ΦYTON
REVISTA INTERNACIONAL DE BOTÁNICA EXPERIMENTAL
INTERNATIONAL JOURNAL OF EXPERIMENTAL BOTANY
FUNDACION ROMULO RAGGIO
Gaspar Campos 861, 1638 Vicente López (BA), Argentina
www.revistaphyton.fund-romuloraggio.org.ar

Molecular biology techniques as new alternatives for medicinal plant identification

Las técnicas de biología molecular como una nueva alternativa para la identificación de plantas medicinales

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Abstract. Mexico bears witness to a long-standing tradition in the use of plants for medicinal purposes; nevertheless, standardized methodological approaches for the proper identification of these are still needed. The problem is especially noticeable during the validation of ingredients in herbal preparations, as so many of them are sold as powders. It is for these reasons that the present study aims to compare the results of classic taxonomical assessments with those obtained using more modern molecular techniques (*e.g.* PCR-amplified 18S ribosomal RNA gene analysis) in one ethnobotanical case-study carried out in the "*Desierto de los Leones*" National Park in Mexico City, Mexico. Molecular identification resulting from the comparison of PCR-amplified 18S rRNA genes from 7 different plant species to those deposited in the GenBank database was performed. Genus-level identification by molecular techniques and database searches coincided with results obtained using classic taxonomical approaches in 6 of the 7-species analyzed. Only one (*Eupatorium*) could not be identified in the GenBank database and has therefore been described in this study. In a further phytochemical analysis, the plant commonly known as "*Avena del campo*" presented the highest content of total flavonoids, while the plants "*Sienecilla*", "*Jarilla amarilla*" and "*Jarameo*" showed abundant levels of alkaloids. Our results support the idea of using molecular biology techniques such as 18S rRNA gene comparisons for plant identification at the genus-level. However, if this is to become a viable alternative for the large-scale assessment of herbal medicines, the need to expand current 18S rRNA gene databases is made patently obvious.

Keywords: *Senecio salignus*; *Capsella bursa-pastoris*; *Raphanus raphanistrum*; *Avena fatua*; *Ribes ciliatum* and *Malva parviflora*.

Resumen. México cuenta con una larga tradición en el uso de plantas medicinales. Sin embargo, el desarrollo de metodologías para la identificación y manejo de ellas son escasos. Uno de los principales problemas es el análisis de las especies presentes en las preparaciones herbales debido a que la mayoría de los ingredientes son polvos. Por este motivo este estudio se centró en comparar los resultados de identificación de las especies colectadas, en un estudio etnobotánico llevado a cabo en el Desierto de los Leones en la Ciudad de México, por técnicas de taxonomía clásica contra técnicas de amplificación por PCR de la fracción del gen ribosomal 18S. Se amplificaron los fragmentos de la fracción 18S rRNA de las 7 especies colectadas se buscaron en la base de datos GenBank para obtener el nombre científico, el que se comparó con el nombre asignado por taxonomía clásica. El estudio indicó que de las 7 especies de plantas colectadas, 6 coincidieron con el nombre científico a nivel de género en ambos métodos. Solo una especie (*Eupatorium*) no fue encontrada en la base de datos del GenBank. De manera paralela se obtuvieron algunos datos fitoquímicos encontrando que la especie conocida como "Avena del campo" presentó alto contenido de flavonoides totales, mientras que las plantas conocidas como "Sienecilla", "Jarilla amarilla" y "Jarameo" mostraron alto contenido de alcaloides. Nuestros resultados apoyan la idea del uso de técnicas de biología molecular como la amplificación del gen 8S rRNA solo a nivel de género. Sin embargo, es importante mencionar que hace falta trabajo para incrementar la base de datos del gen 18S rRNA.

Palabras clave: *Senecio salignus*; *Capsella bursa-pastoris*; *Raphanus raphanistrum*; *Avena fatua*; *Ribes ciliatum* y *Malva parviflora*.

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Received 7.IV.2018. Accepted 25.VII.2018.

INTRODUCTION

Mexico is home to approximately 3,000 to 5,000 species of herbal plants with medicinal applications (Lozoya, 1994; Schlaepfer & Mendoza, 2010; Aarland et al., 2015); however, one of the main problems limiting their use is the lack of standardized tests that certifies their presence in commercialized products. For example, well known is the fact that due to the increased demand for certain species, some are combined with other plant material in adulterated preparations that are then sold to consumers as authentic products, potentially putting them at risk because of unintended side effects or more serious health consequences (Schlaepfer & Mendoza, 2010).

