HISTO-ANATOMICAL AND PRELIMINARY TLC INVESTIGATIONS ON *CYNOGLOSSUM OFFICINALE* L. (*BORAGINACEAE*) SPECIES

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ABSTRACT

The cross-sections through root, aboveground stem, leaf and sepal of Cynoglossum officinale L. species, Boraginaceae family, harvested from the geographical area of Valea Cernei, Vâlcea County (Romania), were obtained and interpreted using microphotography technique. In addition, the polyphenols content of the aerial parts (Cynoglossi herba) was analyzed by thin layer chromatography and established for the reason of pharmacognostic expertise. One caffeic acid derivative was identified from the 15 distinctive chromatographic bands.

INTRODUCTION

Cynoglossum officinale L., Houndstongue, Dog's tongue, *Boraginaceae* family, is a biennial herbaceous plant, 30–60 cm tall, frequent in ruderal zones, grasslands, scrubs, forest edges, acacia plantations, in Europe, Asia, North America [5].

It is often considered as invasive and toxic plant for livestock, wildlife and humans, because of its content in pyrrolizidine alkaloids. In fact, majority of *Boraginaceae* species contain toxic pyrrolizidine alkaloids, highly significant from the chemotaxonomic point of view, together with flavonoids, tannin, polysaccharides, terpenoids [3, 6, 8, 9, 11, 13, 14, 17–20].

However, some *Cynoglossum* species are used as medicinal due to their specific actions: diuretic, anti-inflammatory, analgesic, cicatrizing and wound healing, emollient, antimicrobial, antitumor [12, 21].

In the specialty papers, there are scarce and incomplete data concerning *C. officinale* histo-anatomy [7, 16] and chemical composition [21].

The aim of our paper was the histo-anatomical investigation of the root, aboveground stem, leaf and sepal of *Cynoglossum officinale* species and the preliminary analysis of the polyphenols content from the aerial parts (*Cynoglossi herba*).

MATERIAL AND METHOD

Histo-anatomical investigation

The vegetal material was harvested from *C. officinale* plants in blossom, in May 2016, from the geographical area of Valea Cernei (Cerna Valley), Vâlcea County, Romania.

The fixation and preservation of roots, aboveground stems, leaves and sepals were achieved in 70% ethanol.

The cross-sections and longitudinal-radial sections were obtained using botanical razor. After washing with distilled water, the sections were clarified using 10% sodium hypochlorite solution (Javel water). Then, the clarifying agent was removed by washing with distilled water. Congo red–chrysoidine mixture (Genevese reagent) was used for the staining of sections. Depending on the chemical composition of cell membranes, the reactive induced various stains: pink to red for cellulose and mucilage, pale red for cytoplasm, yellow for suberin and brown for lignin [2].

Stained and mounted sections were analyzed on a Krüss binocular photon microscope (objectives ×4, ×10, ×40) and then photographed using a Sony DSLR-A380 digital system adapted to the microscope.

The description of microscopic sections has been made according to some classical authors [15].

Thin-layer chromatography (TLC) analysis

Preliminary analysis of polyphenols was performed on the aerial parts of *C. officinale* species (*Cynoglossi herba*), using a CAMAG (Muttenz, Switzerland) system, in the following experimental conditions [1, 4, 10]:

▪ stationary phase: TLC silica gel 60 F_{254} 20×10 cm precoated glass plates (Merck, Darmstadt, Germany) pre-washed with chloroform–methanol (1:1, *v/v*);

▪ mobile phase: chloroform–ethyl acetate–toluene–formic acid–methanol (15:20:10: 10:1, in volumes) in a vapor-equilibrated chromatographic tank (20×10 cm twin trough chamber, CAMAG);

▪ sample: 20% methanolic extract of *Cynoglossi herba*;

▪ standards (Merck): 0.05% methanolic solutions of caffeic acid, chlorogenic acid, quercetin and rutin;

▪ migration distance: 80 mm;

▪ application of sample (1–10 µL) and standards (2 µL): CAMAG Linomat 5 semi-automatic system (spray gas nitrogen, dosage speed 150 nL/s, band length 8 mm);

▪ detection: CAMAG TLC Scanner 3 photodensitometer, deuterium–wolfram lamp, UV 254 nm, without derivatization, scanning speed 20 mm/s, data resolution 100 µm/step;

▪ measurement mode: absorption;

▪ spectra acquisition, processing and quantification analysis: winCATS ver. 1.4.3 software package.

