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ORIGINAL ARTICLE

Zornia latifolia: a smart drug being adulterated by *Stylosanthes guianensis*

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Abstract

Dried herbal preparations, based on "*Zornia latifolia*," are commonly sold on web, mainly for their supposed hallucinogenic properties. In this work, we demonstrate that these commercial products contain a different Fabacea, i.e., *Stylosanthes guianensis*, a cheaper plant, widely cultivated in tropical regions as a fodder legume. We were provided with plant samples of true *Zornia latifolia* from Brazil, and carried out a thorough comparison of the two species. The assignment of commercial samples was performed by means of micro-morphological analysis, DNA barcoding, and partial phytochemical investigation. We observed that *Z. latifolia* contains large amounts of flavonoid di-glycosides derived from luteolin, apigenin, and genistein, while in *S. guianensis* lesser amounts of flavonoids, mainly derived from quercetin, were found. It is likely that the spasmolytic and anxiolytic properties of *Z. latifolia*, as reported in traditional medicine, derive from its contents in apigenin and/or genistein.

Keywords Smart drugs · Morphologic analysis · DNA barcoding · Luteolin · Apigenin · Genistein

Introduction

Nowadays, the Internet web market is flooded with many different phytotherapic products, sold as dietary natural compounds, or as pharmacologic preparations; however, the control of their identity, and/or their efficiency is rather loose. At the same time, several eco- and smart drugs derived from vegetal sources have been introduced in the digital market as legal substitutes of abused drugs. In particular, a new generation of legal substitutes of cannabis has recently emerged: these products, often referred to as "herbal highs," are mixtures sold on the web under the brand names of "spices" (for instance, Spice gold, Silver, Dream, etc). The herbal components of vaunted "spices" were chosen because some of them were traditionally known as "marijuana substitutes," in order to obtain a cannabis-like effect, which increases their popularity and appeal to young people seeking new experiences. Such herbal mixtures may lead to potentially injurious drug combinations [1], and may even contain synthetic cannabinoids (JWH-018 and analogues, CP-47,497-C8; AB-CHMINACA, UR144; PB-22, etc.) that are hazardous to human health [2], giving rise to accidental overdosing, requiring hospitalization [3].

One of the most popular herbs in these mixtures is *Zornia latifolia* Sm., aka *Z. diphylla* (L.) Pers. (Fabaceae),[1] popularly known as *maconha brava* or white marijuana [7]. The common name given to this species refers to its pretentious effects similar to those of THC. The history of the supposed hallucinogenic effects of *Z. latifolia* probably dates back to Native Americans; in fact, it was included in Schultes & Hoffman's famous ethnical work, as a "Plant of the Gods" [8]. According

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[1] From a taxonomical point of view, Fortuna-Perez & Tozzi [4] established under synonymy of *Z. latifolia* Sm. the following taxa: *Z. gemella* Willd. ex Vogel (an invalid name), *Zornia gracilis* DC. (under *Z. diphylla* (L.) Pers. var. *gracilis* (DC.) Benth.), *Z. diphylla* (L.) Pers. var. *bernardinensis* Chodat & Hassl., *Z. maranhamensis* G. Don, and *Z. surinamensis* Miq.. These taxa were frequently identified in herbaria as *Z. latifolia*. Before this, *Zornia latifolia* (as *Z. latifolia* DC.) had already been considered as a variety of *Z. diphylla* (L.) Pers. by Bentham [5], but Mohlenbrock [6] reestablished the valid name for *Z. latifolia* Sm..

to The New York Botanical Garden, Herbarium Collection Sheet of *Z. latifolia* from Rondonia State, collected by Prance et al. 8917 (NY 01523113), leaves of *Z. latifolia* from Brazil are "dried and smoked as a hallucinogenic substitute for Cannabis" [9]. Finally, Schultes and Farnsworth [10] cited *Z. latifolia* among plants alleged to have hallucinogenic properties.

Conversely, it should be remarked that no psychoactive constituent has ever been isolated from this plant, and generally speaking, phytochemical studies of *Z. latifolia* are scant [11, 12]. Igwe et al. [12] report the presence of undefined alkaloids in the water extract of the plant, but they erroneously assign this species to family Solanaceae instead of Fabaceae.

Anyway, there are some reports of pharmacologic effects of the plant, and they seem mainly concern the intestinal smooth muscle. *Z. diphylla* has been used by the Otomi Indians of Querétaro (México) for its spasmolytic activity [13]. In the District of Kerala State, India, this species is employed in the treatment of cancer and fungal disease [14], for dysentery, and to induce sleep in children [15]. More recently the anticonvulsant activity of the methanolic extract of *Zornia diphylla* has been reported by Geetha et al. [16]. Juice from *Z. latifolia* has been used in French Guiana to calm inflamed intestines [17].

We bought dried herbal preparations of *Z. latifolia* ("Maconha brava") from different web retailers. However, our analyses showed that, in all cases, herbal samples did not correspond to *Z. latifolia*, but they were instead *Stylosanthes guianensis* (Aubl.) Sw., another legume forage native to South America. Also for this species, both chemical characterization and pharmacologic uses have been scarcely or not reported. We recently found, in the lipophilic fraction of *S. guianensis* extracts, some hydroxy-fatty acids with antiproliferative effect on HeLa and A431 tumor cells [18].

We compared the above material of *S. guianensis,* with autenthic samples of *Z. latifolia* collected in the field, using micro-morphological, molecular, and phytochemical analyses. These data could represent a reference for the correct identification of these plants from the pharmacognostic point of view, especially when they are combined in herbal mixtures sold in the Internet under generic brand names of "spice". In addition, the chemical fingerprint characterization firstly reported in this study for both these species, provides information on herbal products of concern to public health.

Materials and methods

Herbal samples and reference plant species

Dried plant material of *Stylosanthes guianensis* (Aubl.) Sw. was sold under the name of "*Zornia latifolia*" or "Maconha brava," on the following websites: Shaman's Garden Exotic Botanicals "Gaia's Delights" (www.shamansgarden.com) Chicago Illinois; "Bouncing Bear Botanicals" Lawrence, Kansas (www.bouncingbearbotanicals.com); and Herbalfire Botanics, USA (www.herbalfire.com).

Labels on packaging indicated that the products contained plants sampled harvested either from Perú and/or Brazil. This material consisted of aerial parts, in particular leaves and flowers, small branches, and some fruits and seeds. The pods present in one herbal mixture contained seeds that were collected, sown in pots, and grown in greenhouse at the Genoa University Botanical Garden, to obtain fresh plants used for species macro-morphological identification. Voucher samples of plants grown from seeds were determined and then deposited at the Herbarium of DISTAV (GE *sn*).

Dried samples of *Z. latifolia* Sm. were obtained from Fortuna-Perez collections deposited in Herbarium BOTU – Dept. Botânica/Instituto de Biociências de Botucatu, UNESP, Brazil. The plants were collected from natural populations of Minas Gerais and São Paulo States, Brazil. Voucher information for the studied material updated - Fortuna-Perez 834; Fortuna-Perez 2278 (BOTU).

