than a mixture of relatively low amounts of the four MIAs separately. In terms of applied science, this approach would be more reasonable than the random increase of the total alkaloid content through the overexpression of TDC and/or STR. Farrow et al. (2018) recently made a major contribution to this goal by publishing a complete transcriptome of *T. iboga*. In consequence, the authors were able to identify two enzymes, ibogamine 10-hydroxylase (I10H) and noribogaine-10-O-methyltransferase (N10OMT), responsible for the methoxylation of ibogamine, thus converting the latter to ibogaine. Figure 2 summarizes the existing enzymatic information on the ibogan type alkaloid pathway of Apocynaceae species.

Unfortunately, *Tabernaemontana*, *Tabernanthe*, and *Voacanga* species are still poorly consolidated as biological models, and a comprehensive library with standardized protocols for their in vitro cultivation and genetic transformation has yet to be created. The establishment of *C. roseus* as a biological model to study MIA biosynthesis could serve as a blueprint for this endeavor. Cutting-edge research conducted with the last species comprises cell immobilization (Almagro et al. 2013, 2015), identification and manipulation of MIA transport proteins (Payne et al. 2017; Roytrakul and Verpoorte 2007; Yu and de Luca 2013), and the expression of MIA pathway genes in microorganisms, e.g. *Saccharomyces cerevisiae* Meyen ex EC Hansen (Geerlings et al. 2001; Jiang et al. 2017; Lin et al. 2018; Nam et al. 2007). Endophytic bacteria and fungi from *C. roseus* have been shown to be able to biosynthesize some alkaloids characteristic of their host plant (Koul et al. 2013). Kumar et al. (2013) found vinblastine and vincristine in a strain of *Fusarium oxysporum* Schlecht. emend. Snyder & Hansen isolated from the leaves of this plant. An endophyte belonging to the same genus was obtained from *T. alba* (Krengel 2015), but has not been chemically evaluated.

In conclusion, we suggest the following strategies in order to advance towards the goal of commercially feasible in vitro production of the antiaddictive alkaloids of the CIVI-complex from non-model plants of the Apocynaceae family, such as *Tabernaemontana*, *Tabernanthe*, and *Voacanga* species:

1. The mostly qualitative research conducted on the simplest types of cell cultures during the 1980s and 1990s (Table 1) should serve as a basis for modern quantitative studies evaluating the potential of CSCs and CCs

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of different species and genotypes to biosynthesize significant amounts of the CIVI-complex in bioreactors. In other words, the qualitatively proven feasibility of the in vitro production of these compounds should be complemented by quantitative data.

2. More differentiated tissue structures, such as HRCs, shoot, and embryo cultures should also be examined and compared to CSCs and CCs. It should be noted that biosynthesis of the CIVI-complex has only been reported in the case of the latter, but not the former (with the exception of Krengel et al. 2016). Hence, the establishment of differentiated, alkaloid-producing PTCs from *Tabernaemontana*, *Tabernanthe*, and *Voacanga* species offers attractive research opportunities.

3. The future of commercial secondary metabolite production may well lie in genetic engineering. Therefore, the genomes and transcriptomes of appropriate CIVI-complex-producing species should be elucidated in order to completely reveal their respective MIA pathways. This has already been achieved in the case of *C. roseus*, and the published data can be conveniently utilized to search for homologous genes and enzymes in non-model species like *T. iboga* (Farrow et al. 2018).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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DISCUSIÓN GENERAL

Contribución al análisis cualitativo y cuantitativo de especies productoras de alcaloides del tipo ibogano

Los capítulos anteriores ilustran el desarrollo y optimización de un protocolo para el análisis fitoquímico de especies vegetales productoras de alcaloides del tipo ibogano (Krengel et al., 2019a, 2019b, 2016). Se hizo énfasis en posibilitar la obtención de resultados cuantitativos confiables, ya que los perfiles alcaloideos de la mayoría de estas especies solamente han sido determinados de manera cualitativa. Únicamente la comparación cuantitativa entre especies podrá arrojar información relevante para identificar potenciales fuentes naturales de compuestos farmacéuticamente importantes, como los agentes antiadictivos coronaridina, ibogamina, voacangina e ibogaína, entre otros. La triple microextracción de 100 mg de material vegetal con metanol bajo sonicación durante 20 min, seguido por la separación del sobrenadante por centrifugación y su análisis por cromatografía de gases acoplada a espectrometría de masas (GC-MS) mostró ser un método eficaz y conveniente para procesar un gran número de muestras simultáneamente (Krengel et al., 2019b). Al someter los rendimientos alcaloideos obtenidos a análisis multivariante, se pueden comparar las especies y/o grupos experimentales examinados de forma visual y expedita (Krengel et al., 2019a).

