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Morphological, anatomical and micromorphological characterization of *Rhamnus microcarpa* (Rhamnaceae)

Rhamnus microcarpa (Rhamnaceae)'nın morfolojik, anatomik ve mikromorfolojik karakterizasyonu

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ABSTRACT

In this study, general morphological characteristics, the leaf and wood anatomical characteristics and, leaf micromorphological features of *Rhamnus microcarpa*, which is native in Artvin were investigated in detail. Leaves broadly ovate, 3.0-5.5 x 1.9-3.9 cm; stipules 4-7 mm; pinnately 7-10 veined; petiol 6-15 mm. Bud scales dark brown transverse band at the base. Wood was semi-ring porous to ring porous, rays heterocellular, fibers thick-walled especially in latewood. In leaves, dorsiventral mesophyll, hypostomatic type stomata and simple unicellular trichomes observed. Also, epicuticle scales in the lower leaf surface observed.

ÖZ

Bu çalışmada, Artvin'de doğal olarak yayılış gösteren *Rhamnus microcarpa*'nın genel morfolojik özellikleri, yaprak ve odun anatomik özellikleri ve yaprak mikromorfolojik özellikleri detaylı olarak incelenmiştir. Yapraklar geniş yumurtamsı, 3.0-5.5 x 1.9-3.9 cm; stipüller 4-7 mm; damar sayısı 7-10; yaprak sapı 6-15 mm'dir. Tomurcuk pullarının tabanında koyu kahverengi enine şeritler bulunur. Odun yarı halkalı veya halkalı traheli, özışınları heteroselüler, özellikle yaz odununda lifler kalın çeperlidir. Yapraklarda dorsiventral mezofil, hipostomatik tip stoma ve basit üniselüler tüyler tespit edildi. Ayrıca, yaprak alt yüzeylerinde epikütikular pullar gözlendi.

Keywords:
Atadinus, Rhamnus microcarpa, morphology, anatomy, micromorphology, Artvin, Turkey

Anahtar kelimeler:
Atadinus, Rhamnus microcarpa, morfoloji, anatomi, mikromorfoloji, Artvin, Türkiye

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1. INTRODUCTION

The family of Rhamnaceae, which has more than 50 genera and approximately 900 species on the earth, consists of trees and shrubs (Jud et al., 2017).

Rhamnaceae family was represented by 6 genera (*Atadinus* Raf., *Paliurus* Mill., *Ziziphus* Mill., *Rhamnus* L., *Sageretia* Brongn., *Frangula* Mill.) in Turkey (Davis & Yaltırık, 1967; Alkan, 2011; Akkemik et al., 2014; Hassler, 2019). *Atadinus* has been recently described by Hauenschild (2019) and differentiated from the genus *Rhamnus*. Although five species of *Rhamnus* genus (as follow; *Rhamnus fallax* Boiss., *R. depressa* Grubov, *R. imeretinus* Booth et al., *R. libanoticus* Boiss., *R. microcarpus* Boiss.) have been evaluated under the genus *Atadinus*, this decision continues to be discussed.

Rhamnus species are typically found in temperate and sub-tropical climates of the Northern Hemisphere (Richardson et al. 2000; Bolmgren & Oxelman 2004) with greater representation in the tropics of the Old World than in the Neo-tropics (Johnston & Johnston 1978), 57 species which most of them are endemic in China and six species in Pakistan (Hassler, 2019), one species, *R. caroliniana* in Georgia (Coder, 2010), and 22 taxa are native in Turkey, six of them are endemic (Davis & Yaltırık, 1967). Some of Rhamnus species are evergreen (e.g.

Rhamnus alaternus L.), others are deciduous, some barbed, some without thorns. The buds are scaly, spiral or opposed (Davis & Yaltırık, 1967; Akkemik et al., 2014). The genus *Rhamnus* is one of 11 genera of Rhamnaceae possessing two to four seeds per fruit (Medan & Schirarend, 2004).

