



Artículo | Article

Morphoanatomical and phytochemical studies for the quality control of *Neurolaena lobata* (L.) R.Br. ex Cass. (Asteraceae)

[Estudios morfoanatomicos y fitoquimicos para el control de calidad de *Neurolaena lobata* (L.) R.Br. ex Cass. (Asteraceae)]

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Abstract: *Neurolaena lobata* (L.) R.Br. ex Cass. (Asteraceae) is a popular folk remedy for in Central America. The plant is of commercial value in Guatemala but so far there is not any monograph to guide regional laboratories on ensuring identity and chemical tests for this species. As identity test we here run macro and micro morphoanatomical studies of the characters of the vegetative organs. We also developed standard chemical tests for quality by both TLC and HPLC for infusions and tinctures of varying alcoholic strength. Their radical scavenging activities in DPPH and NO were also measured. Macro and micro morphoanatomical characters of the vegetative organs present a set of characteristics to facilitate the identification of dry powdered samples of this species. We developed optimal conditions for the TLC and HPLC phytochemical fingerprints of the 4 most common pharmacopoeial liquid herbal preparations from this herbal drug, namely infusion, 70%, 45% and 20% hydroalcoholic tinctures. Our work provides the Latin-American industry with a set of analyses to establish the identity and chemistry of *N. lobata* samples for quality control purposes..

Keywords: HPLC; TLC; Microscopy; Macroscopy; *Neurolaena lobata*; Guatemala.

Resumen: *Neurolaena lobata* (L.) R.Br. ex Cass. (Asteraceae) es un remedio popular en América Central. La planta tiene un valor comercial en Guatemala, pero hasta el momento no existe una monografía que guíe a los laboratorios regionales para garantizar la identidad y las pruebas químicas para esta especie. Como prueba de identidad proponemos estudios macro y micro morfoanatómicos de los caracteres de los órganos vegetativos. También desarrollamos pruebas químicas de calidad mediante CCF y CLAR para infusiones y tinturas de grado alcohólico variable. También se midieron sus actividades de captación de radicales en DPPH y NO. Los caracteres macro y micro morfoanatómicos de los órganos vegetativos presentan un conjunto de características para facilitar la identificación de muestras de polvo seco de esta especie. Desarrollamos condiciones óptimas para las huellas dactilares fitoquímicas de CCF y CLAR de las 4 preparaciones herbales líquidas farmacopéicas más comunes de esta droga herbal, a saber, infusión, 70%, 45% y 20% tinturas hidroalcohólicas. Nuestro trabajo proporciona a la industria latinoamericana un conjunto de análisis base para establecer la identidad y la química de las muestras de *N. lobata* con fines de control de calidad.

Palabras clave: CLAR; CCF; Microscopia; Macroscopia; *Neurolaena lobata*; Guatemala.

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INTRODUCTION

Tres puntas is a medicinal plant species commonly used in Guatemala but growing in many other countries of the Central American and Caribbean region, where it gets as many different common names. According The Plants List (The Plant List, 2018) the accepted name for this species is *Neurolaena lobata* (L.) R.Br. ex Cass. Other illegitimate names and/or Synonyms include assignments to the genera *Calea*, *Conyza*, *Critonia*, *Eupatorium* and *Pluchea*, as well as several other *Neurolaena* species.

The leaf is the herbal drug. In the wider region constitutes a popular folk remedy for diabetes, colds, gonorrhea, itch, malaria, and is sometimes used as a tick repellent (Gupta *et al.*, 1984; Blair & Madrigal Calle, 2005; U.S. Department of Agriculture Agricultural Research Services, n.d.)

In Guatemala, properties like antibiotic, antimalware, carminative, appetitive, spasmolytic, febrifuge, diuretic, hypoglycemic, hypotensive and tonic are attributed to it. Its antibacterial, antiprotozoal, antifungal, anti-inflammatory, antiulcer and anti-diabetic activities were pharmacologically demonstrated (Cáceres *et al.*, 2009).