Potencial de especies mexicanas de *Tabernaemontana* como fuentes alternativas de alcaloides antiadictivos

Entre los principales resultados del procedimiento arriba descrito aplicado a cuatro especies mexicanas de *Tabernaemontana*, así como *T. iboga* y *V. africana*, las únicas dos especies hoy día relevantes para la producción comercial de ibogaína (Dickinson, 2016), destacan las semejanzas entre los perfiles alcaloideos de las primeras y las últimas, reflejando su estrecha relación quimotaxonómica (Krengel et al., 2020a, 2019a). En particular

la corteza radical de *T. arborea* demostró tener características fitoquímicas favorables para la obtención de grandes cantidades de ibogaína, ya que su perfil alcaloideo fue asombrosamente similar al de *V. africana*, con concentraciones altas de voacangina e intermedias o bajas de ibogaína y vobasina (Krengel et al., 2020a). *Tabernaemontana amygdalifolia*, en cambio, podría ser una excelente fuente de coronaridina e ibogamina (Krengel et al., 2019a). Los efectos antiadictivos de estos dos compuestos se han investigado en menor grado, sin embargo, como ya se mencionó en la introducción, podrían ser complementarios o incluso superiores a los de la ibogaína (Glick et al., 1994). Por ende, si en algún momento se generara demanda de coronaridina e/o ibogamina a nivel nacional o internacional, la corteza de raíz de *T. amygdalifolia* sería una opción altamente viable para obtener la materia prima necesaria. Referente a *T. alba*, se determinaron dos quimiotipos, uno conteniendo principalmente voacangina e ibogaína, y el otro coronaridina e ibogamina. En consecuencia, sería posible extraer los cuatro alcaloides de una sola especie. *Tabernaemontana donnell-smithii* presentó contenidos relativamente bajos de MIAs del tipo ibogano, aunque podría proporcionar cantidades razonables de voacangina (Krengel et al., 2019a).

Los distintos patrones de uso etnobotánico de especies productoras de alcaloides del tipo ibogano en México y África, explicados con base en aspectos químicos, farmacológicos, ambientales, socioculturales e históricos

La ausencia de registros etnobotánicos relacionados con las potenciales propiedades enteógenas de especies mexicanas de *Tabernaemontana* sorprende, en vista del amplio conocimiento que las culturas prehispánicas tenían acerca de plantas y hongos con efectos sicodélicos. La única mención que sugiere algún efecto sobre el sistema nervioso central es el de "estimulante" en una colecta del Dr. Javier Caballero hace varias décadas (Caballero et

al., 1978). Los perfiles alcaloideos de *T. alba*, *T. amygdalifolia*, *T. arborea* y *T. donnell-smithii* por un lado, así como *T. iboga* y *V. africana* por otro lado, muestran más similitudes que diferencias (Krengel et al., 2020a, 2019a), y por consiguiente, no permiten hacer conclusiones contundentes acerca del porqué de la aparente contradicción entre la enorme importancia que la medicina tradicional centroafricana le ha dado a los efectos sicoactivos de las últimas dos especies, y el virtualmente nulo uso enteogénico de las primeras cuatro especies en México. Por ello, en esta tesis se sugiere que la explicación del fenómeno requiere de una revisión desde el punto de vista de las ciencias sociales, particularmente considerando aspectos socioculturales e históricos intrínsecos a las dos regiones. Es posible que los efectos que los alcaloides del tipo ibogano ejercen sobre el sistema nervioso central simplemente nunca se descubrieron en la época prehispánica. Tampoco se puede excluir que el conocimiento referente a las propiedades estimulantes y/o enteógenas de especies mexicanas de *Tabernaemontana* sí existió, pero que se perdió a causa de la conquista de México y la subsecuente represión de las tradiciones indígenas, así como por la erosión cultural sufrida en regiones como Los Tuxtlas, Veracruz, a partir de 1940. Alternativamente, la represión pudo haber ocasionado que el conocimiento correspondiente se mantuviera en secreto por las comunidades correspondientes. Otra posibilidad consiste en una preferencia cultural de las culturas prehispánicas – pero no las centroafricanas – por los denominados "alucinógenos clásicos", como *ololiuqui* (*Turbina corymbosa* (L.) Raf.), tlitliltzin (*Ipomoea violacea* L.), *peyotl* (*Lophophora williamsii* (Lem. ex Salm-Dyck) J.M. Coult.) y teonanácatl (*Psilocybe* spp.), entre otros (Rätsch, 2007; Schultes et al., 2001). Cualitativamente hablando, los efectos de los principios activos de estas plantas y hongos distan mucho de los alcaloides del tipo ibogano, ya que los primeros son principalmente agonistas de los receptores serotoninérgicos 5-HT$_{2A}$, mientras que la ibogaína, por ejemplo, interactúa además con otros sistemas de neurotransmisores, como los receptores de N-metil-D-