In order to protect consumers and promote the development of the medicinal herb industry in Mexico, a reliable way to certify plant material is of paramount importance. Nevertheless, although some degree of quality control can be achieved using a combination of different techniques, no universal certification method currently exists that is applicable to all herbal plants or their products. For example, even though both macro- and microscopic observations permit the identification of different herb species, these require the participation of highly-qualified personnel in order to successfully do so. Another complication arises when working with fragmented and/or pulverized plant material, as phytochemical analyses by TLC, HPLC, IR, NMR and X-rays hardly result in the conclusive identification of specific plants (Smillie & Khan, 2010).

One possible solution could be the application of molecular biology techniques for the identification of herbal components at the genus level. As early as the nineteen-nineties, researchers began working with certification methods that incorporated the analysis of plant genomes in order to do this (Shaw et al., 2002; Techen et al., 2004; Sucher & Carles, 2008; Marcial et al., 2015). Such approaches were aided by PCR amplification techniques and the introduction of thermostable DNA polymerases. Nowadays, such methods constitute powerful tools that have permitted the acquisition of many DNA "fingerprints" so that authentic medicinal herbs can be better distinguished from their mixed (adulterated) counterparts, thereby guaranteeing the safety of the products that incorporate them (Hollingsworth et al., 2009; Chen et al., 2010; Marcial et al., 2015). In this respect, our research group has successfully used PCR-amplified 18S rRNA gene sequence analysis for the authentication of plants claimed to treat diabetes that were being sold on a popular urban market in Mexico City (Aarland et al., 2015).

With this in mind, a floristic study of the "Desierto de los Leones" National Park – also in Mexico City, Mexico – was performed, where a total of 7 different plant species were collected. The objective was to taxonomically classify all 7 plants using both modern PCR-based methods and older classical techniques in order to compare the results of the two approaches. This would also allow us to evaluate the effectiveness of the molecular techniques for their incorporation into future floristic surveys. At the same time, and in order to enrich our phytochemical database, the pharmacological potential and principal chemical constituents of the 7 different plant species were likewise assessed.

MATERIALS AND METHODS

Biological material and sample preparation. The biological material obtained formed part of an ethnobotanical study involving the "Desierto de los Leones" National Park and surrounding areas, which was undertaken by personnel from our research group at the *Universidad Autónoma de la Ciudad de México* (UACM). A total of 7 different herbaceous plants were selected – all of which are used by the local communities to treat various diseases. The plant material was then divided into two samples. The first one was taken to the UACM for taxonomic identification and preparation for storage at the University´s herbarium. The results of this first taxonomic identification using classical approaches were not revealed until molecular analyses were concluded at the *Universidad Autónoma Metropolitana-Iztapalapa* (UAM-I).

Chemical analysis of extracts. Preparation of plant extracts: 200 g of dried material originating from each of the seven plants species to be analyzed were weighed and macerated in 500 mL of methanol for a total of 7 days. The mixture was then filtered through cotton fiber inside a funnel and dried at a constant pressure by means of a rotatory evaporator. Dried material was then stored at -40 °C for further analysis.

Qualitative analysis

a) ***Determination of saponins.*** To determine the presence of saponins in the different plant extracts, the technique described by Aarland et al. (2015) was used. Briefly, 0.02 g of dried extract were mixed with 10 mL of distilled water inside a test tube and then incubated in a water bath at 80 °C for 30 min. Afterwards, the tube was allowed to cool down to room temperature, stirred vigorously and left to stand for 15 to 20 minutes. The presence and content of saponins were assessed by measuring the height of the foam formed (Kalimuthu et al., 2014; Zarza et al., 2017).

b) ***Determination of anthraquinones.*** The presence of anthraquinones was evaluated using thin layer chromatography (TLC). For this, a 0.1 mL aliquot from each of the plant extracts was applied onto a 60F254 silica gel plate (3 × 5 cm). The eluent consisted of a 95:5 (v/v) mixture of dichloromethane and methanol. Yellow or red fluorescent spots under UV-light indicated the presence of anthraquinones (Aarland et al. 2015; Zarza et al., 2017).

c) ***Determination of alkaloids.*** 0.1 mL aliquots from each of the plant extracts were applied into 60F254 silica gel plates (3

× 5 cm). These were then eluted with the same eluent mixture used for anthraquinones and revealed by applying Dragendorff's reagent. The formation of red-brown spots was indicative of the presence of alkaloids (Kalimuthu et al., 2014; Aarland et al. 2015; Zarza et al., 2017).

