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MORPHOANATOMICAL AND PHYTOCHEMICAL STUDIES FOR THE QUALITY CONTROL OF *EUPHORBIA LANCIFOLIA* SCHLTDL. (EUPHORBIACEAE)

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ABSTRACT

Introduction: *Euphorbia lancifolia* Schltdl. (Euphorbiaceae) is a medicinal specie of central America origin, known as Ixbut in Guatemala, widely recognized for its important natural galactogogue activity. Methods: 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. Results: 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. Conclusions: Our work provides the Latin-American industry with a set of analyses to establish the identity and chemistry of E. lancifolia samples for quality control purposes.

Keywords: HPLC; TLC; Microscopy; Macroscopy; *Euphorbia lancifolia*.

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1. INTRODUCTION

Euphorbia lancifolia Schltdl belongs to Euphorbiaceae family, to date, ixbut's active ingredient has never been isolated [1]. However, due to the presence of a white latex, this medicinal herb is believed to be a natural galactagogue and antiophidic of Guatemalan origin [2]. A decoction or infusion made of ixbut leaves has been used for centuries by postpartum and lactating women and is now commercialized in the country to stimulate and increase the flow of breast milk [3].

Ixbut means in Pokom language 'which augments the volume of liquid in women' It is the popular name of a medicinal plant species which leaves are infused and commonly used as galactagogue both in women and cattle in Guatemala and other countries of the Central American region. Other ancient popular uses include the application of latex to snake bites and cases of jaundice, the infusion of leaves in cases of male impotence, fevers, colics and general pain [2]. It is also used for stimulation and cold. [4] and the fresh leaves are edible if included in salads. According The Plants List [5] the accepted name for this species is *Euphorbia lancifolia Schltdl.* (Euphorbiaceae). Other Illegitimate names and/or Synonyms include *Euphorbia lancifolia* var. *villicaulis* Fernald and *Poinsettia lancifolia* (Schltdl.) Klotzsch & Garcke

This medicinal plant is native to Guatemala, Belize and Honduras. It is a perennial herb that is somewhat fleshy, has dark green leaves that span 5 to 9 cm long and grows as tall as 2 metres high. The plant is normally found growing in damp areas. However, it can also be found in pine forests or in open fields in south-eastern Mexico, Guatemala, Belize, El Salvador and Honduras. The infusion of the aerial parts are the herbal drug. *E. lancifolia* is used for several centuries as herbal tea by postpartum Mayan women in Guatemala to stimulate and increase the flow of mother's milk. It was found that it does not induce lactation in non-lactating women, does not produce breast pain or congestion in lactating women between the second and fourteenth month of lactation and does not produce any appreciable change in breast volume or in the mammary glands of nursing mothers. *E. lancifolia* seems to have higher activity as a galactagogue in Guatemala during the month of November, i.e. in the early part of the dry season in Guatemala City shortly before the plant blossoms. It was also found that lactogenic properties of *E. lancifolia* have been considerably exaggerated - not only in the case of women, but also in female cats. Presently, *E. lancifolia* is rarely used as a galactogogue in Guatemala City. This decline in interest is mainly due to the availability of many commercially prepared infant formulas, decreasing most women's dependence

on breast-milk. In other areas, it is only taken when the nursing mothers are facing difficulties producing breast milk, rather than as a general way to supplement milk output [1].

E. lancifolia is found to be non-toxic to insects and when mixed with cattle fodder, it can increase milk yields in cows. tried feeding chicks with several different plants, including *E. lancifolia*. The carotene content and vitamin A activity of dehydrated forage meals prepared from different ingredients were studied. It was found that *E. lancifolia* contained the highest carotene content of the four forages and maintained the highest serum levels of vitamin A [1].

The active principles of *E. lancifolia* remain elusive. [6] found that *it has no activity in* ERα and β and PR binding assays, COX-2 binding and functional assays, serotonin (5-HT_{1A}) binding assays and cell based SEAP reporter gene assays in transiently transfected MCF-7 cells and endogenous gene expression in MCF-7 cells.

