

Pericarp Ultrastructure of *Salvia* Section *Hemisphace* (Mentheae; Nepetoideae; Lamiaceae)

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Abstract: The genus *Salvia* L. (sage), which belongs to the tribe Mentheae of the subfamily Nepetoideae within the family Lamiaceae, is well-known for its medicinal, ornamental, culinary and hallucinogenic uses. The section *Hemisphace* Benth. of this genus is represented in Turkey by three species. The present study is conducted on two morphologically similar *Salvia* species belonging to this section: *Salvia napifolia* Jacq. and *S. russellii* Benth. (excluding *S. verticillata* L.). For this purpose, the pericarp ultrastructure of these species is investigated in detail with the help of light and transmission electron microscopy (TEM). Morphometric characters are analyzed using one-way Analysis of Variance (ANOVA) with Tukey's honestly significant difference (HSD) post-hoc test for multiple comparisons. The taxonomic potential of pericarp characteristics is discussed. The most prominent traits are the thickness of the pericarp, mesocarp and sclerenchyma region that permit the separation of the species studied. Myxocarpy (mucilage formation) is recognized on the surface of the wetted mericarps of both species. Mucilaginous cells reveal a moderate reaction but *S. napifolia* is of a somewhat thicker mucilaginous layer, about 0.20-0.34 mm. This study represents the first comprehensive findings on the pericarp ultrastructure of the species examined.

Keywords: Lamiaceae, pericarp ultrastructure, *Salvia*, section *Hemisphace*, TEM, Turkey.

Salvia Cinsi *Hemisphace* Seksiyonun (Mentheae; Nepetoideae; Lamiaceae) Perikarp İnce Yapısı

Özet: Lamiaceae familyası içindeki Nepetoideae alt familyasının Mentheae oymağına ait olan *Salvia* L. (adaçayı) cinsi tıbbi, süs bitkisi, gıda ve halüsinojenik kullanımları nedeniyle iyi bilinmektedir. Bu cinsin *Hemisphace* Benth. seksiyonu Türkiye'de üç tür ile temsil edilir. Bu güncel çalışma, ilgili seksiyona ait morfolojik olarak birbirine benzeyen iki *Salvia* türü üzerine yürütülmüştür: *Salvia napifolia* Jacq. ve *S. russellii* Benth. (*S. verticillata* L. hariç). Bu amaç için, söz konusu türlerin perikarp ince yapısı ışık ve geçirimsiz elektron mikroskobu (TEM) yardımıyla detaylı olarak araştırılmıştır. Morfometrik karakterler çoklu karşılaştırmalar için tek yönlü Varyans Analizi (ANOVA) ile Tukey'in tam olarak önemli fark (HSD) post-hoc testi kullanılarak analiz edilmiştir. Perikarp özelliklerinin taksonomik önemi tartışılmıştır. Çalışılan türlerin ayırımına izin veren en önemli özellikler; perikarp, mesokarp ve sklerenkima bölgesinin kalınlığıdır. Her iki türün ıslatılmış merikarplarının yüzeyinde miksokarpi (müsilaj oluşumu) görülmüştür. Müsilajlı hücreler orta düzeyde bir reaksiyon göstermiştir, fakat *S. napifolia*'daki müsilajlı tabaka biraz daha kalındır (yaklaşık 0.20-0.34 mm). Bu çalışma incelenen türlerin perikarp ince yapısı üzerine ilk detaylı bulguları sunmaktadır.

Anahtar kelimeler: Lamiaceae, perikarp ince yapısı, *Salvia*, *Hemisphace* seksiyonu, TEM, Türkiye.

1. Introduction

In Turkey, *Salvia* L. is called "adaçayı" and is often consumed as tea (Baytop, 1999). In the traditional Turkish folk medicine, it is used as antibacterial, stomachic, diuretic, spasmolytic, antiseptic, hemostatic, stimulant and carminative and in the treatment of sore throats, cough, colds and wounds (Ulubelen, 2003; Kotan et al., 2008; Topcu et al., 2008; Kirmizibekmez et al., 2012; Er, Tugay, Özcan, Ulukuş, & Al-Juhaimi, 2013).

