Use of Anatomical, Chemical, and Molecular Genetic Characteristics in the Quality Control of Medicinal Species: A Case Study of Sarsaparilla (*Smilax* spp.)[1]

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Species of the genus *Smilax*, popularly known as sarsaparilla, are used in folk medicine as a tonic, an anti-rheumatic, and an anti-syphilis treatment, and are sold in Brazilian drugstores without any quality control regarding their origin and efficacy. The origin of the material is mainly based on wild extraction. Quality control of herbal drugs should include a more reliable identification of the source involving characterization and definition of their anatomical and chemical characteristics. The current study aimed to verify whether the combined use of anatomical, chemical, and molecular genetic characteristics might be useful in the quality control of medicinal plants, specifically the sarsaparilla sold in compounding drugstores in the state of São Paulo, Brazil. Root samples were subjected to conventional light microscopy and scanning electron microscopy. To determine the chemical profile, thin-layer chromatography (TLC) was applied to ethanol extracts of the roots. The chemical profile of the chemical material sold in stores was compared with the previously determined profiles of medicinal *Smilax* species (*S. goyazana*, *S. rufescens*, *S. brasiliensis*, *S. campestris*, *S. cissoides*, *S. fluminensis*, *S. oblongifolia,* and *S. polyantha*). Although there was considerable similarity between the anatomical structure of the commercial sarsaparilla and the structure reported in the literature for the *Smilax* species, there were differences in the phloem organization and in the presence of a series of idioblasts containing raphides, phenolic idioblasts, and metaxylem in the center of the plant structure. TLC analysis of the commercial ethanol extracts revealed spots with colors ranging from yellow to green. In addition, the same spots showed components with the same retention factor (Rf), indicating chemical similarity between the different samples. However, the distribution pattern of the spots,

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as well as the Rf of the commercial samples, differed from those obtained for the eight species of *Smilax*, which were very similar to each other. Comparing the groups examined in the present study with regard to microsatellite markers and DNA barcoding revealed that commercial sarsaparilla is genetically different from the eight species of *Smilax* known for their medicinal properties in Brazilian ethnobotanical surveys. This seriously calls into question their effectiveness. This case study of sarsaparilla demonstrates the utility of anatomical, chemical, and molecular genetic characteristics in the quality control of medicinal plants.

As espécies do gênero *Smilax*, conhecidas popularmente como salsaparrilha, são empregadas na medicina popular como fortificante, contra o reumatismo e anti-sifilítico e são vendidas em farmácias brasileiras sem que exista um controle de qualidade de sua origem e eficácia. A origem do material comercializado no Brasil é baseada principalmente no extrativismo. O controle de qualidade de plantas medicinais deve basear a identificação da droga vegetal em fontes confiáveis que envolvam sua caracterização anatômica e química. O objetivo do trabalho foi verificar se o uso combinado de características anatômicas, químicas e genético-moleculares poderiam ser úteis no controle de qualidade de plantas medicinais, usando como exemplo, a salsaparrilha vendida em farmácias do Estado de São Paulo, Brazil. Amostras de raízes foram submetidas à técnicas convencionais de microscopia de luz e microscopia eletrônica de varredura. Para determinar o perfil químico dos extratos etanólicos das raízes, foi utilizada cromatografia em camada delgada (TLC). A composição química do material comercializado foi comparada com os perfis químicos de espécies de *Smilax* previamente identificadas (*S. goyazana* A. De Candolle, *S. rufescens* Grisebach, *S. brasiliensis* Sprengel, *S. campestris* Grisebach, *S. cissoides* Martius ex Grisebach, *S. fluminensis* Steudel, *S. oblongifolia* Pohl ex Grisebach and *S. polyantha* Grisebach). Embora haja uma grande semelhança entre a estrutura anatômica da salsaparrilha comercializada e a descrita na literatura para as espécies de *Smilax*, foram observadas diferenças na organização do floema, na presença de idioblastos em série contendo ráfides, idioblastos fenólicos e presença de elementos de metaxilema no centro da estrutura radicular. As análises em TLC dos extratos etanólicos do material comercializado revelaram manchas com cores que variam de amarelo a verde. Além disso, as mesmas manchas mostraram que seus componentes apresentam o mesmo índice de retenção (Rf), indicando semelhança química entre as diferentes amostras comercializadas. No entanto, o padrão de distribuição das manchas, assim como o Rf das amostras comerciais, diferiram daqueles obtidos das oito espécies de *Smilax* identificadas, que por sua vez, eram muito semelhantes entre si. Comparando-se os grupos analisados no presente estudo em relação aos marcadores microssatélites e *DNA barcoding* observou-se que a salsaparrilha comercial é geneticamente diferente das oito espécies de *Smilax* conhecidas por suas propriedades medicinais em levantamentos etnobotânicos brasileiros, o que torna questionável a eficácia do material comercializado, uma vez que o perfil químico de tais amostras comerciais também é diferente. Sendo assim, através deste estudo de caso envolvendo a salsaparrilha, foi possível verificar que a utilização conjunta de características anatômicas, químicas e genético- moleculares é eficiente no controle de qualidade de plantas medicinais.

Key Words: Thin-layer chromatography, histology, ethnobotany, microsatellite markers, DNA barcoding, *Smilax*.

Introduction

In Brazil, the use of the medicinal *Smilax* species (Smilacaceae, Liliales), popularly known as sarsaparilla, dates back to the 19th century (Medeiros et al. 2007), and it is currently one of the best-selling drugs in pharmacies throughout Brazil. The medicinal importance of the genus has been globally recognized since ancient times. The leaf and root extracts are used to treat health problems such as syphilis, gout, rheumatism, skin problems, asthma, toothaches, injuries, and even eye pain (Vandercolme 1947).

Of the 32 Brazilian species of this genus (Andreata 1997, 2009), only 10 (*Smilax brasiliensis* Spreng. [= *S. pruinosa* Griseb.], *S. campestris* Griseb. [= *Smilax marginulata Mart. ex Griseb., Smilax montana Griseb., Smilax rubiginosa Griseb., Smilax*

scalaris Griseb., S. viminea Griseb.], *S. cissoides* M. Martens & Galeotti, *S. cognata* Kunth. [= *S. montevidensis* hort. ex Steud.], *S. hilariana* A.DC., *S. longifolia* Rich. [= *S. acuminata* Willd.], *S. muscosa* Toledo, *S. polyantha*, *S. rufescens* Griseb. [= *S. nitida* Griseb.], *S. siphilitica* Humb. & Bonpl. ex Willd. [= *S. duidae* Steyerm.]) are known as sarsaparilla, and only seven (*S. brasiliensis*, *S. longifolia*, *S. siphilitica*, *S. polyantha*, *S. rufescens*, *S. campestris*, and *S. cissoides*) of these have records of their medicinal properties in ethnobotanical surveys (Andreata 1997).