Micromorphological analysis

Light microscope observations were carried out by a Leica M205 C stereomicroscope, coupled to EC3 camera and LAS EZ V1.6.0 image analysis software, and by a Leica DM 2000 transmission-light microscope, coupled to DFC 320 camera and IM 1000 and QWin software (Leica Microsystems, Wetzlar, Germany).

For bright field microscopy, representative median portions of the adult leaflets were processed. Samples of *S. guianensis* were fixed in formalin–acetic acid alcohol (FAA) for 24 h [19], dehydrated in an ethanol series, and embedded in JB4 resin. Cross sections were cut at 8-mm intervals, mounted serially, and stained with 0.05% Toluidine Blue O (TBO) in acetate buffer, pH 4.4, for 1 min, as a metachromatic stain [20]. Observations were made using a Leica DM 2000 optical microscope, equipped with Leica IM 500 image processing software 4.0.

Adult leaflets of *Z. latifolia* fixed in FAA for 24 h [21], in buffered neutral formalin (BNF) for 48 h [22], were placed under low vacuum to ensure penetration of the fixatives. Leaflets were then stored in 70% ethanol. The material was dehydrated through a *t*-butanol series [21], embedded in paraffin, serially sectioned with a rotary microtome, and then stained with safranin O and astra blue [23]. The samples were also embedded in plastic resin [24], and then sectioned. Transverse sections were cut at a thickness of 10–12 μm and stained with Toluidine Blue O (TBO) at pH 4 [25]. Samples were observed with an Olympus BX51 microscope (Olympus

America, Inc.), and images were taken using Kodak Pro Image 100 ASA film (Eastman Kodak, Rochester, NY, USA).

Scanning electron microscopy (SEM) observations were carried out using Vega3 Tescan type LMU microscope equipped with X-ray energy dispersive system EDS Apollo XSD (Tescan USA Inc., Cranberry Twp, PA, USA). Samples were fixed and dehydrated as reported above, and then they were critical-point-dried and mounted on aluminum SEM stubs. Stubs were sputter-coated with 10-nm gold or carbon. Plant fragments and sections with crystalline idioblasts were also elementally analyzed by EDS to verify crystal composition.

DNA barcoding analysis

The three samples were identified by the DNA barcoding approach to integrate morphological and chemical analyses [2]. Specifically, using the BLAST algorithm [26], we compared the barcode sequences obtained from our samples to reference data deposited in the international GenBank database (https://www.ncbi.nlm.nih.gov/). Genomic DNA was isolated from the three samples using the DNeasy Plant Mini kit (Qiagen, Milan, Italy) to obtain high-quality DNA, free of polysaccharides or other metabolites that might interfere with DNA amplification. The concentration of extracted DNA for each sample was estimated spectro-fluorometrically. DNA barcoding analysis was performed at three plastidial (*rbcL*–RuBisCo large subunit-, *matK*–maturase K-, and the intergenic spacer trnH-psbA) and one nuclear (ITS) loci. These loci represent standard DNA barcoding markers, generally used to characterize plant species: they have been adopted in many studies dealing with medicinal and toxic plants, see for example [27].

PCR amplification for each candidate marker was performed using puReTaq Ready-To-Go PCR beads (Amersham Bioscience, Italy) in a 25-μl reaction, according to the manufacturer's instructions. PCR cycles consisted of an initial denaturation for 7 min at 94 °C, 35 cycles of denaturation (45 s at 94 °C), annealing (30 s at 53 °C), extension (1 min at 72 °C), and final extension at 72 °C for 7 min. The *rbcL* was amplified using rbcL1F and rbcL4R primer pair [28]; the *matK* was amplified using KIM_1R and KIM_3F primers [29], while for non-coding regions trnH-psbA and ITS, the primer pair psbA-trnH [30] and ITS1-ITS4 [31] were respectively used. The PCR products obtained from reference species were submitted for sequence analysis to Macrogen Inc., Korea (www.macrogen.com). Heavy DNA strands were bi-directionally sequenced using an ABI 3730XL automated sequencer at Macrogen.

The resulting sequences were compared with the GenBank DNA database, using Basic Local Alignment Search Tool (BLAST) [26]. Each sequence was assigned to the plant species showing the maximum identity close to 100%, according to Barcode of Life Database Identification System (BOLD-IDS) guidelines (http://www.boldsystems.org/index.php/IDS_OpenIdEngine), and to the criteria adopted by Mezzasalma et al. [27]. Sequence data were submitted to the European Bioinformatics Institute of the European Molecular Biology Laboratory (EMBL-EBI).

Phytochemical analysis: preparation of the crude extract

In order to make a rigorous phytochemical comparison, dried samples of both *Z. latifolia* and *S. guianensis* were minced by means of an electric blender, and then extracted in the same way, i.e., with 90% aq. 2-propanol. Seven hundred fifty milliliters of solvent were employed for every 100 g of plant; the extraction was carried out at RT for 3 h under stirring. After filtration, the solutions were dried under vacuum at 38 °C. One milligram of crude extract was used for HPLC-MS analysis, while the rest of the extract underwent column chromatographic fractioning, and then NMR and MS analysis of a few selected fractions.

All the solvents and reagents employed for chemical analyses were purchased from VWR International S.r.l. (Milan, Italy), unless differently specified.

LC-MS and HR-ESI-MS

LC-MS analysis of crude extract was carried out in an Agilent 1100 HPLC-MSD Ion Trap XCT system, equipped with an electrospray ion source (HPLC-ESI–MS) (Agilent Technologies, Palo Alto, CA, USA). Separations were performed on a Symmetry C18 column 1 × 150 mm with 3-μm particle size (Waters Corporation, Milford, MA, USA). Eluents used were water (eluent A) and methanol (eluent B), both added with 0.1% formic acid. The gradient employed was: 15% eluent B for 3 min, then linear to 95% eluent B in 25 min and finally hold at 95% eluent B for other 15 min. The flow rate was set to 30 μL/min and the column temperature was set at 25 °C. The injection volume was 8 μL. Ions were detected in ion charged control with a target ion value of 200,000 and an accumulation time of 300 ms, using capillary voltage, 3300 V; nebulizer pressure, 15 psi; drying gas, 8 L/min; drying gas temperature, 325 °C; rolling averages, 2; averages, 5. Mass spectra were acquired in negative ionization mode, *m/z* 100–1000, consistent with expected mass charge ratios, and analyzed using integrated Agilent Data Analysis software (LC/MSD Trap Software).