The genus *Salvia* is assigned to the tribe Mentheae of the subfamily Nepetoideae within the family Lamiaceae. *Salvia* is distinguished from the other 72 genera in the Mentheae by the presence of two aborted posterior stamens and a markedly elongated connective tissue separating thecae of the two expressed stamens that may form a lever mechanism of pollination for which the genus is best known (Walker & Systma, 2007). It is widely known as the largest genus of the Lamiaceae, consisting of approximately 1000 species restricted to five

regions throughout the world: central and south America (approx. 550 spp., the most significant center of species diversity), western Asia (approx. 220 spp., mainly in Turkey, Iran, Russia, and Afghanistan), eastern Asia (approx. 100 spp., mainly in China and Japan), east and south Africa (approx. 60 spp.), and Europe (approx. 36 spp.) (Walker & Systma, 2007).

In western Asia, the most significant center of species diversity of the genus is found in Turkey with 100 species recognized until recently. The rate of Turkish endemic to *Salvia* species is 53%. These species are divided into seven sections: *Salvia* Hedge (synonym *Eusphace* Benth.), *Aethiopsis* Benth., *Plethiosphace* Benth., *Drymosphace* Benth., *Horminum* Benth., and *Hemisphace* Benth. (Boissier, 1879; Doğan et al., 2007). Leaf (pinnatisect, trisect or simple), calyx (membranous or thick textured, upper lip 2-sulcate or not, concave or not), corolla (upper lip falcate or not, tube squamulate or not, annulate or not) and stamen (type A, B or C) characteristics are the most significant means for

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separating these sections from each other. In Turkey, the section *Hemisphece* is represented by three non-endemic species: *Salvia napifolia* Jacq., *S. russellii* Benth., and *S. verticillata* L. (Hedge, 1982).

It has been demonstrated that mericarp (nutlet) characters can be used successfully at many taxonomic levels, depending on the characters chosen and the variation present (Guerin, 2005). Ultrastructural studies on the pericarp have been carried out in various genera of the family Lamiceae and have proved the usefulness of pericarp characters for species and generic level in the family (Wagner, 1914; Bilimovitsch, 1935; Wojciechowska, 1958, 1961a, b, 1966, 1972; Makarova, 1967; Hedge & Lamond, 1968; Hedge, 1970; Witztum, 1978; Ryding, 1992a, b, 1993a, b, 1995, 2001, 2010; Budantsev, 1993a, b; Budantsev & Lobova, 1997; Duletić-Laušević & Marin, 1999; Mosquero, Juan, & Pastor, 2002; Moon & Hong, 2006). However, there have been only a few studies on the pericarp ultrastructure of the genus *Salvia* (Wojciechowska, 1958, 1966; Hedge, 1970; Oran, 1997; Ryding, 2010; Büyükkartal, Kahraman, Çölgeçen, Doğan, & Karabacak, 2011; Hassan & Al. Thobaiti, 2014). No detailed data have been recorded on the pericarp ultrastructure of *S. napifolia* and *S. russellii* belonging to the section *Hemisphece* of this genus. Therefore, this study aims to investigate the pericarp ultrastructure of these species that have not been comprehensively studied to date; by light and transmission electron microscopy and to assess the taxonomic importance of pericarp characters in their delimitation.

2. Material and Methods

Specimens of *S. napifolia* and *S. russellii* in the section *Salvia* of the genus *Salvia* were collected from their natural habitats during field investigations in Turkey. The collected specimens were pressed and dried on the basis of standard herbarium techniques (Davis & Heywood, 1973) and kept in the herbarium of the Department of Biological Sciences, at the Middle East Technical University (METU) in Ankara. The source of each species is as follows; *S. napifolia*: Hatay, Samandağ to Yayladağ, F. Celep 1646 and *S. russellii*: Van, Van to Gürpınar, A. Kahraman 1451.

