

RESEARCH ARTICLE

## Morphological and anatomical study of the genus *Sambucus* L. (Adoxaceae) in Iran

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### Abstract

*Sambucus* (Adoxaceae) is a tall tree-like shrub distributed throughout temperate and subtropical regions in Europe, Asia, North Africa and North America. Since there is not any morphological and anatomical comprehensive study on *Sambucus* species in Iran and *Sambucus nigra* is located in the Arasbaran region with very limited distribution, we have encouraged studying the patterns of morphological variation and anatomical structure among *Sambucus* taxa.

In this study, the morphological and anatomical structure of 18 populations from two species of *Sambucus* (*S. ebulus* and *S. nigra*) has been considered to evaluate the relationships in this genus. In total, nine quantitative and nine qualitative morphological features were evaluated and measured. Ward's dendrogram showed two main clusters. The first cluster is composed of a population of *S. nigra*. The second cluster was composed of two subsets and contained populations of *S. ebulus* in Golestan, Mazandaran and Gilan provinces. There were two types of epidermal cells, puzzle-shaped in *S. ebulus* and oblong cells in *S. nigra*. The stems are rounded shape in both species. The margin is quite wavy in *S. ebulus* and straight in *S. nigra*. Both species showed a rounded shape with a wavy margin in petiole cross-sections. Pedicel cross-sections of both species are similar to each other in the general shape of cross-sections and margins. Both are rounded in shape and the margin is quite sinuous shapes in *S. ebulus* and wavy in *S. nigra*. The present study showed that a combination of morphological and anatomical data provides reliable evidence for the differentiation of two species of *S. nigra* and *S. ebulus*.

**Keywords:** Adoxaceae, anatomical structure, Iran, morphological, *Sambucus*.

### Introduction

*Sambucus* L. (Adoxaceae) with 25 extant taxa is a tall tree-like shrub and can be found growing in temperate and subtropical regions in Europe, Asia, North Africa and North America (Mabberley 2008). *Sambucus* was previously classified in the Caprifoliaceae family but due to new genetic research and reclassification (Donoghue 2003), it now belongs to Adoxaceae family. A large number of variants are known in horticulture (Bean 1951). This genus includes two species in Iran namely, *S. ebulus* L. and *S. nigra* L. that distributed in the North, Northwest and Northeast of Iran (Jamzad 1993). In this concept, *Sambucus* L. is widely known for its species properties, for example, *S. nigra* L. had its immunomodulatory functions pointed out by Barsett et al. (2012) and *S. ebulus* L. has anti-inflammatory activity (Ebrahimzadeh et al. 2006). Habitats of *Sambucus* include places near forests, rivers

and as a non-cultivated plant at higher altitudes. It is common in Iran and distributed in moist grasslands or forest margins of Turkey (Kaya et al. 2019). This plant can grow in a vast variety of soil conditions wet or dry, but it can grow in partial shade conditions or under moderate sunlight. Various parts of this plant for example bark, roots, leaves, flowers and fruits have medicinal properties and therefore they are economically used in the food, pharmaceutical and cosmetic industry (Hejný 2001). *Sambucus* has long been used to treat various illnesses as a diaphoretic, diuretic, astringent, laxative and emetic (Uncinimanganelli et al. 2005; Merica et al. 2006).

It is a perennial plant characterized by a long, creeping and branched rhizome. Young plants of *Sambucus* have numerous branches arising from the base. Many of these branches subsequently branch again and form new shoots. It's characterized by opposite pinnate leaves

and the stems terminate in a cluster of flowers. The inflorescences are composed of aromatic white flowers which are clustered in umbrella cymes (Jančovičová 2011). Stems of inflorescences (Pedicels) are generally short, tomentose with two bracteoles (Watson and Dallwitz 2007). The adaxial surface of the leaf is devoid of stomata. Stalk-like extrafloral nectaries reaching up to 10 mm long occur in the nodal regions of the stem between the bases of the leaves and also at the bases of leaflets (Fahn 1987). Stipules are absent on the first two pairs of leaves which develop from hibernated buds in spring (Neubauer 1977).