Anatomical and micromorphological characteristics have been used in several taxonomical investigations (Ozcan et al., 2015; Erşen Bak & Merev, 2016). A few of morphological and anatomical studies have been conducted in *Rhamnus* species in the world. In earlier studies, wood anatomical characteristics of some *Rhamnus* species were examined in Turkey: *R. imeretinus* Boot., *R. microcarpus* Boiss. and *R. catharticus* L. (Merev, 1998), *R. thymifolius* Bornm. (Başaran & Yaman, 1998), *R. pyrellus* O. Schwarz, *R. nitidus* Davis, *R. pichleri* Schneider & Bornm. ex Bornm., *R. thymifolius* Bornm., *R. hirtellus* Boiss. (Akkemik et al., 2007) and *R. petiolaris* Boiss., *R. oleoides* subsp. *graecus* (Boiss & Reut.) (Akgün et al., 2010). Fahn et al. (1986) and Schweingruber et al. (2011) have wood anatomical studies of different *Rhamnus* species in Israel and Europea.

Mantese & Medan (1992) compared leaf anatomies of four species in the genus *Retanilla* (DC.) Brongn. belonging to the family Rhamnaceae and reported dorsiventral leaf mesophyll, mucilaginous epidermises, hypostomatic type stomata, simple, undulate hairs, tannin composition and idioblast for these species. Serdar et al. (2007) examined anatomical characteristics of *Frangula alnus* Mill. subsp. *pontica* (Boiss.) P.H.Davis & Yalt. and observed secretory canals in this taxon. They compared two subspecies of *F. alnus* and reported some differences between them. Shisode & Patil (2011) determined petiole anatomy and leaf epidermal characteristics of 13 species belonging to the family Rhamnaceae, and indicated that petiole outline, epidermal structures, cell layers in the cortex, pattern of vascular system and occurrence of crystals can be used as distinguishing characters among the taxa. It has given information in another study that leaf surface micromorphological characteristics are also useful and could be used to distinguishing features among the taxa in species and also subspecies level classifications (Hui et al. 2008). Efe et al. (2005) studied morphological features and leaf anatomy of five endemic *Rhamnus* species in Turkey.

Rhamnus microcarpa distributed in Giresun, Gümüşhane, Rize, Artvin and Erzincan (Eminağaoğlu & Anşin, 2002, 2003, 2004, 2005; Eminağaoğlu et al., 2007; Eminağaoğlu, 2009, 2012; Güner et al., 2012; Akkemik et. al., 2014; Eminağaoğlu, 2015; Yüksel & Eminağaoğlu, 2017; Eminağaoğlu et al., 2018; Akyıldırım Beğen & Yüksel, 2018; Yüksel & Akyıldırım Beğen, 2018). It has minor morphological differences from the other taxa within the genus, and no detailed study was performed on its anatomy. In this context, the purpose of the present study is to investigate wood and leaf anatomical characteristics, and micromorphological features of the species and to distinguish more precisely from the other species.

2. MATERIAL AND METHOD

2.1. Morphological Analysis

Materials used in this study were collected in the years of 2016 and 2017. Plant specimens were photographed and GPS coordinates were taken in the field. The plant specimens were dried by using herbarium methods, and identified using identification keys given in Flora of Turkey (Davis, 1965-1985; Davis et al., 1988; Güner et al., 2000). Plant samples were prepared by herbarium techniques and kept at the Herbarium of Artvin Coruh University (ARTH), Artvin, Turkey. The plant name was checked using Türkiye Bitkileri Listesi, Damarlı Bitkiler (Güner et al., 2012), The International Plant Names Index (IPNI, 2015) and the Plant List (PL, 2013). The important taxonomic characters involved in the diagnosis of the taxa were determined and leaf, seed, petiole, flower and fruit measurements were performed.

2.2. Anatomical Preparation

Wood sections in three directions, transversal, tangential and radial, were taken by using a sliding microtome. Sections stained with safranine O and alcian blue combination (Ruzin, 1999; Ives, 2001). Macerations were prepared using Schultze's method (Normand, 1972). All wood terms were determined according to the International Association of Wood Anatomists Committee on Nomenclature (Wheeler et al., 1989). For all features, mean values were based on 30 measurements or counts.

Anatomical observations of leaf were performed in living specimens. Plant leaf samples were fixed in FAA (Formal 5 ml + glacial acetic acid 5 ml + %70'lik ethyl alcohol 90 ml) and stored in 70% alcohol for anatomical studies. Transverse sections of leaf and paradermal sections of upper and lower epidermis of leaves were prepared

manually using commercial razor blades and stained in Hematoxylin for about 15 min. To remove the excess stain, sections were washed in water several times (Algan, 1981). Semi-permanent slides were mounted in glycerin or permanent slides were covered with glycerin-gelatin (Vardar, 1987). Well-stained sections were examined under a light microscope and photographed using an Olympus BX-53 microscope with digital camera attachment DP-73.

