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Evaluation of *Lonicera etrusca* var. *etrusca* (Caprifoliaceae) Stem and Leaf in Terms of Anatomical Structures and Some Phenolic Compounds

Lonicera etrusca var. *etrusca* (Caprifoliaceae)'nın Gövde ve Yaprağının Anatomik Olarak ve Bazı Fenolik Bileşikler Açısından İncelenmesi

***Lonicera etrusca* var. *etrusca* (Caprifoliaceae)**
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ABSTRACT
Objectives: The *Lonicera* genus includes medicinally important plants. Two varieties of *Lonicera etrusca* is recorded in Turkey. Anatomical structures and phytochemical contents are important in the diagnosis and identification of medicinal plants. This study includes stem and leaf anatomy of *Lonicera etrusca* var. *etrusca* and HPLC analysis of methanol extracts obtained from these parts.
Materials and Methods: Plant materials were collected from Ankara. Methanol extracts were prepared from stems and leaves by ultrasonic bath. The amounts of chlorogenic acid and caffeic acid which are major compounds in the stem and leaves were determined by HPLC. For anatomical studies, specimens were preserved in 70% alcohol. Transverse and surface sections were prepared by hand. Detection of tissues was performed with Sartur reagent. Anatomical examinations were performed using a light microscope and microphotographed.
Results: In HPLC analysis the highest amount of chlorogenic acid was determined in the leaf (1.148%), and the highest amount of caffeic acid (0.156%) was determined in the stem. In the anatomical analysis, it was observed that the stem was disc-shaped and hollow; pericycle is in a ring form, consists of fibre-like cells with thick walls and wide lumina; cork occurs adjoining pericyclic fibres; thin-walled pith cells contain dense druse crystals. The leaf lamina is bifacial in the transverse section; palisade and spongy parenchyma, both contain abundant starch grains; solitary druse crystals are sparse in the leaf mesophyll; the stomata were observed only on the lower surface with 3-5 subsidiary cells. As a result of this study, *L.*

etrusca var. *etrusca* has been clarified in terms of anatomical structures and phenolic compounds.
Conclusion: The chemical contents and anatomical structures of the plant may contain important information that can be used in classification. This study may support in taxonomically classification for the *L. etrusca* var. *etrusca*.
Keywords: *Lonicera etrusca* var. *etrusca*, Caprifoliaceae, HPLC, plant anatomy, Turkey

ÖZ
Amaç: *Lonicera* cinsi tıbbi açıdan önemli bitkileri içerir. Türkiye'de *Lonicera etrusca*'ya ait iki varyete kayıtlıdır. Tıbbi bitkilerin teşhisinde ve tanımlanmasında anatomik yapılar ve fitokimyasal içerikler önemlidir. Bu çalışma, *Lonicera etrusca* var. *etrusca*'nın gövde ve yaprak anatomisini ve bu kısımlardan elde edilen metanol ekstrelerinin HPLC analizini içermektedir.
Gereç ve Yöntemler: Bitki materyalleri Ankara'dan toplanmıştır. Metanol ekstreleri, ultrasonik banyo ile gövde ve yapraklardan hazırlandı. Gövde ve yapraklarda bulunan ana bileşikler olan klorojenik asit ve kafeik asit miktarları HPLC ile belirlendi. Anatomik çalışmalar için örnekler %70'lik alkolde korundu. Enine ve yüzeyel kesitler el ile hazırlandı. Dokuların boyanması Sartur reaktifi ile gerçekleştirildi. Anatomik incelemeler ışık mikroskobu kullanılarak yapıldı ve mikrofotoğraflandı.
Bulgular: HPLC analizinde en yüksek klorojenik asit miktarı yaprakta (%1.148), en yüksek kafeik asit miktarı (%0.156) ise gövdede tespit edilmiştir. Anatomik analizde gövdenin disk şeklinde ve içi boş olduğu; perisiklin halka şeklinde, kalın çeperli ve geniş lümenli lif benzeri hücrelerden oluştuğu; mantarın iç kısımda perisiklik liflere bitişik oluştuğu; ince çeperli öz hücrelerinin yoğun druz kristalleri içerdiği gözlendi. Yaprak bifasiyaldir; palizat ve sünger parenkiması yoğun nişasta içerir; druz kristalleri yaprak mezofilinde seyrektir; stomalar sadece alt yüzeyde ve 3-5 stoma komşu hücrelerine sahiptir. Bu çalışma sonucunda *L. etrusca* var. *etrusca* anatomik yapı ve fenolik bileşikler açısından aydınlatılmıştır.
Sonuç: Bitkinin kimyasal içeriği ve anatomik yapısı sınıflandırmada kullanılabilecek önemli bilgiler içerebilir. Bu çalışma *L. etrusca* var. *etrusca* için taksonomik sınıflandırmayı destekleyebilir.
Anahtar kelimeler: *Lonicera etrusca* var. *etrusca*, Caprifoliaceae, HPLC, bitki anatomisi, Türkiye