The morphological and anatomical studies of this genus are not so extensive. Sedláčková et al. (2018) determined the basic morphological characteristics and antioxidant activity of *Sambucus nigra* L. inflorescences. Alerico et al. (2016) characterized the morphoanatomy and identify the compounds present in the internal part of the stem bark of *S. australis* through chemical and histochemical methods. Micrographs and descriptions of the anatomy of the root of *Sambucus nigra* are given by Cutler et al. (1987) and of the stem wood by Metcalfe (1948). Petiole anatomical studies by Amini et al. (2019) show that vascular bundles and the existence of druse crystals are diagnostic characters in species of *Sambucus*.

Since there is not any morphological and anatomical comprehensive study on *Sambucus* species in Iran and *Sambucus nigra* is located in the Arasbaran region with very limited distribution, we have encouraged to study the patterns of morphological variation and anatomical structure among *Sambucus* taxa. Moreover, due to different populations of *Sambucus nigra* and *S. ebulus* collected from different parts of Iran, we expect to resurrect subspecies or varieties in these two species and find diagnostic morphological and anatomical characters about this genus.

The specific objectives of this study were as follows: (1) to find morphological characters that could be useful for the diagnosis of taxa; (2) to study the species relationship; (3) to assess the value of anatomical characters in two species of *Sambucus* in Iran.

## Materials and Methods

### Morphological methods

In the present study, 18 populations of two *Sambucus* species were collected from different locations in Iran and preserved in the Gonbad Kavous University Herbarium (GKUH). Identification of populations was carried out based on Flora Iranica (Wendelbo 1965). A list of voucher specimens of the corresponding species is given in Tab. 1.

Nine quantitative and nine qualitative morphological

**Table 1.** List of the Iranian species of *Sambucus* used in the present study along with their localities and vouchers preserved in the Gonbad Kavous University Herbarium (Gonbad Kavous, Iran).

Taxa	Collection data
<i>Sambucus nigra</i> L.	Gilan: Arasbaran, Vayghan, Isazade-Araei (803308)
<i>S. nigra</i>	Gilan: Arasbaran, Aynalu, Isazade-Araei (803307)
<i>S. nigra</i>	Gilan: Arasbaran, Shabkhaneh, Isazade-Araei (803309)
<i>S. ebulus</i> L.	Golestan: Gorgan, Tuskestan forest, Isazade-Araei (803315)
<i>S. ebulus</i>	Golestan: Golestan National Park, Isazade-Araei (803314)
<i>S. ebulus</i>	Golestan: Nowkandeh, Isazade-Araei (803311)
<i>S. ebulus</i>	Golestan: Bandar Gaz, Isazade-Araei (803310)
<i>S. ebulus</i>	Golestan: Kordkuy, Isazade-Araei (803313)
<i>S. ebulus</i>	Mazandaran: Babol, Isazade-Araei (803318)
<i>S. ebulus</i>	Mazandaran: Nur, Nurforest, Isazade-Araei (803319)
<i>S. ebulus</i>	Mazandaran: Sari, Isazade-Araei (803317)
<i>S. ebulus</i>	Mazandaran: 6 km to Daryakenar, Isazade-Araei (803326)
<i>S. ebulus</i>	Gilan: Lahijan, near to Rasht, Isazade-Araei (803316)
<i>S. ebulus</i>	Gilan: Arasbaran, Berenjanestak, Isazade-Araei (803321)
<i>S. ebulus</i>	Gilan: Rasht, Isazade-Araei (803322)
<i>S. ebulus</i>	Gilan: Lefur, Isazade-Araei (803320)
<i>S. ebulus</i>	Gilan: Vayghan, Isazade-Araei (803323)
<i>S. ebulus</i>	Gilan: Lahijan, Isazade-Araei (803324)