2.3. Micromorphological Analysis

Micromorphological features of the fruits were studied using a stereomicroscope (Leica M60 with a digital camera attachment DFC 295) and a scanning electron microscope (Zeiss Evo LS 10, ACU-Biltekmer). The leaves were first examined using a stereomicroscope to determine shape, color and maturity. For scanning electron microscopy, dried and mature upper and lower leaf parts were separately placed on stubs using double-sided adhesive tape, and coated with gold in a Cressington sputter coater 108 auto coating apparatus for 2 minutes. They were examined and photographed from the same region (from the middle part and margin of the leaf).

3. RESULTS AND DISCUSSION

Results were given based on qualitative and quantitative data as follows:

3.1. Morphology

Rhamnus microcarpa Boiss., Fl. Orient. 2: 20 (1872)

≡*Oreoherzogia microcarpa* (Boiss.) W.Vent in Feddes Repert. Spec. Nov. Regni Veg. 65: 52, 103 (1962).

Low shrub, deciduous to 0-0.30 m, usually much branched. Branches opposite or alternate, unarmed, glabrous; winter buds with scales. Young twigs puberulent or glabrous, bud scales 5-7, glabrous, margins brush-hairy, dark brown transverse band at the base, 1-4mm (Figure 1a).

Leaves fasciculate, broadly ovate, 3.0-5.5 x 1.9-3.9 cm, acute; stipules 4-7mm, mainly subulate, caducous, rarely persistent; leaf blade always undivided, pinnately 7-10 veined, margin crenate- serrulate, upcurved, glabrous on both surfaces, dark green above, yellowish green below, usually subcordate at base, petiol 6-15 mm, strigose (Figure 1e, f).

Inflorescence of solitary or few fascicled in axillary cymes, Flowers 2.5-4 mm, mostly yellowish green, small, bisexual, rarely polygamous, pedicel 2.5-7 mm, puberulent. Calyx tube campanulate to cup-shaped; sepals 4 or 5, ovate-triangular, adaxially ± distinctly keeled. Petals 4, rarely absent, shorter than sepals, cucullate to hooded, often enfolding stamens, base shortly clawed, apex often 2-fid. Stamens 4, surrounded by and shorter than petals; anthers dorsifixed. Disk thin, adnate and lining calyx tube. Ovary superior, globose, free, 2-4-loculed; styles ± deeply 2-4-cleft. Fruit 3-5 x 3-5 mm, red-berrylike drupe, obovoid-globose or globose; pedicel 2.5-5 mm, Seeds 3-4 x 2-3 mm, obovoid or oblong-obovoid, unfurrowed or abaxially or laterally with a ± long, narrow to gaping, often distinctly margined furrow; endosperm fleshy. Seed 3-4. On dry slopes and adpressed to rocks, 1400-2050 m (Figure 1a, b, c, d).

Flowering period: June; *fruiting period*: August.

Figure 1. Morphological appearances of *Rhamnus microcarpa.* a: habitus; b, c: branches with flower buds or fruits; d: flowers; e, f: leaves and fruits.

Specimens examined: Turkey- Artvin, Şavşat, Meydancık, Çermik village, rocky slope, 2022 m, 41° 22' 49" 42° 07'

27", 25.06.2016; *Ö.Emin.* 22316, ARTH 11922; 1954 m, 41° 22' 49" 42° 07' 29", 23.08.2016; 08.05.2017, *Ö.Emin.* 22244, 22245, ARTH 11918, 11919; Yusufeli, Sütlüce, rocky place, 2251 m, 40° 35' 38" 41° 21' 06", 16.08.2016, *Ö.Emin*. 22367, ARTH 11946; Yusufeli, Barhal, rocky slope, 1426 m, 40° 57' 18" 41° 18' 30", 07.06.2017, *Ö.Emin.* 22368, ARTH 11947.

3.2. Anatomical Results

3.2.1. Wood Anatomy

Wood semi-ring porous to ring porous. Growth ring distinct. Vessels mostly solitary, earlywood vessels sometimes in pairs or radial and tangential multiples, a more or less continuous band in annual ring boundaries; latewood vessels mostly diagonal and dendritic patterns, slightly radial and tangential multiples, or in clusters, surrounded by vasicentric vascular tracheids, rounded to angular in cross-section (Figure 2a).