Compounds isolated from *E. lancifolia* include several terpenes such as lupeol acetate, germanicol pentanoate, α- and β-amyrin, α- and β-amyryl *p*-hydroxycinnamate, α- and β-amyryl ferulate, amyryl ferulate, and the ubiquitous β-sitosterol as well as an unidentified porphyrin-type compounds, phytol and ethyl linolene [7]. None of the compounds identified are known to be toxic or harmful within a biologically relevant dose range. Cates *et al*. [4] found that acetone and methyl extracts of *E. lancifolia* do not show inhibition in microbes or cancer cell lines.

Accordingly, The Guatemalan Vademécum of Medicinal Plants describes the leaves of this species as of medicinal is indicated as galactogogue, tonic and antiseptic. It seems safe in cattle, although the anecdotal toxicity reported for cows and horses seems to be due to the ingestion of the seeds and other species together with Ixbut. The long term observations in clinical trials and the popular perception for this remedy as safe for breast feeding mothers and their newborns [2] give credence to its relative safety at normal doses and regimes. Traditionally the average dose of ixbut per cup is 5 leaves or 5 sections of stem (about 5 grams); and up to 6 cups of ixbut tea are given daily to breastfeeding women [1]. The Vademecum recommends infusions (2-4g) or 10% decoction or tinctures (1:10, 35% Ethanol) (2-3 a day/ 6 weeks) but emphasizes that the plant is not officially accepted in any pharmacopoeia so this hampers its commercialization in the form of proper herbal medicines, thus remaining sold as loose plant material which is consumed as a domestic level.

The recent regulatory framework introduced in the Region makes the quality control of herbs such as *E. lancifolia* compulsory and more stringent [8]. A recent seminar-workshop to discuss the required adaptations of both industry and regulators to successfully implement this framework [9]

resulted in a series of collaborations to develop affordable methods for the quality control of local plants. This is the second of a series of studies to provide with open access protocols and data to ascertain the identity and quality of Central American medicinal plants.

2 MATERIALS AND METHODS

2.1 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.

2.1.1 Plant material

For the morphoanatomical studies, whole and fresh wild plants of *E. lancifolia* were collected and 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 Cytohistology Department, both from the Faculty of Chemical & Pharmaceutical Sciences of the San Carlos of Guatemala University.

For phytochemical analyses, a commercial sample of Ixbut (*Euphorbia lancifolia* Schltdl., Euphorbiaceae) consisting on 300g of dried (<12% Humidity) leaves and stems were kindly provided by Quinfica S.A. (Guatemala) (Lot EL8117-075). Macroscopically the material is green-greyish with characteristic flavours and taste. The plant material complied with the microbial specifications of the European Pharmacopoeia 7 (European Directorate for the Quality of Medicines & HealthCare, 2010) and the limits in heavy metals (<5 ppm on Pb, As, Hg and Cd). Samples are available at the Herbarium of the UCL School of Pharmacy (NL2017002).

2.1.2 Extraction

Samples of 10g of the pulverized plant material (Salter Grinder, UK) were extracted with 100mL of ethanol 70%, 40%, 20% or distilled water according pharmacopoeial methods [10] filtered sequentially through gauze and Qualitative filter paper (Whatman, UK).

2.1.3 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 [3] . For microscopical analysis, handmade transverse sections from aerial parts of the species were performed and stained with Safranin, leaves were cleared, stained and mounted by conventional methods according to Solis et al. [11] and Gattuso & Gattuso [12]. These sections were mounted with gelatin-glycerin and observed with a Micromaster® microscope, photo graphed using a Westover^{TM} camera, and digitalized with Micron (USB) program.

2.1.4 Physicochemical analysis

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

2.1.5 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.

2.1.6 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 choose 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.

2.1.7 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 werewere 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.

2.1.8 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 [13].

2.1.9 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 [14]. 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).