**Table 2.** Quantitative and qualitative morphological characters in *Sambucus* species.

No.	Character	No.	Character
1	Plant height (mm)	10	Life form (shrub 0; herbaceous 1)
2	Leaf length (mm)	11	Anther trichome (glandular 0; linear 1)
3	Peduncle length (mm)	12	Anther color (pale 0; purple 1)
4	Peduncle width (mm)	13	Leaflet number (multi-leaved 0; single-leaved 1)
5	Leaflet length (mm)	14	Petiole trichome (glandular 0; linear 1)
6	Leaflet width (mm)	15	Petal color (yellow 0; purple 1)
7	Pedicle length (mm)	16	Leaf trichome (glandular 0; linear 1)
8	Pedicle width (mm)	17	Stipule (present 0; absent 1)
9	Petiole length (mm)	18	Flower number (less 0; many 1)

features were evaluated and measured (Tab. 2). These features were life form, plant height, petiole length, petiole trichome, leaflet number, stipule, leaflet length, leaflet width, pedicle length, pedicle width, anther color, anther trichome, petal color, peduncle length, peduncle width, leaf trichome and leaf length.

Grouping of the species was obtained by using UPGMA (Unweighted Paired Group Method with Arithmetic mean) and Ward (Minimum spherical cluster method) as well as PCoA (Principal Coordinate Analysis) (Podani 2000). Statistical analysis including ANOVA (Analysis of Variance) and CVA (Canonical Variance Analysis) test was performed to show significant morphological differences. To show variable morphological characters, PCA (Principle Component Analysis) was investigated to identify the most variable morphological characters

(Podani 2000). Three times measurements were performed for statistical analysis. These analyses were done by PAST software v.2.17 (Hammer et al. 2012).

### Anatomical methods

Fresh leaf materials of the second or third nodes of the stem were fixed in the field with formalin-acetic acid-alcohol (FAA, or removed from herbarium specimens at GKUH). Four cross-sections were measured for each sample to assess the consistency of anatomical characters. All materials were boiled for 15 minutes, then fixed in Carnoy solution (alcohols to acetic acid in proportion 3:1). Materials were kept warm in a tube with previous lotions (the above-mentioned solutions) for 4 hours then the leaves were cleaned. Handmade cross-sections were obtained from petioles, pedicels and stem using commercial razor blades. The cross-sections were obtained using the carmine and methylene green double staining method. Subsequently, the materials were washed in distilled water (1 minute) and dehydrated through ethyl alcohol (70%) and were mounted on microscopic glass slides. Slide sections were studied and photographed.

For leaf epidermal anatomical features, all materials were boiled for 15 minutes, then fixed in Carnoy solution (alcohols to acetic acid in proportion 3:1). The epidermis was separated with H<sub>2</sub>O<sub>2</sub> and acetic acid (1:1), to prepare the leaves. Materials were kept warm in a tube with previous lotions (the above-mentioned solutions) for 4 hours then the leaves were cleaned. After cleaning, the materials were washed in distilled water. Epidermis separation followed. Epidermal samples were stained with 2% acetocarmine and were mounted on microscopic glass slides.

Slide sections were studied and photographed with the help of an Olympus light microscope by using an Olympus DP12 Digital Camera (Japan). Some characters (stomata length/weight, number of stomata, number of epidermal cells) were measured with Image Tools ver. 3.0 and Axio Vision 4.8. (Tab. 3).

Irr: Irregular; Obl: Oblong; Puz: Puzzle-shaped; Wa: Wavy; Str: Straight.

Some of the most important anatomical characters of epiderms, stems, petioles and pedicels in these species are as follows: transverse section shape, the thickness of collenchyma and sclerenchyma, collenchyma and sclerenchyma layer number, parenchyma thickness, the thickness of phloem and xylem, number of vascular bundles, the thickness of pith, trichome type.