HR-ESI-MS analysis of selected fractions was carried out in a Hybrid Quadrupole-Orbitrap mass spectrometer (Q Exactive, Thermo Fisher Scientific, Waltham, MA, USA) in negative ion mode. The acquisition parameters were set time by time in direct infusion analysis (DIA) to assess the

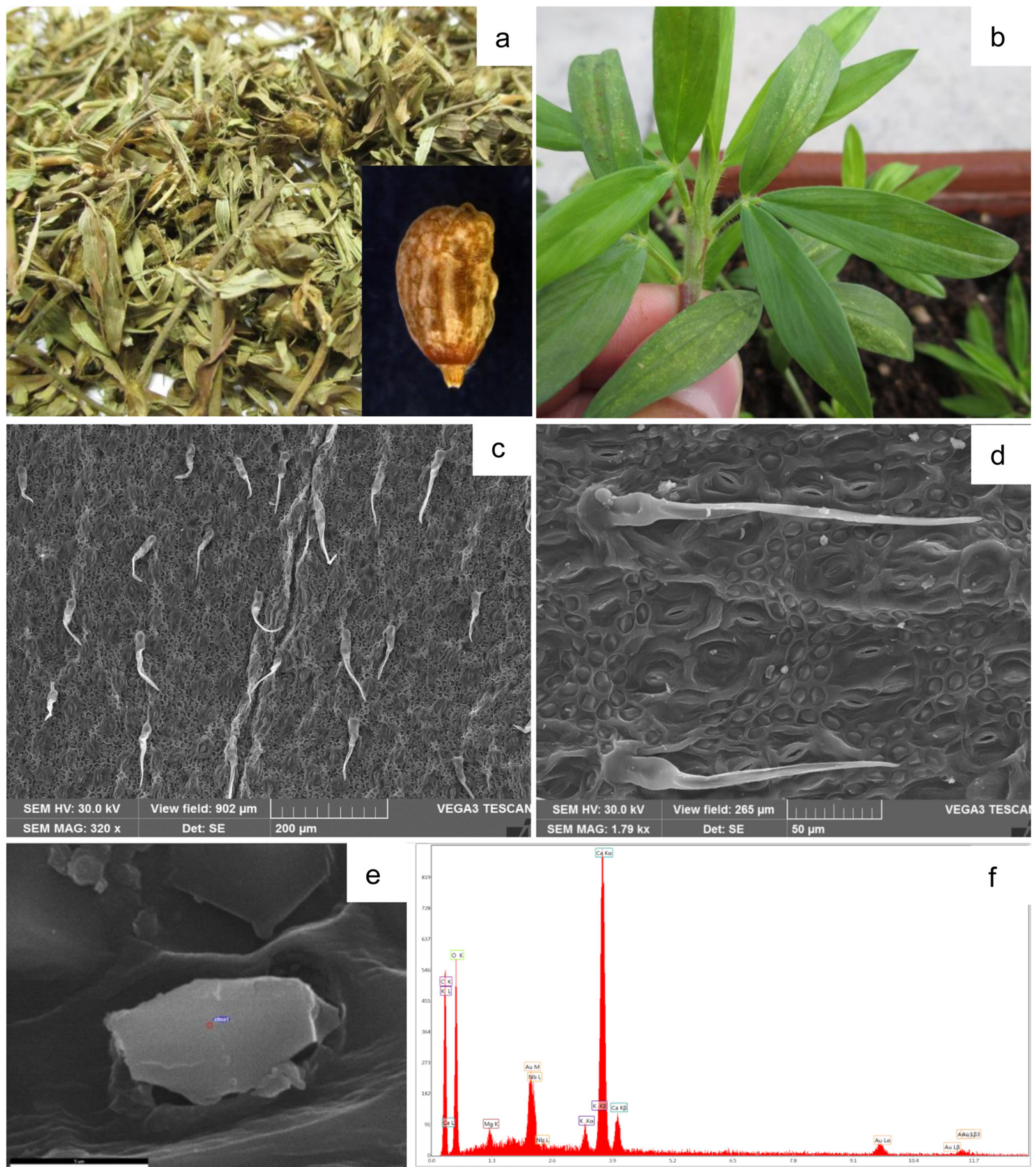

Fig. 1 *Stylosanthes guianensis*. **a** An herbal sample bought on Internet. In the inset: one fruit found in the package. **b** Plant with typical trifoliate leaves, grown in the Genoa Botanical Garden from seeds found in the commercial material. **c–e** Scanning electron microscopy (SEM) of the adaxial leaflet epidermis, showing abundance of stomata, trichomes, and collapsed crystalliferous idioblasts, at different magnification. **e** a single calcium oxalate crystal is shown. **f** SEM-EDS analysis of the crystal shown in **e**, where the calcium peak confirms the crystal composition

elemental composition. The calculations were performed using a tool integrated in the dedicated software (Thermo Xcalibur 4.0.27.10), and the calculated elemental formulas were unambiguous for all the compounds.

Preparative liquid chromatography

Crude extracts were fractionated by a medium pressure (MPLC) liquid chromatography system, consisting of an Alltech 426 HPLC pump (Grace Alltech, Columbia, MD, USA), equipped with a VWR LaPrep 3101 detector. Both direct-phase (Fluka silicagel 100, 15–25 μm) and reversed phase (Merck LiChroprep RP-18, 25–40 μm) modes were employed. In addition, gel-filtration chromatography on a Sephadex LH-20 stationary phase (GE Healthcare Life Sciences) was performed.

NMR analysis

NMR spectra were recorded on a Bruker Avance III 500 MHz instrument (Bruker Co. Italia, Milan, Italy), operating at 499.802 MHz (^{1}H) and 125.687 MHz (^{13}C). Solvent peaks were used as internal standards, and for CD_3OD, this was set at 3.32 ppm (^{1}H) and 49.0 ppm (^{13}C).

Results

Morphological analysis

Commercial samples of *S. guianensis* (Fig. 1a) and herbarium samples of *Z. latifolia* (Fig. 2a) were analyzed by both microscopic and molecular techniques to confirm identification at the species level.

S. guianensis leaves are trifoliate (Fig. 1b), and the fruit is a small one-seeded pod with a reduced rostrum (Fig. 1a inset, and Fig. 3a). In mature leaflets both adaxial and abaxial epidermis shows the occurrence of paracytic stomata, uni- and multiseriate hairs, broad at the base and tapering above, and a large amount of crystalliferous idioblasts (Fig. 1c, d). Collapsed crystal idioblasts form networks, the interstices of which are occupied by accompanying epidermal cells and single stomates (adaxial surface) or clusters of stomates (abaxial surface). SEM-EDS analysis confirmed the elemental composition of crystals as calcium oxalate (Fig. 1e, f), in accordance with data reported by Brubaker and Horner [32]. In transversal sections of leaflets, mucilaginous idioblasts in the palisade parenchyma (Fig. 4a, b, arrowheads), proximal to the adaxial surface, and idioblasts containing phenolic substances in the abaxial one were detected (Fig. 4a, b, arrows). These features are in agreement with previous anatomical observations on different *Stylosanthes* species, including *S. guianensis* [33].