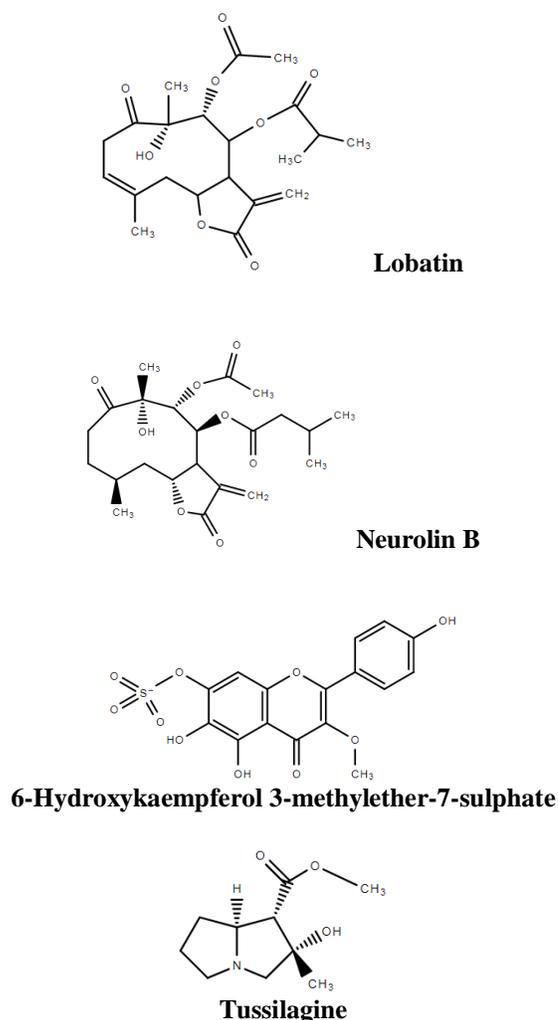
In Panama is used as an antipyretic and for gastrointestinal complaints (ulcers, parasite) by the Guaymi Indians in the form of infusion of the ground stem bark (Joly *et al.*, 1987; Joly *et al.*, 1990). Several preclinical pharmacological studies have validated these popular uses (Gracioso *et al.*, 2000; Fujimaki *et al.*, 2005; García *et al.*, 2007)

Its phytochemistry has been reasonably explored. Eleven flavonoids from the dichloromethane, ethyl acetate and water extracts of the leaf of the plant have been isolated including five quercetagenin derivatives, four kaempferol derivatives and two luteolin derivatives (Kerr *et al.*, 1981). The leaf also contains neurolenins and furanoheliangolides (sesquiterpene) lactones such as neurolin A and neurolin B, lobatin A and lobatin B, neurolin C-F, lobatin C, 8 β -isovalerianoyloxy-9a-hydroxy-calyculatolide, and 8 β -isovalerianoyloxy-9a-acetoxycalyculatolide (Manchand & Blount, 1978; Borges-del-Castillo *et al.*, 1982; Passreiter *et al.*, 1995). These compounds have been extensively researched as anti-inflammatory agents (Walshe-Roussel *et al.*, 2013; McKinnon *et al.*, 2014; Kiss *et al.*, 2015;) which may well be on the basis of its preclinical in vivo antinociceptive activity (Gracioso *et al.*, 1998). Finally, *N. lobata* contains non-toxic pyrrolizidine

alkaloids, including the untypical methyl ester alkaloids tussilagine, isotussilagine and their possible biosynthetic precursor 2-pyrrolidineacetic acid in the methanolic leaf extract (Passreiter, 1998a).

The recent regulatory framework introduced in the Region makes the quality control of herbs such as *N. lobata* compulsory and more stringent (MINECO, 2011). Some of the authors participated in a seminar-workshop to discuss the required adaptations of both industry and regulators to successfully implement this framework (Prieto, 2017) and from this event a series of collaborations to develop affordable methods for the quality control of local plants was born. Therefore, this is the first one of a series of studies to provide with open access protocols and data to ascertain the identity and quality of Central American medicinal plants.

Figure No. 1
Some of the characteristic compounds described in *N. lobata*



MATERIALS AND METHODS

Reagents and Chemicals

Water and acetic acid (50%) for HPLC, and butanol are purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Acetic acid (glacial) analytical reagent grade, ethyl acetate laboratory reagent grade and methanol were from Fisher chemicals (UK). Formic acid 98% by VWR. Caffeic acid, rutin and quercetin by Sigma-Aldrich. 2-aminoethyl-diphenyl borinate (98%) by Lancaster Laboratories (UK). DPPH, ascorbic acid, Griess reagent, sodium nitrite from Sigma-Aldrich. Sodium Nitroprusside (SNP) from Fisher chemicals.

Plant material

For the morphoanatomical studies, whole and fresh wild plants of *N. lobata* were collected from "Ecoparcela el Kakawatal", Suchitepéquez, Guatemala; localized at 539 meters under the sea level at 14°33'6.66"N; 91°21'53.19"O. The identification and deposit of the specimens take place at the "Herbario de Biología de Guatemala (BIGU)"; dry drug samples were also made and deposited in the Cytology Department, both from the Faculty of Chemical & Pharmaceutical Sciences of the San Carlos of Guatemala University.

For the phytochemical studies, a commercial sample of *c.a.* 300 g of Tres Puntas (Lot NL00147-002) was provided by Quinfica S.A. (Guatemala) consisting on dried (<8% humidity) leaves and stems were and analyzed. Samples are available at the Herbarium of the UCL School of Pharmacy (NL2017001).