INTRODUCTION

Caprifoliaceae Juss. family includes herbs and small trees, its native range is mainly north temperate and the medically important genera of the family are *Viburnum* L., *Lonicera* L. and *Sambucus* L.[1-2]
The genus *Lonicera* spreads from temperate and subtropical Northern Hemisphere to Malaysia and includes 200 accepted species and six species of *Lonicera* are naturally grown in Turkey.[1-3] *Lonicera etrusca* Santi grows naturally in Turkey and is known for yellowish corolla, red berries, obovate-oval leaves, hollow young branches. Two varieties of *L. etrusca* are in Turkey, *L. etrusca* var. *etrusca* Santi and *L. etrusca* var. *hispidula* Boiss. *L. etrusca* var. *etrusca* is with the widest distribution in the genus *Lonicera* is grown in Turkey. *L. etrusca* var. *etrusca* has young shoots, upper leaves, flowers glabrous and lower leaves sparsely hairy to glabrous features while *L. etrusca* var. *hispidula* are young shoots, upper leaves, flowers

densely glandular-pubescent and lower leaves pubescent, eglandular.[3] Determination and identification are important for the plants as traditional medicinal used. The chemical contents and anatomical structures of the plant may contain important information that can be used in classification. Previous studies have generally been done on *L. etrusca*, regardless of varieties. *Lonicera* is one of the most important genera in Caprifoliaceae family, especially for its use in Traditional Chinese Medicine. *L. japonica* Thumb. are used for the treatment of febrile illnesses, sores and swellings in Traditional Chinese Medicine.[4-7] *L. caerulea* L. has therapeutic uses for hypertension, bacterial infection and gastrointestinal disorder in northern Russia, China and Japan.[8] *L. quinquelocularis* Hard. is used for its anti-inflammatory effect.[9] In Turkey, *L. etrusca var. etrusca* is used for diuretic effect.[10] *L. caprifolium* L. is used as a laxative and emetic.[11]

The family contains valerianic acid, aucubin glycosides, saponins, coumarins and cyanogenetic glycosides.[2] In phytochemical studies with *Lonicera* species, iridoids, bis-iridoids, triterpene saponins, phenolic acids, flavonoids, coumarins, anthocyanins, and monoterpene alkaloids were determined.[12-15] Fruits of *Lonicera* species (*L. altaica* Pall., *L. caerulea* L., *L. edulis* Turcz. Ex Freyn) have major phenolic compounds such as flavonoids (rutin, quercetin, isoquercetin), anthocyanins (cyanidin, peonidin, delphidin glucosides), phenolic acids (gallic acid, vanilic acid, caffeic acid, ferrulic acid, chlorogenic acid, genistic acid).[16] It is known that phenolic compounds in plants have important biological and pharmacological effects. Studies have shown that these compounds have significant antioxidant, antimicrobial, anti-inflammatory and anticancer effects.[17-18]

In this study, the leaf and stem anatomy of *L. etrusca* var. *etrusca*, which is widely distributed in Turkey, was examined in detail and its characteristic structures were revealed. It was also evaluated in terms of phenolic compounds with significant biological activity. This is the first report on this species in which both anatomical features are evaluated and phenolic compound quantification is performed.