Zornia latifolia is morphologically characterized by leaves with two leaflets (Fig. 2b), bracteoles linear to lanceolate, and loment fruit, with 4–8 articles, pubescent to villous, with retroserly hairy bristles 2–3 mm long (Fig. 3b, c). Both leaflet epidermis show the occurrence of anisocytic stomata; a few scattered uniseriate trichomes are mainly located on the vein and on the leaflet margins (Fig. 2c, d). The secretory structures

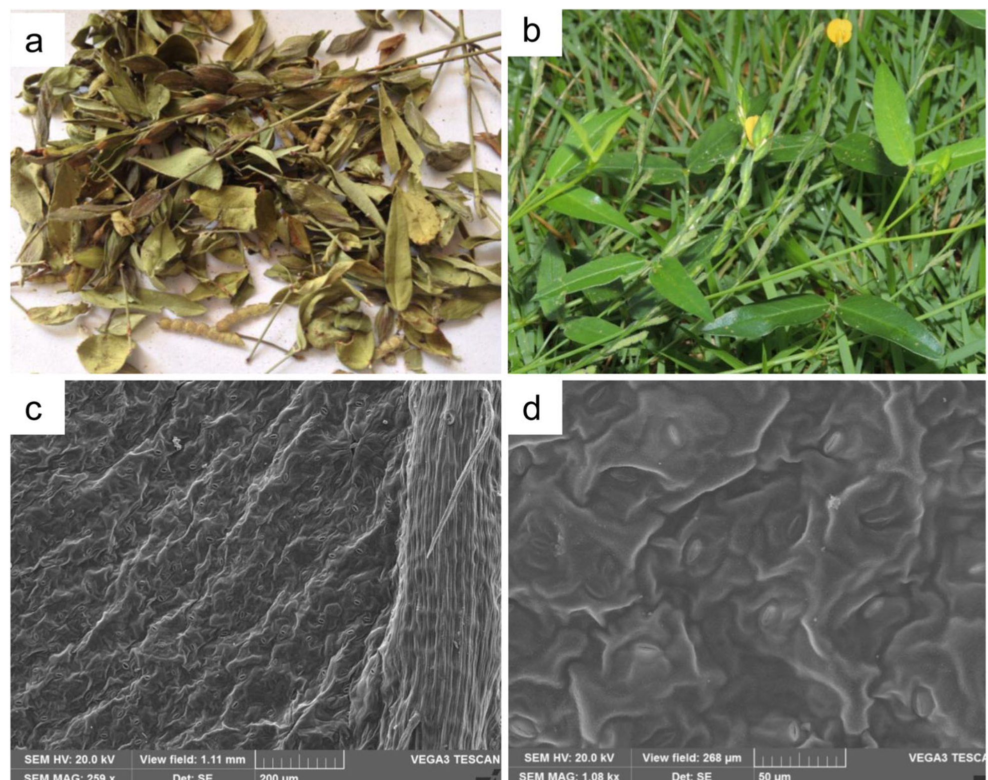

Fig. 2 *Zornia latifolia.* **a** Dried sample from Herbário BOTU, Brazil. **b** Plant with bifoliate leaves (from Brazil). **c** Abaxial leaflet epidermis with abundant stomata, and a few scattered uniseriate trichomes, mainly located on the vein. **d** Adaxial leaflet epidermis, with abundant stomata

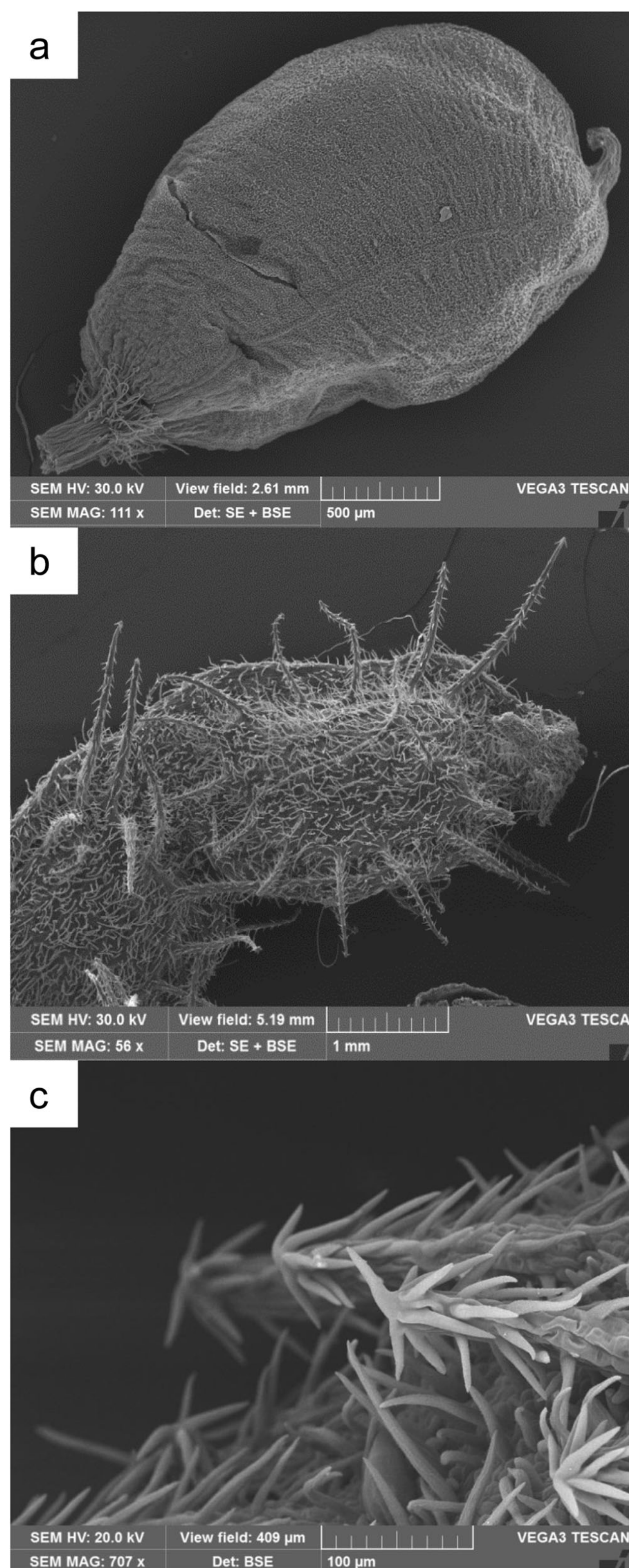

Fig. 3 **a** SEM comparison between one-seeded pod of *S. guianensis* and **b** *Z. latifolia* articles of the loment, with **c** retroserly hairy bristles

found in leaflets are mucilage epidermis and cavities (Fig. 4c, d (arrow)) and idioblasts secreting phenolic compounds (Fig. 4c), present in the mesophyll, as previously reported by Fortuna-Perez et al. [34].

Molecular results

Molecular identification through the DNA barcoding approach involved the use of four different markers: *rbcL*, *matK*, trnH-psbA, and ITS (Table 1).