RESULTS AND DISCUSSIONS

Histo-anatomical investigation

Root

In cross-section, into the lower third, the root has circular shape and secondary structure due to the presence of two secondary meristematic areas (phellogen and libero-ligneous cambium). In cross-section, from the outside towards the inside of the root, the following histological sequence was observed. Periderm is made of suber, phellogen and phelloderm. Suber is made up of 3–4 layers of large, flattened, suberin-impregnated cells. From point to point, suber is exfoliated. Subero-phellodermic cambium consists of a single layer of anterior-posterior flattened cells, with thin walls, the radial walls being slightly undulated. Phelloderm is made up of 2–3 cellular layers, with thin cellulosic walls. Into the cortical parenchyma of the primary structure, ergastic substances are stored. Conducting tissues are arranged in two concentric rings. Phloem tissue forms a thin, external ring, made of sieve tubes, phloem parenchyma and annex cells. The libero-ligneous cambium is found between xylem and phloem tissues. Xylem tissue forms the internal ring, consisting of many metaxylem vessels of different sizes, disorderly arranged and accompanied by some xylem parenchyma, pushing to the center protoxylem vessels with small diameter. In longitudinal-radial sections, xylem vessels exhibited reticulate thickenings. Protoxylem is under-represented, accompanied by xylem parenchyma. Medullary rays are multicellular, one- or pluriseriate, cellulosic, both into the phloem and xylem rings. Medullary parenchyma is missing (Figures 1–3).

In cross-section, into the upper third, the root has circular shape and secondary structure due to the presence of phellogen and libero-ligneous cambium. In cross-section, from the outside towards the inside of the root, the following histological sequence was observed. Periderm consists of suber, phellogen and phelloderm. Suber is composed of

3–4 layers of large, anterior-posterior flattened cells, impregnated with suberin. Sometimes, suber is exfoliated. Subero-phellodermic cambium is made up of a single layer of anterior-posterior flattened cells, with thin walls, the radial walls being slightly undulated. Phelloderm consists of 5–6 cellular layers, with thin cellulosic walls. Into the cortical parenchyma of the primary structure, ergastic substances are stored. Conducting tissues are disposed in two concentric rings. Phloem tissue forms a thin, external ring, made up of sieve tubes, phloem parenchyma and annex cells. Between the xylem and phloem tissues stands the libero-ligneous cambium. Xylem tissue forms the tick, internal ring, made up of many metaxylem vessels of different calibers, disorderly placed and accompanied by some libriform tissue, pushing to the center the protoxylem vessels with small diameter. In this area, meatus-type storage parenchyma appears very developed. Xylem vessels show reticulate thickenings, in longitudinal-radial sections. Metaxylem is abundant near the libero-ligneous cambium, to the rest being dispersed into the storage parenchyma. Protoxylem is under-represented, accompanied by xylem parenchyma. Medullary rays are multicellular, one- or pluriseriate, cellulosic, both into the phloem and xylem tissue rings. Medullary parenchyma is missing (Figures 1–3).

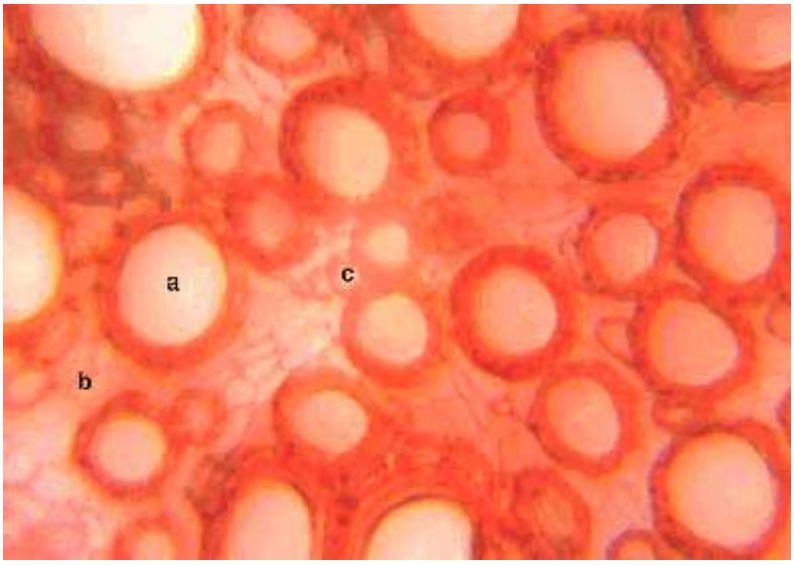

Figure 1. Cross-section through C. officinale root: (a) metaxylem; (b) xylem parenchyma; (c) protoxylem (Congo red–chrysoidine staining, ×400).

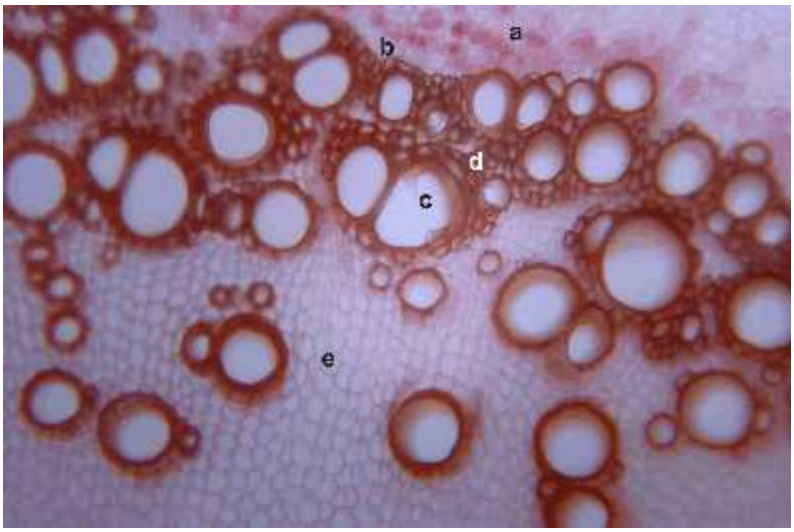

Figure 2. Cross-section through C. officinale root: (a) secondary phloem tissue; (b) libero-ligneous cambium; (c) metaxylem; (d) libriform tissue; (e) storage parenchyma (Congo red–chrysoidine staining, ×100).