MATERIALS AND METHODS

Plant materials

The plant material was collected from Beynam Forest (Ankara/Turkey) in 2020 (Figure 1). A voucher specimen was deposited in the Ankara University Faculty of Pharmacy Herbarium (AEF 30738) in Turkey.

High Performance Liquid Chromatography (HPLC) analysis

For HPLC analysis, methanol extracts of stem and leaf were prepared. Stem and leaf of *L. etrusca* var. *etrusca* were air-dried. Powdered samples were extracted with methanol (Merck) using an ultrasonic bath. After the extracts were filtered and then concentrated with an evaporator.[19] HPLC analysis was performed with a Waters Spherisorb C18 column (25 cm x 4.6 mm, 5µm) maintaining at 40 ºC. In a gradient, 0.01% formic acid (Sigma-Aldrich) (A) and acetonitrile (Sigma-Aldrich) (B) were used as the mobile phase, with a flow rate of 1 ml/min. The detection wavelength was 300 nm and the injection volume was 20 µl.[20] After analysis, method validation was performed. Sample extracts (4 mg/ml) and stock standard solutions of each compound (500 µg/ml) were prepared by dissolving methanol. For the calibration curve, chlorogenic acid (Sigma-Aldrich) and caffeic acid (Sigma-Aldrich) standards were investigated by injecting different concentrations, in triplicate. The precision of the method was determined by carrying on intra-day and inter-day variation and these variations were expressed by the relative standard deviation (RSD). LOD and LOQ were established at a signal/noise of 3 and 10, respectively. For LOD and LOQ, 10 injections of chlorogenic acid and caffeic acid were made and averaged. For recovery assay, It was carried out by spiking 3 different known concentrations of standards into the sample solution. The mixtures were analyzed by the same method used. For the robustness of the method, It was

evaluated by changing the mobile phase composition, column temperature, flow rate, and detector wavelength.

Light microscope analysis

The samples for anatomical studies were preserved in 70% alcohol. The transverse and surface sections were cut by hand with a razor blade in microscopic preparat form. The Sartur solution was used in microscopic examinations.[21] The anatomical analysis and the microphotographs were taken using the Leica DM 4000B.

RESULTS

High Performance Liquid Chromatography (HPLC) analysis

In this study, stem and leaf of *L. etrusca* var. *etrusca* were analyzed quantitatively for chlorogenic acid, and caffeic acid by using HPLC.

Yields of stem and leaf extracts are 8.59% and 13.69%, respectively. The chlorogenic acid and caffeic acid contents of stem and leaf of *L. etrusca* var. *etrusca* extracts are shown in Table 1 and given HPLC chromatograms (Figure 2, 3).

For calibration table, the ranges of 5 to 100 µg/ml the calibration plots for chlorogenic acid and caffeic acid were linear. The LOD values for chlorogenic acid and caffeic acid were determined as 0.093 µg/ml and 0.068 µg/ml; the LOQ values were determined 0.311 µg/ml and 0.229 µg/ml, respectively (Table 2).

Intra-day and inter-day variations were used to determine the precision. The result showed that RSD values were always less than 2% (Table 3).

For a recovery assay, 3 different known concentrations of chlorogenic acid and caffeic acid were spiked into the sample solution. The mean extraction recovery of chlorogenic acid and caffeic acid was in the range of 97.638-99.795% and 97.260-102.092, respectively (Table 4).

It was determined that some changes (mobile phase composition, column temperature, flow rate, and detector wavelength) made for the robustness of the test method did not have a significant effect on chromatographic resolution.