2.1.10 Nitric oxide (NO·) radical scavenging assay

The experimental protocol is based on the Griess reaction and follows closely a previously described protocol [15]. In microtitre plates (96 wells) 200µl of sodium nitroprusside (5mM) and 50µl of sample are mixed. At 1-hour intervals, Pipette 50µl supernatant onto a second plate, add 50µl 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 Excel2007, USA) using a calibration curve built up with Sodium Nitrite.

2.2 Results

2.2.1 Morphoanatomical of the description fresh drug

Euphorbia lancifolia (Ixbut) is a perennial herb usually fleshy and succulent with white latex. Shows very large cylindrical and articulated pale green stems, whit very short petiolate, alternate and lanceolate almost rhomboid leaves. The sheets present entire margins, green and glabrous on the upper side, pale and little hairy on the back. Flowers involucres arranged in small terminal naked cyme of turbinate-campanulate yellowish flowers (Fig. 1A).

The dry material is conforming by short petiolate green yellowish leaves, darker and glabrous on the upper side and pale and little velvety on the back, with entire and curved margins, sweet and pleasant odor and non-easily broken. Small parts of stems can also be found (Fig. 1B).

2.2.2 Diagnostic micromorphological characteristics

Stem transverse section shows an unstratified epidermis with irregular quadrangular cells, and lacunar collenchyma under epidermal layer (Fig. 1C), the pith usually hollows with laticifers and surrounded by a ring of twenty open collateral vascular bundles, each one with a small cap of sclerenchyma (Fig. 1D and 1E).

Petiole in transverse section appears reniform, with three collateral vascular bundles, unstratified epidermis, lacunar collenchyma and laticifers (Fig. 1F).

Leaf transverse section shows a bifacial sheet, a glabrous, 1-celullar epidermal layer with rectangular and irregular cells and an evident cuticle on upper side (Fig. 2A) and an unstratified pubescent and papillose epidermis on the lower side (Fig. 2B). Palisade parenchyma cells are one or two layered, large and irregular. Spongy mesophyll shows small rounded cells and large cylindrical discontinuous cells forming the aerenchyma and occupying two-thirds of the thickness (Fig. 2C). The lower epidermis shows non-glandular hairs: multicellular with thin walls, warted cuticle and pointed end (Fig. 2D), bicellular with thick walls and warted cuticle (Fig. 2E), multicellular with pronuounced terminal cell, and unicellular ones, multicellular are abundant under low magnification and shows basal cells, of 8-10 subsidiary ones in a rosette form (Fig. 2F). The midrib pronounced only to the abaxial side, shows a small collateral vascular bundle in flat

arch under a thick layer of compact parenchyma; an unstratified epidermis and lacunar collenchyma under both epidermal layers but in a small cluster under upper side (Fig. 3A).

The surface view shows a brochidodromous venation, cells of upper epidermis polygonal in outline with flat anticlinal walls (Fig. 3B), in the lower epidermis sinuous anticlinal walls, anomocitic, anisocitic, tetracitic, actinocitic and paracitic stomata at level and sunk (Fig. 3C-D).

Stem surface view shows large amount of paracitic and anisocitic stomata (Fig. 3E).

Fig.1. Macro and micrographical chart for *Euphorbia lancifolia* showing (A) image of herbarium specimen, (B) dry drug, (C) stem transverse section: unstratified epidermis and lacunar collenchyma, (D) stem transverse section: hollow pith and vascular bundles (E) steam vascular bundles, (F) petiole transverse section