d) ***Determination of tannins.*** 0.02 g from each of the plant extracts were dissolved in 10 mL of distilled water. The solution was divided into 3 test tubes and treated with the following: a 1% (w/v) gelatin solution in the first test tube; a gelatin-salt reagent (1 g of gelatin and 10 g of NaCl dissolved in 100 mL of distilled water) in the second test tube; and a saline solution (10% (w/v) NaCl) in the third test tube. The appearance of a white precipitate in the first and second test tubes and the absence of this precipitate in the third indicated the presence of tannins (Aarland et al. 2015; Zarza et al., 2017; Vashisht et al., 2017).

e) ***Determination of coumarins.*** 0.02 g from each of the plant extracts were combined with 10 mL of distilled water inside test tubes. These were then covered with pieces of filter paper that had previously been moistened in a NaOH solution (1 g in 15 mL) before being subsequently heated until they reached their boiling point. After 5 minutes, the filter paper was removed from the test tubes, dried and exposed to UV-light. Blue fluorescence indicated the presence of volatile (Aarland et al. 2015; Zarza et al., 2017).

Quantitative chemical analysis.

Total flavonoids. These were determined colorimetrically using the aluminum chloride method described by Chang et al. (2002). For this assay, 0.5 mL from each of the plant extracts were mixed with 1.5 mL of a 95% ethanol solution, 0.1 mL of a 10% (w/v) aluminum chloride solution, 0.1 mL of a 1 M potassium acetate solution and 2.8 mL of distilled water. The mixture was incubated at room temperature for 30 minutes and the absorbance was determined at 415 nm. A standard curve was prepared using quercetin, with concentrations that ranged from 10 to 100 μg/mL. The results were then expressed as miliequivalents/g dry weight (mEQ/mLmg).

Taxonomic identification by molecular biology techniques. DNA extraction was performed on 50 mg of dehydrated and pulverized leaf samples from each of the 7-plant species to be analyzed using the Axy prep Multisource Genomic DNA miniprep kit and following the manufacturer´s instructions. The concentration and purity of the extracted DNA were determined from the A260nm / A280nm ratios obtained using a NanoDrop® spectrophotometer. DNA integrity was verified by electrophoresis on a 0.7% (w/v) agarose gel using 100 ng from each sample. A fragment from a conserved region of the 18S rRNA gene was then amplified using the following primer pair [Aarland et. al., 2015]: 5´-GTGGCCTAAACGGCCATAGTCCCTC-3´ (forward) and 5´GGAAACTTACCAGGTCCAG AGATAG-3´ (reverse). Amplification was performed with a vent polymerase enzyme (BioLab New England) and 30 ng of DNA. PCR conditions were the following: an initial denaturation at 94 ˚C for 3 minutes, followed by 30 cycles of denaturation at 94 ˚C for 1 min, hybridization at 54 ˚C for 45 sec and polymerization at 72 ˚C for 1 min, with a final extension at 72 ˚C for 5 minutes.

PCR products were visualized on a 2% (w/v) agarose gel stained with GelRedTM (Biotium, USA) using a Multi-Doc imaging system (UVP, UK). PCR products were cleaned with the Wizard® SV Gel Kit and PCR Clean-Up System (Promega, Madison, USA). The amplicons were then sequenced at the UAM-I's Divisional Molecular Biology Laboratory. The sequences obtained were compared with those deposited at the GenBank-NCBI database by searching the 18S rRNA gene entries using the BLAST Algorithm (Altschul et al., 1990). A scientific name was assigned only when the percent identity between the experimental sequences and those obtained from the database searches was more than 95%.

RESULTS AND DISCUSSION

Features and overview of "Desierto de los Leones" and surrounding areas. This national park, together with the nearby town of San Bartolo Ameyalco (pop. 33,000 inhabitants), are located on the outskirts of Mexico City at an altitude of 2420 meters above sea level or 200 meters higher than the city center. Owing to such a high elevation, the area enjoys one of the most temperate climates of the region. For example, during the winter it becomes one of the coldest localities on the southwest quadrant of the city, even with the occasional reports of snowfall. Its immense forests of cedar, fir, pine and oak trees help improve local air quality and play essential roles in the process of aquifer recharge in the region. The land is fertile and for this reason many of the houses, especially those of the original inhabitants, still have orchards that harbor different species of fruiting trees.

The animals that used to populate these hills were deer, opossums, badgers, rabbits, hares, mountain hens, pocket gophers, buzzards and coyotes. Unfortunately, many of the woodlands adjacent to San Bartolo, including those of the "Desierto de los Leones" National Park, have suffered some degree of habitat loss and degradation, which have gradually resulted in the local disappearance of many of these species. Nevertheless, there is still a great variety of birds in the area, as well as the occasional sighting of rabbits and a few snakes.