Stem anatomy

The transverse section of the stem is broadly grooved, hollow disc-like. The single-layer epidermis consists of square-rectangular cells and is covered by a very thick cuticle. The stomata are rarely observed. The abaxial side of the epidermal layer consists of 8-10 rows of collenchymatous cortex cells and is bordered by the endodermis. The pericycle is in a ring form, consists of fibre-like cells with thick walls and wide lumina. Cork occurs adjoining pericyclic fibres. Phloem is composed of thin-walled cells containing starch grains. Sometimes contains sclerenchymatous cells. The pith is heterogeneous. The pith cells with thickened walls surround the xylem and are elongated within the xylem as arms. The arm cells contain very dense starch grains. Thin-walled pith cells contain dense druse crystals. Dead cells occur towards the middle of the stem (Figure 4).

Leaf anatomy

The transverse section of the leaf is broadly V-shaped. In the midrib, the upper epidermis consists of single-layered epidermal cells with thickened walls. 1-5 rows of collenchyma are located under the epidermal layer. Between the main vein and the collenchyma tissue is filled with parenchymatous cells. The main vein consists of arc-shaped xylem and phloem. The abaxial side of the phloem is lined with 1-2 layers of bundle sheath-like parenchymatous cells containing dense starch grains in a crescent shape. The walls of the midrib lower epidermal cells are very thick, the lumina of the cells is very narrow compared to the upper epidermal cells. The adaxial side of the lower epidermis is powered with 1-9 rows of collenchyma tissue. The abaxial side of the midrib is more protruding than the adaxial side. The cuticle

layer is thinner on the lower epidermis compared to the upper epidermis. The stomata are not observed in the midrib, but contain solitary crystals. The leaf lamina is bifacial in the transverse section. The upper epidermis cells are square with a thick adaxial wall and covered by a thick cuticle layer. Palisade parenchyma consists of a single layer of elongated cells. The spongy parenchyma contains thin walled, 1-8 rows of isodiametric cells. Palisade and spongy parenchyma, both contain abundant starch grains. The lower epidermis cells are rectangular, outer walls are thick and smaller than the upper epidermal cells. The cuticle layer is thinner and stomata are observed. Solitary druse crystals are sparse in the leaf mesophyll (Figure 5). In the leaf lamina surface sections; the stomata were observed only on the lower surface with 3-5 subsidiary cells. The lower epidermal cells are sinuous. The upper epidermis layer is free from stomata and consists of polygonal epidermal cells (Figure 6).

DISCUSSION

In this study, leaf and stem of *L. etrusca* var. *etrusca* were evaluated in terms of anatomical structures and phenolic compounds.

HPLC was used for the quantitation of phenolic compounds (chlorogenic acid and caffeic acid), end of analysis the method was validated. Chlorogenic acid and caffeic acid have important biological activities such as antioxidant and anti-inflammatory activity.[18,22] Since some of *Lonicera* species are used for medicinal purposes, *L. etrusca* var. *etrusca* has also been evaluated in terms of phenolic compounds with biological activity. The results of HPLC analysis, while the amount of chlorogenic acid in stem and leaf was 1.043% and 1.148%, respectively; the amount of caffeic acid was 0.156% and 0.073%, respectively. It has been determined that chlorogenic acid and caffeic acid are among the major compounds in the stem and leaves of *L. etrusca* var. *etrusca*. Phenolic compounds were also detected in studies with other species (*L. japonica, L. confusa, L. fulvotomentosa, L. macranthoides, and L. hypoglauca)*. In the studies, it was determined that the amounts of chlorogenic acid and caffeic acid were similar*,* and it was seen that chlorogenic acid was more than caffeic acid.[14-15] These results are consistent with the results of our study.