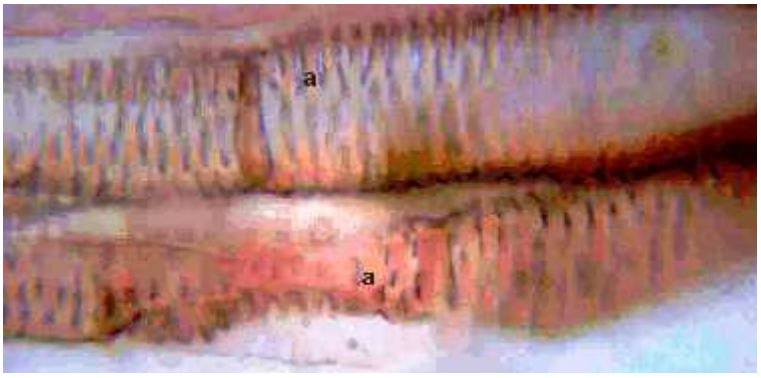

Figure 3. Longitudinal-radial section through C. officinale root: (a) metaxylem with reticulate thickenings (Congo red–chrysoidine staining, ×400).

Aboveground stem

In cross-section, into the upper third, the aboveground stem has circular sinuous shape and secondary structure generated by libero-ligneous cambium. The epidermis consists of a single layer of heterodiametric cells, with thickened external wall covered by thin cuticle. Epidermal cells are tangential elongated, with thin radial walls and thickened tangential external and internal walls. From point to point, it is observed unicellular, long tector hairs and stomata. Cortex is made up of two parts, the external area consisting of 2–3 layers of chlorenchyma. The inner cortex is well represented, parenchymatic, containing ergastic substances. For the cross-sections in the lower third of the aboveground stem, the cortex is organized into an external area consisting of 2–3 layers of tabular collenchyma, followed by 2–3 layers of angular collenchyma, and then into an internal zone made by 4–5 layers of cortical parenchyma bounded on the inside of a single endodermal stratum, of primary type, consisting of large cells. The conducting tissues are organized into two concentric rings due to the activity of libero-ligneous cambium. Phloem tissue is the thin outer ring made of sieve tubes, phloem parenchyma and annex cells. Medullary rays are multi-cellular, pluriseriate, cellulosic. Secondary xylem tissue formed the internal ring, consisting of metaxylem with various calibers and well represented libriform. Xylem vessels have spiral and reticulate thickenings. Medullary rays are wide, multicellular, pluriseriate, and strongly lignified. Primary xylem tissue is poorly represented, consisting of some vessels and parenchyma. Medullary parenchyma is well developed, of meatus type (Figures 4–6).

Figure 4. Cross-section through C. officinale aboveground stem: (a) epidermis; (b) tabular collenchyma; (c) cortical parenchyma; (d) phloem tissue; (e) libero-ligneous cambium; (f) metaxylem; (g) libriform tissue; (h) protoxylem; (i) medullary parenchyma (Congo red–chrysoidine staining, ×100).

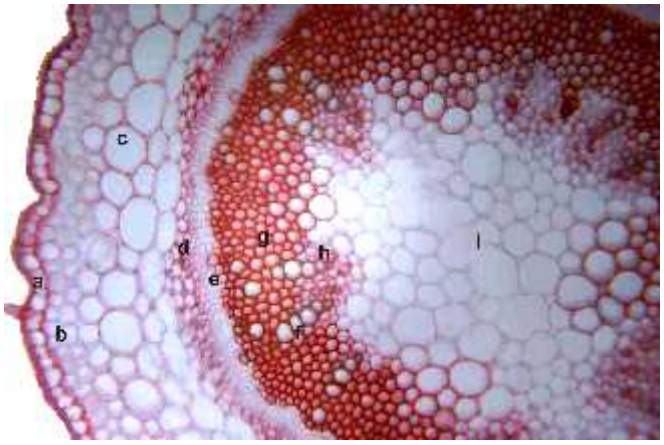

Figure 5. Cross-section through C. officinale aboveground stem: (a) epidermis; (b) chlorenchyma; (c) cortical parenchyma; (d) phloem tissue; (e) libero-ligneous cambium; (f) metaxylem; (g) libriform tissue; (h) protoxylem; (i) medullary parenchyma (Congo red–chrysoidine staining, ×100).