aspartato (NMDA), opioides, acetilcolinérgicos y sigma. Adicionalmente, modula los transportadores de serotonina y dopamina (Alper, 2001; Glick & Maisonneuve, 1998; Sweetnam et al., 1995). Esto hace que los alcaloides del tipo ibogano causen una estimulación central acompañada por cierto grado de toxicidad – algunas veces letal – no presentes en los "alucinógenos clásicos" (Alper, 2001; Nichols, 2004; Ott, 1996; Schultes et al., 2001). En consecuencia, es probable que las culturas originarias de México y de África Central compartieron el afán por trascender el mundo y la existencia físicos con ayuda de preparaciones enteógenas de origen vegetal o fúngico, pero que a la vez apreciaron de manera muy diferente las respectivas propiedades farmacológicas de las distintas clases de principios activos disponibles en cada región (Krengel et al., 2020b).

Actividad farmacológica de extractos alcaloideos y compuestos puros de *T. alba* y *T. arborea*

Los ensayos de acitvidad biológica realizados con extractos de *T. alba* y *T. arborea* revalaron sus propiedades contra *M. tuberculosis* (Guzmán Gutiérrez et al., 2020). Esto resulta interesante por dos razones: Primero, al comprobar que preparaciones obtenidas de especies de *Tabernaemontana* nativas de México poseen cierta eficacia antibiótica, se corroboran los registros de uso etnomedicinal no sicoactivo referente al género en el país (Krengel et al., 2020b). Aunque hay que mencionar que paradójicamente, la mayor actividad antituberculosis se detectó en órganos y especie que no cuentan con antecedentes de aplicación en la medicina tradicional mexicana, específicamente las cortezas de tronco y sobre todo de raíz de *T. arborea*. Visto desde otro ángulo, los resultados son coherentes, ya que las cortezas son los principales sitios de acumulación de MIAs, al menos en las dos especies con las que se llevaron a cabo los ensayos farmacológicos (Krengel et al., 2016), y la comparación de los efectos antibióticos de extractos (de por sí alcaloideos) y de MIAs

puros confirmó que este grupo de compuestos son responsables de dichas propiedades. Como todos los órganos de todas las especies de *Tabernaemontana* contienen alcaloides (Krengel et al., 2016; Van Beek et al., 1984), es válido extrapolar los resultados a las especies y los órganos que sí presentan reportes de uso etnomedicinal en México.

Segundo, los experimentos de Guzmán Gutiérrez et al. (2020) podrían ayudar a desarrollar nuevos fármacos en medio de un estado de emergencia cada vez más preocupante causado por la creciente resistencia de microorganismos a los antibióticos (Prestinaci et al., 2015). En este contexto, cabe destacar que en nuestros experimentos no fueron los alcaloides del tipo ibogano simples los que mostraron mayor actividad contra *M. tuberculosis*, sino un MIA dimérico, la voacamina, compuesta por dos alcaloides monoméricos de los tipos ibogano y corinanteano, respectivamente (Van Beek et al., 1984). Más notable aun es que el extracto alcaloideo de la corteza radical de *T. arborea* presentó una potencia superior a la de la voacamina, a la vez de ser menos citotóxico. Por tanto, la compleja mezcla de alcaloides mayoritarios y minoritarios contenida en el extracto parece inducir efectos sinérgicos que se traducen en un mayor margen de seguridad que los sus componentes individuales. Si se toma en consideración que la voacamina también es capaz de eliminar varios tripanosomátidos (Chowdhury et al., 2017), queda claro que el potencial comercial de especies mexicanas de *Tabernaemontana* no se limita a la producción exclusiva de agentes antiadictivos.