Earlywood vessels tangential diameter 38.8 (20-75) µm and radial diameter 53.9 (30-80) µm, latewood vessels tangential diameter 22.8 (15-32.5) µm and radial diameter 27.3 (15-50) µm, vessels ca. 127 (95-156)/ mm^2, vessel member length 242.4 (145-300) µm. Perforation plates simple in oblique end walls. Vessel pits alternate, round to oval, with slit-like apertures. Vessels and vasicentric vascular tracheids with spiral thickening.

Libriform fibers 584.8 (370-800) µm long, 11.5 (9-15) µm wide, thin- to thick-walls 3.75 (3-6) µm, gelatinous (Figure 2c). Axial parenchyma apotracheal and paratracheal a more or less continuous marginal bands of 1-2 cells.

Rays 10 (7-12)/mm, uniseriate, biseriate and multiseriate, weakly heterocellular, composed of slightly upright and square marginal cells and mostly procumbent cells (Figures 2b, d). Uniseriate ray height 98.7 (40-230) µm and 5 (2-13) cells, biseriate ray height 120.6 (55-225) µm, multiseriate ray height 281.58 (90-584) µm (max. 46 cells) and multiseriate ray width 49 (22.5-75) µm, 2-7 (8) cells.

Perforated ray cells present (Figure 2e). Crystals not observed.

In the study of Merev (1998), *R. microcarpa* wood has been described as having diffuse porous and homocellular rays. In this study, it is determined that as semi-ring porous or ring porous and heterocellular rays. In earlier studies, Fahn et al. (1986), Akkemik et al. (2007) and Akgün et al. (2010) stated that various ray types are found in *Rhamnus.*

The mesomorphy value calculated based on the vessel member characteristics of the *R. microcarpa* is 59.07. The low mesomorphy value (<75) is emphasized as an indicator of xeromorphy (Wheeler & Baas, 1991). The mesomorphy value of *Rhamnus* species grown in Mediterranean climate was calculated between 11 and 23 (Akkemik et al., 2007) and between 12 and 22 (Akgün et al., 2010). Although the mesomorphy value of *R. microcarpa* was found to be higher than *Rhamnus* taxa of Mediterranean origin, it can be said that this species in the form of dwarf shrub, wrapped on rocks, is xerophyte. But, Merev (1998) founded that vessel density (82/mm^2) is less and vessel member length (308 µm) is longer than in our study. Hence, mesomorphy value (135.21) calculated by Merev is higher than from ours. In addition, multiseriate (277 µm) and uniseriate (142 µm) rays height are longer than in our study. Consequently, our results do not coincide with those of Merev's.

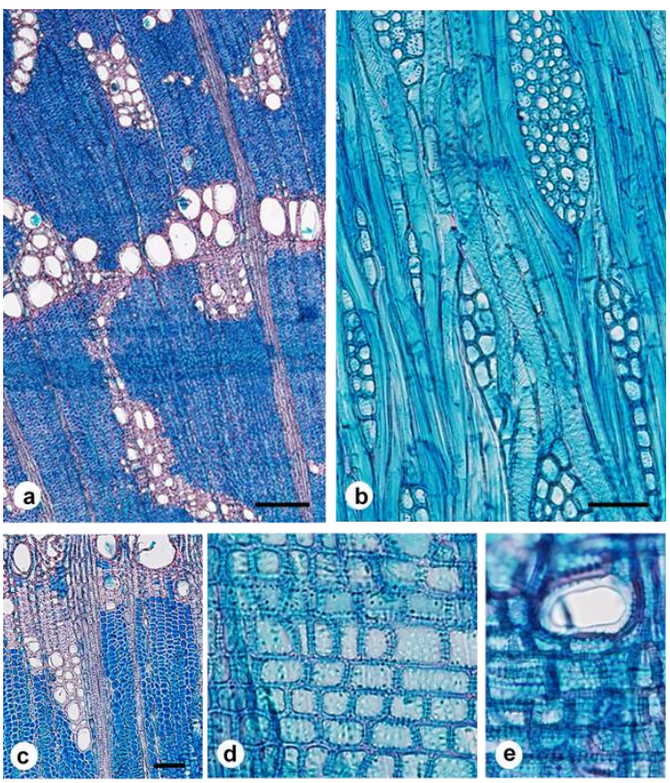

Figure 2. Wood anatomy of *R. microcarpa*. a: semi–ring porous to ring porous xylem, earlywood vessels radial and tangential multiples, a more or less continuous band in annual tangential multiples, b: uniseriate, biseriate and multiseriate, heterocellular rays, c: latewood vessels surrounded by vasicentric vascular tracheids, gelatinous fibres, d: Procumbent, square and upright ray cells, e: Perforated ray cells. Scale bars: a: 100 µm, b, c: 50 µm.

