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Foliar micromorphology of the family *Lythraceae* in Iran with special emphasis on the genera *Lythrum*, *Ammannia*, and *Rotala*

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Abstract

In the present survey, leaf micromorphological characters of 14 species belonging to three genera viz. *Lythrum*, *Ammannia*, and *Rotala* (*Lythraceae*) were analyzed using scanning electron microscopy (SEM). The results revealed two types of trichomes, three types of the papilla, nine types and five sub-types of epicuticular wax ornamentation, three types of anticlinal wall and outer periclinal layer, four types of outer stomatal rim/peristomatal rim pattern on both sides of the leaf surfaces. These characteristics are quite useful from taxonomic point of view. Based on the micromorphological characteristics of the leaves, an identification key is also provided for the studied species. Numerical analyses (unweighted pair-group method with arithmetic averages and principal components analysis) was used to find out the similarities and micromorphological correlations among the studied taxa. The present study revealed the alliance of eight *Lythrum* species, while some traits overlap between *Ammannia*, *Rotala*, and three *Lythrum*. The results also showed that, numerical analyses of leaf micmorphological traits is unable to determine the delimitation of the studied genera.

Keywords: Epicuticular wax, epidermis, multivariate analysis, scanning electron microscopy, taxonomy

مطالعه ریزریخت‌شناسی برگ تیره گل‌حناییان در ایران با تاکید بر جنس‌های *Lythrum*، *Ammannia* و *Rotala**

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خلاصه

در بررسی حاضر، ویژگی‌های ریزریخت‌شناسی برگ ۱۴ گونه از سه جنس *Lythrum*، *Ammannia* و *Rotala* متعلق به گل‌حناییان (*Lythraceae*) با استفاده از میکروسکوپ الکترونی روبشی (SEM) مورد تجزیه و تحلیل قرار گرفت. نتایج، دو نوع کرک، سه نوع پاپیل، نه تیپ و پنج زیرتیپ از تزیینات موم روی کوتیکولی، سه نوع دیواره آنتی‌کلینال و لایه بیرونی پری‌کلینال، چهار نوع لبه بیرونی روزنه/ الگوی لبه درونی در دو طرف سطح برگ را آشکار ساخت. این صفات برای شناسایی جنس‌ها و گونه‌های تیره مذکور مهم و دارای اهمیت تاکسونومیک هستند. برای یافتن شباهت‌ها و همبستگی‌های ریزریخت‌شناسی بین آرایه‌ها، از آنالیز عددی (روش جفت-گروه وزن‌دهی نشده با میانگین‌های حسابی و تحلیل مؤلفه‌های اصلی) استفاده شد. یافته‌های بررسی حاضر، ارتباط و اتحاد هشت گونه *Lythrum* را نشان می‌دهد، در حالی که برخی از صفات بین گونه‌های *Ammannia*، *Rotala* و سه گونه *Lythrum* هم‌پوشانی دارند. نتایج این بررسی همچنین نشان داد که تجزیه و تحلیل عددی صفات ریزریخت‌شناسی برگ قادر به تعیین محدوده سه جنس مورد مطالعه نمی‌باشد.

واژه‌های کلیدی: آنالیز چندمتغیره، اپیدرم، تاکسونومی، موم روی کوتیکولی، میکروسکوپ الکترونی

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Introduction

Lythraceae comprises ca. 31 genera and 620–650 species, mostly herbs, shrubs, and trees (Graham *et al*. 2005, Qin *et al*. 2007, Cuihua *et al*. 2019), classified in the order *Myrtales*, forming a monophyletic group with *Geraniales* (APG IV 2016). It comprises five genera and 28 species in the area covered by Flora Iranica (Polatschek & Rechinger 1968), four genera (*Lawsonia* L., *Lythrum* L., *Ammannia* L., and *Rotala* L.), and 12 species in the Flora of Iran (Yousef Naanaie 2010). They are mainly aquatic and moisture-loving species (except *La. inermis* L. which is cultivated in southern Iran), that occur in N, S, and W Iran (Polatschek & Rechinger 1968, Yousef Naanaie 2010). Among them, *Lythrum* includes 38 herbaceous annual and perennial species (Torres & Puntieri 2015); distributed world-widely, mainly found growing in temperate regions of Europe, Asia, and North America (Graham 2007). Polatschek & Rechinger (1968) reported 30 species of the genus in the Flora Iranica, of which seven grow widely in Iran. *Lythrum* is identified by its long cylindrical hypanthium (Polatschek & Rechinger 1968), and capsule with two dehiscence valves (Afanas'ev 1949), whereas, *Ammannia* is a genus with about 80–95 species of aquatic or marsh-inhabiting herbs distributed in temperate, shady, and humid areas of tropical and rainy zones, especially in marshes, rivers, seas, and roadsides at 1500 m (Graham 1985, Immelman 1991, Cook 1996, Graham *et al.* 2011, Graham & Gandhi 2013, Naqinezhad & Naseri Larijani, 2017).