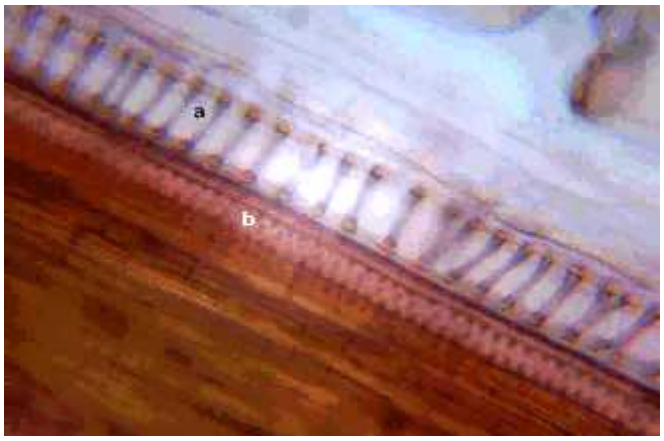

Figure 6. Longitudinal-radial section through C. officinale aboveground stem: (a) metaxylem with spiral thickenings; (b) metaxylem with reticulate thickenings (Congo red–chrysoidine staining, ×400).

Leaf

Leaf's limb

In cross-section, from the outside towards the inside of leaf's limb, the following histological sequence is evidenced. A single layer of upper epidermis is composed of flattened large cells with thickened tangential external and internal walls and thin radial walls. External walls are bulged and covered by a thick cuticle. Mesophyll is organized in a single layer of palisade parenchyma with small, slightly elongated cells, rich in chloroplasts, as well as of 5–7 layers of lacunose parenchyma with small cells, disorderly arranged, leaving aeriferous spaces. Into the mesophyll are found many small libero-ligneous fascicles. The mesophyll has bifacial dorsiventral structure. Lower epidermis is made of a single layer of tangential elongated cells, with thin radial walls and thickened tangential external and internal walls. At this level, there are stomata and long unicellular tector hairs, with sharp peak. In cross-section, the median rib is prominent on the abaxial side as well as into the adjacent secondary ribs. The outer epidermis consists of small cells, slightly flattened anterior-posterior, a thin cuticle covering the wall. At the adaxial pole, it presents rare long unicellular tector hairs with sharp peak. Under the epidermis, at both adaxial and abaxial poles, 2–3 layers of angular collenchyma are found. In the central area are located numerous libero-ligneous conducting fascicles, arranged on a semicircle, into a mass of leaf's parenchyma. These fascicles exhibit various sizes and each of them is flanked in periphloemic position by one collenchyma cap. Into the libero-ligneous fascicles, xylem vessels are seriate disposed and the medullary rays are uniseriate, cellulosic. Leaf's limb has bifacial dorsiventral, hypostomatic structure (Figures 7 and 8).

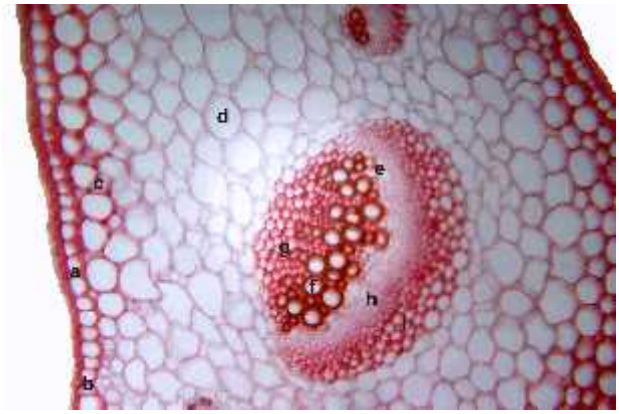

Figure 7. Cross-section through C. officinale median rib: (a) epidermis; (b) stomate; (c) angular collenchyma; (d) leaf's parenchyma; (e) libero-ligneous conducting fascicle; (f) xylem vessel; (g) xylem parenchyma; (h) phloem tissue; (i) collenchyma cap (Congo red–chrysoidine staining, ×100).

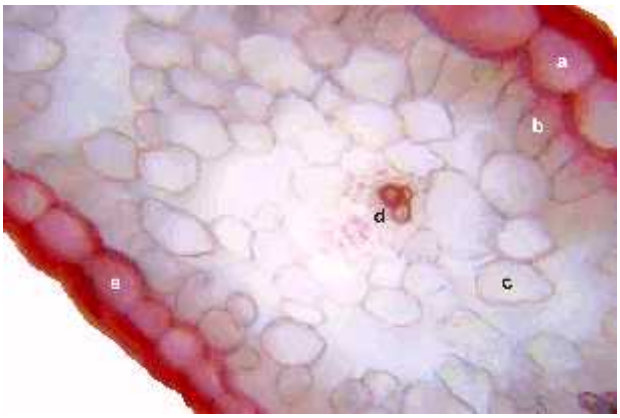

Figure 8. Cross-section through C. officinale leaf's limb: (a) upper epidermis; (b) palisade parenchyma; (c) lacunose parenchyma; (d) libero-ligneous conducting fascicle; (e) lower epidermis (Congo red–chrysoidine staining, ×400).