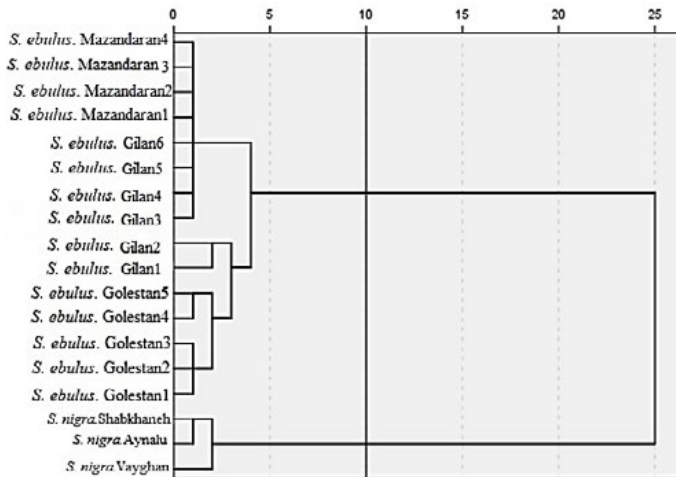
## Results

### Macro morphological study

To define the diagnostic value of morphological characters in species delimitations in studied *Sambucus* species, different clustering methods like UPGMA and Ward produced similar results and both had high cophenetic correlation values (>0.85). Therefore, only the Ward dendrogram is presented here (Fig. 1). The specimens studied in each *Sambucus* species were placed in species-specific clusters, separating the studied species from each other. Ward's dendrogram showed two main clusters (Fig. 1). The first cluster is composed of a population of *S. nigra*. The second cluster was composed of two subsets and contained Golestan, Mazandaran and Gilan populations of *S. ebulus*.

Table 3. Leaf epidermal anatomical features of *Sambucus*.

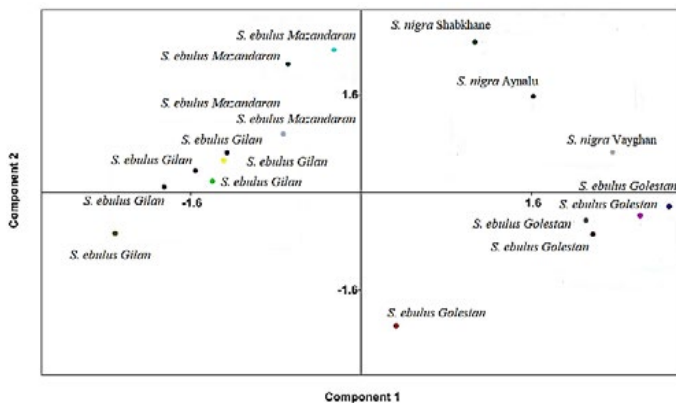
Taxon	Adaxial epidermis			Abaxial epidermis			Stomata type	
	Cell shape	Anticlininal walls	Stomatal apparatus cell	Anticlininal walls	Stomata index (mm <sup>2</sup> )	Stomata density (mm <sup>2</sup> )		Stomata size (µm)
<i>S. nigra</i> L.	Puz	Wa	-	Irr	1.92	131.70 ± 2.3	49.62 × 42.24	Anemocytic
<i>S. nigra</i>	Puz	Wa	-	Irr	1.67	128.50 ± 4.3	46.31 × 40.43	Anemocytic
<i>S. nigra</i>	Puz	Wa	-	Irr	1.59	139.32 ± 3.3	48.41 × 46.34	Anemocytic
<i>S. ebulus</i> L.	Obl	Str	-	Irr	3.31	180.25 ± 2.1	71.32 × 66.24	Anemocytic
<i>S. ebulus</i>	Obl	Str	-	Irr	3.34	177.18 ± 1.4	65.45 × 53.14	Anemocytic
<i>S. ebulus</i>	Obl	Str	-	Irr	3.43	192.23 ± 1.2	62.41 × 54.32	Anemocytic
<i>S. ebulus</i>	Obl	Str	-	Irr	3.17	181.16 ± 3.2	72.43 × 65.30	Anemocytic
<i>S. ebulus</i>	Obl	Str	-	Irr	3.76	186.19 ± 2.5	65.25 × 51.20	Anemocytic
<i>S. ebulus</i>	Obl	Str	-	Irr	3.32	198.14 ± 4.2	60.42 × 52.33	Anemocytic
<i>S. ebulus</i>	Obl	Str	-	Irr	3.97	175.28 ± 1.7	73.40 × 61.30	Anemocytic
<i>S. ebulus</i>	Obl	Str	-	Irr	3.56	180.19 ± 3.5	65.41 × 55.34	Anemocytic
<i>S. ebulus</i>	Obl	Str	-	Irr	3.11	193.14 ± 4.2	72.35 × 66.30	Anemocytic
<i>S. ebulus</i>	Obl	Str	-	Irr	3.05	199.24 ± 6.5	66.40 × 58.34	Anemocytic
<i>S. ebulus</i>	Obl	Str	-	Irr	3.75	185.28 ± 1.7	72.40 × 64.30	Anemocytic
<i>S. ebulus</i>	Obl	Str	-	Irr	3.88	170.19 ± 3.5	65.41 × 53.34	Anemocytic
<i>S. ebulus</i>	Obl	Str	-	Irr	3.45	193.14 ± 4.2	70.35 × 67.30	Anemocytic
<i>S. ebulus</i>	Obl	Str	-	Irr	3.2	189.24 ± 6.5	69.40 × 59.34	Anemocytic
<i>S. ebulus</i>	Obl	Str	-	Irr	3.17	195.28 ± 1.7	71.40 × 62.30	Anemocytic