The results of the anatomical study showed that the transverse section of the stem is hollow, disc-like and broadly grooved. Epidermal tissue consisting of a single layer of cells is covered with a thick cuticle and rarely stomata are observed. The cortex of stem is characterized by collenchymatous cells and bordered by endodermis. The pericyclic layer is ring-shaped and consists of sclerenchymatous cells with wide lumina and thick-walled. The cork is adjacent to the pericycle layer. Vascular tissue is embedded in the sclerenchymatous pith cells. Phloem sometimes contains sclerenchymatous cells. The pith cells branch out between vascular tissue and starch grains are dense in the pith cells. Thin-walled cells of heterogeneous pith contain dense solitary druse crystals. The anatomical features of stem for *Lonicera* genera was reported by Metcalfe and Chalk[23] as cork usually arising in the pericyclic region with wide luminal and thick walled cells, the pericycle contains wide fibres or fibre-like elements with thin walls and very wide lumina, phloem includes thick-walled fibres and xylem is in the form of a continuous cylinder, cluster crystals present. These reports are similar to the findings of our study. *L. etrusca* var. *etrusca* leaf is characterized by bifacial lamina, single-layered epidermis, palisade parenchyma with elongated, 1-row cells, spongy parenchyma with 1-8 rows of isodiametric cells, abundant starch grains and sparse solitary druse crystals. The lower surface includes stomata with 3-5 subsidiary cells and epidermal cells are sinuous. The upper epidermis layer is free from stomata and consists of polygonal epidermal cells. The Leaf features of the Caprifoliaceae family are stated by Metcalfe and Chalk[23] so dorsiventral leaf, a single layer of palisade and solitary crystals are the same anatomical features determined as a result of our study. This report indicated that the family has Ranunculaceous stomata, while our study showed that *L. etrusca* var. *etrusca* has anomocytic stomata.

CONCLUSION

In this study, stem and leaf of *L. etrusca* var. *etrusca* anatomy were studied and important characters were identified. The transverse section of the stem looks disc-shaped and hollow. The pericycle consists of fibre-like cells with thick walls and wide lumina and the cork occurs adjoining pericyclic fibres. The leaf anatomical structure is bifacial, the stomata located characterized by on the lower surface with 3-5 subsidiary cells. The mesophyll contains solitary druse crystals. In addition, the amount of chlorogenic acid and caffeic acid, which are major compounds, were determined by HPLC. Our results are consistent with studies of other *Lonicera* species. Method validation was performed to determine the reliability of the method. Anatomy study coincides with the anatomical features of the genus *Lonicera.* It is important to evaluate this species in terms of anatomical structures and chemical contents, since it is widely grown in our country and is a variety. This study may help minimize confusion of *L. etrusca* var. *etrusca* with other *Lonicera* species, and It may support in taxonomically classification for the *L. etrusca* var. *etrusca*.

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Table 1. Contents of chlorogenic acid and caffeic acid in stem and leaf methanol extracts (n=3).

	Chlorogenic acid (%±SD*)	Caffeic acid (%±SD*)
Stem	1.043±0.009	0.156±0.0009
Leaf	1.148±0.003	0.073±0.001

*SD: Standard Deviation

Table 2. Calibration values for chlorogenic acid and caffeic acid

Standard	Calibration range (µg/mL)	Slope (a±SD*)	Intersection (b±SD*)	Correlation number (r^2±SD*)	LOD (µg/mL)	LOQ (µg/mL)
Chlorogenic acid	5-100	18.733 ±1.547	6.692±1.489	0.995±0,003	0.093	0.311
Caffeic acid	5-100	81.837 ±2.893	20,081±4.751	0.996±0.002	0.068	0.229

*SD: Standard Deviation

Table 3. Intra-day and inter-day precision's data of the method.

Standards	Amount (µg/mL)	Intra-day precision (RSD*%)	Inter-day precision (RSD*%)

	5	1.694	1.425
	10	1.364	1.774
Chlorogenic acid	25	0.516	0.256
	50	0.894	0.497
	100	0.713	0.461
	5	1.649	0.892
	10	1.937	1.780
Caffeic acid	25	1.625	1.417
	50	0.957	1.849
	100	1.370	0.994

*RSD: Relative Standard Deviation

Table 4. Recovery assay's statistical data of the method (n=3).