Due to the similar morphological characteristics and chemical compositions among the species of the genus *Smilax*, taxonomic identification is difficult (Lorenzi 2002). Cunha (1937a, 1937b, 1940) and Stellfeld (1940) mention sarsaparilla counterfeiting, i.e., the use of *Herreria salsaparilha* Mart. (Asparagaceae, Asparagales) for medicinal purposes, attributing to this species the same name and medicinal properties as *Smilax aspera* L.

Alterations and falsifications can be prevented and resolved by anatomically characterizing the plants, contributing to the quality control of the raw material used in the preparation of herbal medicines and guaranteeing the reliability and quality of such preparations (Czekalski et al. 2009; Marques et al. 2007; Ming 1994). There are few current reports about morphological character studies associated with the chemical analysis of medicinal plant research showing their importance in the quality control of medicinal plants. Among these is the case of *Dodonaea viscosa* Jacq. that exhibits taxonomic confusion with *D. thunbergiana* var. *linearis* E. et. S. and *D. attenuate* Cunn var. *linearis* Benth. The distribution and organization of fibers and stomata plus the distinct chemical profile of species of *Dodonaea* are an example of how referential information for the identification of this crude drug can be used (Venkatesh et al. 2008). As the chemical profile of the methanol extracts of *Smilax brasiliensis*, *S. campestris*, *S. cissoides,* and *S. polyantha* are very similar (Martins 2009), the association of this feature with anatomical data may provide important information about the comparison of *Smilax* species with commercial sarsaparilla material. Likewise, molecular markers have also been used to provide quality control for medicinal plants (Ganopoulos et al. 2012; Khan et al. 2012; Kool et al. 2012; Tnah et al. 2011).

DNA barcoding is a method that has been proposed for the identification of species based on unique sequences in specific regions of DNA (Hebert et al. 2003). Many studies have shown that DNA barcoding is an effective tool for plant identification (Kress and Erickson 2007; Lahaye et al. 2008; Newmaster et al. 2008). In this study, we use a candidate DNA barcodes (rbcL) to identify the species of *Smilax* and commercial sarsaparilla material.

Due to the high discriminative power of microsatellite analysis and DNA barcoding, these markers can be used to determine sarsaparilla identity and to identify plant material of unknown varietal origin by comparing the genotype obtained from the sample with reference genotypes of sarsaparillas, confirmed by morphological characteristics and chemical compositions.

For these reasons, the current study aimed to verify whether the combined use of anatomical, chemical, and molecular genetic characteristics could be useful in the quality control of medicinal plants such as the sarsaparilla sold in compounding drugstores in the state of São Paulo, Brazil.

Materials and Methods

Obtaining Botanical Material

The commercial samples of sarsaparilla used in the anatomical, chemical, and genetic analysis were purchased in compounding drugstores and herbal shops in the state of São Paulo. The samples were identified as follows: NPS 7, NPS 8, NPS 9, NPS 10, NPS 11, NPS 13, NPS 14, NPS 15, NPS 17, NPS 18, NPS 19, NPS 20, NPS 21, NPS 22, NPS 23, NPS 24, NPS 25, NPS 26, NPS 27, NPS 28, NPS 29A, NPS 29B, NPS 31, NPS 32, NPS 35, NPS 36, NPS 38, NPS 37, NPS 39, NPS 40, NPS 41, NPS 45, NPS 46, NPS 47, NPS 48, and NPS 50.

The sarsaparilla packages were opened and evaluated for the presence of foreign organic matter or any unidentified fragments or other plant parts, such as stems, leaves, or even parts of other plants.

For comparative analyses, in addition to the commercial samples, the leaves (molecular and chemical analyses) and roots (chemical analyses) of the species *Smilax goyazana, S. rufescens, S. brasiliensis, S. campestris, S. cissoides, S. fluminensis* Steud.(= *Smilax china* Vell., *Smilax syringoides* Griseb.), *S. oblongifolia* Pohl ex Griseb. (= *Smilax coriifolia* A.DC.), and *S. polyantha* were also used. These samples were obtained from the greenhouse of the Department of Biological Sciences of ESALQ/USP,

Piracicaba-SP, Brazil. *S. oblongifolia* was also used for the comparative anatomical analysis of the roots of the species. Specimens were incorporated into the ESA herbarium under the numbers ESA 107635 (*S. brasiliensis*), ESA 107657 (*S. campestris*), ESA 107656 (*S. cissoides*), ESA 107633 (*S. fluminensis*), ESA 107645 (*S. goyazana*), ESA 107643 (*S. oblongifolia*), ESA 107663 (*S. rufescens*), and ESA 107649 (*S. polyantha*).

Scanning Electron Microscope (SEM) Analysis

The sarsaparilla samples were subdivided into smaller fragments using a razor blade, fixed on an aluminium support with carbon double-sided tape, and coated with a gold layer (30 to 40 nm) in a Balzers model SCD 050. The analysis and image digitization were performed using a scanning electron microscope (Zeiss model LEO 435VP), operated at 20 Kv with the scales printed directly on the electron micrographs.

Light Microscopy (LM) Analysis

The root materials obtained from the stores were subjected to hydration and softening with water, glycerine and alcohol (1:1:1) (Kraus and Arduin 1997). The roots of *S. oblongifolia* were fixed in Karnovsky fixative (Karnovsky 1965; 2% glutaraldehyde +0.8% paraformaldehyde in 0.1M phosphate buffer pH 7.2) for 24h.

The samples were then dehydrated in an ethanol series (10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100%) and infiltrated with hydroxyethyl methacrylate (Leica Historesin®). The 7 µm sections were obtained using a Leica RM2245 microtome, stained with 0.05% toludine blue in phosphate-citrate buffer pH 4-6 (Sakai 1973) and mounted between a slide and coverslip with Entellan (Merck) synthetic resin.

The images were captured using a trinocular microscope (Leica, model DMLB) attached to a camcorder (model DC 300 with IM50 software), and the scales for the images were obtained under the same conditions as used for the other images.

Chemical Analysis

The samples were dried at 40°C for five days and pulverized using a knife-type mill (Marconi, model MAO 48). The leaf and root extracts of eight *Smilax* species and commercial sarsaparilla samples were prepared by cold extraction in a commercial blender with ethanol using 10 g of dried, ground plant material. The extraction was performed using a disperser mixer Polytron-Ultra Turrax 175 on rotation 3 (16,000 rotations/min). The solvent was evaporated in the rotor to concentrate the extract. The organic extraction yield was calculated by dividing the weight of total plant used in the process of extraction by weigh of the crude extract in grams (% w/w).