**Figure 1.** Cluster analysis (Ward's method) based on morphological features of *Sambucus*.

The ANOVA showed significant differences ( $p < 0.05$ ) for the quantitative morphological characters among the *Sambucus* species (data not given). Moreover, the CVA revealed significant differences among the studied species.

The principal component analysis revealed that two components were providing more than 74% of the total observed variation in studied morphological characters. Studying the component loading was evident that leaflet number, stipule and life form are the most important features in the first factor and plant height, petal color and flower number are most significant in the second factor. These characters may be focused on in the taxonomy of the genus and the delimitation of *Sambucus* species. PCA ordination based on studied morphological characters separated species into distinct groups (Fig. 2). PCA confirmed the results of cluster analysis by Ward's method based on qualitative and quantitative features of two studied species (Fig. 2).



**Figure 2.** PCA plot of *Sambucus* species based on observed morphological data.

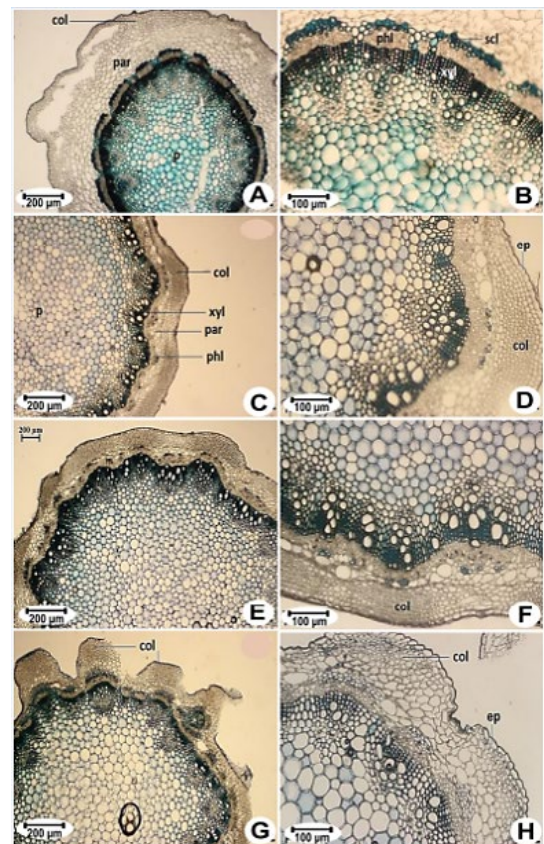
**Anatomical study**

Selected LM micrographs of cross-sections of epiderms, stems, petioles and pedicels are presented in

Fig. 3-Fig. 6. Most characters show significant variability between two species but were constant among different specimens of each species studied.