To date (November 2017), only ITS reference sequences for *Z. latifolia* are available in GenBank, while other *Zornia* species have been characterized for all but trnH-psbA markers. Given these assumptions, the morphologically characterized sample of *Z. latifolia* used in this study as comparison for phytochemical and genetics analyses was correctly assigned (ID) by ITS, therefore confirming the reliability of DNA barcoding in identifying this species.

HPLC-MS

The HPLC-MS chromatograms of the two plants crude extracts, obtained in ESI^- ionization mode, are reported in Fig. 5a as total ion currents (TIC). The detailed comparison of the components found analyzing these chromatograms are reported below.

Zornia latifolia (*Zl*)

In the chromatogram of *Zl* (Fig. 5a, black line), two intense broad peaks were found, centered at retention time (RT) 23.9 and 25.3 min. The former peak (23.9 min) showed a prominent mass peak at *m/z* 593 $[M-H]^-$ (Fig. 5b), and the latter (25.3 min) at *m/z* 577 $[M-H]^-$. Preparative liquid chromatography allowed us to isolate the corresponding metabolites, and to investigate them by means of 1- and 2-D NMR: they turned out to be all flavonoid di-glycosides.

The metabolites corresponding to the peak at 23.9 min were flavonoids derived from the flavone luteolin, in particular luteolin rutinoside (α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranose), relative molecular mass Mr = 594. This finding was confirmed by HR-ESI^--MS (Fig. 5b): peak at *m/z* 593.15118 $[M-H]^-$ ($C_{27}H_{29}O_{15}^-$ requires 593.15116, Δppm = 0.0154), and MS/MS fragment ion at 285.04044 ($C_{15}H_9O_6^-$ requires 285.04046, Δppm = 0.0894), the latter one corresponding to the aglycone luteolin. The attachment position of the sugars on the flavonoid skeletons was not investigated in detail, also because NMR spectra showed that they occur as a mixture of different positional isomers, among which one is likely luteolin 4'-*O*-α-rutinoside [35].

The chromatographic peak at RT 25.3 min corresponded to flavonoids derived from the flavone apigenin or the isoflavone genistein, again as glyco-rhamnosides (Mr = 578); HR-ESI^--MS showed a peak at *m/z* 577.1567 $[M-H]^-$ ($C_{27}H_{29}O_{14}^-$ requires 577.1563, Δppm = 0.332).

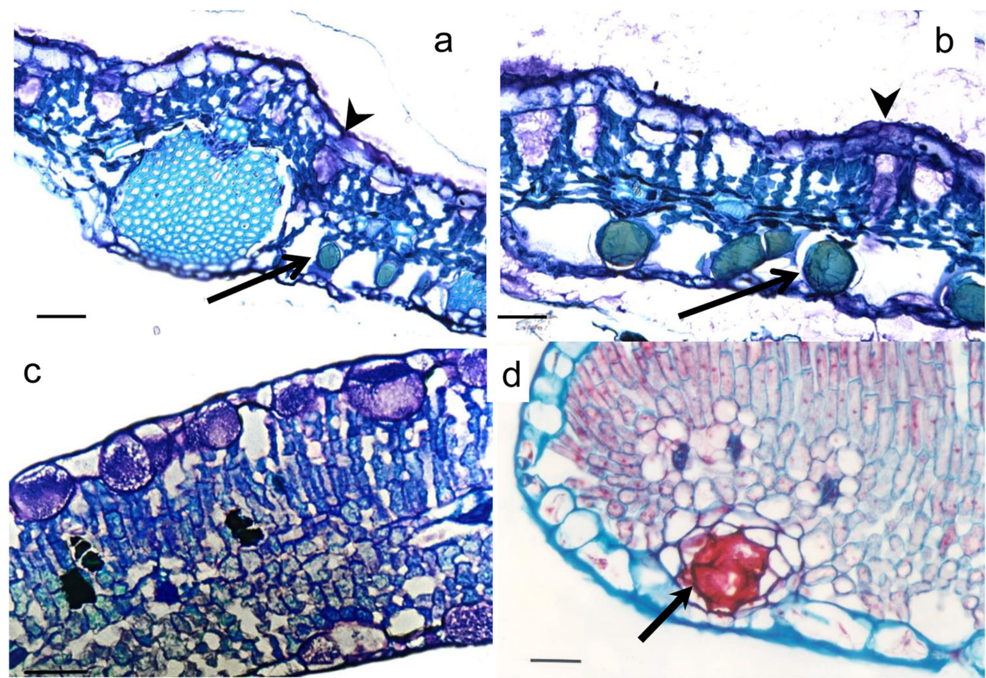

Fig. 4 **a–d** Light microscopy of leaflet cross-sections of *S. guianensis* (**a**–**b**) and *Z. latifolia* (**c**–**d**). *S. guianensis* (**a**-**b**) shows phenolic compounds stained in blue green (arrows) by TBO, while mucilage idioblasts appear purple (arrowheads). **c** *Z. latifolia* leaflet showing phenolic compounds in the mesophyll (green) and secretory epidermis (purple) by TBO staining. **d** Detail of a secretory cavity containing mucilage (arrow), stained by Safranin O and Astra blue. Bars A, 100 μm; B–D, 50 μm

Lesser amounts of mono-glycosylated luteolin, both as glycoside, Mr = 448 (*m/z* 447.0936, $C_{21}H_{19}O_{11}^-$ requires 447.0932, Δppm = 0.884) and as rhamnoside, Mr = 432 (*m/z* 431.0986, $C_{21}H_{19}O_{10}$ requires 431.0983, Δppm = 0.696) could be isolated; however, the corresponding masses could not be evidenced in the chromatogram of the crude extract, probably owing to their low concentration. Luteolin, apigenin, and genistein were also found as aglycons, again in small amounts (when compared to the corresponding diglycosides).

Stylosanthes guianensis (*Sg*)

The chromatogram of *Sg* (Fig. 5a, red line), does not show any intense peak (when compared to that of *Zl*), but rather a series of low-intensity bands. As a consequence, only a few metabolites could be identified, after column chromatography fractioning and NMR analysis. According to our data, the main flavonoid of *Sg* seems to be the flavonol quercetin, mainly found as glyco-rhamnoside (Mr = 610). HR-ESI$^-$-MS showed a peak at *m/z* 609.14618 $[M\text{-}H]^-$, $C_{27}O_{16}H_{29}$ requires 609.14611, Δppm = 0.118. In the chromatogram of the crude extract, the peak at *m/z* 609 is visible at RT 23.3 min. A small amount of quercetin 3-O-glycoside (*m/z* = 463.0885 $[M\text{-}H]^-$, $C_{21}O_{12}H_{19}$ requires 463.08820, Δppm = 0.671) could be isolated by preparative chromatography, but the correspondent mass peak could not be seen in the HPLC of the crude extract. Also quercetin-3-methyl ether was isolated from the crude extract. In addition, *Sg* contained several simple phenols: among the others, we could isolate significant amounts of free caffeic acid.