Extraction

Samples of 10 g of the pulverized plant material (Salter Grinder, UK) were extracted with 100 mL of ethanol 70%, 40%, 20% or distilled water according pharmacopoeial methods (European Directorate for the Quality of Medicines & Health Care, 2010) filtered sequentially through gauze and Qualitative filter paper (Whatman, UK).

Identity tests

Fresh and dried plant materials were used for the macroscopic, organoleptic, micro-morphological and quantitative studies. The macroscopical description was made on specialized literature basis. The morphologic aspects were compared with the ones described in the Flora of Guatemala in order to establish the minimum characteristics for its

identification. For microscopical analysis, handmade transverse sections from aerial parts were performed and stained with safranin, leaves were cleared, stained and mounted by conventional methods according to Solis *et al.* (Solis *et al.*, 2003) and Gattuso & Gattuso (Gattuso & Gattuso, 1999). These sections were mounted with gelatin-glycerin and observed with a Micromaster® microscope, photographed using a Westover™ camera, and digitalized with Micron (USB) program.

Physicochemical analysis

From dried material, total ashes were determined by standard laboratory methods and moisture percent was determined by the thermogravimetric process.

Total Ashes

The percentage of total ashes was performed by quadruplicated, using the weight differences before and after drying it, for one hour in a laboratory muffle at 600° C. One gram of the pulverized sample was placed in a tared crucible. Weighed accurately and ignited gently until completely carbonized, keep it from burning, then gradually increase the temperature to 500-600° C. Continue the ignition until the sample turned into a white ash with constant weight. The ash was weighed and the percentage of total ashes was determinate.

Moisture

Determination of moisture percent was carried out by quadruplicated, using the weight differences of the plant material in a humidity balance (moisture analyzer) 5 g of material, after one hour of drying at 105° C in a humidity balance. Five grams of homogenized plant material (5 g) were chosen and placed on the sample pan, distributed in a thin layer. A program of one hour of drying at 105° C was run and at the end the moisture percent was determinate by the apparatus.

Thin layer chromatography

Manual TLC analyses were performed using TLC silica gel 60 F254 aluminum sheets 20 x 20. (Merck, Germany). A Camag TLC visualizer with the WinCATS software version 2.2 was used to document the plates (Camag, Switzerland).

The extracts and the standard mixture (Rutin and Caffeic acid) diluted to 200µg/mL with methanol were loaded with micropipette. The plates were developed using CAMAG developing chamber. The

method included 20-minute saturation time, using saturation pads. The whole process is done at room temperature (18-22° C). The mobile phase used was ethyl acetate: formic acid: acetic acid: water at ratios 100:11:11:26. (Wagner *et al.*, 1983). During development, the solvent front was allowed to migrate 70mm before allowed to dry. It was revealed with Natural products reagent (NPR), consisting on 250mg of 2-aminoethyl-diphenyl borinate dissolved in 50mL of ethyl acetate, dried on air and dipped in a solution of PEG 4000.

High performance Liquid Chromatography UV analysis

HPLC-UV analysis: Equipment consisted on an Agilent 1200 series HPLC system with UV-VIS PDA detector (Agilent Technologies, UK), Agilent ChemStation software, Phenomenex® C18 column (250 × 4.6 mm id, 5 µm). Solvent A (H₂O + acetic acid 0.2% v/v) and B (methanol + acetic acid 0.2% v/v) were mixed in gradient mode as follows: 0 min 90% A, 0-5 min 80% A, 5-65 min 50% A, 65-75 min 20% A; flow rate 0.8 mL/min. The injection volume and column temperature were set at 10 µL and 40°C, respectively (Giner *et al.*, 1993).

DPPH· Radical scavenging activity

This method evaluates the free- radical scavenging capacity of the extracts by measuring their ability to reduce the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. The protocol was adapted from previously established methods (Burits & Bucar, 2000). Briefly, DPPH· was dissolved in methanol, and the experiments were performed on freshly prepared solution. The assay conditions were as follows: 10 µL of test compound/extract (concentration 100 µM, and its serial double dilutions) was added to 200 µL of methanolic solution of DPPH· in microplates (Corning, UK). After incubation at room temperature for 20 minutes the absorbance at 490 nm was evaluated with a plate reader (TECAN infinite M200, Switzerland) and the data collected and processed using TECAN Magellan version 7.0. (TECAN infinite M200, Switzerland).

Nitric oxide (NO·) radical scavenging assay

The experimental protocol is based on the Griess reaction and follows closely a previously described protocol (Sreejayan, 1997). In microtitre plates (96 wells) 200 µl of sodium nitroprusside (5 mM) and 50

µl of sample are mixed. At 1-hour intervals, Pipette 50 µl supernatant onto a second plate, add 50 µl of Griess reagent (1% sulphanilamide, 0.1%). This was then incubated again at room temperature for an additional 15 minutes. The absorbance was read at 540 nm and the percentage and the EC₅₀ of NO inhibition and total NO remaining in solution was calculated in Excel (Microsoft Excel 2007, USA) using a calibration curve built up with Sodium Nitrite.