2.1. Light microscopy (LM)

The mericarps of *S. napifolia* and *S. russellii* were first examined using a stereomicroscope to ensure that they were of normal size and maturity. For pericarp ultrastructure studies, the ripe mericarps were softened and placed for 24 hours in distilled water. Then, they were fixed in 3% glutaraldehyde (with 0.1 M Na-P buffer) and 1% osmium tetroxide (with 0.1 M Na-P buffer). After that, they were dehydrated through a graduated ethanol series and embedded in Epon 812 (Luft, 1961). Transverse semi-thin sections for LM were obtained by sectioning of the embedded material. After that, these semi-thin sections were stained with methylene blue and toluidine blue and mounted permanently. By means of LM, the sections obtained were examined and photographed for the pericarp characteristics, such as thickness of the pericarp, mesocarp, sclerenchymatous region and endocarp and mucilaginous cells. According to Hedge (1970) and Ryding (2010), the terminology was adjusted for pericarp structure.

The method of Ryding (1992a) was followed to determine whether the formation of mucilage on their surface occurs when they were wetted. The amount of mericarp mucilage production by the species studied was compared. According to this method based on the extent of swelling of mucilaginous cells, there were four main types of reactions: (1) a strong reaction with mucilaginous cells 0.8-1.5 mm long, (2) a moderate reaction with mucilaginous cells 0.1-0.5 mm long, (3) a weak reaction with mucilaginous cells less than 0.1 mm long, and (4) a very weak reaction when no appreciable elongation of mucilaginous cells occurred after swelling in water.

2.2. Transmission Electron Microscopy (TEM)

For TEM, ultra-thin sections were obtained by sectioning of the embedded material. Then, they were stained with uranyl acetate (Stempak & Ward, 1964) and lead citrate (Sato, 1968). Next, they were examined with the aid of JEOL CX-100 TEM and micrographs were taken.

2.3. Statistical Analysis

Quantitative characters were analyzed for their minimum, maximum, mean and standard deviation values and significance using the Statistica version 13.3 software. Importance of differences was tested using one-way Analysis of Variance (ANOVA) with Tukey's honestly significant difference (HSD) post-hoc test for multiple comparisons (Sokal & Rohlf, 1995).

3. Results

The data resulting from morphometric analysis are given in Tab. 1. LM and SEM micrographs showing the pericarp ultrastructure of *S. napifolia* and *S. russellii* are presented in Figs. 1-2. Box-and-whisker plots of quantitative characters, such as the thickness of the pericarp, mucilaginous layer, mesocarp, and sclerenchymatous region and size of endocarp cells, are provided in Fig. 3.

The results obtained from detailed LM and TEM studies on the pericarp ultrastructure of the species examined show that the pericarp varies between 70.71 and 140.44 μm in thickness, apart from mucilage-containing cells. It is distinguished into four main regions: the exocarp (epicarp or outer epidermis), mesocarp, sclerenchyma region, and endocarp (inner epidermis) (Figs. 1-2).

The exocarp, the outermost layer of the pericarp, is epidermal in origin and usually comprises isodiametric to elongated, large, thick-walled, and colourless mucilaginous cells in groups of one or more. Occasionally, it is also composed of smaller and dark brown non-mucilaginous cells. The mucilaginous cells that produce mucilage upon wetting exhibit a moderate reaction. The thickness of the mucilage region ranges from 0.14 to 0.34 mm (Figs. 1a-d; 2 a-d).

The mesocarp is 35.90-70.45 μm thick and consists of a dark amorphous mass of strongly compressed and tangentially elongated, thick-walled sclerenchymatous cells and thin-walled parenchymatous cells. The innermost layer of the mesocarp is formed by prismatic crystals of calcium oxalate (Figs. 1a-c, e, h; 2a, b, e-g)