Loss of flora has likewise occurred, which is why a floristic study and the storage of plant material at UACM's herbarium will allow us to have a historical record of the vegetation of this area. In addition, this small collection can also serve as a repository of viable specimens for future propagation inside gene banks.

Phytochemical analysis. In the ethnobotanical study that was undertaken, the 7 species of herbaceous plants that

Table 1. Ethnobotanical results, scientific names and entry folios for the Herbarium at the Universidad Autónoma de la Ciudad de México (UACM).
Tabla 1. Resultados etnobotánicos, nombres científicos y número de folio en el Herbario de la Universidad Autónoma de la Ciudad de México (UACM).

Family	Scientific name	Common name	Local uses*	Folio**	Reports***
Asteracea	*Ageratina glabrata*	Sienecilla	Arthritis	702	Analgesic effect of leaf extracts. Hypoglycemic effect of extracts from aerial parts (García et al., 2011a; García et al., 2011b; Bustos-Brito et al., 2016).
Asteracea	*Senecio salignus*	Jarilla amarilla	Stress	700	Anti-inflammatory effect of leaf extracts (González et al., 2013)
Brassicaceae	*Capsella bursa-pastoris*	Hierba del pastor	Pain /Aches	708	Hepatoprotective effect of extracts from aerial parts. Antibacterial, anti-inflammatory, antioxidant and anticancer effects (AlqaSoumi et al., 2008; Al-Snafi 2015).
Brassicaceae	*Raphanus raphanistrum*	Jarameo	Immune system function	710	Antiproliferative effect in cancer cell lines (Marrelli et al., 2015).
Poaceae	*Avena fatua*	Avena de campo	Regulates blood pressure	706	Antioxidant, anti-inflammatory, antidiabetic, anti-cholesterolemic and immunomodulatory activities (Czer-winski et al., 2004; Singh et al., 2013).
Grossulariaceae	*Ribes ciliatum*	Acné	Acne treatment	704	Antithrombotic activity of leaf extracts (Luzak et al., 2014).
Malvaceae	*Malva parviflora*	Malva de quesitos	Herpes and psoriasis	697	Neuroprotective effect against Alzheimer in leaf extracts. Hypoglycemic effect of leaf extracts (Aslam & Sial, 2014; Pérez-Gutiérrez, 2012).

* Medicinal use according to Juana Morales Martínez, local plant user; study conducted by researchers at UACM's Herbarium.
** Registration number assigned at the Herbarium.
*** Literature reports.

Table 2. Phytochemical assays conducted in the extracts of the seven species of plants that were selected for this study.
Tabla 2. Estudios fitoquímicos realizados en los extractos de las siete especies de plantas que fueron seleccionadas para este estudio.

Plants	Chemical groups					
	Anthraquino-nes	Alcaloids	Tannins	Coumarins	Saponins	Flavonoids [a]
Sienecilla	++	+++	+++	-	++	26.08 ± 0.96 A
Jarilla amarilla	+	+++	++	-	++	26.28 ± 4.09 A
Hierba del pastor	+	+	-	-	-	28.68 ± 3.11 A
Jarameo	+	+++	++	-	+	28.77 ± 3.02 A
Avena de campo	+	-	+	-	+	55.14 ± 5.68 B
Acné	++	+	+	-	+++	ND
Malva de quesitos	+++	++	+	-	+	52.68 ± 5.91 B

+ low, ++ moderate, +++ high. Not Observed.
[a] Quercentin miliequivalents/g dry weight mEQ/mL.
The similar capital letters are not significantly different amount each sample to the hypothesis testing "means-two independent samples, $\alpha = 0.005$"

Table 3. Results from alignments of the 18S rRNA query sequences to those found at the GenBank database having the highest percent identities.
Tabla 3. Resultados de los alineamientos de las secuencias contra los encontrados en la base de datos GenBank (banco de genes) que tienen el mayor porcentaje de similitud.

a) Caspella bursa-pastoris

Capsella bursa-pastoris voucher Ahrendsen_9 18S ribosomal RNA gene, partial sequence
Sequence ID: **gb|KT459181.1|** Length: 1809 Number of Matches: 1
Range 1: 1250 to 1415

Score	Expect	Identities	Gaps	Strand	Frame
294 bits(159)	3e-76()	164/166(99%)	1/166(0%)	Plus/Plus	