RESULTS

Morphoanatomical description

Tres puntas is an annual or perennial erect median bush, with grooved stem; short petiolate, acuminate, trilobate, glabrous and alternate leaves. Sheets shows dentate margins, dark green color, hirsute and shaggy on the upper side and olive-green color and short hairy at the back; with rough texture, soft smell and bitter taste. (Figure No. 2A). Raw material is conformed by the aerial parts of the plant. Dried and fragmented drug contain the petiolate brownish-dark green leaves, rough, rolled and easily broken, can contain small pieces of stem, keeping their properties unchanged (taste and smell), (Figure No. 2B).

Diagnostic micromorphological characteristics

Stem transverse section shows depressions and protuberances composed of different types of tissue, discontinuous open collateral vascular bundles with vascular cambium, surrounded by parenchyma cells with chloroplasts, angular collenchyma (Figure No. 2C) and one layer of epidermal cells with multicellular non-glandular hairs and unicellular and multicellular glandular ones. Non-glandular trichomes in two types, multicellular uniseriate with wide base and pointed tip and multicellular osteolate ones with thick walls, thin base and pointed end. Glandular ones with multicellular stalk and unicellular head and unicellular short stalk and unicellular globous head (Figure No. 2C & 2D).

Leaf transverse section shows a bifacial blade with quadrangular epidermal cells and thin cuticle in upper epidermis (Figure No. 2E), the lower epidermis shows irregular and slightly papillose, oval cells both epidermal layers are unstratified. Palisade cells in a single row with large cells and compact arrangement. Spongy mesophyll with irregular aspect and distribution, shows small droplets of oil. Many glandular and non-glandular hairs can be seen (Figure No. 2E).

Figure No. 1

Macro and micrographical chart for *Neurolaena lobata* showing (A) image of fresh plant specimen in its habitat, (B) dry leaves, (C) stem transverse section, (D) stem transverse section with glandular and non-glandular hairs, (E) leaf transverse section, (F) leaf transverse section at and mid rib insertion.