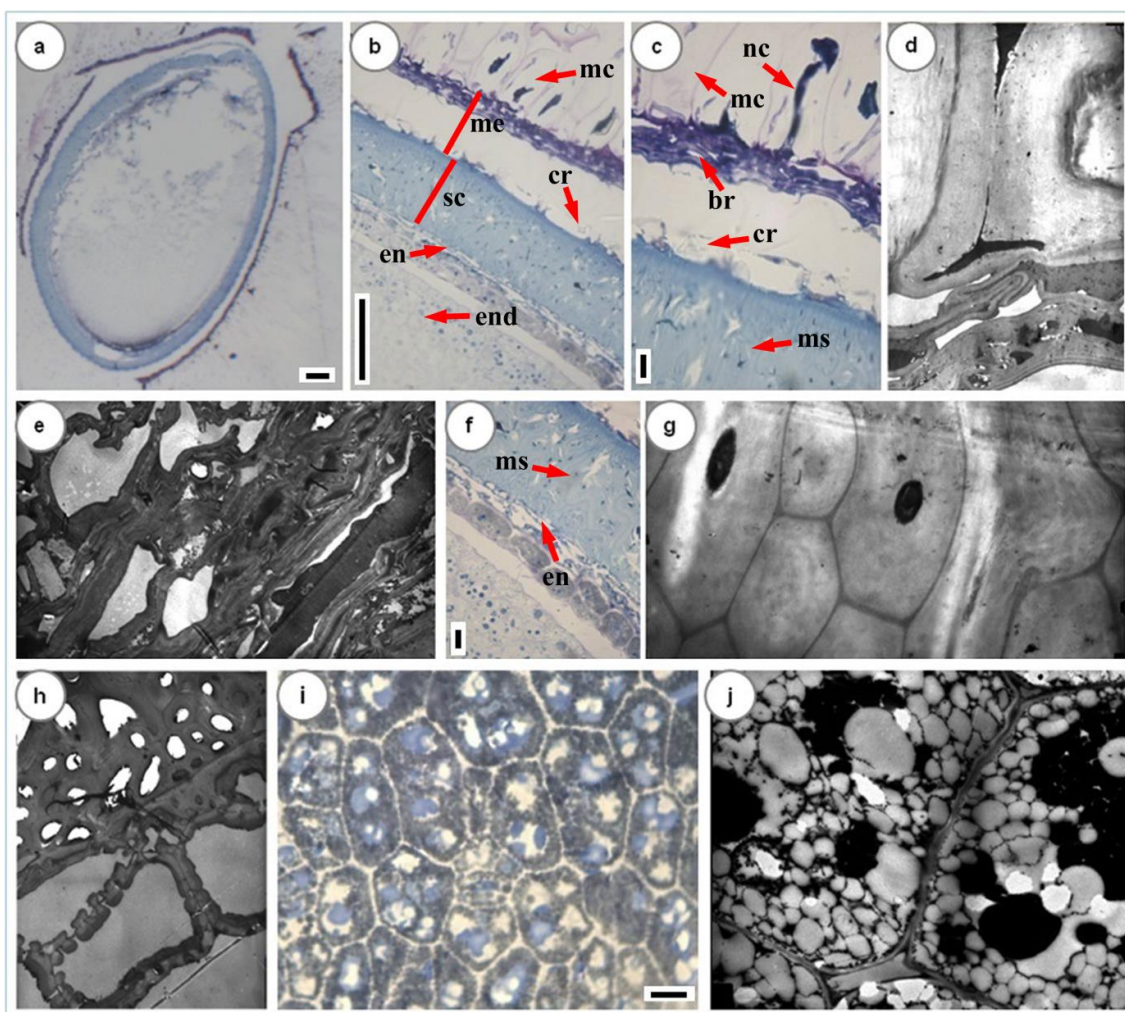


Figure 1: LM and TEM micrographs showing the pericarp ultrastructure of *Salvia napifolia* Jacq. **a.** General appearance of cellular layers forming the mericarp. **b.** General appearance of cellular layers in the pericarp and the endosperm (arrows). **c.** Mucilaginous and non-mucilaginous cells in the exocarp, brachysclereids in the outermost layer of the mesocarp, large crystals in the innermost layer of the mesocarp, and macrosclereids in the sclerenchyma region (arrows). **d.** Mucilaginous cells in the exocarp. **e.** Sclerenchymatous cells in the outermost layer of the mesocarp. **f.** Macrosclereids in the sclerenchyma region and endocarp cells (arrows). **g.** Macrosclereids in the sclerenchyma region. **h.** Bordered pits in the wall of sclerenchymatous cells in the mesocarp. **i-j.** Endosperm cells filled with nutrients. *Abbreviations:* br = brachysclereid, cr = crystal, en = endocarp, end = endosperm, mc = mucilaginous cells, me = mesocarp, ms = macrosclereid, nc = non-mucilaginous cells, sc = sclerenchyma region. *Scale bars for LM micrographs:* a = 100 μm , b, f = 10 μm , c = 50 μm , i = 20 μm .