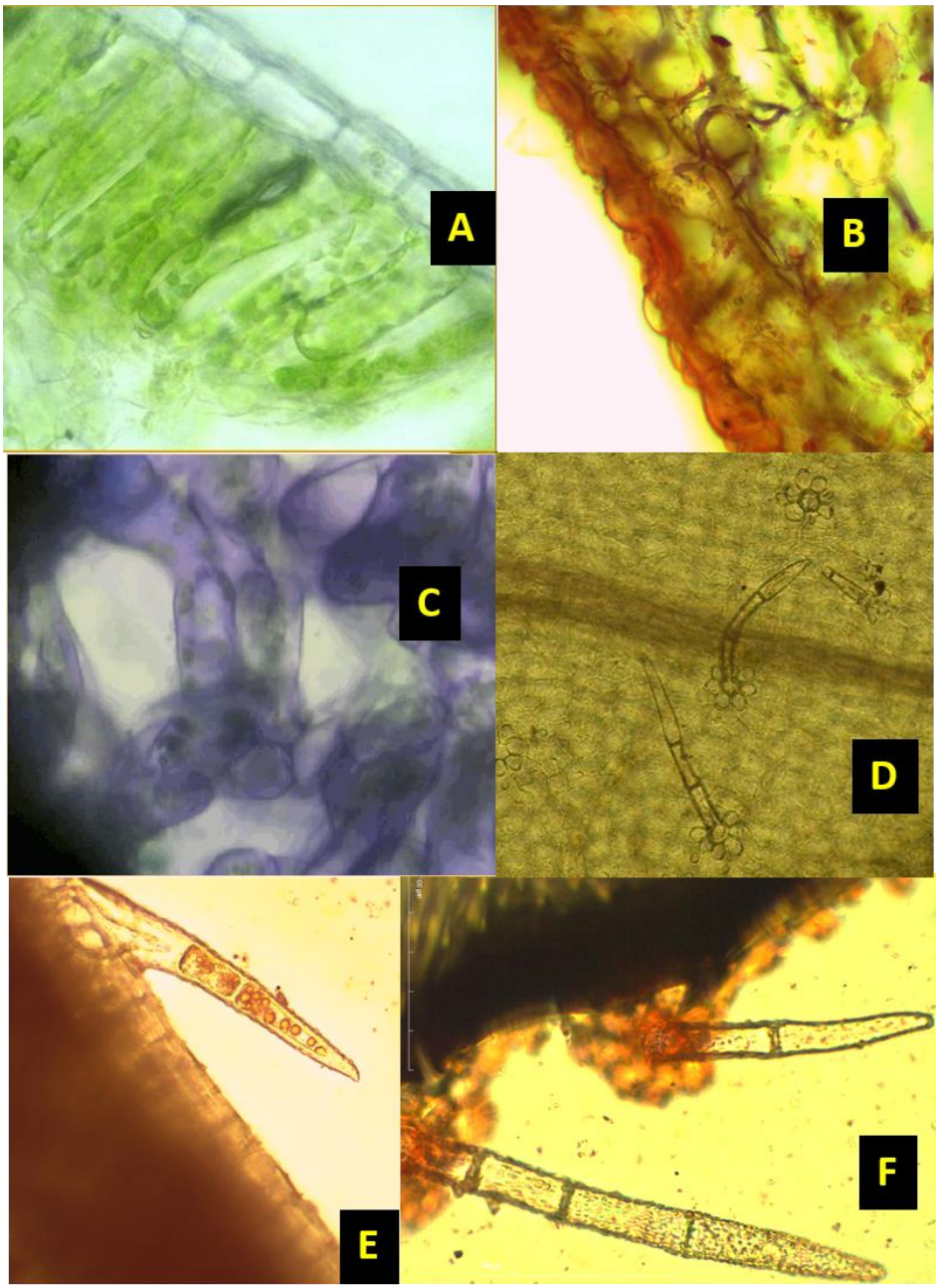

Fig.2. *Euphorbia lancifolia* leaf transverse section showing (A) adaxial epidermal layer, (B) abaxial epidermal layer, (C) aerénquima, (D) non glandular hairs, (E) non glandular hairs with thick walls, (F) non glandular hairs with warted cuticule and multicellular basal cells