Standards	**Concentration in sample (μg/mL)**	**Amount spiked (μg/mL)**	**Mean amount found in mixture (μg/mL)**	**Mean recovery (%±SD*)**	**RSD** **
Chlorogenic acid	0.04	0.02	0.03	99.795±1.516	1.519
		0.04	0.04	97.638±1.563	1.601
		0.08	0.06	99.528±0.297	0.299
Caffeic acid	0.006	0.003	0.0045	97.260±0.507	0.521
		0.006	0.006	102.092±1.325	1.297
		0.012	0.009	101.268±0.406	0.401

*SD: Standard Deviation, **RSD: Relative Standard Deviation

Figure 1. *Lonicera etrusca* var. *etrusca*

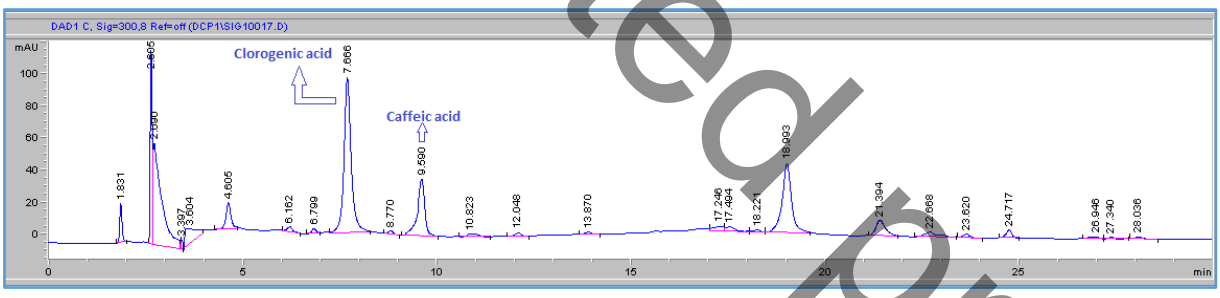

Figure 2. HPLC chromatograms of stem (*L. etrusca* var. *etrusca*)

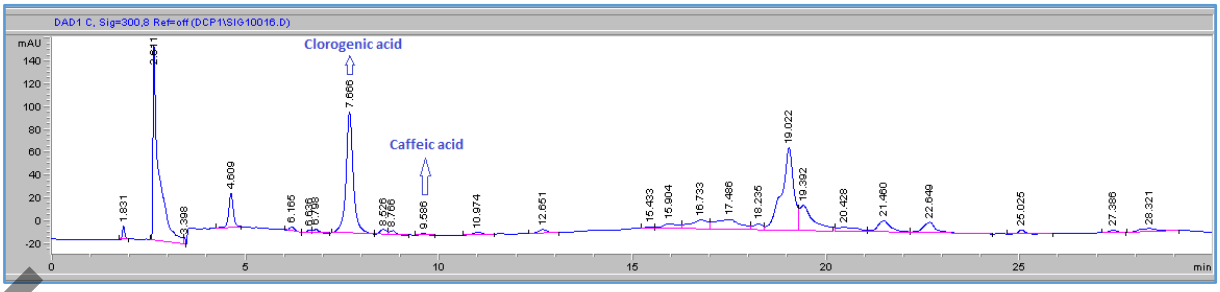

Figure 3. HPLC chromatograms of leaf (*L. etrusca* var. *etrusca*)