Petiole

Petiole has semicircular shape, with flat adaxial side and two adaxial extensions. The epidermis is composed of a single layer of small, heterodiametric cells, with thin radial walls and thickened tangential external and internal walls. On the outside, it has a thin cuticle. Stomata are found from point to point. The angular collenchyma is organized into 2–3 sub-epidermal layers. Foliar parenchyma contains many libero-ligneous conducting fascicles of varying sizes arranged on a semicircle and each one flanked in periphloemic position by a sclerenchyma cap (Figures 9 and 10).

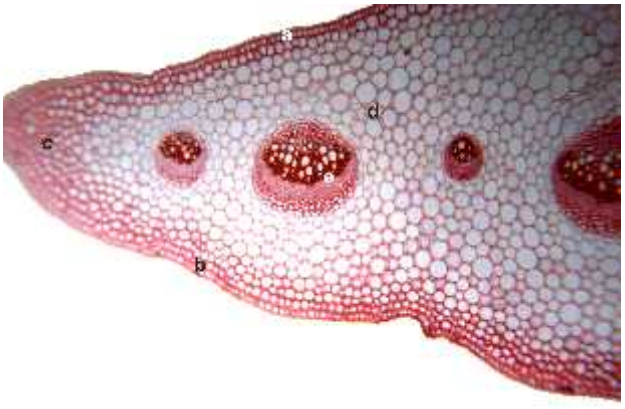

Figure 9. Cross-section through C. officinale petiole: (a) epidermis; (b) stomate; (c) angular collenchyma; (d) foliar parenchyma; (e) conducting fascicle (Congo red–chrysoidine staining, ×40).

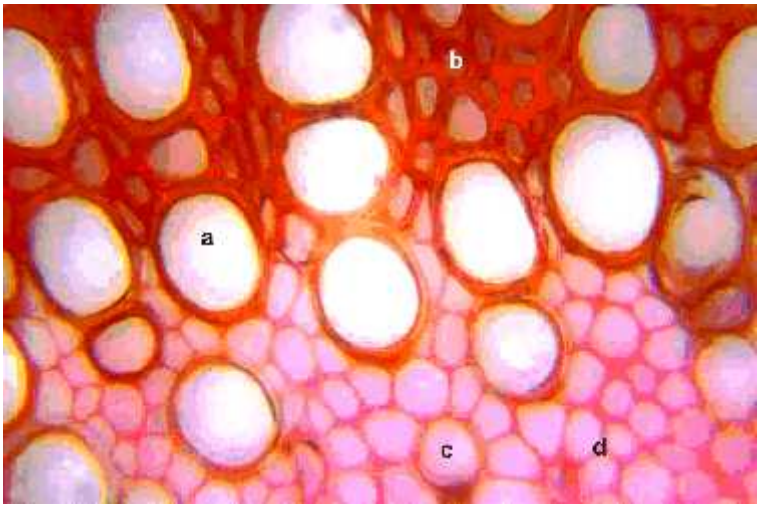

Figure 10. Cross-section through C. officinale petiole: (a) metaxylem; (b) libriform tissue; (c) protoxylem; (d) xylem parenchyma (Congo red–chrysoidine staining, ×400).

Sepal

In cross-section, from the inside to the outside of the sepal, the following histological sequence is evidenced. Upper epidermis is composed of a single layer of large, flattened cells, with thickened tangential external and internal walls and thin radial walls. External walls are bulged and covered with a thin cuticle. Subepidermal palisade parenchyma is made of 1–2 layers of slightly elongated cells rich in chloroplasts. The following 2–3 layers represent foliar parenchyma of meatus type, containing small libero-ligneous conducting fascicles. Above the lower epidermis, 1–2 layers of palisade parenchyma with elongated cells rich in chloroplasts are highlighted. Lower epidermis is made of a single layer of tangentially elongated cells, with thin radial walls and thickened tangential external and internal walls. At this level, there are stomata and fewer long unicellular tector hairs with sharp peak (Figure 11).

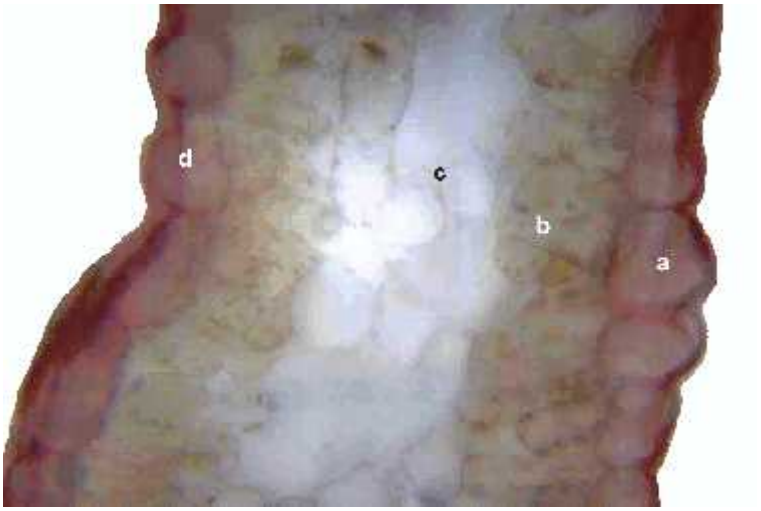

Figure 11. Cross-section through C. officinale sepal: (a) upper epidermis; (b) palisade parenchyma; (c) fundamental parenchyma; (d) lower epidermis (Congo red–chrysoidine staining, ×400).