Features:

```
Query  8     GAACTC-TTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGCGATT  66
Sbjct  1250  GAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGCGATT  1309
Query  67    TGTCTGGTTAATTCCGTTAACGAACGAGACCTCAGCCTGCTAACTAGCTACGTGGAGGCA  126
Sbjct  1310  TGTCTGGTTAATTCCGTTAACGAACGAGACCTCAGCCTGCTAACTAGCTACGTGGAGGCA  1369
Query  127   TCCCTTCACGGCCGGCTTCTTAGAGGGACTATGGCCGTTTAGGCCA  172
Sbjct  1370  TCCCTTCACGGCCGGCTTCTTAGAGGGACTATGGCCGTTTAGGCCA  1415
```

b) Avena fatua

Avena fatua 18S ribosomal RNA gene, partial sequence
Sequence ID: **gb|KT153030.1|** Length: 1734 Number of Matches: 1
Range 1: 1213 to 1382

Score	Expect	Identities	Gaps	Strand	Frame
296 bits(160)	9e-77()	167/170(98%)	2/170(1%)	Plus/Plus	

Features:

```
Query  8     GACTGA-A-CTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGA  65
Sbjct  1213  GACTGAGAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGA  1272
Query  66    GCGATTTGTCTGGTTAATTCCGTTAACGAACGAGACCTCAGCCTGCTAACTAGCTATGCG  125
Sbjct  1273  GCGATTTGTCTGGTTAATTCCGTTAACGAACGAGACCTCAGCCTGCTAACTAGCTATGCG  1332
Query  126   GAGCCATCCCTCCGCAGCTAGCTTCTTAGAGGGACTATGGCCGTTTAGGC  175
Sbjct  1333  GAGCCATCCCTCCGCAGCTAGCTTCTTAGAGGGACTATCGCCGTTTAGGC  1382
```

c) Senecio Salignus

Senecio integerrimus voucher Steele 1274 18S ribosomal RNA gene, partial sequence
Sequence ID: **gb|KT179663.1|** Length: 1810 Number of Matches: 1
Range 1: 1248 to 1414

Score	Expect	Identities	Gaps	Strand	Frame
279 bits(151)	9e-72()	162/167(97%)	2/167(1%)	Plus/Plus	

Features:

```
Query  9     GAACTC-TTC-TGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGCGATT  66
Sbjct  1248  GAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGCGATT  1307
Query  67    TGTCTGGTTAATTCCGTTAACGAACGAGACCTCAGCCTGCTAACTAGCTATGTGGAGGTA  126
Sbjct  1308  TGTCTGGTTAATTCCGTTAACGAACGAGACCTCAGCCTGCTAACTAGCTATGTGGAGGTA  1367
Query  127   TCCCTCCATGGCCAGCTTCTTAGAGGGACTATGGCCGTTTAGGCCAC  173
Sbjct  1368  TCCCTCCACGGCCAGCTTCTTAGAGGGACTATGGCCTTTTAGGCCAC  1414
```

d) Raphanus raphanistrum

Brassica rapa subsp. oleifera x Raphanus sativus voucher B4 18S ribosomal RNA gene, partial sequence
Sequence ID: **gb|KT225360.1|** Length: 1740 Number of Matches: 1
Range 1: 1213 to 1378

Score	Expect	Identities	Gaps	Strand	Frame
289 bits(156)	2e-74()	163/166(98%)	2/166(1%)	Plus/Plus	

Features:

```
Query  10    GAACTC-TTC-TGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGCGATT  67
Sbjct  1213  GAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGCGATT  1272
Query  68    TGTCTGGTTAATTCCGTTAACGAACGAGACCTCAGCCTGCTAACTAGCTACGTGGAGGCA  127
Sbjct  1273  TGTCTGGTTAATTCCGTTAACGAACGAGACCTCAGCCTGCTAACTAGCTACGTGGAGGCA  1332
Query  128   TCCCTTCACGGCCGGCTTCTTAGAGGGACTATGGCCGTTTAGGCCA  173
Sbjct  1333  TCCCTTCACGGCCGGCTTCTTAGAGGGACTATGGCCGTTTAGGCCA  1378
```

e) Ribes cilliatum

Ribes aureum Pursh. 18S ribosomal RNA (18S rRNA) gene
Sequence ID: **gb|L28143.1|RIBRGEA** Length: 1767 Number of Matches: 2
Range 1: 1244 to 1408

Score	Expect	Identities	Gaps	Strand	Frame
287 bits(155)	9e-74()	163/166(98%)	3/166(1%)	Plus/Plus	