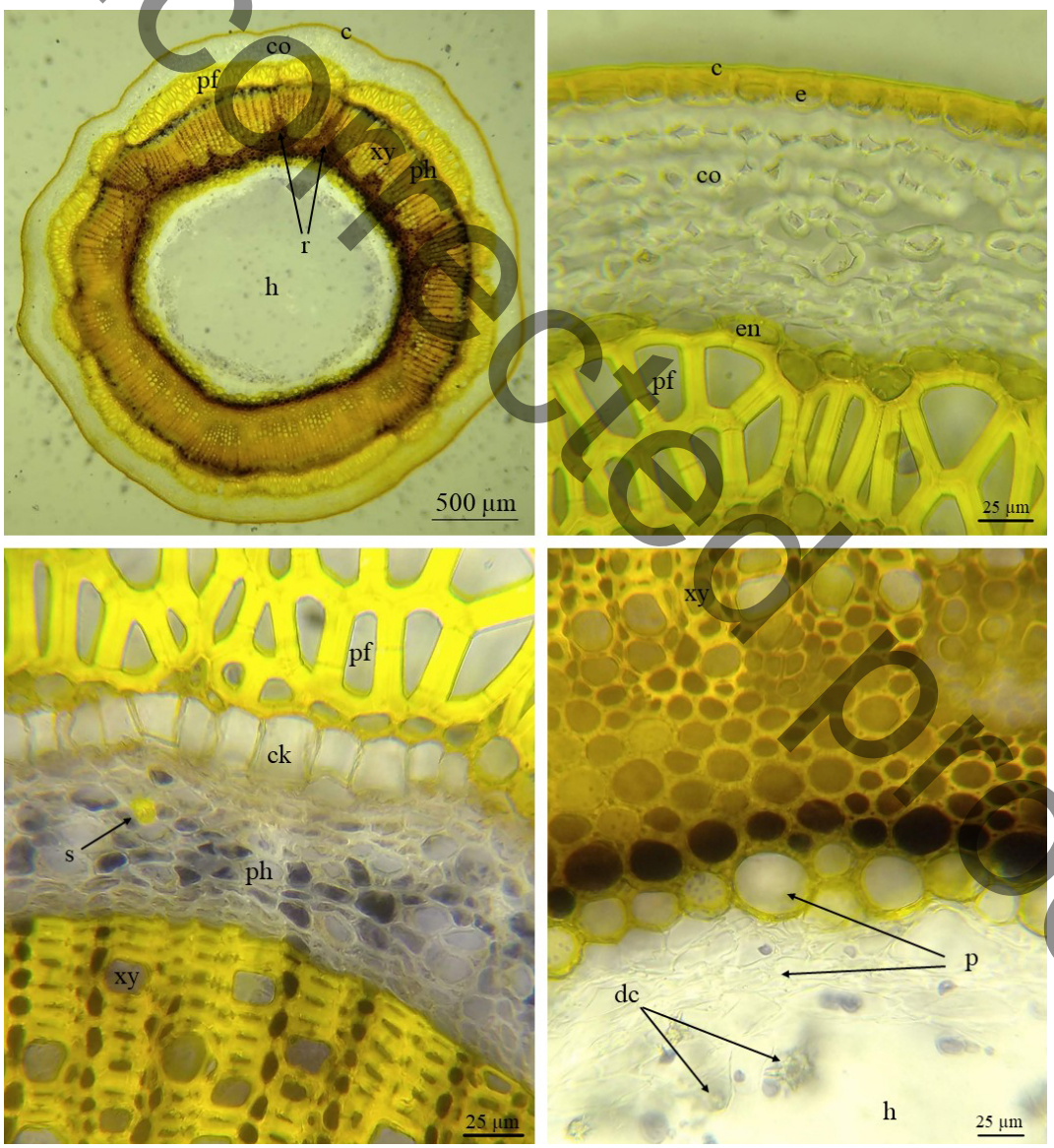

Figure 4. Transverse section of stem
c: cuticle, ck: cork, co: collenchyma, dc: druse crystal, e: epidermis, en: endodermis, h: hollow, p: pith cell, pf: pericyclic fiber, ph: phloem, r: ray, s: sclerenchymatous cell, xy: xylem.