TLC analysis

The experimental results on the preliminary TLC analysis of polyphenols from *Cynoglossi herba* are shown in Figures 12–14. From the 15 distinctive chromatographic bands, one caffeic acid derivative (R_f 0.64) was identified in an amount of 8.23 mg/100 g of dried vegetal product.

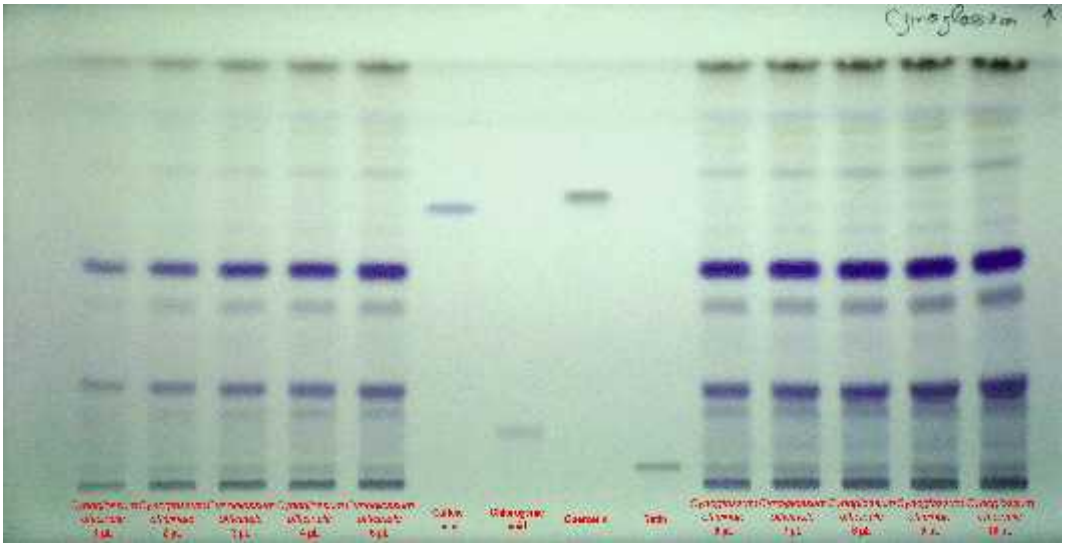

Figure 12. TLC chromatogram of polyphenols from Cynoglossi herba methanolic extract (UV 254 nm, without derivatization). From left to right: first five applications – sample (1–5 µL); subsequent four applications – standards (2 µL); last five applications – sample (6–10 µL).

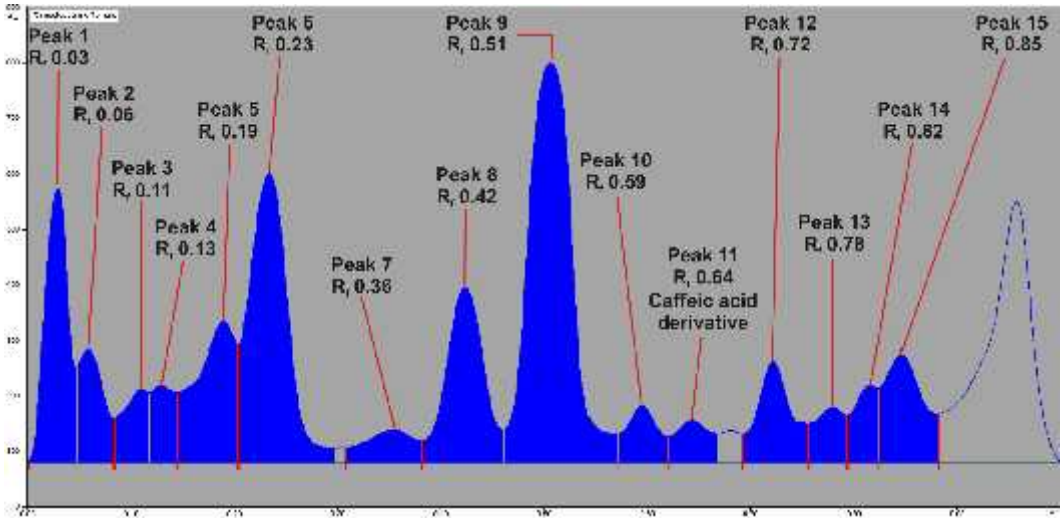

Figure 13. Densitogram of polyphenols (UV 254 nm) separated from Cynoglossi herba methanolic extract.