Table 1: Morphometric data related to the pericarp ultrastructure of the species studied. Numbers refer to minimum-maximum (mean \pm standard deviation). In case of a significant ANOVA, different superscript letters in each column indicate which taxa significantly differ from each other ($p < 0.001$). Taxa sharing identical letters are not significantly different ($P \geq 0.001$).

Species	Pericarp thickness (μm)	Mucilaginous layer thickness (mm)	Mesocarp thickness (μm)	Sclerenchyma region thickness (μm)	Endocarp cell length (μm)	Endocarp cell width (μm)
<i>S. napifolia</i>	100.87-140.44 (123.43a \pm 13.40)	0.19-0.34 (0.25a \pm 0.05)	43.27-70.45 (53.66a \pm 7.61)	50.17-85.70 (67.83a \pm 11.52)	3.08-6.28 (4.81a \pm 1.13)	9.01-18.58 (13.52a \pm 3.07)
<i>S. russellii</i>	70.71-98.28 (81.22b \pm 9.27)	0.14-0.23 (0.18b \pm 0.03)	35.90-48.07 (41.33b \pm 4.10)	38.83-52.09 (45.16b \pm 54)	3.64-5.99 (4.71a \pm 0.81)	7.13-17.32 (12.16a \pm 3.21)

The mesocarp is followed by a sclerenchymatous region mostly composed of vertically arranged and thick-walled macrosclereid cells. In the lumen, there occurs a cavity at the centre or below the centre of the cells. The thickness of the sclerenchyma region varying between 38.83 and 85.70 μm is approximately half of the total pericarp thickness (Figs. 1a-c, f, g; 2a, b, g-i).

Below the sclerenchyma region, there is a single layer of transversely arranged endocarp cells with a length of 3.08-6.28 μm and a width of 7.13-18.58 μm (Figs. 1a, b, f; 2a, b, g, i). The testa (seed coat), enveloping the endosperm and embryo, is composed of 3-5 layers of thick-walled brachysclereids. The endosperm, a nutrient-rich tissue, is composed of parenchymatous cells that are mostly rounded in shape (Figs. 1a, b, i, j; 2a, b, i-k).