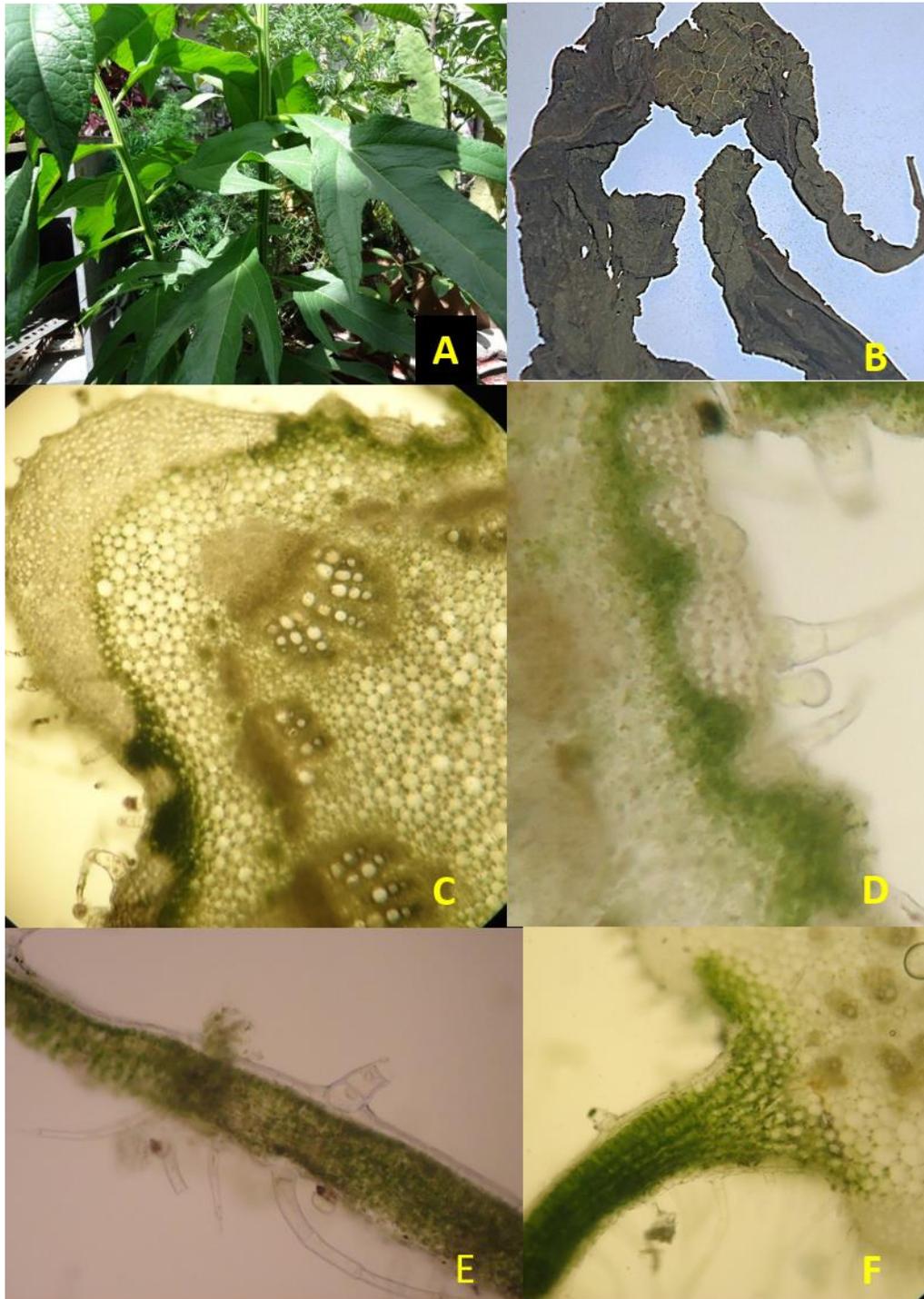


Figure No. 2
Photomicrographical chart for *Neurolaena lobata* showing details: (A-B) Cystolith trichomes, (C) glandular and non-glandular hairs, (D) transverse section at mid rib level, (E-F) diaphanized leaf.