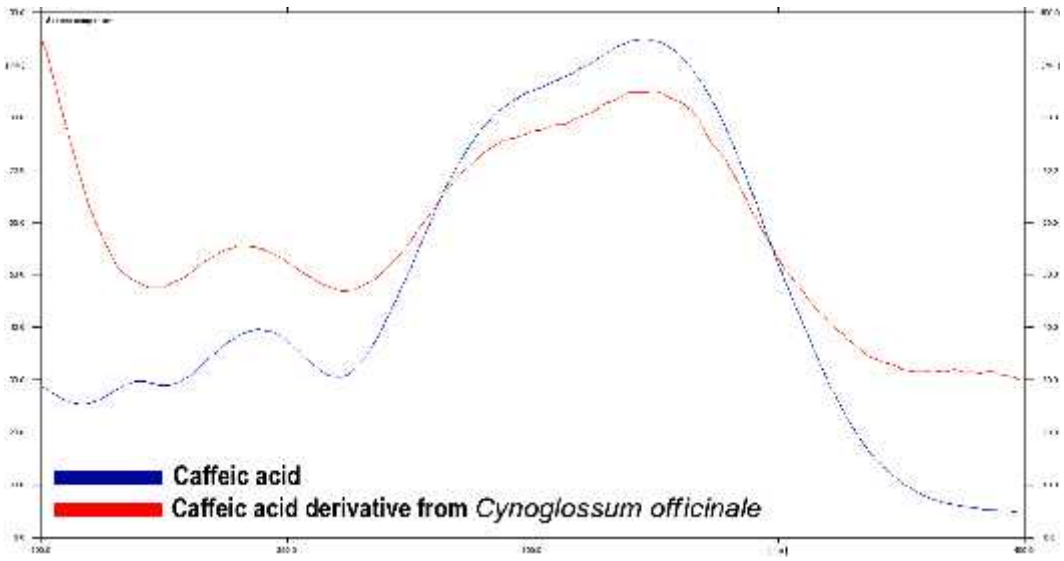

Figure 14. Caffeic acid derivative in situ UV spectra of standard and compound separated from the analyzed sample.

CONCLUSIONS

The histo-anatomical investigation of the root, aboveground stem, leaf and sepal of *Cynoglossum officinale* species and the preliminary TLC analysis of *Cynoglossi herba* polyphenols were achieved. The root has circular shape and secondary structure due to the presence of phellogen and libero-ligneous cambium. The aboveground stem has circular sinuous shape and secondary structure, into the upper third. Leaf's limb has bifacial dorsiventral, hypostomatic structure. Petiole has semicircular shape, with flat adaxial side and two adaxial extensions. Subepidermal palisade parenchyma of sepal is made of 1–2 layers of chloroplast-rich slightly elongated cells. One caffeic acid derivative was identified and quantified from the 15 distinctive chromatographic bands.

BIBLIOGRAPHY

1. **Altemimi, A., Watson, D.G., Kinsel, M., Lightfoot, D.A.**, 2015 – *Simultaneous extraction, optimization, and analysis of flavonoids and polyphenols from peach and pumpkin extracts using a TLC-densitometric method*, Chem. Cent. J. 9:39.
2. **Andrei, M., Paraschivoiu, R.M.**, 2003 – *Microtehnică botanică*, Ed. Niculescu, Bucureşti, 2003, 222 pag.
3. **Asibal, C.F., Glinski, J.A., Gelbaum, L.T., Zalkow, L.H.**, 1989 – *Pyrrolizidine alkaloids from Cynoglossum creticum. Synthesis of the pyrrolizidine alkaloids echinatine, rinderine, and analogues*, J. Nat. Prod. 52(1):109–118.
4. **Bojić, M., Simon Haas, V., Sarić, D., Maleš, Z.**, 2013 – *Determination of flavonoids, phenolic acids, and xanthines in mate tea (Ilex paraguariensis St.-Hil.)*, J. Anal. Methods Chem. 2013:658596.
5. **Ciocârlan V.**, 2000 – *Flora ilustrată a României. Pteridophyta et Spermatophyta*, ediţia a 2-a revizuită şi adăugită, Ed. Ceres, Bucureşti, 1138 pag.
6. **Damianakos, H., Jeziorek, M., Sykłowska-Baranek, K., Buchwald, W., Pietrosiuk, A., Chinou, I.**, 2016 – *Pyrrolizidine alkaloids from Cynoglossum columnae Ten. (Boraginaceae)*, Phytochem. Lett. 15:234–237.
7. **De Jong, T.J., Goosen-De Roo, L., Klinkhamer, P.G.L.**, 1998 – *Is the threshold size for flowering in Cynoglossum officinale fixed or dependent on environment?*, New Phytol. 138(3):489–496.
8. **El-Shazly, A., Sarg, T., Ateya, A., Abdel Aziz, E., Witte, L., Wink, M.**, 1996 – *Pyrrolizidine alkaloids of Cynoglossum officinale and Cynoglossum amabile (family Boraginaceae)*, Biochem. Syst. Ecol. 24(5):415–421.
9. **El-Shazly, A., Wink, M.**, 2014 – *Diversity of pyrrolizidine alkaloids in the Boraginaceae: structures, distribution, and biological properties*, Diversity 6(2):188–282.
10. **Gîrd, C.E., Nencu, I., Costea, T., Duţu, L.E., Popescu, M.L., Ciupitu, N.**, 2014 – *Quantitative analysis of phenolic compounds from Salvia officinalis L. leaves*, Farmacia 62(4):649–657.
11. **Niculescu, Mariana -** *Morfologia şi anatomia plantelor*, VOL. I, Ed. Sitech, Craiova, 2009, 201 p., ISBN 978-606-8021-87-4
12. **Niculescu, M., Făgăraș M.**, 2015 - *The grassland natura 2000 habitats found in Lespezi quarry and the surroundings, Dambovita county,* 15th International Multidisciplinary Scientific GeoConference SGEM 2015, www.sgem.org, SGEM2015 Conference Proceedings, ISBN 978-619-7105-39-1 / ISSN 1314-2704, June 18-24, 2015, Book5 Vol. 1, 877-882 pp, DOI: 10.5593/SGEM2015/B51/S20.116, http://www.sgem.org/sgemlib/spip.php?article6369&lang=en
13. **Niculescu, M., Făgăraș, M., Popescu,C.,** 2016 - *Diversity, distribution and ecology of the invasive alien plants in the ,,Cotmeana platform,, protected area, Romania,* Conference proceedings of the 16th International Multidisciplinary Scientific Geoconference (SGEM2016)