The eluent used for thin-layer chromatography (TLC) was butanol: acetic acid:distilled water (BAW) (40:10:50). After elution, the chromatogram was observed under ultraviolet (UV) light at 254 nm and 366 nm to determine if any substance showed fluorescence. Two chemical developers were used: anisaldehyde solution with sulphuric acid (flavonoids, saponins, terpenoids, steroids, and catechins) and Natural Products/Polyethylene Glycol (NP/PEG) reagent (flavonoids) (Wagner and Bladt 1996). The Rf (Retention factor) and the color of the developed spots in the sulphuric acid solution of anisaldehyde and NP/PEG were used as the evaluation parameters.

Molecular Genetic Studies

Genomic DNA from the fresh leaves of two individuals of *Smilax brasiliensis*, *S. campestris, S. cissoides, S. fluminensis*, *S. polyantha*, *S. goyazana*, *S. rufescens,* and *S. siphilitica* was extracted using the CTAB protocol described by Doyle and Doyle (1990) with modifications according to Martins et al. (2013).

The protocol for the genomic DNA extraction for the 15 commercial samples of sarsaparilla roots was based on Khan et al. (2007) for the preparation of the extraction buffer and Khanuja et al. (1999) for DNA isolation and purification.

In order to obtain good quality DNA for the DNA barcoding technique sequencing, the extraction protocol of Doyle and Doyle (1990) was used, with modifications according to Alves (2013).

After electrophoresis, the quantification of the samples was estimated by the intensity of fluorescence emitted by SYBR® Green under UV in 1% agarose (w/v). The intensity was compared with the standard molecular weights and specific known concentrations (λ phage DNA) using Microsoft Office Picture Manager.

The microsatellite markers were developed according to Billotte et al. (1999), using genomic libraries enriched with SSRs regions and the

genotypes of the *S. brasiliensis* cultivated in the greenhouse of the Department of Biological Sciences ESALQ, USP, Piracicaba-SP, Brazil. Complementary primers to sequences flanking the SSRs were designed with the aid of WEBSAT (Martins et al. 2009), PRIMER 3 (Rozen and Skaletsky 2000), GENE RUNNER (Hastings Software, Inc.), and CLUSTALX, version 2.0 (Larkin et al. 2007). The sequences of the polymorphic loci were annotated and submitted to GenBank (JX070058, JX070059, JX070060, JX070061, JX070062, JX07006, JX070064, JX070065, JX070066, JX070067, JX070068, JX070069, JX070070, JX07007, JX070072, JX070073, JX070074) using Banklt (http://www.ncbi.nlm.nih.gov/Banklt).

Twenty-six primer pairs were designed for the species *S. brasiliensis*, and 17 were selected for genotyping (Martins et al. 2013).

PCR was performed for the 26 microsatellite loci, and the 62 genotypes were evaluated in a 20 µL final volume containing approximately 30 ng of DNA, 0.24 µL of forward primer (10 µM) synthesized with an M13 sequence on the 5'-end (CACGAC GTTGTAAAACGAC), 0.30 µL of reverse primer (10 µM), 0.45 µL of 10 µM IRDye-labeled M13 Primer, 1.2 µL of dNTP solution (250 µM each deoxyribonucleoside triphosphate), 1.5 µL of 1X buffer solution (50 mM KCl; 10 mM Tris-HCl, pH 8.9); 0.6 µL of BSA (bovine serum albumin) (2,5 µg/mL), 0.6 µL of $MgCl_2$ (3 mM), and 1 U of Taq DNA polymerase (Fermentas). For 6 of the 26 loci (Sbr01, Sbr03, Sbr05, Sbr09, Sbr15, Sbr16), the thermocycler was programmed to start with 5 minutes at 95°C for the initial denaturation, followed by 30 cycles of 30 seconds each of denaturation at 94°C, 40 seconds of annealing at 52°C and a 1-minute extension at 72°C, ending with 10 minutes at 60°C for the final extension. For remaining loci, the thermocycler was programmed for a touchdown start with 4 minutes at 94°C for the initial denaturation, followed by 10 cycles at 94°C for 40 seconds, 50°C dropping by 1°C for 40 seconds and 72°C for 1 minute, 30 cycles of 30 seconds each of denaturation at 94°C, 40 seconds of annealing at 40°C and 1 minute of extension at 72°C, and 5 minutes at 72°C for the final extension.

The amplification products were separated under denaturing conditions on 5% polyacrylamide gels (v/v) containing 8 M urea and 1X TBE (0.045 M Tris-borate and 0.001 M EDTA) in an automated sequencer (DNA Sequence Analyser 4300S NEN DNA Analyser-LI-COR Corporate) for approximately 2 hours and 30 minutes at 70 watts. The loci were genotyped using specific SAGA (LI-COR Corporate) computer software for further analysis.

Only six SSR markers (Sbr01, Sbr05, Sbr06, Sbr11, Sbr13, and Sbr16) were successfully amplified in commercial samples of sarsaparilla and were used for clustering Bayesian analysis.

A Bayesian analysis was used to attribute tests for each individual to a cluster, based on its genotype, using the program STRUCTURE (Falush et al. 2007). Twenty independent simulations were performed for each value of *K* clusters. In each simulation, 500,000 resamplings with a burn-in of 100,000 were performed.

For RBLC genes, PCR amplification was performed using ReadyMix Mastermix (Advanced Biotechnologies, Epson, Surrey, UK). We added 3.2% bovine serum albumin (BSA) to all reactions to serve as stabilizer for enzymes, to reduce problems with secondary structure, and to improve annealing (Palumbi 1996). PCR amplification was performed using Veriti® Thermal Cycler (Applied Biosystems).

PCR programs used are as follows: (a) pre-melt at 94°C for 60 s, denaturation at 94°C for 60 s, annealing at 50°C for 60 s, extension at 72°C for 60 s (for 35 cycles), followed by a final extension at 72°C for 7 min. PCR products were purified with ExoSAP-IT™ PCR Purification Kit (GE Healthcare), and cycle sequencing reactions were carried out in a GeneAmp PCR System 9700 thermal cycler using the ABI PRISM® BigDye® Terminator v3.1 (Applied Biosystems, Inc., California, USA). Cycle sequencing products were precipitated in ethanol and sodium acetate to remove excess dye terminators. Then suspended into 10 µL HiDi formamide (ABI) before sequencing on a ABI 3130 *xl* Genetic Analyzer (ABI).

Complementary strands were assembled and edited using Sequencher v3.1 (Gene Codes, Ann Arbor, Michigan, USA). Sequences were edited, using Geneious Pro version 4.8.5 (Drummond et al. 2009).

We use the edited sequences of *Smilax* species and commercial samples of sarsaparilla in order to realize the "blast" in NCBI and in the sequence identify similar species using BOLD System (www.boldsystems.org).