Other peaks in the *Sg* chromatogram are found at 25.6 min (peak at *m/z* 625), and at 21.6 min (*m/z* 595); both of them could not be assigned to a specific metabolite. A summary of the main chromatographic peaks, with the corresponding more abundant mass peaks of the two extracts, is reported in Table 2.

Discussion and conclusion

Macro- and micro-morphologic analysis combined with molecular techniques have already proved to be effective to identify the herbal preparations, in commercial mixtures [2]. In the present case, we could find out that the herbal material sold on the web under the name of "*Zornia latifolia*,"was actually *Stylosanthes guianensis*, a different fodder Fabacea. Considering the dried herbal material, the main diagnostic feature in mature leaflets of *Sg* was the abundance of crystalliferous idioblasts on both surfaces forming a network, the interstices of which are occupied by single stomates (adaxial) or clusters of stomates (abaxial). This feature is easy to check also at low magnification. On the other hand, *Zl* (from herbarium samples) showed leaflet epidermis in which crystalliferous idioblasts were completely missing. When fruits are also present, it can be seen that they are very different from each other: those of *Sg* are smooth one-seeded pods, while those of *Zl* are loments, with 4–8 articles, pubescent to villous.

Molecular analysis, combined with morphological investigation, supported the identification of plant products sold on line. As reported in [2], the preparation of herbal blends does

Table 1 BLAST identification of samples collected in this study using four DNA barcoding regions. Identification results were provided as the species name showing the maximum nearest matches (maximum identity). The BLAST maximum identity matches were grouped into two categories: (1) 'identified' (ID), when the maximum identity scores corresponded to the queried species; and (2) 'not identifiable' (NI), when the BLAST search returned the same maximum identity score with more than one species that might correspond or not to that queried or when the maximum identity scores were consistently below 100% and the correct species did not occur among the entries. The presence of sequence of the species query was indicated with OK (presence) or NO (absence)

Samples	Morphological identification	*rbcL*			*matK*			trnH-psbA			ITS		
		GenBank	Blast-ID	Ident	GenBank	Blast-ID	Ident	GenBank	Blast-ID	Ident	GenBank	Blast-ID	Ident
S1	*Zornia latifolia*	NO (NI)	*Zornia cantoniensis*	99%	NO (NI)	*Zornia bracteata*	100%	NO (NI)	*Poiretia latifolia*	80%	OK (ID)	*Zornia latifolia*	100%
			Zornia bracteata	99%		*Zornia sericea*	100%		*Amicia glandulosa*	78%		*Zornia confusa*	99%
			Pterocarpus floribundus	97%		*Zornia orbiculata*	99%		*Amicia medicaginea*	78%		*Zornia sericea*	99%
S2	*Stylosanthes guianensis*	NO (NI)	*Stylosanthes biflora*	99%	OK (ID)	*Stylosanthes guianensis*	100%	NO (NI)	*Arachis hypogaea*	93%	OK (ID)	*Stylosanthes guianensis*	100%
			Arachis hypogaea	98%		*Stylosanthes gracilis*	99%		*Stylosanthes fruticosa*	93%		*Stylosanthes hippocampoides*	99%
			Chapmannia floridana	98%		*Stylosanthes hamata*	99%		*Dalbergia hupeana*	96%		*Stylosanthes gracilis*	99%
S3	*Stylosanthes guianensis*	NO (NI)	*Stylosanthes biflora*	99%	OK (ID)	*Stylosanthes guianensis*	100%	NO (NI)	*Stylosanthes fruticosa*	93%	OK (ID)	*Stylosanthes guianensis*	100%
			Arachis hypogaea	98%		*Stylosanthes gracilis*	99%		*Arachis hypogaea*	92%		*Stylosanthes hippocampoides*	99%
			Chapmannia floridana	98%		*Stylosanthes hamata*	99%		*Dalbergia balansae*	94%		*Stylosanthes gracilis*	99%

S1 has been sent from Herbário BOTU-Depto Botânica-Instituto de Biologia, Brazil; S2 and S3 were bought at the web market under the name "*Zornia latifolia*" (S2: www.shamansgarden.com, S3: www.bouncingbearbotanicals.com). See Materials and methods *Herbal samples and reference plant species*

Accession numbers in European Bioinformatics Institute of the European Molecular Biology Laboratory (EMBL -EBI) are: for sample S1 (RBCL, LT970860; MATK, LT970854; TRNH-PSBA, LT970857; ITS, LT970851), for sample S2 (RBCL, LT970861; MATK, LT970855; TRNH-PSBA, LT970858; ITS, LT970852) and for sample S3 (RBCL, LT970862; MATK, LT970856; TRNH-PSBA, LT970859; ITS, LT970853)