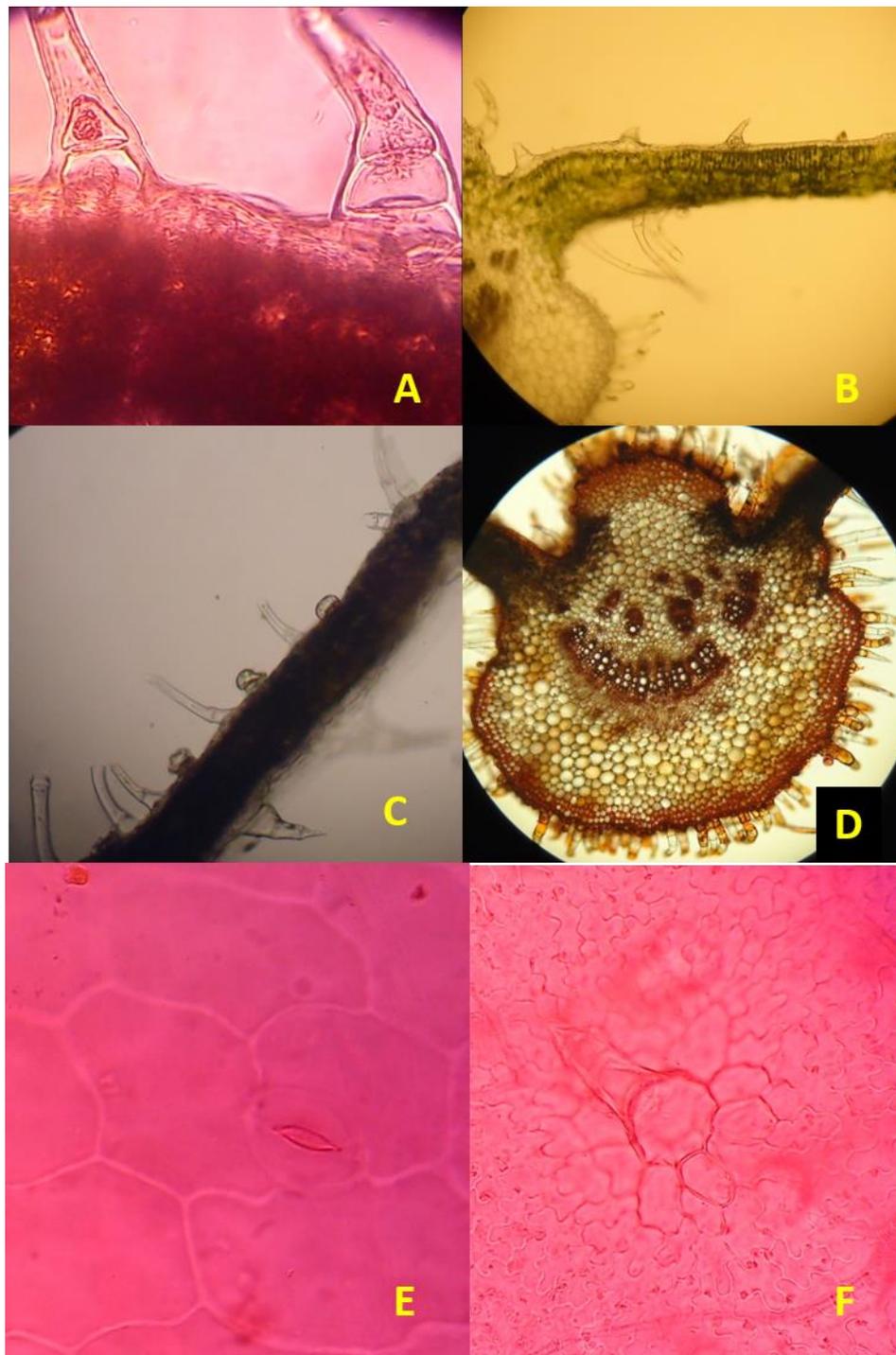


Figure No. 3

TLC analyses of *N. lobata* extracts. Left: TLC of Lane 1: standard compounds rutin, caffeic acid and quercetin (in increasing order of