Sequences were edited, assembled, and multiple sequence alignments (MUSCLE method) were done by using Geneious Pro version 4.8.5 (Drummond et al. 2009). To evaluate the genetic distance among species of *Smilax* and commercial sarsaparilla material, we used a standard phylogenetic technique: neighbor-joining (NJ) with MEGA

version 5.0 (Tamura et al. 2011); nodes validation was performed by 1,000 bootstraps.

Results and Discussion

Scanning Electron Microscopy and Light Microscopy

After removing all of the contamination detected by structural analysis, such as stems, eudicotyledonous roots, and leaves, cross-sections of the commercial sarsaparilla samples for the anatomical studies showed a structure similar to that found in several monocot roots (Arber 1925; Kauff et al. 2000), i.e., with several protoxylem elements surrounding a pith (Fig. 1 A–L).

The protective tissue of these roots was composed of an internal cortex (Fig. 3B) formed by one or two layers of parenchyma cells with thick walls and endodermis (Fig. 1 A–L). This type of protective tissue was described by Stellfeld (1938), who observed a loss of parenchyma in sarsaparilla (*Smilax* sp. – species unidentified) and attributed this effect to a root infection by pathogens. Cunha (1940) also reported cortex loss in Brazilian sarsaparilla (*Smilax* sp. – species unidentified).

Martins and Appezzato-da-Gloria (2006) reported that the young root of *Smilax polyantha* has a white coloration because it still possesses the epidermis and the outer cortex. However, as the root matures, it acquires a brown coloration due to the loss of these tissues and because the protective tissue is then replaced by a thickened inner cortex and the endodermis.

The same phenomenon was observed in six other *Smilax* species (*S. brasiliensis, S. campestris, S. cissoides, S. goyazana, S. oblongifolia,* and *S. rufescens*) by Martins et al. (2010) and in *S. subsessiliflora* Duhamel (Guimarães et al. 2010).

In *S. fluminensis* (Soares 2010) and *S. siphilitica* (Silva 2010), a total loss of the cortical parenchyma was observed, leaving only the inner periclinal walls of the one before the last layer of the cortex, which assumes a protective tissue function along the endodermis. The walls of the two layers of the internal cortex and the endodermis walls exhibit a brown color due to the accumulation of phenolic compounds, corroborating the descriptions reported by Martins et al. (2010).

The shape of the endodermal cells, in cross-section, is rectangular, with higher anticlinal walls. The parietal thickening in "O" (fully thickened endodermis) shows that the lamellae and the lumen are elongated (Fig. 3C). Cunha (1937b) used the characteristics of the endodermis, particularly the shape of the lumen, to distinguish between *Smilax* species. However, Martins et al. (2010) found no differences in the lumen to separate *S. brasiliensis, S. campestris, S. cissoides, S. goyazana, S. oblongifolia,* and *S. rufescens* species.

In all of the species analyzed by the authors mentioned above and in *S. polyantha* (Martins and Appezzato-da-Gloria 2006) and S. *subsessiliflora* (Guimarães et al. 2010), the parietal thickening in "O" was laminated and similar to that observed in the commercial samples. However, the endodermis reinforced in "U" (the endodermis is thickened on the inner and radial walls) can also be observed in *Smilax*, for example, in *S. fluminensis* (Soares 2010), and in *S. siphilitica* (Silva 2010).

The elements of the xylem vein in the samples showed scalariform perforation plates, as described by Gattuso (1995) for a *Smilax campestris* species and by Martins and Appezzato-da-Gloria (2006) for the underground stem of S. *polyantha*. Cheadle (1942), studying types of vessel elements in monocots, observed the occurrence of scalariform perforation plates in five species of *Smilax* but also described this type of plate for Dioscoreaceae, and such plants are often sold as sarsaparilla. In Araceae roots, the vessel elements also have scalariform perforation plates (Grayum 1990), for example in *Monstera deliciosa* Liebm. and in *Philodendron undulatum* Engl. (Huggett and Tomlinson 2010).

The triangular arrangement of the phloem (Figs. 2A and 2B) is similar to that seen in the aerial roots of *Philodendron bipinnatifidum* Schott ex Endl. (Vianna et al. 2001) and *Rhodospatha oblongata* Poepp. (Filartiga 2011), Araceae species, but it was not observed in the *Smilax* species described in the literature (Guimarães et al. 2010; Martins et al. 2010; Silva 2010; Soares 2010), which show a phloem with circular arrangements (Figs. 2D and 2E).

Another difference with regard to the *Smilax* species was the presence of metaxylem elements in the center of the medulla in the commercial samples (Figs. 2A and 2C). This characteristic was not observed in *Smilax* species (Guimarães et al. 2010; Martins et al. 2010; Silva 2010; Soares 2010) (Fig. 2D), but it can be observed in Araceae roots (Huggett and Tomlinson 2010; Vianna et al. 2001).

The medulla is wide and visible and is formed by lignified cells containing starch grains of a spherical