14**. Niculescu, L., Niculescu, M., Soare, R., Bonea, D.,** 2015 - *Plant communities characteristic to the montane and subalpine springs and streams in the Parang Mountains (Southern Carpathians), Romania* - JEPE, Vol. 16, No. 4 (2015), https://docs.google.com/a/jepe journal.info/viewer?a=v&pid=sites&srcid=amVwZS1qb3VybmFsLmluZm98amVwZS1qb3VybmFsfGd4OjgwMzIwZGUwMDQ2N2E2NQ
15. **Pfister, J.A., Molyneux, R.J., Baker, D.C.**, 1992 – *Pyrrolizidine alkaloid content of houndstongue (Cynoglossum officinale L.).*, J. Univ. Arizona 45(3):254–256.
16. **Sharma, R.A., Singh, B., Singh, D., Chandrawat, P.**, 2009 – *Ethnomedicinal, pharmacological properties and chemistry of some medicinal plants of Boraginaceae in India*, J. Med. Plants Res. 3(13):1153–1175.
17. **Stegelmeier, B.L.**, 2011 – *Pyrrolizidine alkaloid-containing toxic plants (Senecio, Crotalaria, Cynoglossum, Amsinckia, Heliotropium, and Echium spp.)*, Vet. Clin. North Am. Food Anim. Pract. 27(2):419–428, *ix*.
18. **Stegelmeier, B.L., Gardner, D.L., Davis, T.Z.,** 2009 – *Livestock poisoning with pyrrolizidine-alkaloid–containing plants (Senecio, Crotalaria, Cynoglossum, Amsinckia, Heliotropium, and Echium spp.)*, Rangelands 31(1):35–37.
19. **Toma, C., Rugină, R.**, 1998 – *Anatomia plantelor medicinale. Atlas*, Ed. Academiei Române, Bucureşti, 1998, 320 pag.
20. **Upadhyaya, M.K., Furness, N.H.**, 1994 – *Influence of light intensity and water stress on leaf surface characteristics of Cynoglossum officinale, Centaurea spp., and Tragopogon spp.*, Can. J. Bot. 72(9):1379–1386.
21. **Upadhyaya, M.K., Tilsner, H.R., Pitt, M.D.**, 1988 – *The biology of Canadian weeds: 87. Cynoglossum officinale L.*, Can. J. Plant Sci. 68(3):763–774.
22. **Van Dam, N.M., Verpoorte, R., van der Meijden, E.**, 1994 – *Extreme differences in pyrrolizidine alkaloid levels between leaves of Cynoglossum officinale*, Phytochemistry 37(4):1013–1016.
23. **Van Dam, N.M., Witte, L., Theuring, C., Hartmann, T.,** 1995 – *Distribution, biosynthesis and turnover of pyrrolizidine alkaloids in Cynoglossum officinale*, Phytochemistry 39(2):287–292.
24. **Xu, Q., Zhang, D., Dang, L., Li, K.**, 2009 – *Triterpene acids from Cynoglossum amabile*, Chem. Res. Chin. Univ. 25(3):404–406.
25. **Yu, C.H., Tang, W.Z., Peng, C., Sun, T., Liu, B., Li, M., Xie, X.F., Zhang, H.**, 2012 – *Diuretic, anti-inflammatory, and analgesic activities of the ethanol extract from Cynoglossum lanceolatum*, J. Ethnopharmacol. 139(1):149–154.