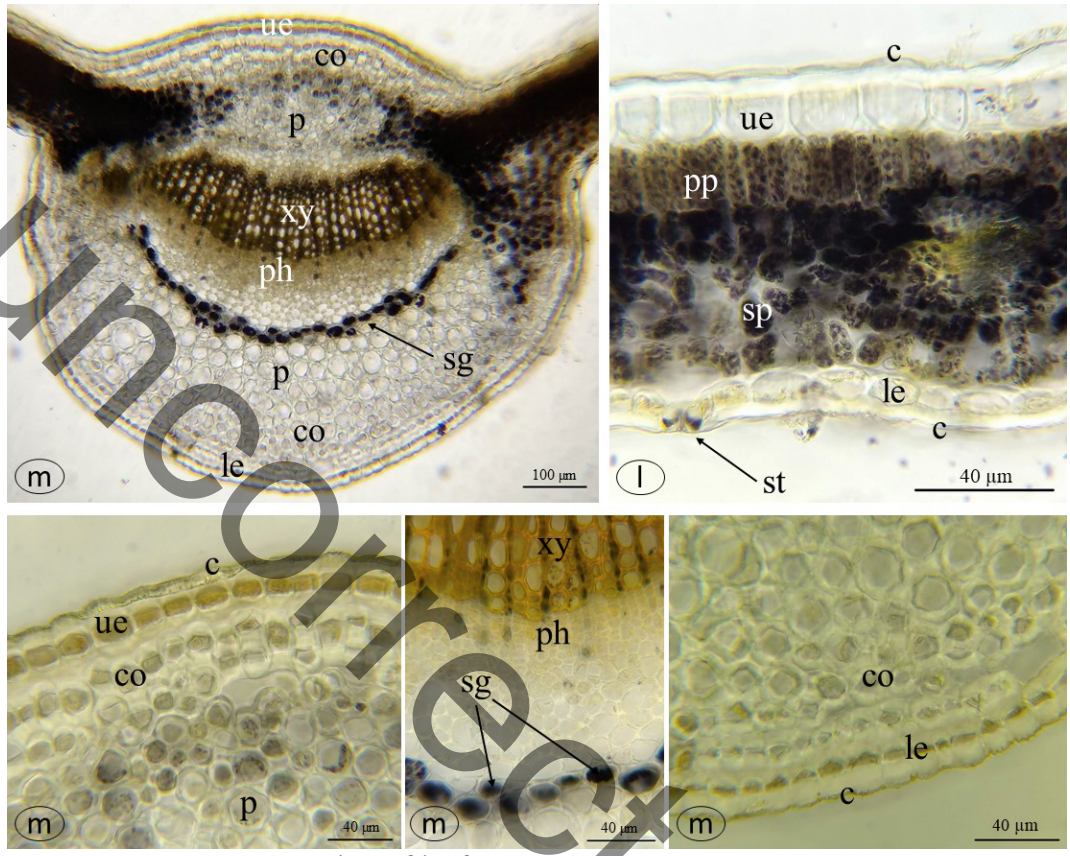

Figure 5. Transverse section of leaf
c: cuticle, co: collenchyma, l: lamina, le: lower epidermis, m: midrib, p: parenchyma, pp: palisade parenchyma, ph: phloem, sg: starch grains, sp: spongy parenchyma, st: stomata, ue: upper epidermis, xy: xylem.

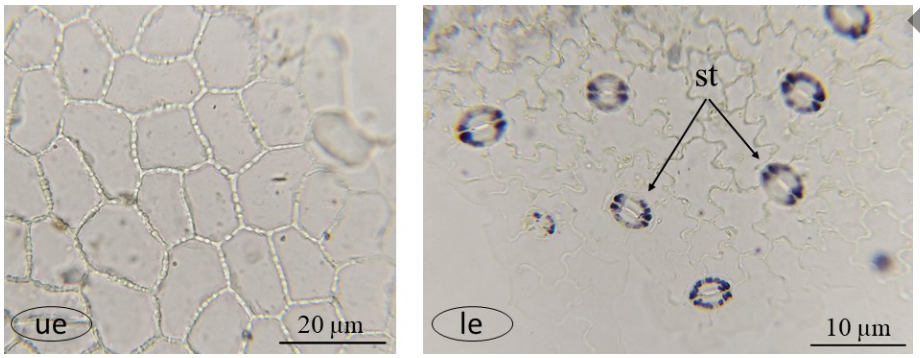

Figure 6. Surface section of leaf
le: lower epidermis, st: stomata, ue: upper epidermis.