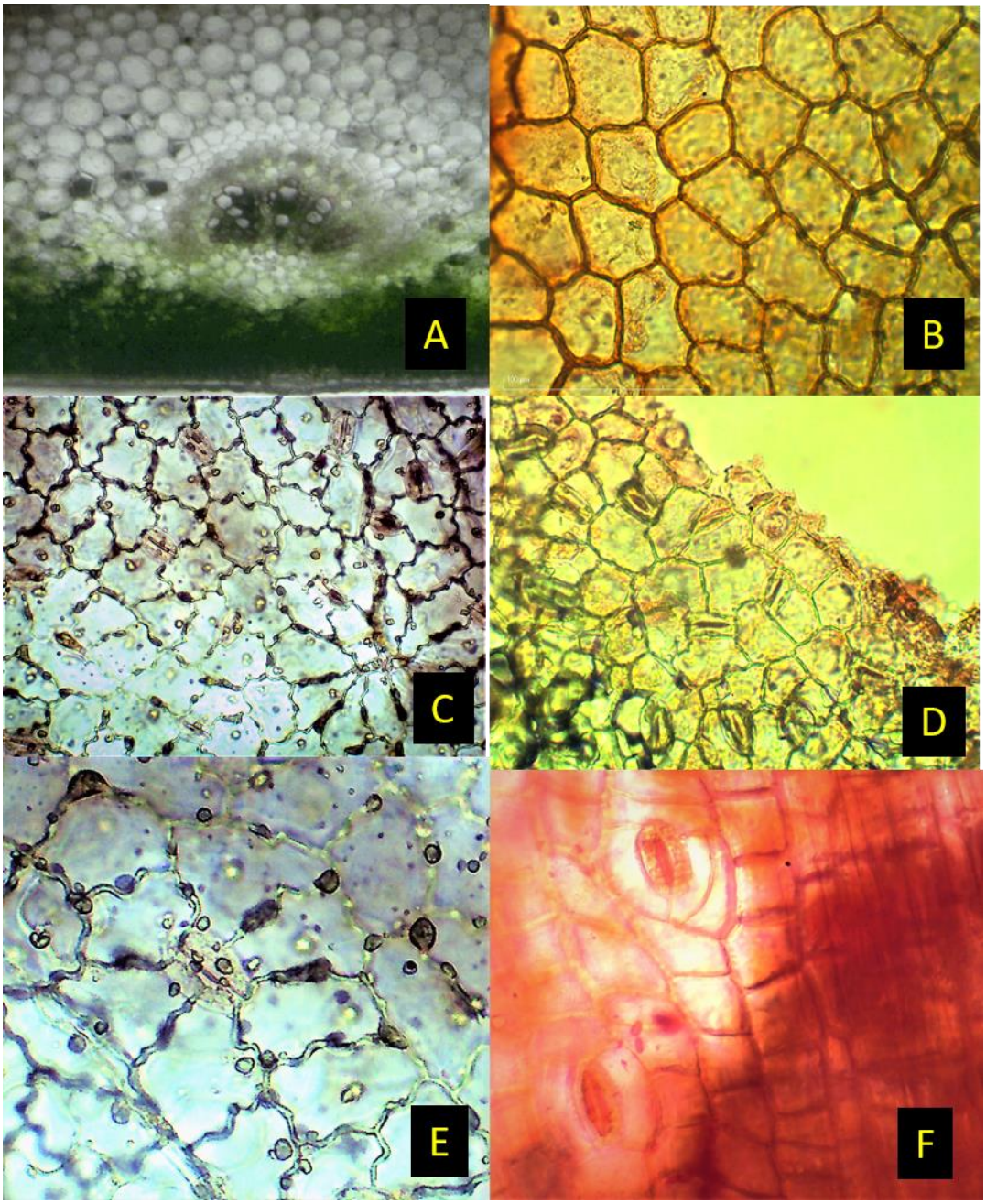

Fig.3. *Euphorbia lancifolia* (A) Mid rib transverse section, (B) adaxial epidermal layer in upper view, (C) abaxial epidermal layer in upper view, anomocitic and anisocitic stomata, (D) epidermal dissection, paracitic and tetracitic stomata, (E) actinocitic stomata, (F) Steam surface view

2.2.3 Macroscopical aspects of the dry commercial sample

The commercial material consists of roughly chopped dry stems and leaves of the plant (Fig. 4).