**Epidermal cell description**

Epidermal and stomata characters of the leaves, such as cell shape, anticlinal wall patterns, stomata index, density, size, and types were examined (Tab. 3). There were two types of epidermal cells: Puzzle-shaped and oblong cells could be seen and anticlinal walls have been observed the wavy and sinuous shapes or straight. There are puzzle-shaped cells with wavy anticlinal walls on the adaxial leaf side of *S. nigra* (Fig. 3A). Oblong cells with straight cell walls were seen in *S. ebulus* (Fig. 3C, 3E and 3G). Abaxial leaf epidermal cells were irregular, with wavy anticlinal walls in *S. nigra* (Fig. 5B). All studied species had stomata on the abaxial leaf surface. All treated populations were of the anemocytic stomata type (Fig. 3B and 3D). The smallest in size stomata were observed in *S. nigra* (Figs. 3B) and the largest were observed in *S. ebulus* (Fig. 3D). The maximum stomatal index and density were registered in *S. ebulus* (Tab. 3).

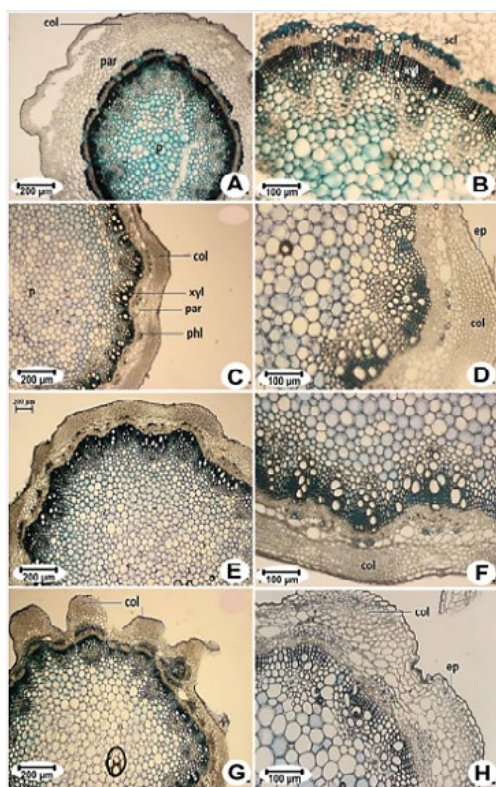


**Figure 3.** Epidermal cells on the adaxial and abaxial side of the specimens' leaves: shape, size, anticlinal wall and stomata under light microscopy. (A, B): *S. nigra*; (C, D, E, F, G and H): *S. ebulus*.

**Stem section**

Transverse sections from the middle part of the stem revealed the following elements (Fig. 4). The stems

are rounded shape in both species. The margin is quite wavy in *S. ebulus* (Fig. 4G) and straight in *S. nigra* (Fig. 4A). The epidermis is composed of single-layered cells. The collenchyma tissue is located under the epidermis of the stem. Collenchyma tissue thickness ranging from 279.70  $\mu\text{m}$  in *S. ebulus* to 154.30  $\mu\text{m}$  in *S. nigra*. Sclerenchyma tissue thickness in *S. nigra* is 79.84  $\mu\text{m}$  and that is the maximum sclerenchyma thickness (Fig. 4B). Vascular bundles are arranged in a single circle. The number of vascular bundles is different. There are 16 vascular bundles in *S. nigra* (Fig. 5A) and 13 in *S. ebulus* (Fig. 5C). Pith is composed of large orbicular parenchymatous cells with intercellular spaces and pith thickness in *S. ebulus* (2880  $\mu\text{m}$ ) is larger than *S. nigra* (2596  $\mu\text{m}$ ).