Features:

```
Query  14    CTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGCGATTTGT  73
Sbjct  1244  CTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGCGATTTGT  1303
Query  74    CTGGTTAATTCCGTTAACGAACGAGACCTCAGCCTGCTAACTAGCTATGTGGAGGATCAC  133
Sbjct  1304  CTGGTTAATTCCGTTAACGAACGAGACCTCAGCCTGCTAACTAGCTATGTGGAGGATCAC  1363
Query  134   CTTCACGGCCAGCTTCTTAGAGGGACTATGGCCG-TTTA-GGCCAC  177
Sbjct  1364  CTTCACGGCCAGCTTCTTAGAGGGACTATG-CCGATTTAAGGCCAC  1408
```

f) Malva parviflora

Malva sylvestris partial 18S rRNA gene
Sequence ID: **emb|AM071397.1|** Length: 1743 Number of Matches: 1
Range 1: 1227 to 1392

Score	Expect	Identities	Gaps	Strand	Frame
296 bits(160)	1e-76()	164/166(99%)	0/166(0%)	Plus/Plus	

Features:

```
Query  8     GAACTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGCGATT  67
Sbjct  1227  GAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGCGATT  1286
Query  68    TGTCTGGTTAATTCCGTTAACGAACGAGACCTCAGCCTGCTAACTAGCTACACGAAGGTG  127
Sbjct  1287  TGTCTGGTTAATTCCGTTAACGAACGAGACCTCAGCCTGCTAACTAGCTACACGAAGGTG  1346
Query  128   ATCCTTCGTGGCTAGCTTCTTAGAGGGACTATGGCCGTTTAGGCCA  173
Sbjct  1347  ATCCTTCGTGGCTAGCTTCTTAGAGGGACTATGGCCGCTTAGGCCA  1392
```

g) Sienecilla (*Eupatorium glabratum*)

GAGTGCGACTCAACTCTTTCTGATCTATGGGTGGTGGTGCATGGCCGTTCTTAGTT-GGTGGAGCGATTGTCTGGTTAATTC
CGTTAACGAACGAGACCTCAGCCTGCTAACTAGCTATGCG-GAGGTATCCCTTCGCGGCCAGCTTCTTAGAGGGACTATGG
CCGTTTAGGCCAC

were selected represented a total of 5 families – all of which are used to treat various diseases by the local inhabitants of San Bartolo, as shown in Table 1. Phytochemical analyses revealed that the species commonly known as "*Sienecilla*", "*Jarilla amarilla*" and "*Jarameo*" all possessed relatively high levels of alkaloids, making them interesting candidates for future studies aimed at identifying and purifying such compounds from them (Table 2). On the other hand, the plants known as "*Malva de quesitos*" and "*Avena de campo*" presented the highest contents of total flavonoids (Table 2), a class of compounds that are often reported as having distinct pharmacological properties. Accordingly, all plants collected in this study have been credited with possessing at least one type of pharmacological effect (Table 2).

Molecular biology techniques. In order to identify the 7 different plant species using molecular biology techniques, total DNA was extracted and a conserved region of the 18S rRNA gene was amplified by PCR. The sequences obtained were then aligned and compared with other 18S rRNA gene sequences deposited at the GenBank database – the genetic sequence repository of the United States' National Institutes of Health. Thus, it was possible to determine the scientific names of the closest defined neighbors (taxa with the highest identities to the query sequences) and compare them to those inferred by the taxonomist at UACM. In all cases, the 18S rRNA gene sequences of the closest defined taxa shared more than 97% identity with those of the query sequences.

Genus-level identification by molecular techniques and database searches coincided with the results obtained using classic taxonomical analysis in 6 of the 7-species analyzed. Only one (*Eupatorium*) could not be identified in the GenBank database (Table 3). This result highlights the limits of using molecular biology techniques and bioinformatics to validate the identity of certain plants whose 18S rRNA gene sequences are still not found in publicly-available databases. Nevertheless, such methods are still useful for identifying taxa at the level of genus, as was the case for the remaining plants in our study. This is an important point because some herbaceous species lack flowers or, as mentioned before, are sold as powders, so it is not always possible to identify them using classic taxonomical approaches.

The 18S rRNA gene sequences obtained were uploaded to the GenBank database, along with pertinent information concerning the plants found in the area studied. Importantly, our research group has continuously worked to expand the 18S rRNA gene sequence inventory at the GenBank database, having deposited many new sequences in previous studies (Aarland et al., 2015).

Once the 7 species of plants had been properly identified, we proceeded to search the literature for evidence of their historical use, finding that all species had at one point been used for medicinal purposes. These however, did not coincide with the ones ascribed to them by the villagers of San Bartolo Ameyalco, which opens the door for future pharmacological assays that could be used to corroborate the effects that the locals claim the herbs possess. Additionally, if such effects can be authenticated, then the identity of the bioactive compounds responsible for such activities could also be determined.