Fig.4. Dry stems and leaves of *E. lancifolia* as presented in commercial products.

2.2.4 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. Values for Moisture were 9.61 ± 0.15 (Median: 9.64; Range 9.31-9.91) and Total ashes 2.76 ± 0.09 (Median 2.71; Range 2.68-2.88).

2.2.5 Antioxidant Assays

The scavenging properties of the three preparations in two different radicals (DPPH$^{\bullet}$ and Nitric Oxide) are shown in Table 1. They are mildly active against DPPH$^{\bullet}$ but largely inactive against nitric oxide. There is not a significant difference between the infusion and the 45% tincture against these radicals.

Table 1. Solid Residue of the different extracts of *E. lancifolia* and their effective concentration 50% (EC50) in two antioxidant assays

Extract	Solid Residue (mg/mL)	DPPH$^{\bullet}$ (EC50) µg/mL	NO$^{\bullet}$ (EC50) µg/mL
E. lancifolia 70%	5.5	105 ± 28	913 ± 260
E. lancifolia 45%	5.3	49 ± 13	766 ±173
E. lancifolia infusion	5.0	30 ± 11	637 ± 222
Ascorbic Acid	-	4.67 ± 1.2	190 ± 65

2.2.6 HPLC Analysis

The chromatograms are presented in Figure 6. The chromatograms are presented in Figure 6. The infusion predictably being busier with very polar components in the first minutes and the tinctures presenting a cleaner baseline throughout. There is the presence of three common peaks at Rt = 7.4 (1), Rt = 15.5 min (2) and 34.2 min (3) with UV spectra consistent with flavonoids in all preparations.