3.2.2. Petiole Anatomy

Petiole is narrowly sulcate in upper part and horseshoe shape in outline. Vascular bundle is U-shape. Epidermal cells are narrow or ovate shape. Single vascular bundle comprises a wide area in the middle. Below the upper and lower epidermises 3 to 6 layers of collenchyma cells are present (Table 1). Collenchyma layers cover a large area in the lower part and surround of the petiole. Many parenchymatous cells containing druse crystals are settled in the surrounding of vascular bundle. Uniseriate and unicellular simple trichomes cover all petiole, but glandular trichomes are not present. Sclerenchymatous crystals do not occur in phloem part of vascular bundles (Figure 3a, b).

Table 1. Petiole anatomical features of *R. microcarpa.*

Character	Measurements (µm)
Xylem thickness	219.29±05.93
Phloem thickness	232.01±5.93
Vascular bundle thickness	623.93±35.56
Vascular bundle breadth	776.91±24.12
Trachea size	19.57±0.49
Cortex thickness	247.97±34.67
Collenchyma thickness (upper surface)	77.87±13.03
Collenchyma thickness (lower surface)	55.37±11.51
Petiole thickness	1236.41±11.20
Petiole breadth	1432.06±8.53

Few leaf anatomical reports are present in literature. In previous researches, anatomical characteristics of *R. wighti* L. was determined from India (Shisode & Patil, 2011). They reported horseshoes petiole and arc-shape vascular bundle in *R. wighti*. In addition, Efe et al. (2005) investigated leaf characteristics of five *Rhamnus* species and determined general anatomical characters. They reported orbiculate petiole for *Rhamnus pichleri* Schneider and Bornm. ex Bornm. Our results are in accordance to their findings. Secretory cavities, however, are not observed in the petiole of *R. microcarpa*.

3.2.3. Leaf Anatomy

3.2.3.1. Midrib

It is semi-circle, sulcate and covers a large area. Adaxial part is concave, while abaxial one is convex. Under the upper and lower epidermis, several layers of collenchyma cells are observed. Collateral vascular bundle is single, arc shape and covers a large area. Sparsely scattered sclerenchymatous cells can be visible in phloem part of vascular bundle (Figure 3c, d).

In previous studies, Shisode & Patil (2011) reported petiole anatomies of 13 species belong to Rhamnaceae family. They also determined stem and leaf epidermal structures, vascular bundles characteristics, presences of crystals and proposed that these features could be used as additional taxonomical characteristics in their systematic. They reported hypodermal layer in petioles. Mantese & Medan (1992) compared leaf anatomies of four species in the genus Retanella belong to the family Rhamnaceae and reported dorsiventral leaf mesophyll, mucilaginous epidermises, hypostomatic type stomata, simple, undulate hairs, tannin composition and idioblast for these species. Serdar et al. (2007) examined anatomical characteristics of *Frangula alnus* subsp. *pontica* and observed secretory canals in this taxon. Efe et al. (2005) reported leaf characteristics of five *Rhamnus* species and dorsiventral mesophyll was found in the species. Our results are generally in accordance with these previous reports in the family Rhamnaceae. However, hypodermal layer in petiole, idioblastic structure in mesophyll and secretory ducts have not been encountered in *R. microcarpa*. On the other hand, collenchymas circle was observed in the petiole in agreement with Shisode & Patil's report (2011).

3.2.3.2. Lamina

The leaves show dorsiventral type (bifacial) mesophyll. It is included of two-three palisade layers in the upper part and five spongy layers (Figure 3e, f). The palisade tissue covers slightly many areas than spongy parenchyma. A thick cuticle covers epidermis. Upper epidermis cells with straight walls are equal to lower ones (Figure 4a, b). Similar to our findings, Varone & Gratani (2009) investigated leaf expansion of *R. alaternus* L. and reported bifacial leaf mesophyll for this species. It was mentioned that spongy parenchyma (97.2±0.2 µm) covers slightly large area than palisade parenchyma (82.0±1.0 µm) in this species (Table 2).