Desmetoxicarbonilación de coronaridina y voacangina

Se comprobó por primera vez que la coronaridina y la voacangina se pueden convertir en ibogamina e ibogaína, respectivamente, mediante su desmetoxicarbonilación directa en extractos crudos metanólicos obtenidos de especies que biosintetizan MIAs del tipo ibogano (en este caso de las cortezas radicales de *T. alba* y *T. arborea*; Krengel et al., 2019b), lo que

simplifica significativamente la purificación y el aislamiento de los alcaloides individuales. El procedimiento químico correspondiente consiste de una saponificación seguida por una reacción de descarboxilación, ambas de rutina. No obstante, solamente se ha aplicado con éxito a compuestos puros. Intentos anteriores de realizar el tratamiento semisintético con extractos alcaloideos de *V. africana* fracasaron (Jenks, 2002). Resultó además sumamente interesante que tanto coronaridina como voacangina se desmetoxicarbonilaron por completo sin necesidad de descarboxilar los respectivos productos de la saponificación. Como no se encontraron antecedentes en la literatura científica, se propusieron dos posibles mecanismos de reacción: uno siendo una descarboxilación de Krapcho, y el otro una desprotonación catalítica de la unidad indólica, seguida sucesivamente por una transferencia intramolecular del grupo acilo hacia el nitrógeno del grupo indólico, una protonación del resultante anión carbamato, y finalmente una hidrólisis del grupo uretano formado. Cabe aclarar que el segundo mecanismo parece ser más probable, debido a que las descarboxilaciones de Krapcho no suelen ocurrir bajo condiciones tan suaves como las inducidas durante el experimento descrito. De cualquier forma, después de someter los extractos metanólicos de la raíz de *T. arborea* al tratamiento de saponificación, sus perfiles alcaloideos dejaron de asemejarse a los de las cortezas de *V. africana* para adquirir una composición química característica de la corteza radical de *T. iboga*, presentando primordialmente ibogaína y, en menor grado, ibogamina (Krengel et al., 2019b). Si se considera que las terapias antiadictivas basadas en ibogaína se pueden realizar tanto con el compuesto puro como con extractos alcaloideos complejos de la última especie (Alper et al., 2008; Dickinson, 2016), se hace evidente que *T. arborea* podría aprovecharse para la elaboración de ambos tipos de productos.

Cultivo *in vitro* de especies mexicanas de *Tabernaemontana*

Ahora bien, si el objetivo fuera obtener suficiente cantidad de alcaloides del tipo ibogano de especies mexicanas de *Tabernaemontana* para cubrir parte de la demanda global, se requeriría de considerables volúmenes de material vegetal, preferiblemente provenientes de genotipos altamente productores del compuesto en cuestión. Por supuesto sería posible lograr lo anterior mediante métodos convencionales de cruzamiento y cultivo agrícola-forestal, tal como se está llevando a cabo de manera incipiente o consolidada en los casos de *T. iboga* y *V. africana*, respectivamente (Brako Danquah, 2012; Dickinson, 2016; Krengel et al., 2019c). Sin embargo, se deberían aprovechar las técnicas biotecnológicas cada vez más sofisticadas para hacer los procesos de producción más eficientes. Por ejemplo, se comprobó que *T. alba* puede ser propagada mediante el CTV y, más específicamente, embriogénesis somática indirecta inducida en callos. Así, de un explante foliar, de tallo o radical se pueden reproducir miles de clones de la planta donadora que en el caso ideal se seleccionó previamente con base en sus características genéticas y fitoquímicas. Las plantas completas regeneradas biosintetizaron niveles de MIAs comparables con plantas silvestres maduras, tanto en condiciones *in vitro* (medio nutritivo artificial estéril y ambiente aséptico) como *in vivo* (tierra para macetas y ambiente no estériles). Los mismos callos sobre los cuales se formaron los embriones somáticos, en cambio, no produjeron alcaloides. Ambas formas de organización vegetal comparten el mismo genoma, y la principal diferencia entre ellas reside en sus niveles de diferenciación (sub)celular (simple en el primer y compleja en el segundo caso). Por lo tanto, se concluyó que el factor determinante de la biosíntesis de MIAs en especies de *Tabernaemontana* era el grado de diferenciación existente (Krengel et al., 2016). Krengel et al. (2019c) vislumbran tres vías que podrían conducir hacia la integración de herramientas biotecnológicas en la producción comercial de alcaloides del tipo ibogano utilizando especies mexicanas de *Tabernaemontana*: Primero, la propagación