CONCLUSIONS

The use of molecular biology techniques proved to be a viable alternative for the genus-level identification of plants without flowers, as was the case for 6 of the 7 herbs species studied. Moreover, the identities determined using classic taxonomical analysis coincided with those obtained by molecular biology methods. The phytochemical assays indicated that the plants known as "*Sienecilla*", "*Jarrilla amarilla*" and "*Jarameo*" presented relatively high levels of alkaloids, making them interesting candidates for future studies aimed at identifying and purifying such substances from them. On the other hand, "*Malva de quesillos*" and "*Avena de campo*" presented the highest contents of total flavonoids, a class of compounds that have often been reported as having distinct pharmacological properties.

ACKNOWLEDGEMENTS

This work was partially financed by both the Universidad Autónoma Metropolitana-Iztapalapa (UAM-I) and the Universidad Autónoma de la Ciudad de México (UACM). The authors would like to thank B. Sc. (Hons.) Leonardo Castillo Pelayo for his assistance during the revision of the English-language manuscript.

AUTHORS' CONTRIBUTIONS

Juan Manuel Villa-Hernández, Bernarda García-Ocón and Edgar del Carmen Sierra-Palacios designed and performed the experiments. Laura Josefina Pérez-Flores and José Alberto Mendoza-Espinoza directed the study. All authors contributed substantially to the analysis of the data, as well as to the revision of the manuscript.

REFERENCES

Aarland, R.C., S. Peralta, C. Morales, F. Parra, J.M. Villa, F. Díaz de León, L.J. Pérez, F. Rivera & J.A. Mendoza (2015). A pharmacological and phytochemical study of medicinal plants used in Mexican folk medicine. *Indian Journal Traditional Knowledge* 14: 550-557.

Aa-Snafi, A.E. (2015). The chemical constituents and pharmacological effects of *Capsella bursa-pastoris*- a review. *International Journal of Pharmacology & Toxicology* 5: 76-81.

Alqasoum, S.I., A.J. Al-Rehaily, A.M. Alsheikh & M.S. Abdel (2008). Evaluation of the hepatoprotective effect of *Ephedra foliate*, *Alhagi maurorum*, *Capsella bursa-pastoris* and *Hibiscus sabdariffa* against experimentally induced liver injury in rats. *Natural Product Sciences* 14: 95-99.

Altschul, S.F., W. Gish, W. Miller, E.W. Myers & D.J. Lipman (1990). Basic local alignment search tool. *Journal of Molecular Biology* 215: 403-410.

Aslam, M. & A.A. Sial (2014). Neuroprotective effect of ethanol extract of leaves of *Malva parviflora* against amyloid-B- (AB) Mediated Alzheimer's disease. *International Scholarly Research Notices* 2014: 1-5.

Bustos, C., A. Asdrade, J.D. Giraldo, A.D. Moreno & L. Quijano (2016). Acute hypoglycemic effect and phytochemical composition of *Ageratina petiolaris*. *Journal of Ethnopharmacology* 185: 341-346.

Chang, C.C., M.H. Yang, H.M. Wen & J.C. Chern (2002). Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *Journal of Food and Drug Analysis* 10: 178-182.

Chen, S., H. Yao, J. Han, C. Liu, J. Song, L. Shi, Y. Zhu, X. Ma, T. Gao, X. Pang, K. Luo, Y. Li, X. Li, X. Jia, Y. Lin & C. Leon (2010). Validation of the ITS2 region as a novel DNA barcode for identifying medicinal plant species. *PLoS ONE* 5: e8613. doi. org/10.1371/journal.pone.0008613.

Czerwinki, J., E. Bartnokowska, H. Leontowicz, E. Lange, M. Leontowicz, E. Katrich, S. Trakhtenberg & S. Gorinstein (2004). Oat (*Avena sativa* L.) and amaranth (*Amaranthus hypochondriacus*) meals positively affect plasm lipid profile in rats fed cholesterol-containing diets. *The Journal of Nutritional Biochemistry* 15: 622-629.

García, P.G., T.R.E. Del Río, M.R. Guzmán, G.M.I. Martínez & T.R.F. Scior (2011). Estudios preliminares sobre el efecto analgésico del extracto de hojas de *Ageratina glabrata* en dos modelos térmicos de dolor agudo. *Revista Mexicana de Ciencias Farmacéuticas* 42: 45-51.

García, P.G., S.E. García, G.I. Martínez, T.R.F. Scior, J.L. Salvador, P.M.M. Martínez & R.E. del Río (2011). Analgesic effect of leaf extract from *Ageratina glabrata* in the hot plate test. *Brazilian Journal Pharmacognosy* 21: 928-935.