The genus is identified by its opposite, sessile leaves (Polatschek & Rechinger 1968), flowers arranged in three (sometimes one) to many flowers in axillary cymes; calyx with four teeth and four appendages (Afanas'ev 1949). *Ammannia* consists of four representatives in the Flora Iranica and the Flora of Iran (Polatschek & Rechinger 1968, Yousef Naanaie 2010), and to this Naqinezhad & Naseri Larijani (2017) added *A. coccinea* Rottb. Among them, *A. baccifera* L. and *A. verticillata* (Ard.) Lam. are the most common in Iran. *Rotala* is an aquatic or amphibious, tropical, and subtropical genus with 44 species and considerable phenotypic plasticity (Cook 1979, Mabberley 2005). A few species of *Rotala* are cultivated and the rest are sources of timber, natural dyes and have medicinal applications (Egolf & Andrick 1978). Polatschek & Rechinger (1968) reported two species of *Rotala* in the Flora Iranica, while Yousef Naanaie (2010) reported *R. indica* (Willd.) Koehne. for the Flora of Iran as a weed of rice fields. The genus is distinguished by its opposite leaves, leathery capsule with transverse narrowly striate valves (Afanas'ev 1949, Polatschek 1968, Yousef Naanaie 2010). Leaf structure characters have been extensively used to discriminate different taxa (Siddiqi *et al.* 1991, Carpenter 2005, Shaheen *et al.* 2009, Ghahremaninejad *et al.* 2012). The most prominent studies conducted on different genera of the family *Lythraceae* are as follows: stem and leaf anatomy (Metcalfe & Chalk 1950, Baas 1986, Rao *et al.* 1987, Little *et al*. 2004, Kshirsagar & Vaikos 2012), palynology (Graham *et al*. 1987, Perveen & Qaiser 2005), phytochemistry (Manayi *et al*. 2014), karyology (Graham & Cavalcanti 2001), genetic diversity (Noormohammadi *et al*. 2010), and phylogeny (Conti *et al.* 1996, 1997, Sytsma *et al.* 2004, Graham *et al.* 2005, 2006, Narzary *et al*. 2016, Cuihua *et al*. 2019). However, micro-morphological data of leaves were poorly known in the family. Concerning the importance of leaf structure characteristics in differentiating different species and genera, the aims of this study were to investigate the micromorphological characteristics of leaves of the *Lythraceae* family in Iran and to determine how these traits support the generic and specific relationships.

Materials and Methods

In the present survey, 13 Iranian species plus *L. portula* (L.) D.A. Webb. from Morocco, belonging to three genera of *Lythraceae* were used for leaf micromorphological analysis. Dried specimens were used from Guilan (GUH), and Tehran (TUH) universities herbaria, as well as herbarium of the Research Institute of Forests and Rangelands (TARI) of Tehran, Iran (Table 1). Flora Iranica (Polatschek & Rechinger 1968), Flora of Iran (Yousef Naanaie 2010), and Flora of Turkey (Davis 1972,

1988) were the main primary references for identification. The terminology of leaf micromorphological characteristics followed those of Barthlott *et al*. (1998), Wilkinson (1979), Ergen Akin *et al*. (2013), and Kumar & Murugan (2015). The leaf surfaces of all samples were examined and evaluated prior to stereomicroscopy with electron microscope. Leaves of each species were examined from 2–5 populations of each (except for two species) (Table 1). For scanning electron microscopy (SEM), fully developed leaves were picked out and washed in water detergent for about 20 min. (in a water bath at 45 °C), and then stored for drying. Part of the dried leaves were mounted on SEM stubs using silver double-sided adhesive tape and finally covered by gold in a sputter coater. Scanning electron microscopy was performed using WEGA-TESCAN scanning electron microscopy, operating at 10 KV, at Razi Metallurgical Research Center (Tehran, Iran).