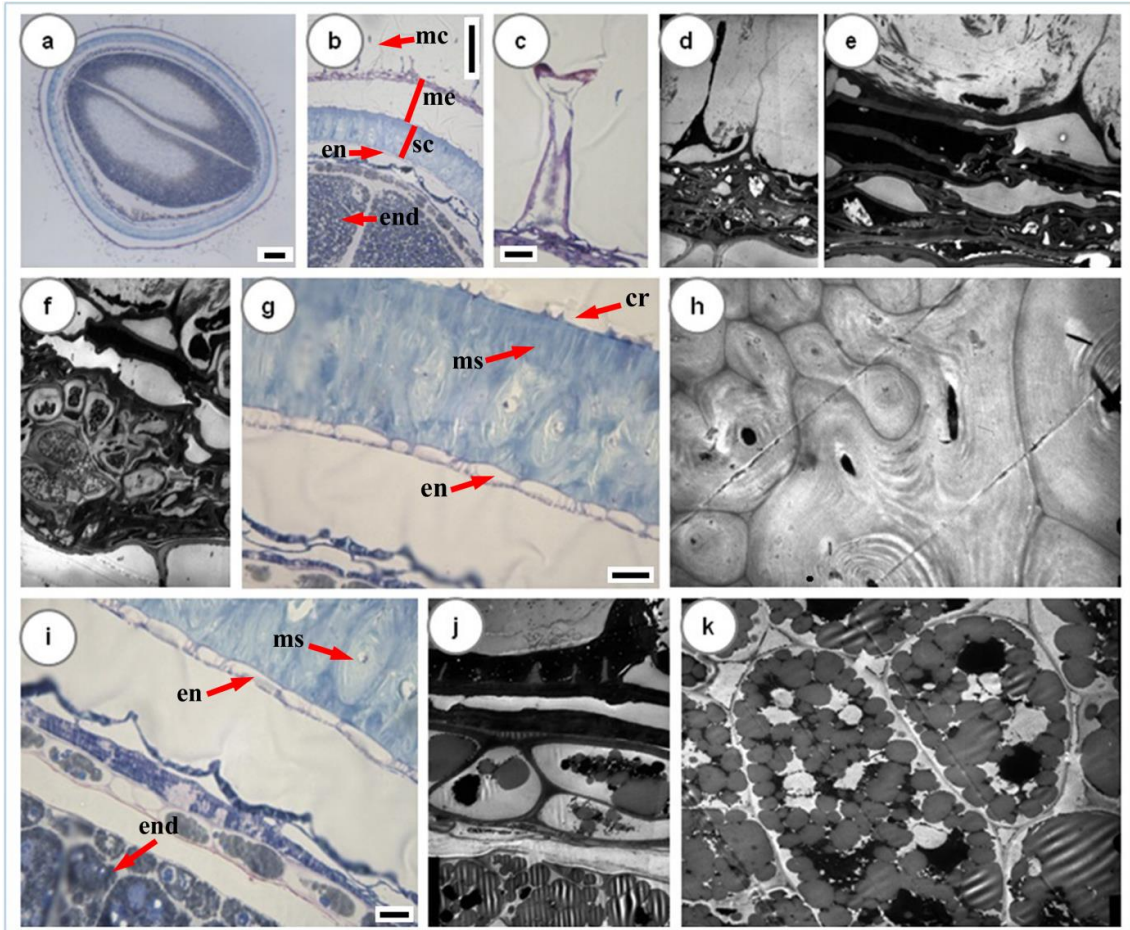


Figure 2: LM and TEM micrographs of the pericarp ultrastructure of *Salvia russellii* Benth. **a.** General appearance of cellular layers forming the mericarp. **b.** General appearance of cellular layers in the pericarp and the endosperm (arrows). **c-d.** Mucilaginous cells in the exocarp. **e-f.** Sclerenchymatous cells in the outermost layer of the mesocarp. **g.** Large crystals in the innermost layer of the mesocarp, macrosclereids in the sclerenchyma region, and endocarp cells (arrows). **h.** Macrosclereids in the sclerenchyma region. **i.** Macrosclereids in the sclerenchyma region, endocarp cells, and the endosperm (arrows). **j-k.** Endosperm cells filled with nutrients. *Abbreviations:* cr = crystal, en = endocarp, end = endosperm, mc = mucilaginous cells, me = mesocarp, ms = macrosclereid, sc = sclerenchyma region. *Scale bars for LM micrographs:* a = 100 μ m, b = 50 μ m, c = 20 μ m, g, i = 10 μ m.