Hollingsworth, P.M., L.L. Forrest, J.L. Spouge, M. Hajibabaei, S. Ratnasingham, M. van der Bank , M.W. Chase, R.S. Cowan, D.L. Erickson, A.J. Fazekas, S.W. Graham, K.E. James, K.J. Kim, W. J. Kress, H. Schneider, J. van AlphenStahl, S.C.H. Barrett, C. van den Berg, D. Bogarin, K.S. Burgess, K.M. Cameron, M. Carine, J. Chacón, A. Clark, J.J. Clarkson, F. Conrad, D.S. Devey, C.S. Ford, T.A.J. Hedderson, M.L. Hollingsworth, B.C. Husband, L.J. Kelly, P.R. Kesanakurti, J.S. Kim, Y.D. Kim, R. Lahaye, H.L. Lee, D.G. Long, S. Madriñán, O. Maurin, I. Meusnier, S.G. Newmaster, C.W. Park, D.M. Percy, G. Petersen, J.E. Richardson, G.A. Salazar, V. Savolainen, O. Seberg, M.J. Wilkinson, D.K. Yi & D.P. Little (2009). A DNA barcode for land plants. *Proceedings of the National Academy of Sciences of the USA* 106: 12794-12797.

Kalimuthu, K., R. Prabakaran & V. Preetha (2014). Phytochemical screening and GC-MS studies on the ethanolic extract of *Turnera ulmifolia* L. *International Journal of Pharmaceutical and Phytopharmacological Research* 4: 179-181.

Lozoya, X. (1994). Two decades of Mexican ethnobotany and research on plant derived drugs In: Ethnobotany and the Search for New Drugs. *Ciba Foundation Symposium* 185, 130 p.

Luzak, B., M. Bencher, J. Rywaniak, D. Dudzinska, M.Rozalski, U. Krajewska, E. Balcerczak, A. Podsedek, M. Redzynia & C. Watala (2014). Extract from *Ribes nigrum* leaves *in vitro* activates nitric oxid synthase (eNOS) and increase CD39 expression in human endothelial cells. *Journal of Physiology and Biochemistry* 70: 1007-1019.

Marcial, J., J.A. Mendoza & E. Sierra (2015). DNA Barcoding: an alternative to for the identification of the medicinal plants employed in Mexico. *Journal of Plant Sciences* 10: 116-127.

Marrelli, M., B. Cristaldi, F Menichini & F. Conforti (2015). Inhibitory effects of wild dietary plants on lipid per oxidation and on the proliferation of human cancer cells. *Food and Chemical Toxicology* 86: 16-24.

Pérez, C., R. Serrano, M. González, M.A. Zavala & S. Pérez (2013). Anti-inflammatory activity and composition of *Senecio silignus* Kunth. *Biomed Research International* 2013: 1-4.

Pérez, R.M. (2012). Evaluation of hypoglycemic activity of the leaves of *Malva parvi*flora in streptozotocin induced diabetic rats. *Food & Function* 4: 420-427.

Schlaepfer, L. & J.A. Mendoza (2010). Las plantas medicinales en la lucha contra el cáncer, relevancia para México. *Revista Mexicana de Ciencias Farmacéuticas* 41: 18-27.

Shaw, P.C., J. Wang & P.P.H. But (2002). Authentication of Chinese medicinal materials by DNA technology. Singapore: World Scientific: 229.

Singh, R., S. De & A. Belkheir (2013). *Avena sativa* (Oat), A potential nutraceutical and therapeutic agent: an overview. *Critical Reviews in Food Science and Nutrition* 53: 126-144.

Smillie, T.J. & I.A. Khan (2010). A comprehensive approach to identifying and authenticating botanical products. *Clinical Pharmacology & Therapeutics* 87: 175-86.

Sucher, N.J. & M.C. Carles (2008). Genome-based approaches to the authentication of medicinal plants. *Planta Medica* 74: 603-623.

Techen, N., S.L. Crockett, I.A. Khan & B.E. Scheffler (2004). Authentication of medicinal plants using molecular biology techniques to compliment conventional methods. *Current Medicinal Chemistry* 11: 1391-1401.

Vashisht, S., M.P. Singh & V. Chawla (2017). A comparative pharmacognostic evaluation of different extracts of *Shorea robusta* Gaertn. f. Resin. *International Journal of Pharmaceutical and Phytopharmacological Research* 7: 1-5.

Zarza, A.L., E. Sauri, D. Raddatz, L.F. Cuevas, L.L. Pinzón, F. Rivera & J.A. Mendoza (2017). Pharmacological, phytochemical and morphological study of three Mayan accessions of *Bixa orellana* L. leaves. *Emirates Journal of Food and Agriculture* 29: 163-169.