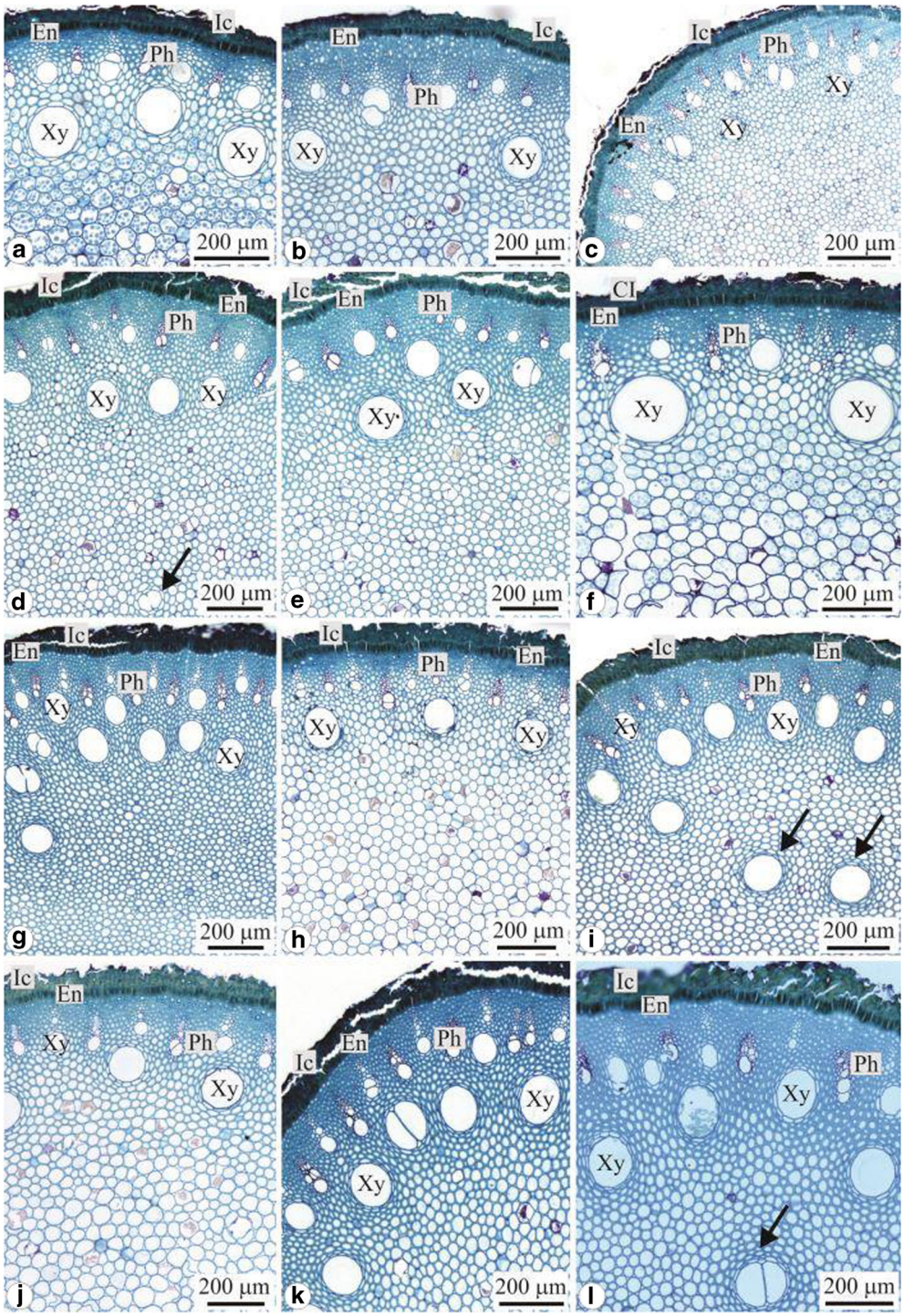

Fig. 1. Photomicrographs of commercial samples of sarsaparilla. **A–L**. Overview of the roots of the commercial samples in cross-section. Observe in **D, I, and L** the metaxylem (arrow) in the center of the organ of (**A**) NPS 29A, (**B**) NPS 22, (**C**) NPS 39, (**D**) NPS 28, (**E**) NPS 29B, (**F**) NPS 36, (**G**) NPS 40, (**H**) NPS 45, (**I**) NPS 46, (**J**) NPS 47, (**K**) NPS 48, and (**L**) NPS 50. Ic = Internal cortex, En = endoderm, Ph = phloem and Xy = xylem.

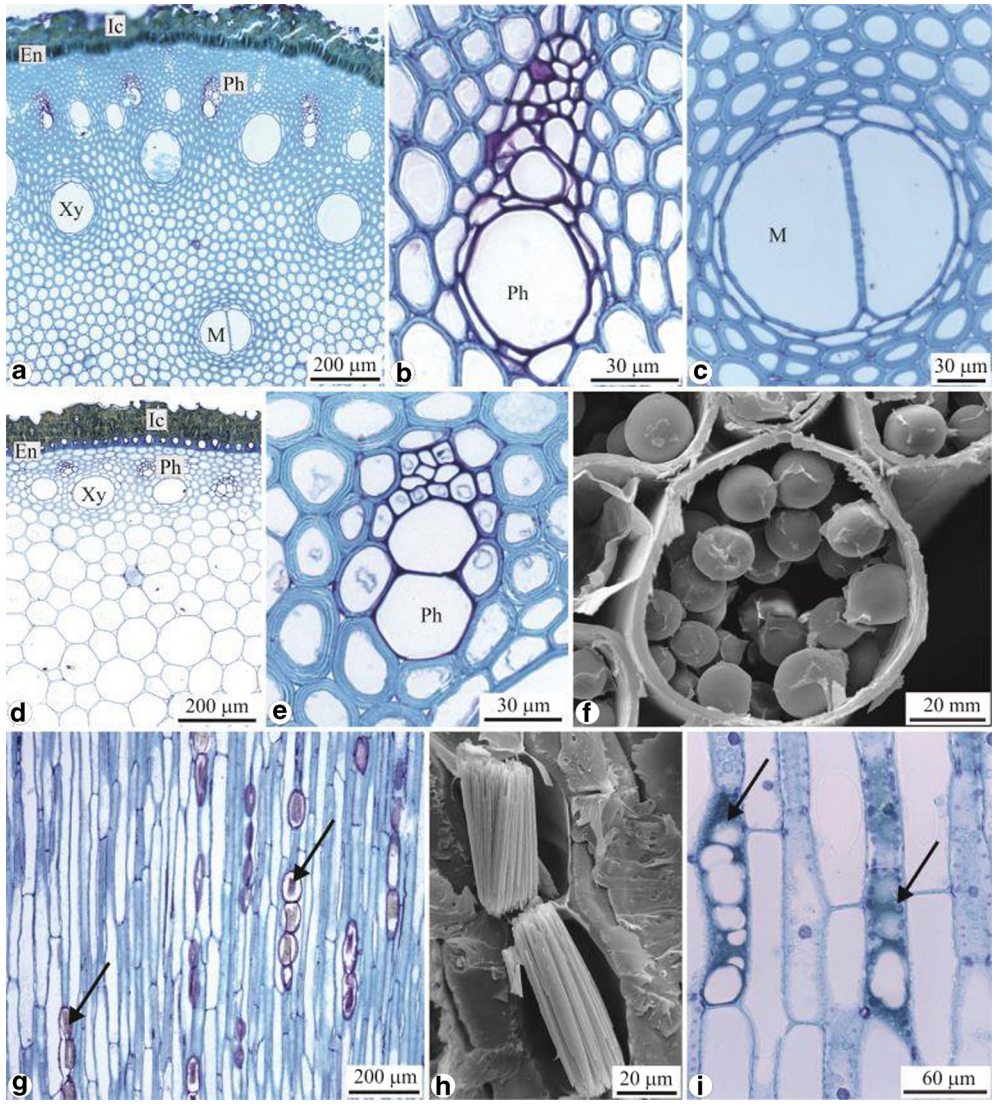

Fig. 2. Photomicrographs **(A–E, G, I)** and electron micrographs **(F, H)** of commercial sarsaparilla samples (**A–C, F–H**) and of *Smilax oblongifolia* (**D, E, I**). **A–C**. Root of sample NPS 36. **A.** Overview. **B**. Phloem details. **C**. Details of vessel elements in the center of the structure. **D, E**. Overview and phloem details, respectively. **F.** Medullar parenchyma of the sample NPS 8 containing starch grains. **G**. Root of sample 28 showing idioblasts in series containing raphides (arrows). **H**. Detail of two idioblasts containing raphides. **I**. Idioblasts containing phenolic compounds (arrows) **A–E.** In cross-section **G, I** in longitudinal section. Ic = Internal cortex, En = endoderm, Ph = phloem and Xy = xylem, M = metaxylem.

and simple shape (Fig. 2F). Starch grains in the medullary parenchyma, similar to the ones found in the commercial samples, were observed in *Smilax goyazana* and *S. brasiliensis* (Martins et al. 2010). However, in the *Smilax*, polygonal starch grains can be observed in *S. oblongifolia*, *S. campestris,* and *S. cissoides* (Martins et al. 2010) and in *S. fluminensis* (Soares 2010) and *S. siphilitica* (Silva 2010); polyhedral and spherical starch grains can be observed in the medullary parenchyma.