Table 1. Plant samples used in the current study along with related data

Taxon	Locality & herbarium No.
Lythrum	
L. silenoides	Lorestan prov.: Khorramabad, Jam , Divan, 1000 m, 30.5.1999, Veis Karami, 24113 & 24113-1 (TUH)
L. virgatum	W Azerbaijan prov.: Between Salmas and Ghoshchi, Khan-takhti, 1400 m, 27.6.1994, Ghahraman & Mozaffarian, 17444 (TUH)
	W Azerbaijan prov.: Urmia, Ghoshchi pass, 1970 m, Tarighi & Kazempour, 17287 (TUH)
L. salicaria	Mazandaran prov.: 40 km to Amol, Andvar village, 2500 m, 6.6.2000, Attar, Okhovvat & Mehdigoli, 26355 (TUH)
	E Azerbaijan prov.: Tabriz, unknown collector, 1949 (TUH) Kurdistan prov.: Between Kamyaran and Sanandaj, Attar & Deljou, 14228 (TUH) Gilan prov.: Rasht, around of Pir-bazar, Meshkati 5779 (GUH) Gilan prov.: Rasht, Lakanshahr, Rahmati 5780 (GUH)
L. junceum	Kohgiluyeh and Boyer-Ahmad prov.: Dogonbadan, Cheshm-e Belgheys, 1400 m, 24.4.1997, Ghahraman, Attar & Sheykhi, 20328 (TUH) Morocco: Azilal (Beni-Mellal), Mittlerer Atlas, Cascades douzoud, 960–1060 m, Podlech, 47700 (TUH)
L. hyssopifolia	Gilan prov.: Langarod, Chamkhaleh, -25 m, 25.5.2000, Naqinezhad, 21459 (TUH) Gilan prov.: Anzali, Mozaffarian & Maassoumi 6901 (TUH) Sistan and Baluchestan prov.: Sarbaz, Ghahraman & Mozaffarian, 14068 (TUH) Khuzestan province: 12.4.1992, Attar & Ghahraman, 19023 (TUH)
L. thymifolia	Gilan prov.: Anzali, -20 m, Mozaffarian & Maassoumi, 6901 (TUH)
L. portula	Morocco: Tetouan, 9 km to SW Souk-e Araba-Ayacha, 120 m, 5.5.1987, Podlech, 43652 (MSB) Spain: Murcia, 25383(TUH)
L. thesioides	Fars prov.: 6 km to Jahrom, between Hood and Kore village, 800 m, Asadi & Akhani, 61867 (TARI)
	Razavi Khorassan prov.: East of Torbat-e Jam, between Golbanoo mine and Sharshari polis station, 29.6.1988, unknown herbarium No. (TARI)
L. tribracteatum	Fars prov.: 30 km to S Jahrom, near Heram village, 800 m, 28.11.1987, Assadi & Akhani, 61852 (TARI) Fars prov.: 60 km to S Jahrom, near Heram village, 800 m, 29.11.1987, Assadi & Akhani, 61864 (TARI)
Ammannia	
A. verticillata	Lorestan prov.: Khorramabad, Cham-divan, 1000 m, 6.8.1998, Veis Karami, 24111 (TUH)
A. auriculata	Lorestan prov.: Khorramabad, Cham-divan, 1000 m, 1.8.1999, Veis Karami, 24108 (TUH)
A. multiflora	Qazvin prov.: Alamut, Moallem Kela-ye Shahrak, 11.8.1991, unknown collector, 9966 (TUH) Gilan prov.: Someh Sara, district Hendekhal-e, Nokhal-e Akbari village, -21 m, 21.8.2015, Ashori, 8553 (GUH) Lorestan prov.: Khorramabad, Cham-Divan, 27.9.1999, Veis Karami, 24110 (TUH)
A. baccifera	Gilan prov.: Rudbar, Rahimabad, Resht-e Rud, 211 m, 21.8.2015, Ashori, 8554 (GUH) Lorestan prov.: Khorramabad, Cham-Divan, 1.8.1999, Veis Karami, 24109 (TUH) Khuzestan prov.: road from Ahvaz to Andimeshk, between El Rud, 29.10.1993, Ghahraman, Mozaffarian & Sheikhol Eslam, 17633 (TUH)
Rotala	
R. indica	Gilan prov.: Siahkal, Kharrarud, Sels village, 55 m, 16.9.2015, Ashori, 8555 (GUH) Gilan prov.: 4 km from Asalem towards Khalkhal, 20.9.1989, Zehzad & Taheri, 2013 (TARI) Gilan prov.: 2 km of E Rezvandeh, unknown collector, 14879 (TARI)