masiva de genotipos apropiados mediante embriogénesis somática y posiblemente organogénesis. Después de una fase de aclimatación, las plantas completas regeneradas se transferirían a plantaciones convencionales, sea en condiciones de campo o de invernadero. Segundo, el cultivo *in vitro* de tejidos vegetales con niveles de diferenciación adecuados para la biosíntesis de MIAs, como por ejemplo brotes, raíces no transformadas o bien, raíces pilosas. Tercero, el cultivo *in vitro* de callos o células en suspensión, siempre y cuando se logre seleccionar, aislar y propagar líneas celulares que a pesar de sus bajos grados de diferenciación sean capaces de biosintetizar alcaloides del tipo ibogano. En la literatura científica se pueden consultar varios ejemplos de la viabilidad de este vía con respecto de los géneros *Tabernanthe* y *Tabernaemontana*, incluyendo *T. tomentosa*, una especie mexicana del último (Basile et al., 1999; Pawelka & Stöckigt, 1983; Sierra et al., 1991; Stöckigt et al., 1982; Van der Heijden et al., 1988, 1986). La optimización de las condiciones de cultivo, sobre todo en lo concerniente a la calidad y las concentraciones de reguladores de crecimiento y nutrimentos, pero también a el fotoperiodo, la tempertaura y el intercambio gaseoso, entre otros, podría ser esencial para aumentar el rendimiento de los cultivos *in vitro*. Éstos tienen la gran ventaja de permitir con relativa facilidad el estudio de la ruta biosintética de los alcaloides del tipo ibogano y otros MIAs, ya que por definición se mantienen en condiciones controladas. Además, si se conocen las enzimas claves que favorecen la formación de determinados compuestos, éstas se pueden sobreexpresar en los cultivos *in vitro* para mejorar la producción de alcaloides o bien, para dar origen a plantas completas genéticamente modificadas. En concreto, se propone sobreexpresar – o posiblemente silenciar – las esterasas/descarboxilasas e/o hidroxilasas/O-metiltransferasas descubiertas por (Farrow et al., 2019, 2018) en *T. iboga*, las cuales en conjunto convierten coronaridina en ibogamina, voacangina o ibogaína. De esta manera, sería posible obtener cultivos *in vitro* o plantas completas de especies mexicanas de *Tabernaemontana* que biosinteticen uno solo

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de los cuatro alcaloides mencionados o bien, cualquiera de los compuestos intermedios hidroxilados.

CONCLUSIONES Y PERSPECTIVAS GENERALES

Durante el proyecto de investigación descrito en esta tesis, se desarrolló un protocolo simple y eficiente para analizar cualitativa y cuantitativamente los perfiles alcaloideos de plantas que biosintetizan MIAs del tipo ibogano. La aplicación del protocolo al estudio fitoquímico de cuatro especies mexicanas de *Tabernaemontana* ilustró su estrecha relación quimiotaxonómica con *T. iboga* y *V. africana*, permitiendo las siguientes conclusiones: Por un lado, las marcadas discrepancias respecto de los usos etnobotánicos de las primeras y las segundas probablemente no se debe a factores químicos, sino socioculturales e históricos particulares de cada región, es decir México y África Central. Por otro lado, especies mexicanas de *Tabernaemontana* no solamente guardan un gran potencial para la producción comercial de las sustancias antiadictivas coronaridina, ibogamina, voacangina e ibogaína, sino también para el desarrollo de nuevos fármacos antimicrobianos basados, por ejemplo, en MIAs diméricos como la voacamina. La actividad antibiótico determinada en extractos de *T. alba* y *T. arborea* reafirma algunas aplicaciones externas del género en la medicina tradicional mexicana. Se comprobó que los compuestos esterificados de este conjunto de alcaloides estructuralmente relacionados pueden ser desmetoxicarbonilados directamente en extractos crudos mediante un procedimiento semisintético de un solo paso. Con vistas a la producción sustentable de alcaloides del tipo ibogano utilizando especies apocináceas, se hizo una amplia revisión de técnicas biotecnológicas y de cultivo *in vitro* potencialmente viables.

Referente a las perspectivas que surgen del conocimiento recopilado en esta tesis, se sugiere continuar con la determinación fitoquímica del gran número de especies de *Tabernaemontana* que existen en México, sobre todo las endémicas, así como profundizar en el desarrollo de protocolos de producción *in vitro* de alcaloides del tipo ibogano. La elucidación de la ruta biosíntetica de los últimos mediante estudios genómicos, transcriptómicos, proteómicos y metabolómicos merece especial atención, con el objetivo

final de permitir la sobreexpresión dirigida de enzimas claves, y por ende, asegurar la generación sustentable, económicamente viable e internacionalmente competitiva de nuevos agentes antiadictivos.

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