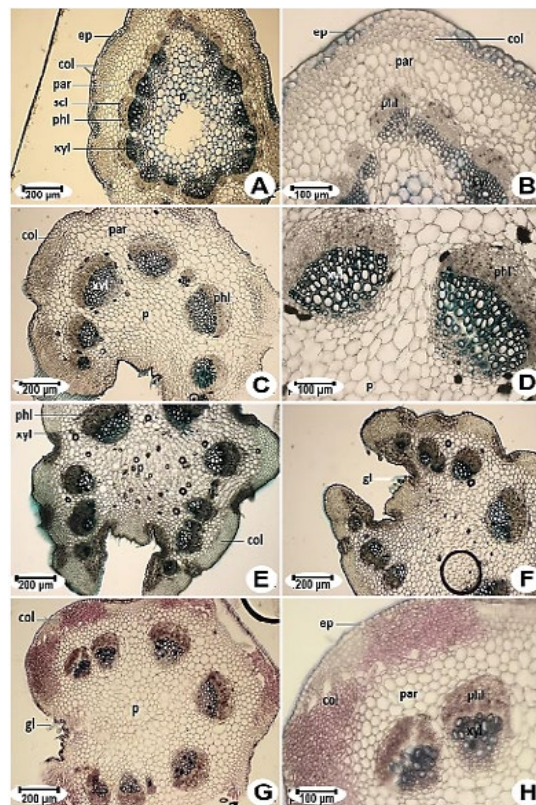


**Figure 4.** Transverse section of the stem in *Sambucus*, (A, B):*S. nigra*; (C, D, E, F, G and H):*S. ebulus*. (ep: epidermis; phl: phloem; xy: xylem; col: collenchymas; par: parenchyma; p: pith; scl: sclerenchyma; c: covering trichome).

### Petiole section

Petiole cross-sections showed a rounded shape with a wavy margin (Fig. 5). The epidermis is composed of single-layered cells. The collenchyma tissue is located under the epidermis of the stem. They are 4-12 layered in both species. The cortex tissue is composed of 4-5 layered parenchyma cells. The fiber composed of sclerenchyma cells is present in *S. nigra*. Vascular bundles are arranged in a single circle. The shape and number of vascular bundles are different. The number of vascular bundles are 8-16 in two sizes with V-shaped outlines in two species. There are 16 vascular bundles in *S. nigra* (Fig. 5A) and 8

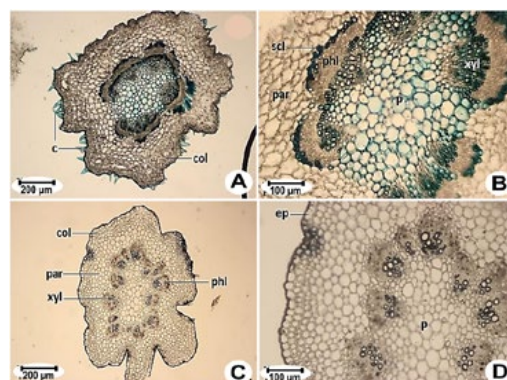
in *S. ebulus* (Fig. 5C). Pith thickness in *S. nigra* (1118  $\mu\text{m}$ ) is smaller than *S. ebulus* (1424  $\mu\text{m}$ ).



**Figure 5.** Transverse section of petiole in *Sambucus*, (A, B):*S. nigra*, (C, D, E, F, G and H):*S. ebulus*.

### Pedicle section

Pedicle cross-sections of both species of *Sambucus* are studied. These two species are similar to each other in the general shape of cross-section and margins. Both are rounded in shape and the margin is sinuous shapes in *S. ebulus* (Fig. 6C) and wavy in *S. nigra* (Fig. 6A). The epidermis is composed of single-layered cells. Collenchyma tissue thickness ranging from 140.60  $\mu\text{m}$  in *S. ebulus* to 168.20  $\mu\text{m}$  in *S. nigra*. The number of vascular bundles is different. Vascular bundles in *S. nigra* are 7 (Fig. 6A) and in *S. ebulus* are 11 (Fig. 6C). Pith thickness in *S. ebulus* (678.20  $\mu\text{m}$ ) is smaller than *S. nigra* (721.5  $\mu\text{m}$ ).