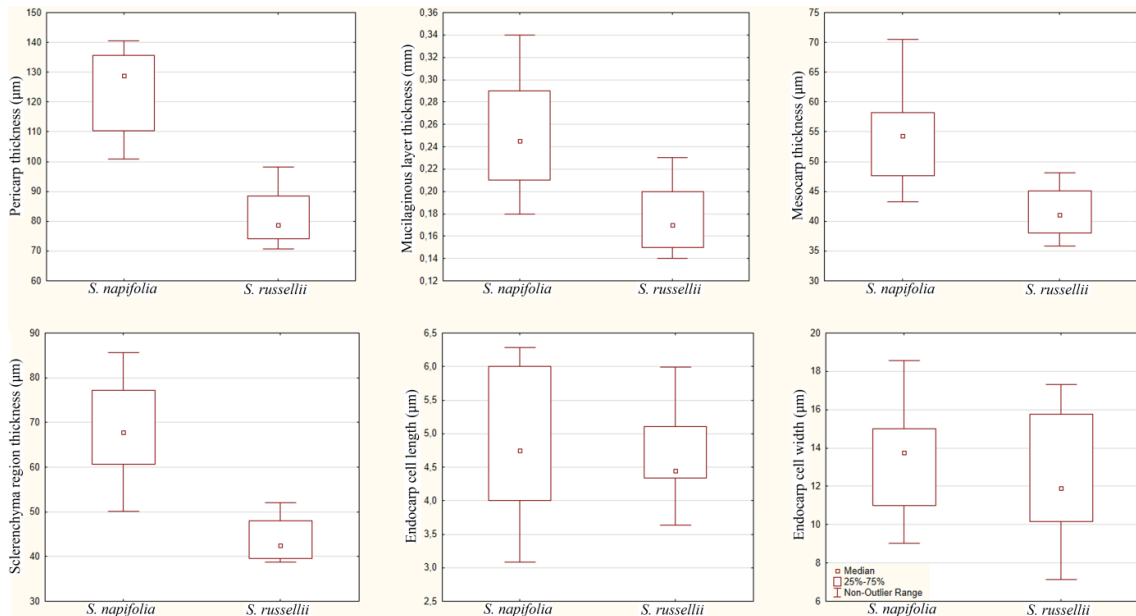


Figure 3: Box-and-whisker plots of quantitative characters of the species studied.

4. Discussion

The pericarp ultrastructure of various genera in the family Lamiaceae has been investigated and the valuable of the pericarp characteristics in the classification of this family has been pointed out by some authors (i.e. Wojciechowska, 1958, 1961a, b, 1966; Makarova, 1967; Ryding, 1992a, b, 1993a, b, 1995, 2001, 2010; Budantsev & Lobova, 1997; Duletić-Laušević & Marin, 1999; Moon & Hong, 2006). Differences in anatomical structure of the mericarps between some genera of the tribe Nepeteae are recognized (Makarova, 1967). The Nepeteae is also divided into three informal generic groups on the basis of a combination of mericarp morphology, pericarp structure, and vegetative and floral features (Budantsev & Lobova, 1997). The groups of *Prunella* L. and *Cleonia* L. plus *Lepechinia* Willd. and *Chaunostoma* Donn. Sm. are separated by differences in pericarp structure from other genera in the Lamiaceae (Ryding, 1995). Mericarp morphological and anatomical characters of *Lycopus* are useful as diagnostic characters at the specific and interspecific levels (Moon & Hong, 2006). Much of the variation in pericarp structure of the tribe Mentheae has been remarked to be strongly correlated to the variations in DNA and gross morphology (Ryding, 2010). In spite of the above-mentioned information on the pericarp structure of the family Lamiaceae and its taxonomic significance, the data on that of the genus *Salvia* are still incomplete. To date, the pericarp ultrastructure of *Salvia* has been rarely studied (Wojciechowska, 1958, 1966; Hedge, 1970; Oran, 1997; Ryding, 2010; Büyükkartal et al., 2011; Hassan & Al. Thobaiti, 2014). Characters of the pericarp ultrastructure, such as the presence of myxocarpy, the thickness of the pericarp as well as the proportions of its individual layers, have been found to be useful for the systematics of *Salvia* (Hedge, 1970). The groups of two-staminate genera, as in *Salvia*, and four-staminate genera of Salviinae, as in *Lepechinia* and *Chaunostoma*, can be separated from each other and from other Lamiaceae genera by differences in pericarp structure (Ryding, 2010). Variations in the pericarp structure have been pointed out to be helpful in the identification of morphologically similar *Salvia* species (Büyükkartal et al., 2011). In this study, the pericarp of *S. napifolia* is observed to be significantly thicker than that of *S. russellii*. According to Duletić-Laušević and Marin (1999), the pericarp thickness in the tribe Nepetoideae of the Lamiaceae usually correlates with dimensions of mericarps. However, *Mentha aquatica* L. is a very thick pericarp (ca. 95 µm) though it has very small mericarps (ca. 0.8 mm x 0.6 mm). Mericarps of *S. napifolia* and *S. russellii* are similar in size (ca. 2.5 x 1.5 mm; Hedge, 1982) but there is an important variation between them in terms of the pericarp thickness.