Results

The information obtained from the morphological study could not reveal the structural details of the leaf surface (except for the presence or absence of hairs). Therefore, only the electron microscopic results are discussed here.

Based on the current scanning electron microscopy, two types of leaf trichomes were identified (Fig. 1 A-C, Table 2): Type I: Short and long, flat, acute, verrucate, appressed to semi-erect hairs: e.g., leaf surfaces and midrib of *L. salicaria* L. (Fig. 1 A-B). Type II: Short, flat, acute, smooth, and appressed hairs: e.g., on adaxial surface of *L. tribracteatum* Salzm. ex Sprengel. (Fig. 1 C). In addition, in some species, papillae were observed in both leaf surfaces.

In general, three types of papillae were distinguished (Fig. 1 D-I, Table 2): Type I: Triangular, flat, acute, with striate epicuticular folding in *L. silenoides* Boiss. & Nöe (Adaxial surface, Fig. 1 D), *L. junceum* Banks & Sol. (Adaxial surface and leaf margin, Fig.1 E), and *L. thymifolia* L. (adaxial surface, Fig. 1 F). Type II: Cylindrical, obtuse with striate epicuticular folding in *L. thesioides* M.B. (leaf margin and adaxial surface, Fig. 1 G). Type III: Lanceolate-oblong, flat, obtuse with striate epicuticular folding viz. *A. verticillata* (adaxial surface Fig. 1 H), *A. multiflora* Roxb. (leaf margin and on adaxial surface Fig. 1 I), and *A. baccifera* (leaf margin and adaxial surface) (Table 2).