**Figure 6.** Transverse section of pedicle in *Sambucus*, (A, B):*S. nigra*; (C, D):*S. ebulus*.

## Discussion

*Sambucus* has little attention in previous morphological and anatomical studies, hence, this study presents the first comprehensive research of this genus in Iran. Species differentiation is the first step toward understanding the evolution of plants and mechanisms of various characteristics and approaches. In our study, morphological and anatomical character data could delimit the studied *Sambucus* species.

In taxonomy, the use of quantitative morphological characters is traditionally limited to the existence of acondyulous diagnostic characters (Amini et al. 2018; Amini et al. 2019). The presence of distinguished combinations of morphological characters supports the existence of two different species, which emphasize previous molecular (Jacobs et al. 2009; Amini et al. 2019). Our morphological investigations on different species of *Sambucus* confirmed the importance of morphological characters for distinguishing taxa. As it is evident in the PCA graph species are separated by their morphological features. The data on the morphology of *Sambucus* is relatively poor and only a few works are available. Our results were per Pišťanková (2002), which also found medium-sized, more compact clusters with creamy white medium-sized flowers in the variety Bohatka. In consistence with Sedláčková et al. (2018), our morphological investigations on different species of *Sambucus* confirmed the importance of the length of inflorescences. Results of the study show that there are some anatomical variations between *S. nigra* and *S. ebulus*. However, many features are identical. Our observations are as per previous records in other Dipsacales elements (Ruginá and Toma 2007; Gundersen 1910; Petit 1887), While the number of vascular bundles and existence of the crystals of calcium oxalate isa diagnostic character in species studied. Oxalic acid and its salts are usually formed as secondary products of ascorbic and glyoxylic acid metabolism (Williams andWandzilak 1989). They are present in a number of higher plants, including some commonly consumed crop and food plants (Noonan and Savage 1999). Our finding confirms the result of Imenšek et al. (2020) that show *Sambucus* fruits are a good source of oxalates and interspecific hybridization significantly improves the production and the nutritional quality of fruits.

Our results show that the epidermis is made up of only one cell layer with polygonal cells with thin lateral walls. Stomata have an important role as valuable differentiating characters at different levels of plant taxonomy, ecology, and physiology. Two species of *Sambucus* show an anemocytic type of stomata. Stomata

are present on the abaxial surfaces of leaves only. Habitats and the locality of the species also significantly affect the stomata density. Some scientific reports have shown that genetic and habitat factors and some ecological factors such as water availability, leaf size, stomata size, and density play a significant role for stomata (Blum et al. 1981; Scheettleand Rochelle 2000). *S. nigra* is a shrub and grows in a dry soil condition, whereas *S. ebulus* is herbaceous and occupies grasslands or forest margins with high humidity. So high stomatal index and density were observed in *S. ebulus* and low density in *S. nigra*. The findings in the current study are in accord with those in other studies relating to the stomata and structure (Miller 1983). The anatomical structure of the stem is in concordance with previous records by Alerico et al. (2016) that the phloem is composed of axial parenchyma cells and radial parenchyma cells, sometimes more elongated than the axial cells, sieve tube elements, companion cells and angle fibers.

As not been studied before *Sambucus* petiole and pedicel cross-sections, observations could not be compared with other findings. The present study confirmed the leaf anatomical structure by Amini et al. (2019) that vascular bundles and the existence of druse crystals are diagnostic characters. These two species are sympatric species in Iran. These taxa differ in taxonomically important morphological and anatomical characteristics. The genera such as *Sambucus* seem to have extended their distribution rather recently. So there is a hypothesis that the high similarities in the two species studied can be due to the recent divergence of these species.

In conclusion, the present study provided additional evidence for taxonomists in future studies that they could differentiate two species of *S. nigra* and *S. ebulus* by using a combination of morphological and anatomical data.

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