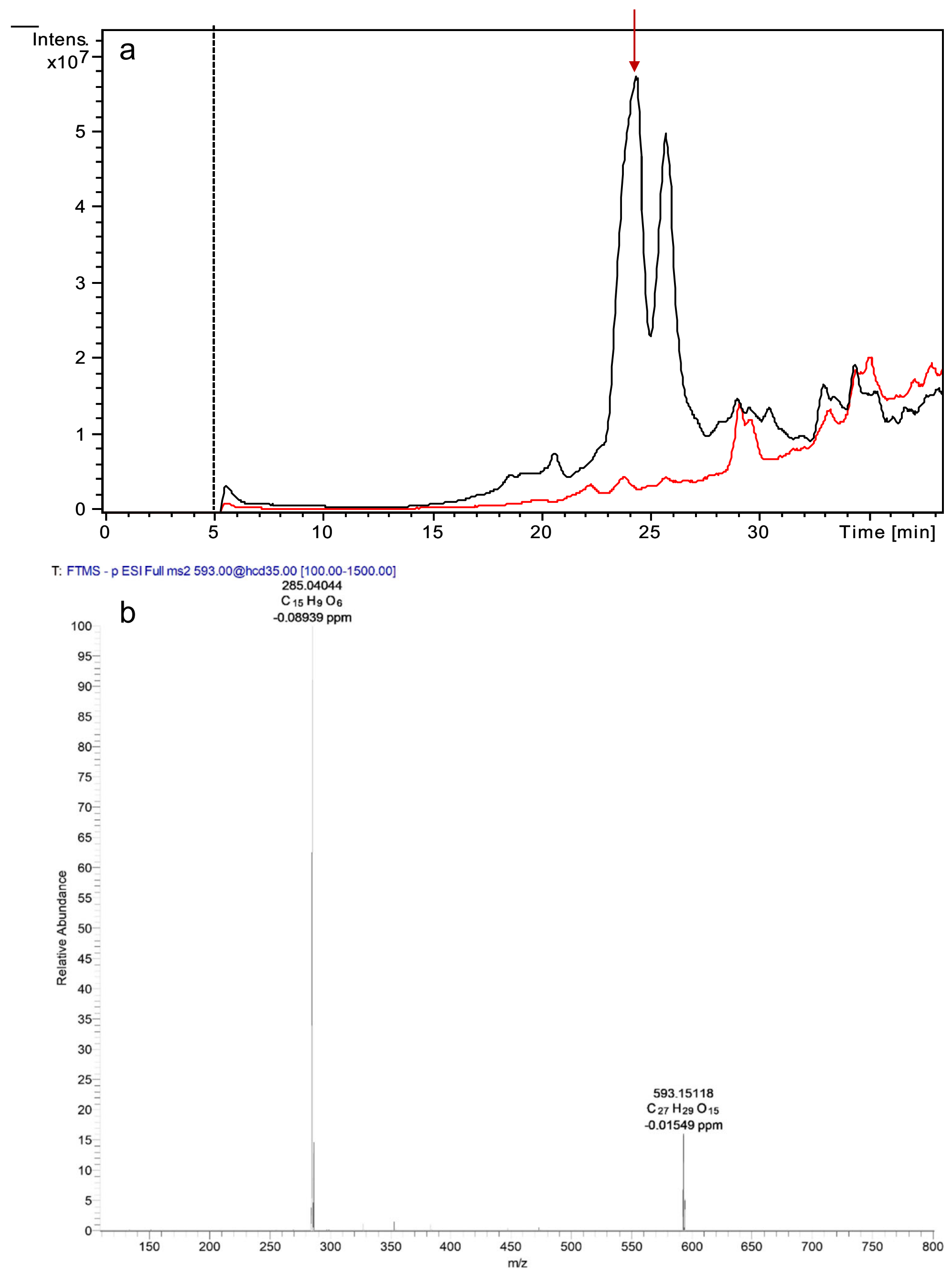

Fig. 5 **a**–**b** LC-MS analysis of the crude extracts. **a** Total ion currents (TIC, ESI⁻) of *Zornia latifolia* (black line), and of *Stylosanthes guianensis* (red line). The arrow corresponds to the RT where the mass spectrum of Fig. 5b was recorded. **b** MS/MS HR spectrum (ESI⁻) of the chromatographic peak at 23.9 min: the fragment ion at *m/z* 285 corresponds to $[M-H]^-$ of the aglycone luteolin

Table 2 Main mass peaks (*m/z*, ESI⁻) in the HPLC-MS chromatograms of *Zornia latifolia* (*Zl*) and *Stylosanthes guyanensis* (*Sg*). For chromatographic conditions, see Experimental Section

RT (min)	*Zl* main peaks, *m/z*	*Sg* main peaks, *m/z*
16.7	611 (ND)	–
21.6	–	595 undetermined quercetin glycoside
23.3	–	609 quercetin glyco-rhamnosides
23.6	–	651, 629, 613, 607 (all ND)
23.9	593 luteolin glyco-rhamnosides	–
25.3	577 apigenin and genistein glyco-rhamnosides	–
25.6	–	625 (ND)

ND not determined

not affect the success rate of DNA barcoding, for which small plant fragments are needed. However, a limit of DNA barcoding approach in plants is the availability of reference databases. The accessions deposited in GenBank still lack of some pivotal sampling details [36, 37]. This deficiency has been overcome by integrating molecular and morphological identification with chemical analysis. The combination of these three approaches validates the recognition and characterization of products sold online.

From a phytochemical point of view, the differences between the two plants are significant: the high concentration of luteolin, apigenin, and genistein di-glycosides (rutinosides) is a good marker of *Zl*. On the other hand, *Sg* contains rather low amounts of different flavonoids, mainly derived from quercetin.

It is therefore evident that in this case the web market distributes herbal products that do not match the specification list. In addition, not only the effects on health of such products are not known, but they do not even respond to the consumers' demand. At present, given the paucity of scientific data on both chemical composition and pharmacological activity of these two plants, their use as legal alternative to Cannabis is not recommended.

Our study constitutes a first investigation aimed at filling this lack of knowledge. We have found a significant content of apigenin and genistein in the *Zl* extract, mainly as rutinosides, but also as free aglycones. These constituents could be related to the anxiolytic effects of this plant, in agreement with data reported by Viola et al. [38] for *Matricaria recutita*, and by Suresh & Anupam [39] for *Turnera aphrodisiaca*. In addition, Salgueiro et al. [40] have reported that some natural flavonoids, in particular chrysin and apigenin bind specifically to benzodiazepine sites of $GABA_A$ receptor, exerting anxiolytic effects. As concerns genistein, Rodrìguez-Landa et al. [41] reported the anxiolytic-like effect of this flavonoid in rats, and Huo et al. [42] suggest the potential application of genistein for the treatment of insomnia.

Further studied are in progress to confirm if apigenin and/or genistein are responsible for the Cannabis-like effects of *Zornia latifolia*.