The medulla samples were also observed to have a high frequency of idioblasts in series (Figs. 2G and 2F), consisting of relatively larger cells than their neighbors that were filled with the raphides of acicular crystals of calcium oxalate (Fig. 2H). Idioblasts in series containing raphides had been described in

the cortex of *Smilax* roots (Martins et al. 2010) and in the stem cortex of *Smilax muscosa* Toledo and *S. pilosa* Andreata & Leoni (Dias-Neto 2012) but not in the medulla.

Idioblasts containing phenolic compounds were not present in the commercial samples (Fig. 2G), but they were present in the medulla of the roots of *Smilax oblongifolia* (Fig. 2I) and in other species of the genus studied by Martins et al. (2010), Guimarães et al. (2010), Silva (2010), and Soares (2010).

Chemical Profile

The best results for leaf crude extract yield were found for the species *S. rufescens* and *S. brasiliensis* (7.86%), whereas the best crude extract yield for the root extract was obtained from *S. oblongifolia* (7.6%). The leaf extracts that had the lowest yields were obtained from *S. fluminensis and S. polyantha* (4.35% and 3.42%, respectively), while the lowest root extracts were from *S. brasiliensis* (2.2%). The crude extract yield of the commercial samples varied between 3.97% and 7.10%. Therefore, we observed that there is no standard crude extract yield for these samples due to the large variation observed.

The TLC analysis showed that the chromatographic profiles obtained for the leaf and root extracts of the *Smilax* species were quantitatively similar with respect to the appearance of spots on the chromatograms developed with anisaldehyde sulphuric acid solution (Figs. 3A and 3B) and NP/PEG (Figs. 4A and 4B), but they were different from the ones found in the commercial samples (Figs. 3C and 4C).

According to TLC, the leaf samples of the *Smilax* species (*S. goyazana*, *S. rufescens*, *S. brasiliensis*, *S. campestris*, *S. cissoides*, *S. fluminensis*, *S. oblongifolia,* and *S. polyantha*) that were developed by anisaldehyde sulphuric acid solution showed spots with retention factors (Rf) (Table 1) of 0.13 (brown coloration) and 0.27 (green coloration) (Fig. 3A).

When developed with NP/PEG and observed under UV at 365 nm, an Rf of 0.39 (brilliant white) was observed, except in *S. fluminensis*; an Rf of 0.51 (red) was more intense in *S. brasiliensis*, an Rf of 0.65 (red) was found only in *S. rufescens,* and an Rf of 0.75 (brilliant white) was found except in *S. cissoides* and *S. fluminensis* (Fig. 4A).

In the samples of the roots identified as *Smilax* species mentioned above, Rf values of 0.13 (brown coloration), 0.23 (green coloration), 0.29 or 0.39 (yellow), and 0.67 (blue) were observed in the TLC plates developed with anisaldehyde sulphuric acid solution (Fig. 3B).

For the TLC developed with NP/PEG and observed under UV light at 365 nm, an Rf of 0.35 (brilliant white) was observed for all of the species, although the intensity varied; additionally, an Rf of 0.55 (intense brilliant white) was observed only in *S. cissoides*, and an Rf of 0.88 (intense brilliant white) was observed in all of the species (Fig. 4B).

The commercial samples developed with anisaldehyde sulphuric acid solution showed Rf values of 0.16, 0.29, 0.35, 0.52, and 0.73 with different green shades, as shown in Fig. 3C. When developed with NP/PEG and observed under UV light at 365 nm, no spot was observed, confirming the absence of phenolic compounds in these samples (Fig. 4C).

The distribution pattern and coloration of the TLC spots for the identified species seem to follow the same pattern, indicating chemical similarities among the eight analyzed species. The same result was found when we analyzed the group of root samples from the pharmacy. However, when we compare this group with the eight *Smilax* species identified, the chromatographic pattern is different, indicating that the two analyzed groups are substantially different with regard to their chemical compositions.

The extracts of the commercial samples did not show spots with white fluorescence at Rf 0.39 or 0.75 (leaf), red at Rf 0.51 or 0.75 (leaf), or white at Rf 0.35, 0.47, or 0.88 (roots). These results indicate that the commercial samples are different from the reference samples used for *Smilax*.

According to Cáceres et al. (2012), steroidal saponins are the main component of medicinal properties attributed to *Smilax* extracts. The authors report the presence of saponins in the ethanol extract of *Smilax domingensis* Willd. Their results showed Rf values of 0.32–0.88 (indicating anthocyanin), 0.27–0.81 (indicating flavonoids), and 0.8 (indicating saponins). The same authors confirmed the presence of saponins by the stirring test and the verification of persistent foam. In the present study, Rf of 0.8 was not detected because we didn't obtain satisfactory results using the same mobile phase of Cáceres et al. (2012).

There are few studies that emphasize the importance of using TLC techniques in identifying and describing species of *Smilax* (Smilacaceae is a small family with only two genera), but such techniques are commonly used in the quality control of several other families. Barbosa et al. (2009) used TLC in