In the present study, we observed simple trichomes in the leaf of *R. microcarpa* (Figure 3). Unicellular trichomes were reported by Shisode & Patil's report (2011) in *R. wighti* L. and by Serdar et al. (2007) in two subspecies of *Frangula alnus.* Furthermore, it was also mentioned from stellate scales in *Pomaderris apetala.* Labill. by Shisode & Patil's report (2011). We also determined many

epicuticular scales in the abaxial surface of leaf of *R. microcarpa*.

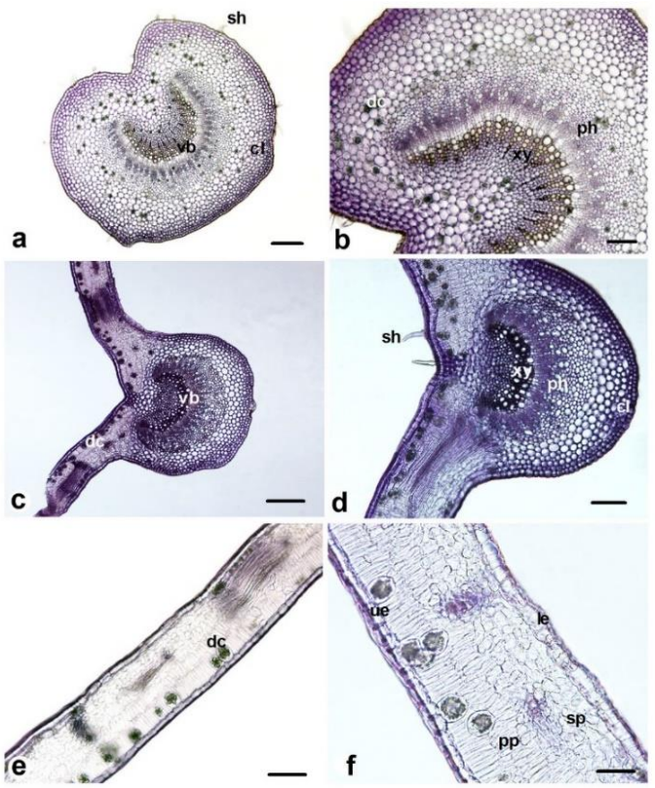

Figure 3. Cross sections of *R. microcarpa* a, b: petiole, c, d: midrib; e, f: lamina. cl: collenchyma, dc: druse crystals, le: lower epidermis, ph: phloem, pp: palisade parenchyma, sh: simple hair (trichome), sp: spongy parenchyma, ue: upper epidermis, vb: vascular bundle, xy: xylem. Scale bars: 200 µm (a, c); 100 µm (b, d, e); 50 µm (f).

3.2.3.3. Stomata

It has occurred only in abaxial surface (hypostomatic type) of leaf. They are anomocytic and many more in lower epidermis (Figure 4a, b). Adjacent epidermal cells to the stomata are more or less with straight walls in adaxial surfaces, but undulate walls in lower ones. From previous reports, several species in the family have hypostomatic type stomata, except for *Colubrina asiatica* (L.) Brongn. (Hui et al., 2011). Efe et al. (2005) reported hypostomatic leaf type was found in the species, except for *Rhamnus thymifolius* Bornm. (amphistomatic leaf type). We also found the same result for *R. microcarpa* in accordance with these reports.

3.2.3.4. Crystals

Druse crystals compounds are observed in some palisade cells of all leaf upper parts and also circle the petiole (Figure 3).

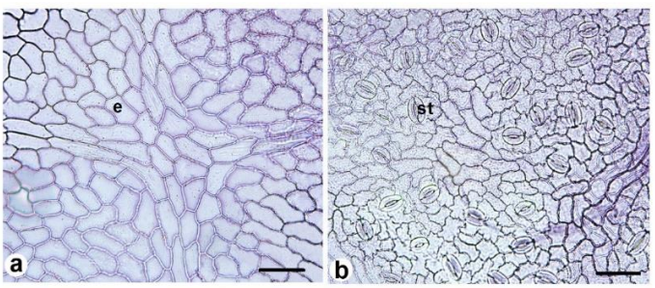

Figure 4. Paradermal section of leaf. a, adaxial surface, b: abaxial surface. e: epidermis; st: stomata. Scale bars: 50 µm.