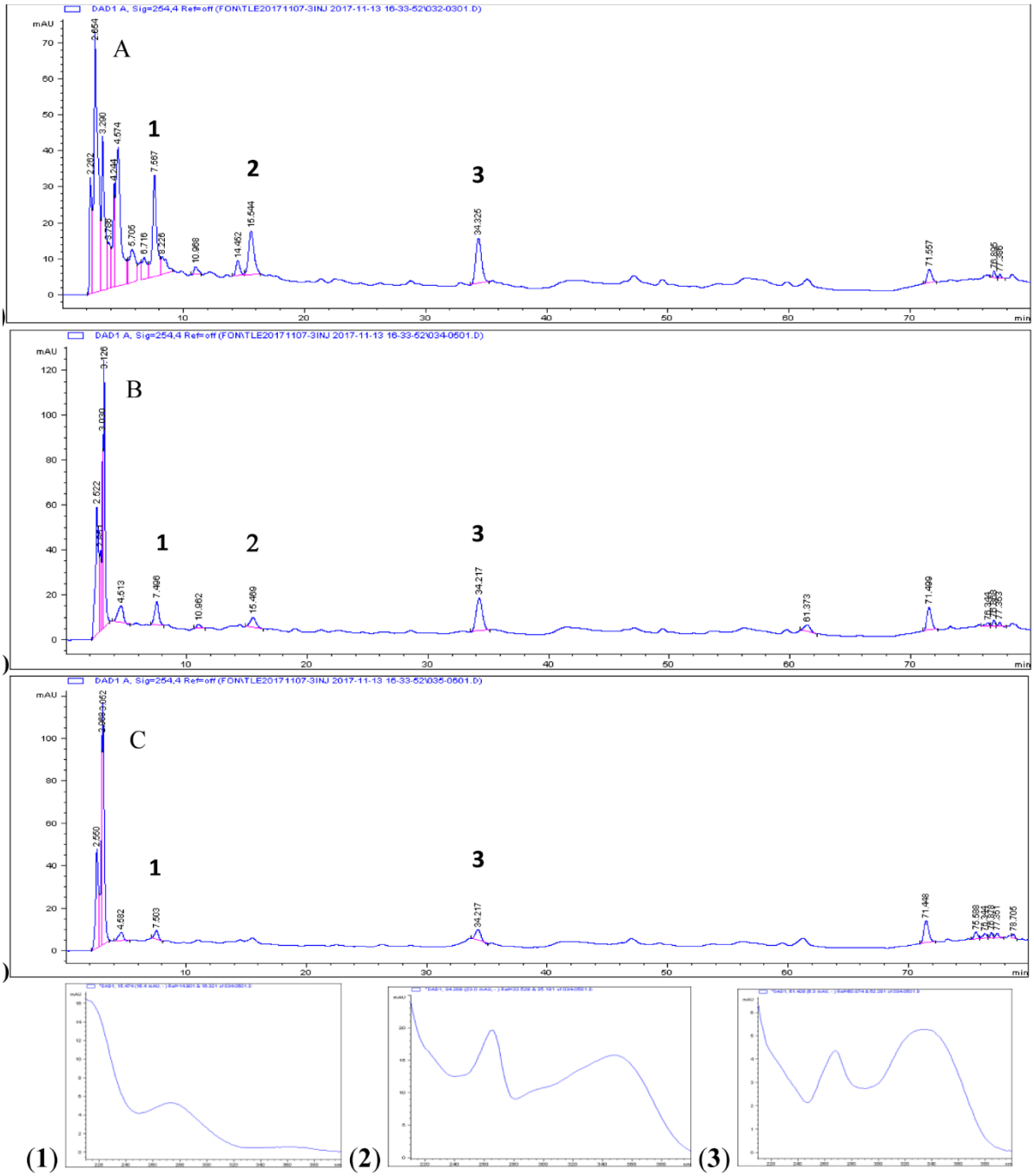

Fig.1. HPLC Chromatogram (λ = 254 nm) of *E. lancifolia* (A) infusion and hydroethanolic tinctures 45%(B) and 70%(C) and UV spectra of peaks (1-3)

3. DISCUSSION

This study was performed with the main purpose of providing the industry with a set of analyses to establish the identity and chemistry of *E. lancifolia* samples for quality control purposes, and recommends which preparations are more recommendable in terms of yield and antioxidant capacity, two parameters of industrial importance [16].

One of the goals of this study was to discuss whether organoleptic, macro- and micromorphological characters can provide a fundamental tool which help in the identification of raw material of *E. lancifolia*, especially when this is pulverized of fragmented. The investigation of lamina abaxial and adaxial surface and stomata types reveal additional characteristics that might be useful for identification and assessment of quality control standards.

The organoleptic characteristics of dried plant material found in this study, were not previously reported. The hollows pith and the presence of laticiffers in the stem were also reported for other members of *Euphorbia* genera, like *Euphorbia pulcherrima* [17]. The characteristics observed on the leaf blade, like the unstratified epidermis, the presence of a smooth cuticle on the upper side and the papillose epidermis on the lower side matches with the findings for *Euphorbia davidii* [18]. Despite the fact that there are no reports about the presence of aerenchyma in other species of *Euphorbia*, the finding is consistent with the description given in Flora of Guatemala, which describes the leaf been fleshy and succulent [3]. This kind of leaves being related to this kind of tissue in other families. The presence of many types of stomata in Euphorbiaceae, like the ones found for *E. lancifolia* in this study, were also reported in a study of some other members of the euphorbiaceous family [19]. The type of verrucous non-glandular hairs was also reported in the Euphorbiaceae family and can be very useful to the identification of this plant [20].

HPLC analyses show the presence of three common peaks at Rt = 7.4 (1), Rt = 15.5 min (2) and 34.2 min (3) with UV spectra consistent with flavonoids in all preparations. These patterns could be used as quality control of such preparations, TLC analyses were not conclusive.

The antioxidant assays show that there is not a significant difference between infusion and the 45% tincture either against radicals, however the preparations are largely inactive against nitric oxide. 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.

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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Conflict of Interest

Authors declare no conflict of interests.

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