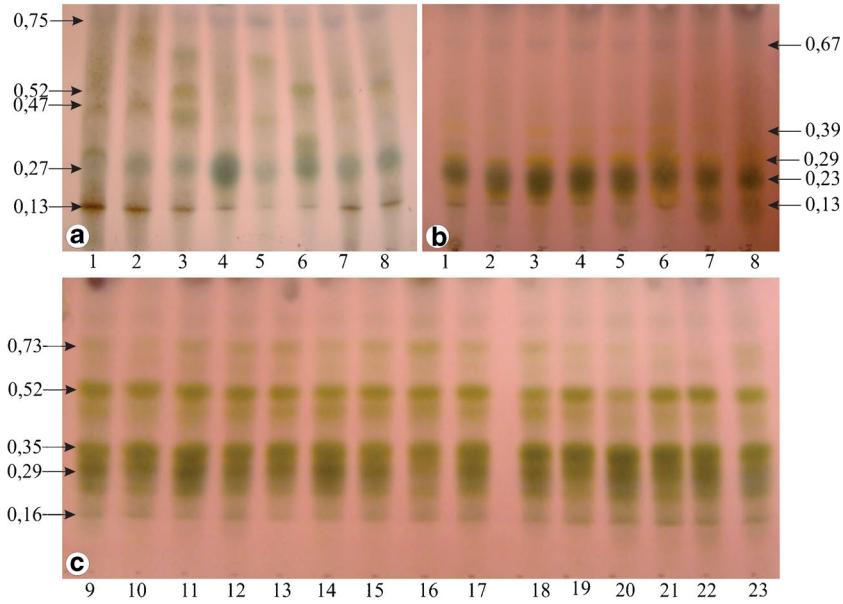

Fig. 3. TLC of the extracts showing the chemical components developed with anisaldehyde sulphuric acid solution using the eluent BAW (butanol:acetic acid:water) **A**. *Smilax* leaves **B**. *Smilax* roots **C**. Roots of commercial sarsaparilla samples. Numbers 1–8 (*S. goyazana, S. rufescens, S. brasiliensis, S. campestris, S. cissoides, S. fluminensis, S. oblongifolia,* and *S. polyantha*) and from 9 to 20 (NPS 9, 11, 18, 24, 29A, 32, 40, 45, 46, 47, 48, and 50).

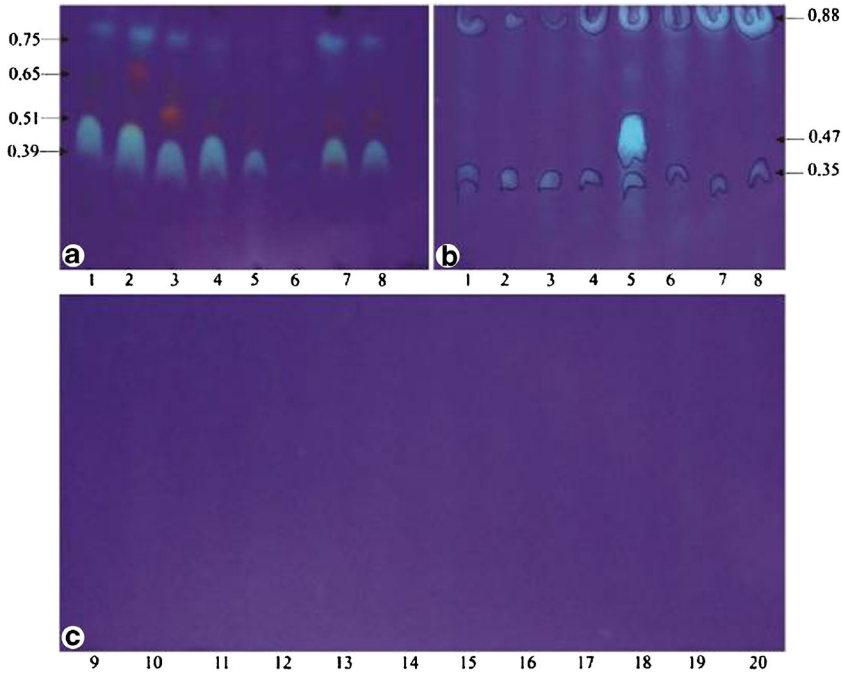

Fig. 4. TLC of the extracts showing the chemical compounds developed with NP/PEG using the eluent BAW (butanol:acetic acid:water) **A**. *Smilax* leaves, **B**. *Smilax* roots, **C**. Roots of commercial samples of sarsaparilla. Numbers 1 through 8 (*S. goyazana, S. rufescens, S. brasiliensis, S. campestris, S. cissoides, S. fluminensis, S. oblongifolia,* and *S. polyantha*) and from 9 to 23 (NPS 9, 11, 18, 24, 29A, 32, 40, 45, 46, 47, 48, and 50).

Table 1. Rf values for the leaves and roots of *Smilax* species and commercial samples of sarsaparilla with different developing solutions.

	Leaf (*Smilax*)	Root (*Smilax*)	Commercial samples
Anisaldehyde	0.13 (brown)	0.13 (brown)	
			0.16 (green)
		0.23 (green)	
	0.27 (green)		
		0.29 (yellow)	0.29 (green)
			0.35 (green)
		0.39 (yellow)	
			0.52 (green)
		0.67 (blue)	
			0.73 (green)
NP/PEG		0.35 (brilliant white)	No spot
	0.39 (brilliant white)		
	0.51 (red)		
		0.55 (brilliant white)	
	0.65 (red)		
	0.75 (brilliant white)		
		0,88 (brilliant white)	

the quality control of 12 commercial samples of copaiba oil and found that only three of the samples analyzed showed an Rf that was similar to those of identified samples of *Copaifera.* The same authors, with the aid of other techniques, were able to detect the presence of soybean oil in the commercial samples.

In addition to the control performed by comparing the Rf similarity of the crude extracts, TLC can also be used to identify major compounds or compounds that can act as specific chemical markers by applying standard commercial chemicals and crude extracts to the chromatographic plate simultaneously. This methodology was used by Camargo and Vilegas (2010) to differentiate between *Turnera* species. The commercial standard used was p-arbutin, which is found only in the aqueous extract of *Turnera diffusa* Willd. ex Schult., allowing for its distinction from *T. ulmifolia* L.

According to Wagner and Bladt (1996), the ethanol extracts of *Smilax regelii* Killip & C.V.Morton (= *Smilax ornata* Lem.) and *Smilax aristolochiifolia* Mill. roots show between 1.8 and 3.0 % steroidal saponins. The same authors characterized these extracts in TLC using chloroform:glacial acetic acid: methanol:water (60:32:12:8) as the mobile phase with a anisaldehyde sulphuric developer. The TLC showed six spots with coloring ranging from yellow to brown, corresponding to Rf ranges that are characteristic of saponins (Rf 0.2–0.75), such as heterosides and sarsasaponin.

Molecular Genetic Studies

The number of alleles observed for each locus ranged from 2 to 16, with an average of 8.06 alleles per locus. The observed and expected heterozygosity for each locus ranged from 0.000 to 0.97 (0.474 on average) and 0.112 to 0.915 (0.643 on average), respectively.

The results of the cluster analysis based on Bayesian statistics executed in the STRUCTURE (Pritchard et al. 2000) software showed the lowest K (groups) value = 2. The analysis (Fig. 5) shows that the individuals were arranged in two groups that correspond exactly to sarsaparilla commercial samples (1–15) and the eight *Smilax* species (16–30). Each bar represents the presence of a color variation, but when we analyzed K = 2, we verified that there is a pattern in the color distribution in each of the three groups analyzed.

The formation of two distinct groups is consistent with the result obtained from anatomic and chemical data. We can detect with microsatellite markers that there are sharing alleles between the species of the genus *Smilax* forming a cluster (16–30), and it was clear that the commercial samples of sarsaparilla present some genetic similarities but they are not closer to the genus *Smilax*.