References

1. Zuba D, Byrska B, Maciow M (2011) Comparison of "herbal highs" composition. Anal Bioanal Chem 400:119–126. https://doi.org/10.1007/s00216-011-4743-7.
2. Cornara L, Borghesi B, Canali C, Andrenacci M, Basso M, Federici S, Labra M (2013) Smart drugs: green shuttle or real drug? Int J Legal Med 127(6):1109–1123
3. Auwärter V, Dresen S, Weinmann W, Müller M, Pütz M, Ferreirós N (2009) "Spice" and other herbal blends: harmless incense or cannabinoid designer drugs? J Mass Spectrom 44(5):832–837. https://doi.org/10.1002/jms.1558
4. Fortuna-Perez AP, Tozzi AMGA (2011) Nomenclatural changes for *Zornia* (Leguminosae, Papilionoideae, Dalbergieae) in Brazil. Novon 21(3):331–337. https://doi.org/10.3417/2010040
5. Bentham G (1859) Papilionaceae. Pp. 80–85 in C. F. P. de Martius & A. G. Eichler (editors), Flora Brasiliensis, Vol. 15, Parte 1. F. Fleischer, Leipzig
6. Mohlenbrock R (1961) A monograph of the leguminous genus *Zornia*. Webbia 16(1):1–141. https://doi.org/10.1080/00837792.1961.10669720
7. Sedefov R, Gallegos A, King L, Lopez D, Auwarter V, Hughes B, Griffiths P (2009) Understanding the spice phenomenon. European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) Portugal
8. Schultes RE, Hofmann A (1979). Plants of the Gods, McGraw-Hill, New York. Reprinted in 1992, Healing arts, Rochester
9. Von Reis S, Lipp FJ (1982) New plant sources for drugs and foods from the NY botanical garden herbarium. Harvard University Press
10. Schultes RE, Farnsworth NR (1980) Ethnomedical, botanical and phytochemical aspects of natural hallucinogens. Bot Mus Leafl Harv Univ 28(2):123–214
11. López J (1981) Isolation of coumarin in *Zornia diphylla* L. Ing Cienc Quim 5:96–97
12. Igwe SA, Okawa ANC, Akunyili DN (2001) Preliminary phytochemical and pharmacological studies of *Zornia latifolia* extracts. Journal of Health and Visual Science 3:12–19
13. Rojas A, Rojas JI et al (1999) Spasmolytic activity of some plants used by the Otomi Indians of Queretaro (Mexico) for the treatment of gastrointestinal disorders. Phytomedicine 6(5):367–371. https://doi.org/10.1016/S0944-7113(99)80061-0
14. Arunkumar R, Ajikumaran Nair S, Subramoniam A (2012) Effectiveness of *Zornia diphylla* (L.) Pers, against fungal diseases. Ann Phytomed 1:81–89
15. Khare CP (2007) Indian medicinal plants: an illustrated dictionary. Springer-Verlag, Heidelberg
16. Geetha KM, Bhavya S, Murugan V (2012) Anticonvulsant activity of the methanolic extract of whole plant of *Zornia diphylla* (Linn) Pers. J Pharm Res 5(7):3670–3672
17. DeFilipps RA, Maina SL, Crepin J (2004) Medicinal plants of the Guianas (Guyana, Surinam, French Guiana). Natural Museum of Natural History. Smithsonian Institution, Washington DC
18. Clericuzio M, Burlando B, Borghesi B, Salis A, Damonte G, Ribulla S, Cornara L (2017) Antiproliferative hydroxy-fatty acids from the fodder legume *Stylosanthes guianensis*. J Pharm Biomed Anal 141:157–164
19. Pearse AG (1985) Histochemistry, Theoretical and applied, analytical technology. 4th Ed. Churchill Livingstone, New York
20. O'Brien TP, Feder N, Mccully ME (1964) Polychromatic staining of plant cell walls by toluidine blue O. Protoplasma 59:368–373
21. Johansen DA (1940) Plant microtechnique. McGraw–Hill Book Co., New York
22. Lillie RD (1965) Histopathologic technic and practical histochemistry. McGraw–Hill Book Co, New York
23. Gerlach D (1969) Botanische Mikrotechnik: Eine Einführung. Georg Thieme, Stuttgart
24. Meira RMSA, Martins FM (2003) Técnica de inclusão de material herborizado em historesina. Rev Árvore 27(1):109–112. https://doi.org/10.1590/S0100-67622003000100015
25. Vidal BC (1977) Acid glycosaminoglycans and endochondral ossification: microespectrophotometric evaluation and macromolecular orientation. Cell Mol Biol 22:45–64
26. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215(3):403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

27. Mezzasalma V, Ganopoulos I, Galimberti A, Cornara L, Ferri E, Labra M (2017) Poisonous or non-poisonous plants? DNA-based tools and applications for accurate identification. Int J Legal Med 131(1):1–19. https://doi.org/10.1007/s00414-016-1460-y
28. Fay MF, Bayer C, Alverson WS, de Bruijn AY, Chase MW (1998) Plastid rbcL sequence data indicate a close affinity between Diegodendron and Bixa. Taxon 47(1):43–50. https://doi.org/10.2307/1224017
29. Dunning LT, Savolainen V (2010) Broad-scale amplification of matK for DNA barcoding plants, a technical note. Bot J Linn Soc 164(1):1–9. https://doi.org/10.1111/j.1095-8339.2010.01071.x
30. Newmaster SG, Ragupathy S, Janovec J (2009) A botanical renaissance: state-of the- art DNA barcoding facilitates an automated identification technology system for plants. Int J Comput App Technol 35(1):50–60. https://doi.org/10.1504/IJCAT.2009.024595
31. Kress WJ, Wurdack KJ, Zimmer EA, Weigt LA, Janzen DH (2005) Use of DNA barcodes to identify flowering plants. Proc Natl Acad Sci U S A 102(23):8369–8374. https://doi.org/10.1073/pnas.0503123102
32. Brubaker CL, Horner HT (1989) Development of epidermal crystals in leaflets of *Stylosanthes guianensis* (Leguminosae; Papilionoideae). Can J Bot 67(6):1664–1670. https://doi.org/10.1139/b89-210
33. Da Silva Matos D, Leme FM, Dias ES, Arruda RCO (2013) Anatomia foliar de três espécies de *Stylosanthes* SW. e sua associação com a composição e formação potencial de fitobezoares em bovinos. Ciência Rural 43(11):2049–2055. https://doi.org/10.1590/S0103-84782013001100021
34. Fortuna-Perez AP, Castro MM, Tozzi AMGA (2012) Leaflet secretory structures of five taxa of the genus *Zornia* J.F. Gmel. (Leguminosae, Papilionoideae, Dalbergieae) and their systematic significance. Plant Syst Evol 298(8):1415–1424. https://doi.org/10.1007/s00606-012-0647-z
35. Pushpa B, Dayal R (1993) A flavone glycoside from *Dalbergia stipulacea* leaves. Phytochemistry 33(3):731–732
36. De Mattia F, Bruni I, Galimberti A, Cattaneo F, Casiraghi M, Labra M (2011) A comparative study of different DNA barcoding markers for the identification of some members of Lamiaceae. Food Rev Int 44(3):693–702. https://doi.org/10.1016/j.foodres.2010.12.032
37. De Mattia F, Gentili R, Bruni I, Galimberti A, Sgorbati S, Casiraghi M, Labra M (2012) A multi-marker DNA barcoding approach to save time and resources in vegetation surveys. Bot J Linn Soc 169:518–529
38. Viola H, Wasowski C, Stein MLD, Wolfman C, Silveira R, Dajas F, Medina JH, Paladini AC (1995) Apigenin, a component of *Matricaria recutita* flowers, is a central benzodiazepines receptors-ligand with anxiolytic effects. Planta Med 61(03):213–216. https://doi.org/10.1055/s-2006-958058
39. Suresh K, Anupam S (2006) Apigenin: the anxiolytic constituent of *Turnera aphrodisiaca*. Pharm Biol 44(2):84–90
40. Salgueiro JB, Ardenghi P, Dias M, Ferreira MBC, Izquierdo I, Medina JH (1997) Anxyolitic natural and synthetic flavonoid ligands of the central benzodiazepine receptor have no effect on memory tasks in rats. Pharmacol Biochem Behav 58(4):887–891. https://doi.org/10.1016/S0091-3057(97)00054-3
41. Rodrìguez-Landa JF, Hernàndez-Figueroa JD, Hernandez-Calderón BC, Saavedra M (2009) Anxiolytic-like effect of phytoestrogen genistein in rats with long-term absence of ovarian hormones in the black and white model. Prog Neuro-Psychopharmacol Biol Psychiatry 33(2):367–372. https://doi.org/10.1016/j.pnpbp.2008.12.024
42. Huo X-J, Liu W, Qiu M-H, Huang Z-L, Qu W-M (2012) Genistein induces non-rapid eye movement sleep in mice. Sleep Biol Rhytms 10(4):278–286. https://doi.org/10.1111/j.1479-8425.2012.00571.x