Rf); Lane 2: 70% extract (UV=360 nm after treatment with NPR + PEG 4000). Right: sketch for quality control purposes

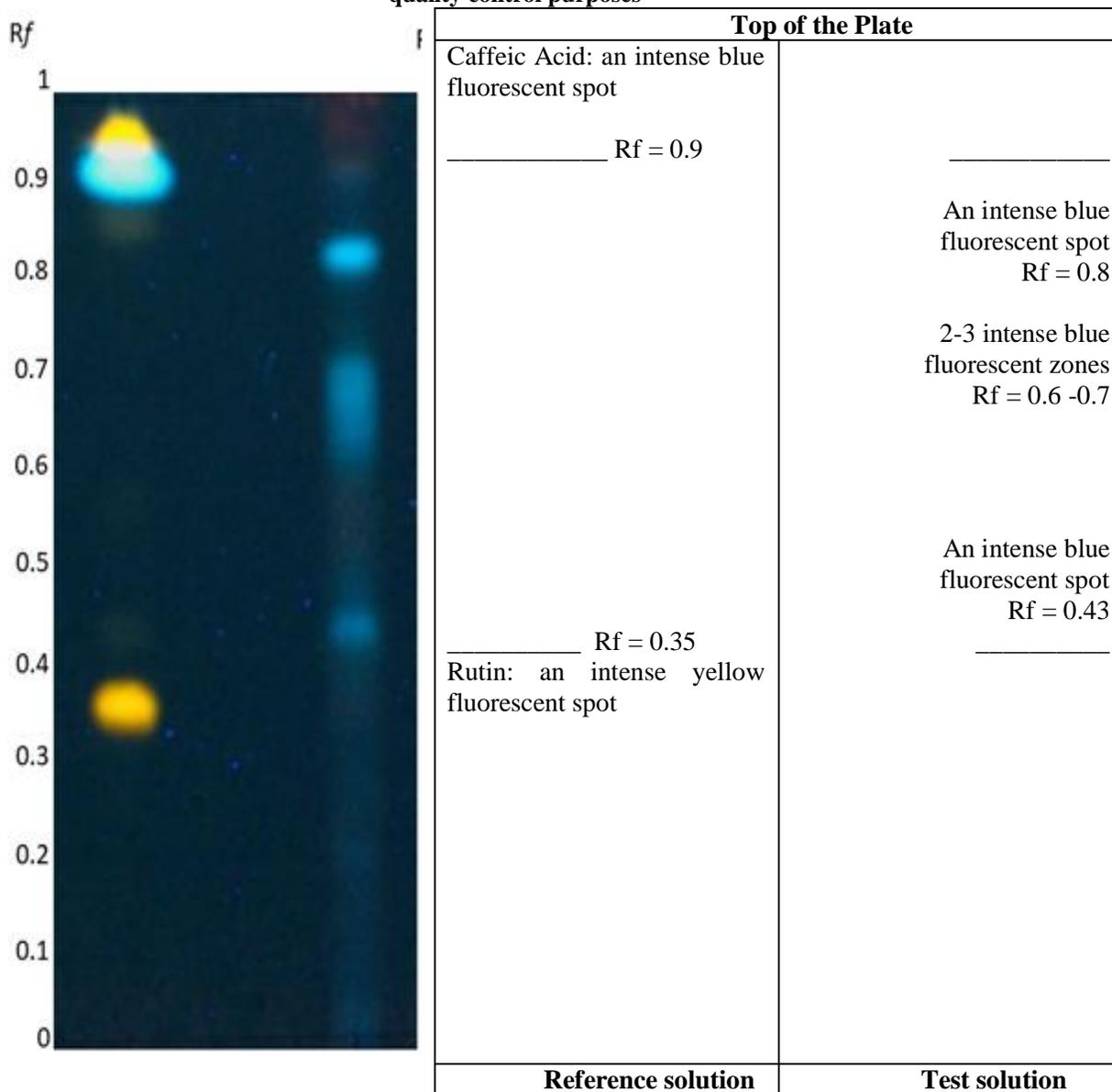
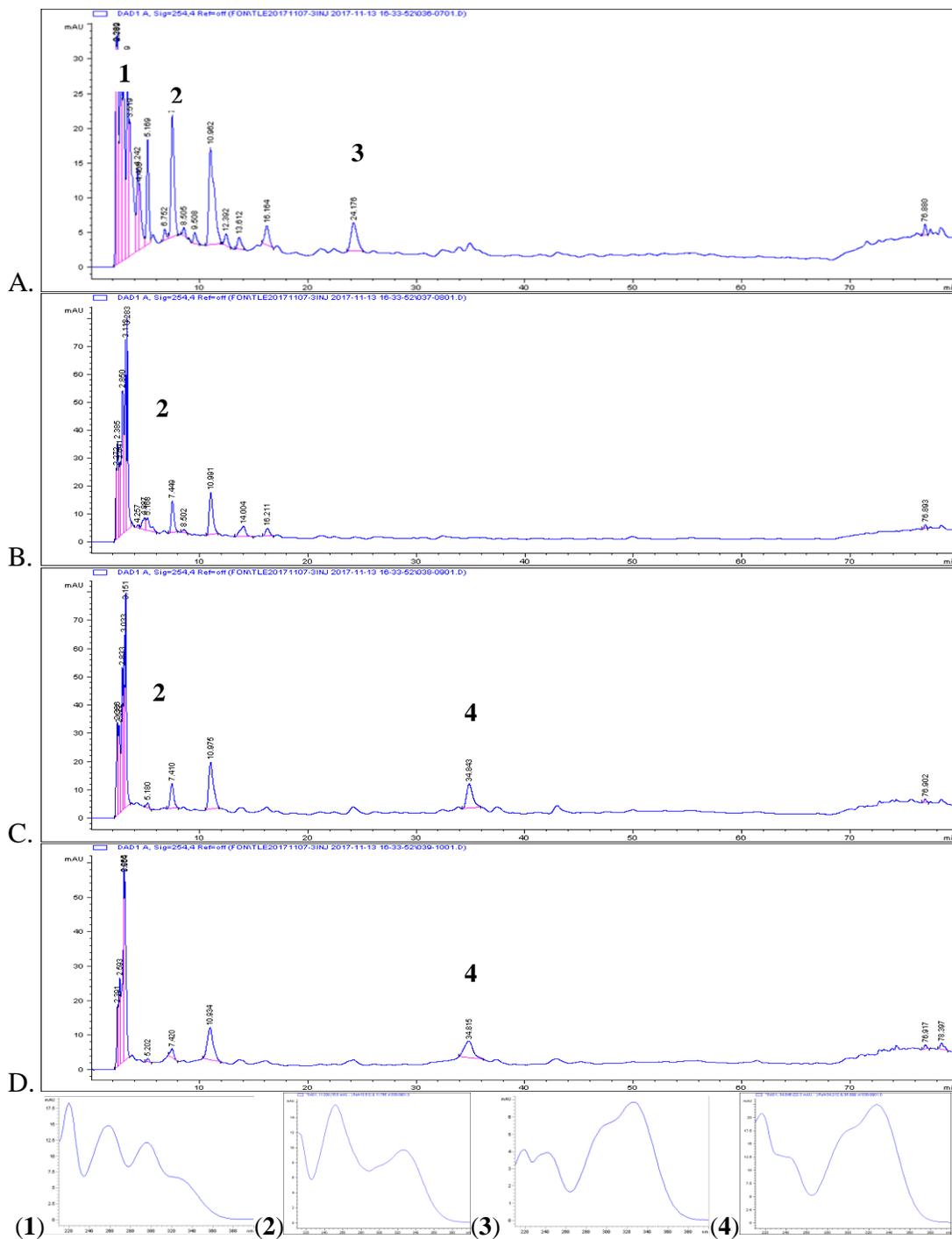