For the DNA barcoding analysis we obtained success of amplifications and sequencing for only six species of *Smilax* (*S. brasiliensis*, *S. campestris, S.*

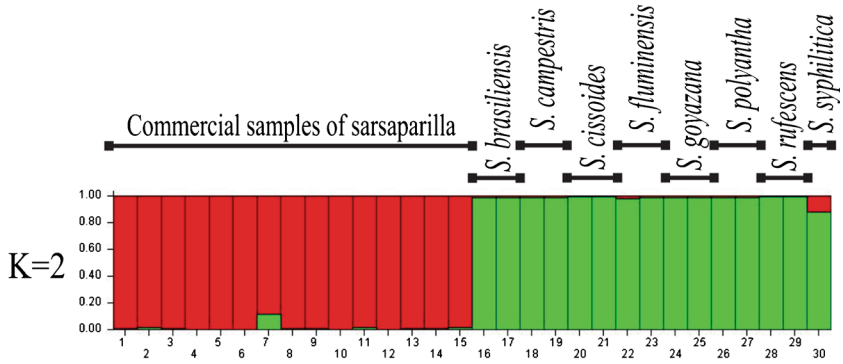

Fig. 5. Attribution tests for commercial samples of sarsaparilla (1–15) and for eight *Smilax* species (16–17 = *Smilax brasiliensis*, 18–19 = *S. campestris*, 20–21 = *S. cissoides*, 22–23 = *S. fluminensis,* 24–25 = *S. goyazana*, 26–27 = *S. polyantha*, 28–29 = *S. rufescens,* and 30 = *S. siphilitica*) were evaluated in two and three clusters (K =2). The sampled individuals are represented by colored vertical bars (Q values). The same color in different samples indicates that they belong to the same group. Different colors on the same bar indicate the percentage of the genome that is shared with each group.

cissoides, S. goyazana, S. polyantha, and *S. rufescens*) and six commercial samples of sarsaparilla (NPS 14, NPS46, NPS24, NPS45, NPS09, NPS47). According to the neighbor-joining tree obtained (Fig. 6), the group with the *Smilax* species present separated from the commercial samples of sarsaparilla. The commercial samples of sarsaparilla form a group that is genetically distinct from the *Smilax* genus analyzed.

Smilax species and the commercial samples of sarsaparilla were compared to sequences in GenBank through a BLAST search. For *Smilax* species, online BLAST searches using *rbcL* yielded the same identification of *Smilax pamensis* (99.6% of similarity), and the commercial samples of sarsaparilla resulted in multiple options like *Rhipogonum elseyanum* F.Muell. (98% of similarity), *Dioscorea guerrerensis* R.Knuth (100% of similarity), and

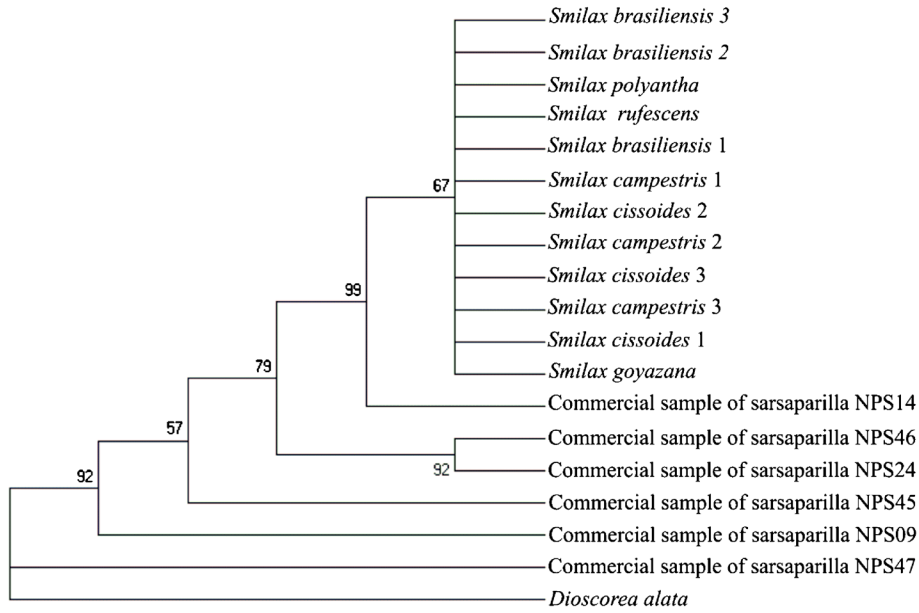

Fig. 6. Neighbor-joining tree of *rbcL* sequences from six *Smilax* species and six commercial sarsaparilla samples, using K2P distances. Bootstrap values greater than 50 shown. *Dioscorea alata* was used as the outgroup.

Table 2. Estimates of the percentage of amplification of the SSR markers for the commercial sarsaparilla samples and eight *Smilax* species.

Species	N	Amplification (%)
Commercial sarsaparilla samples	15	16.7
S. brasiliensis	2	92.9
S. campestris	2	89.3
S. cissoides	2	89.3
S. fluminensis	2	50.0
S. goyazana	2	92.9
S. polyantha	2	78.6
S. rufescens	2	92.9
S. siphilitica	1	57.1

N: Number of individuals sampled.

Heterosmilax yunnanensis Gagnep. (98% of similarity).

The average number of alleles was approximately 2.12 for the *Smilax* species and 4.5 for the commercial sarsaparilla samples (Table 2). The commercial sarsaparilla samples showed a higher than expected heterozygosity (0.866).

A lower proportion of SSR markers that are amplified in the commercial sarsaparilla samples (16.7%) were observed when compared to other species of the *Smilax* genus analyzed in this study (57.1–92.9%). By comparing the examined groups using microsatellite markers, we verified that there is a genetic characterization of the *Smilax* species, as shown in Fig. 5. However, using molecular markers, it was possible to verify that the commercial samples are genetically distant from the eight *Smilax* species studied. Other authors have also developed and characterized microsatellite markers for use in the identification of medicinal species that are difficult to distinguish using morphological and chemical markers (Khan et al. 2012; Tnah et al. 2011).

According to Techen et al. (2014), increasing demand for herbal remedies requires authentication of the medicinal plant material. Molecular barcoding methods are reliable tools for the identification of medicinal plants, their substitutes, and adulterants at the genus and species level. The present study of DNA barcoding analysis was essential to separate the groups of species of *Smilax* and commercial samples of sarsaparilla (Fig. 6), confirming the indicative data of morphological, chemical, and microsatellite markers analysis. These different types of data demonstrate that commercial samples of sarsaparilla are a heterogeneous group unrelated to the eight Brazilian *Smilax* species known for their medicinal properties. Therefore, this case study of sarsaparilla verifies that the combined use of anatomical, chemical, and molecular genetic characteristics are useful in the quality control of medicinal plants.

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