Table 2. Leaf anatomical features of *R. microcarpa.*

Character		*Measurement* (µm) */Calculation*
Upper epidermis length		13.27±0.40
Upper epidermis width		18.88±1.43
Lower epidermis length		13.40±0.55
Lower epidermis width		24.44±1.40
Midrib mesophyll thickness		659.23±14.77
Midrib mesophyll breadth		775.57±22.43
Lamina mesophyll thickness		181.84±1.45
Trachea size		12.58±0.11
Xylem thickness		190.06±4.83
Phloem thickness		204.43±4.84
Vascular bundle thickness		396.38±9.55
Vascular bundle breadth		552.89±14.36
Adaxial surface	Cuticle thickness	5.57±0.15
	Stomatal length	0
	Stomatal index	0
	Stomata number per mm^2	0
Abaxial surface	Cuticle thickness	6.20±0.15
	Stomatal length	27.68±0.48
	Stomatal index	11.57±0.84
	Stomata number per mm^2	30.8±4.22

Shisode & Patil (2011) reported these types' crystals in *R. wighti* L.&A.Gray (*Toxicodendron diversilobum* (Torr.& A.Gray) Greene). Serdar et al. (2007) also mentioned different amounts of Druse crystals in petiole of *Frangula alnus*. The authors also reported secretory cavities in *R. wighti* and also some other species in the family Rhamnaceae. Efe et al. (2005) reported calcium oxalate crystals in the palisade cells of five *Rhamnus* species. Our results are in agreement with these reports except for secretory cavities.

3.2.4. Leaf Micromorphology

Upper and lower leaf surfaces are undulate and surface sculpture is wavy in adaxial parts (Figure 5a, b). Especially abaxial leaf surfaces are densely covered with epicuticular scaly structures (Figure 5c, d). Sparsely simple trichomes can be visible in the middle part of leaf (Figure 5e, f).

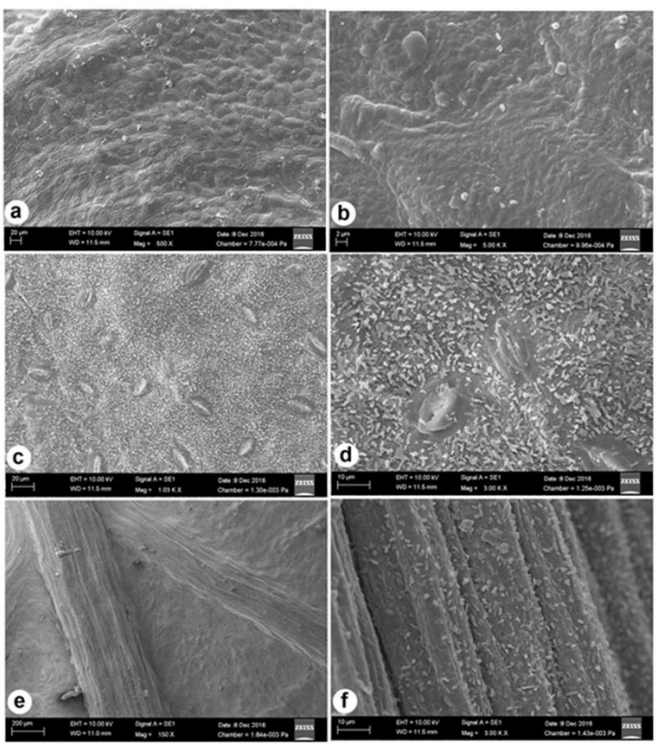

Figure 5. Leaf micrographs of *R. microcarpa*. a, b: Adaxial surface, c, d: Abaxial surface, e, f: midrib.

In previous investigations, leaf micromorphological features of 41 species and four varieties belonging to the Rhamnaceae family were examined, and undulate leaf surface determined. According to surface ornamentation, smooth and striate adaxial epidermal cells or epidermal cells having scaly structures were reported Hui et al. (2011). These authors also mentioned that leaf micromorphological characteristics could be used at the species or subspecies level classifications in Rhamnaceae.

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