Table No. 1
Solid Residue of the different extracts of *N. lobata* and their EC₅₀ in antioxidant assays

Extract	Solid Residue (mg/mL)	DPPH (EC ₅₀) µg/mL	NO• (EC ₅₀) µg/mL
<i>N. lobata</i> 70%	6.8	155 ± 28	1231 ± 397
<i>N. lobata</i> 45%	7.5	117 ± 13	1139 ± 220
<i>N. lobata</i> infusion	6.95	184 ± 33	1235 ± 233
Ascorbic Acid	-	5 ± 1	190 ± 65

Figure No. 4

HPLC chromatograms ($\lambda = 254$ nm) of *N. lobata* infusion (A) and hydroethanolic tinctures 20% (B), 45% (C) and 70% (D). Below the UV spectra of peaks (1-4).



Covering trichomes in different types, Cystolith trichomes are multicellular uniseriate with wide base and pointed tip, showing very thick walls (Figures No. 3A y 3B); multicellular osteolate ones with thick walls, thin base and pointed end; multicellular osteolate ones with thin base, pointed end and thin walls and multicellular osteolate ones with wide base surrounded by 8-13 elevated epidermal cells. The glandular ones with multicellular stalk and unicellular head; unicellular stalk and unicellular globous head and biseriate stalk and unicellular globous head. The osteolate hairs in upper epidermis are shorter than the lower ones, with small amount of cells but wider bases (Figures No. 3C y 3D). At the midrib level, transverse section shows abundant hairs specially on lower epidermis; angular collenchyma under both unstratified epidermal layers; parenchymatous cells with secretory ducts, open bicollateral vascular bundles, these are arranged in one open arch with three to six accessory bundles, the arch is discontinuous near the apex and almost circular near the base of the leaf (Figure No. 3D).

The surface view shows an hypostomatic leaf and open and reticulate venation. Lower epidermal cells with polygonal and slightly sinuous anticlinal walls with anisocytic and anomocytic stomata at epidermis level, numerous covering trichomes and some glandular hairs (Figure No. 3E). Cells of upper epidermis polygonal in outline with sinuous and beaded anticlinal walls, shows large trichomes bases, glandular and non-glandular hairs (Figure No. 3F).

HPLC Analysis

The chromatograms are presented Figure No. 4. The infusion predictably being busier with very polar components in the first minutes and the tinctures presenting a cleaner baseline from the beginning. There is the presence of two common peaks at $R_t = 7.4$ (**1**) and $R_t = 10.9$ min with UV spectra consistent with a flavonoid derivative (most possibly glycosylated). The water extract (infusion) additionally will present a characteristic peak with $R_t = 24.2$ min (**3**) with a UV spectrum consistent with a phenolic acid. Increasing the percentage of ethanol will result in the disappearance of peaks **1** and **3** and the appearance of a lower polarity component ($R_f = 34.8$ min, peak **4**) with a UV spectrum consistent with a phenolic acid. This patterns could be used as

quality control of such preparations and to differentiate between them.

Physicochemical tests

Moisture and total acid ashes were made by quadruplicate, and the results demonstrated quality of plant materials used in this study, considering that all were between the OMS standards. Moisture were $6.79\% \pm 0.32$ (Range 6.40-7.12) and Total ashes 8.57 ± 0.58 (range 7.79-9.07).

TLC Analysis

The tinctures present a distinctive pattern consisting on three blue zones between the R_f of the standards rutin ($R_f = 0.35$) and caffeic acid ($R_f = 0.9$) (Figure No. 4). This pattern could be used as quality control of such preparations. Figure No. 4 presents an image of a real plate and a sketch with quality control parameters.

DISCUSSION

This study was performed with the main purpose of provides the Latin-American industry with a set of analyses to establish the identity and chemistry of *N. lobata* samples for quality control purposes, and recommends which preparations are more recommendable in terms of yield and antioxidant capacity, two parameters of industrial importance (Prior et al., 2005).

The organoleptic characteristics of plant material found in this study, was previously reported in Guatemala (Granados, 2007). The dorsiventral and hypostomatic leaves, the unstratified epidermis, the anomocytic stomata's and the parenchymatic sheets around the vascular boundless had been reported like common to other member of Asteraceae family (Metcalfe et al., 1970).

The presence of many different glandular hairs, similar to the ones found in this study, were previously reported in *Helianthus* from the Asteraceae family (Aschenbrenner et al., 2013).

There is a previous report on a quantitative HPLC method to analyse *N. lobata* neurolenins and furanoheliangolides (sesquiterpene) lactones (Passreiter, 1998b). However, these compounds are not commercially supplied as available standards. Notwithstanding future developments, we believe that a fingerprint is a faster and better option for the QC analysis of this species and certainly more suited to the infrastructure of the regional industry.

The antioxidant assays show that there is not a significant difference between infusion and different tinctures either against oxygen or nitrogen-based radicals. Therefore, any of them will provide virtually the same antioxidant capacity. We advise that addition of ascorbic acid could be considered to preserve the liquid formula and extend their shelf life. All extracts remain liquid down to 20° C except for the 45% ethanol extract which becomes a jelly. This may indicate the presence of some anti freezing molecules -not visible in HPLC analyses- which are only significantly extracted by this alcoholic strength.

As a safety remark, we suggest that preparations containing this herb should not be administered concomitantly with anticoagulants or in case of gastric ulcers, as previous pharmacological studies shown that the infusion is endowed with significant in vitro antiaggregant activity of washed human platelets induced by thrombin (Villar, 1997). The presence of pyrrolizidine alkaloids -although considered not toxic (Passreiter, 1998a)- may hamper international trade for products made of this plant species and certainly warrant further research to completely discard the presence of other more deleterious pyrrolizidine alkaloids.

We hope that these methods will help to improve the Quality control of Guatemalan Medicinal Plants thus contributing to the local, national and regional economy.

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