Myxocarpy is widely observed in the subfamily Nepetoideae (Wagner, 1914; Hedge, 1970; Swarbrick, 1971; Witztum, 1978; Ryding, 1992a, 2001, 2010; Duletić-Laušević & Marin, 1999; Harley et al., 2004). Morphologically allied species have similar mucilage properties (Hedge, 1970). In this study, both species produce mucilage on their mericarps but *S. napifolia* seems to have a somewhat thicker mucilaginous layer than *S. russellii*. The mucilage formation is detected in nearly all of over forty *Salvia* species in Southwest Asia, except for only three species (Hedge, 1970). These

mucilage-producing species can be grouped into four basic types according to the extent of swelling of mucilaginous cells (Ryding, 1992a). A moderately strong mucilaginous reaction is seen in *Elsholtzia blanda* Benth. in the tribe Elsholtzieae but there is no mucilage on the mericarp surface of *Lycopus* taxa (Moon & Hong, 2006). Our results show that a moderately mucilaginous reaction is recognized on the pericarp surface of both species but stronger in *S. napifolia*. According to Oran (1997), the occurrence of mucilage is observed in most of *Salvia* species in Jordan but *S. napifolia* sometimes produces a very small amount or no visible mucilage. According to our detailed study carried out to determine the presence/absence of the mucilage formation, we conclude that Oran's (1997) report is obviously incorrect.

In *Lycopus europaeus* and *Mentha aquatica*, the mesocarp consists of aerenchymatous cells (Ryding, 2010). However, these cells are absent in the mesocarp of the *Salvia* species examined. Two-staminate Salviinae, except a few of the species (i.e. *Salvia splendens* F. Sellow ex Roem. & Schult. and *Perovskia abrotanoides* Kar.), can be distinguished from four-staminate Salviinae and other Mentheae by having large crystals in the innermost cell layer of the mesocarp (Ryding, 2010). Our investigation reveals that the species have large prismatic crystals in the innermost cell layer of the mesocarp. The sclerenchyma region is found in most genera of the Lamiaceae (Ryding, 1995). The presence of such a region in the *Salvia* species disagrees with the situation in some genera, such as *Chelonopsis* Miq., *Renschia* Vatke, and *Scutellaria* L. The Salviinae with four stamens differs from the two-staminate Salviinae (3-9 µm) and other Mentheae (2-12 µm) in having a thicker endocarp (17-40 µm) (Ryding, 2010). In this study, the endocarp of the species is similar in thickness (ca. 3-6 µm).

Though the species studied have similarities in their gross morphology, important differences in morphometric characters, such as the thickness of the pericarp ($p = 0.000$), mesocarp ($p = 0.000$), sclerenchyma region ($p = 0.000$), and mucilaginous layer ($p = 0.000$), are found between them (Tab. 1). They have leaves simple, calyces thick-texture, upper lip not concave and not 2-sulcate in fruit, upper lip of corolla more or less straight, corolla tube annulate and not squamulate, and stamens type C (staminal connectives longer than the filaments, lower theca subulate and stamens not articulating). Although these species agree in having thick endocarp and crystals in the sclerenchyma region, *S. napifolia* are obviously distinct from *S. russellii* based on other pericarp characteristics, the particularly thicker pericarp, mesocarp, and sclerenchyma region.

In summary, there have been no detailed investigations on the pericarp of *S. napifolia* and *S. russellii* even though ultrastructural studies can be used as a powerful tool in delimitation of species. Thus, this study is the first comprehensive report on their pericarp ultrastructure. We hope that the present study further puts emphasis on the value of the pericarp ultrastructure for plant systematics and will encourage future investigations of *Salvia* species. In view of a lack of knowledge of pericarp ultrastructure and differentiation in *Salvia*, this is definitely a research field requiring further study.

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