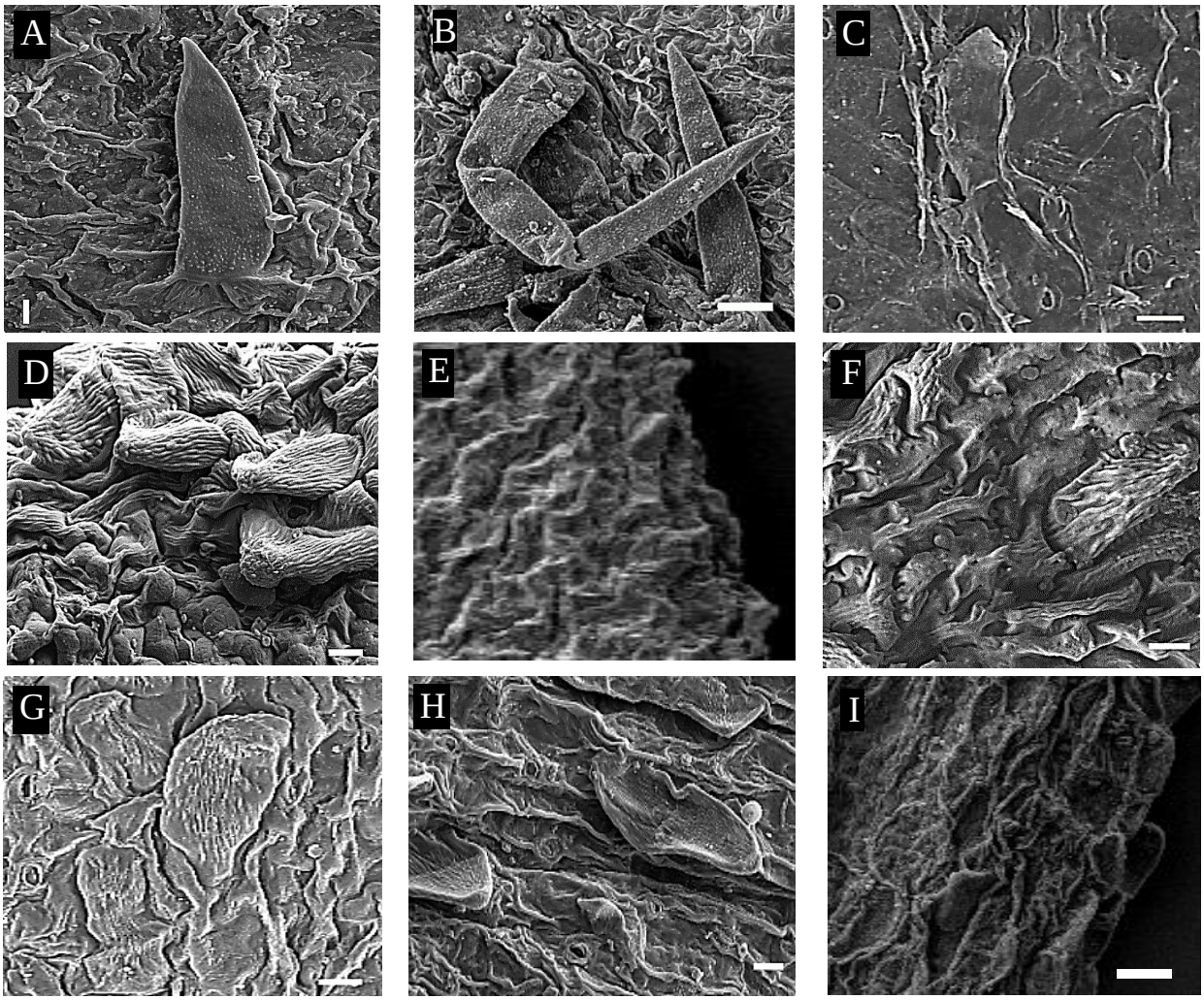

Fig. 1. SEM micrographs showing trichomes and papillae: A & B. *L. salicaria* (adaxial surface A, abaxial surface B), C. *L. tribracteatum* (adaxial surface), D. *L. silenoides* (adaxial surface), E. *L. junceum* (adaxial surface), F. *L. thymifolia* (adaxial surface), G. *L. thesioides* (adaxial surface), H. *A. verticillata* (adaxial surface), I. *A. multiflora* (adaxial surface) (Bars = 50 µm).

Table 2. Trichomes and papillae characteristics of the studied species of *Lythrum*, *Ammannia*, and *Rotala*

Taxon	Papilla position on daxial/abaxial surface	Papilla type in adaxial surface	Papilla ornamentation
L. silenoides	Often in margin	Type I	Striate
L. virgatum	-/-	-	-
L. salicaria	-/-	-	-
L. junceum	-/-	Type I	-
L. hyssopifolia	-/-	-	-
L. thymifolia	Leaf surface	Type I	Striate
L. portula	-/-	-	-
L. thesioides	Often in margin	Type II	Verrucate
L. tribracteatum	-/-	-	-
A. verticillata	Leaf surface	Type III	Striate
A. auriculata	-/-	-	-
A. multiflora	In margin	Type III	Striate
A. baccifera	Often in margin	Type III	Striate
R. indica	-/-	-	-
R. indica	-/-	-	-

Taxon	Presence or absence of trichome on adaxial/abaxial surface	Hair type in adaxial/abaxial surface	Hair position in relation to epidermal cell in adaxial/abaxial surface
L. silenoides	-/-	-/-	-/-
L. virgatum	-/-	-/-	-/-
L. salicaria	+/+	Short and long, flat and verrucate/short and long, flat and verrucate	Appressed to semi-erect/ appressed to semi-erect
L. junceum	-/-	-/-	-/-
L. hyssopifolia	-/-	-/-	-/-
L. thymifolia	-/-	-/-	-/-
L. portula	-/-	-/-	-/-
L. thesioides	-/-	-/-	-/-
L. tribracteatum	+/-	Short, flat, acute, smooth	Appressed hairs
A. verticillata	-/-	-/-	-/-
A. auriculata	-/-	-/-	-/-
A. multiflora	-/-	-/-	-/-
A. baccifera	-/-	-/-	-/-
R. indica	-/-	-/-	-/-

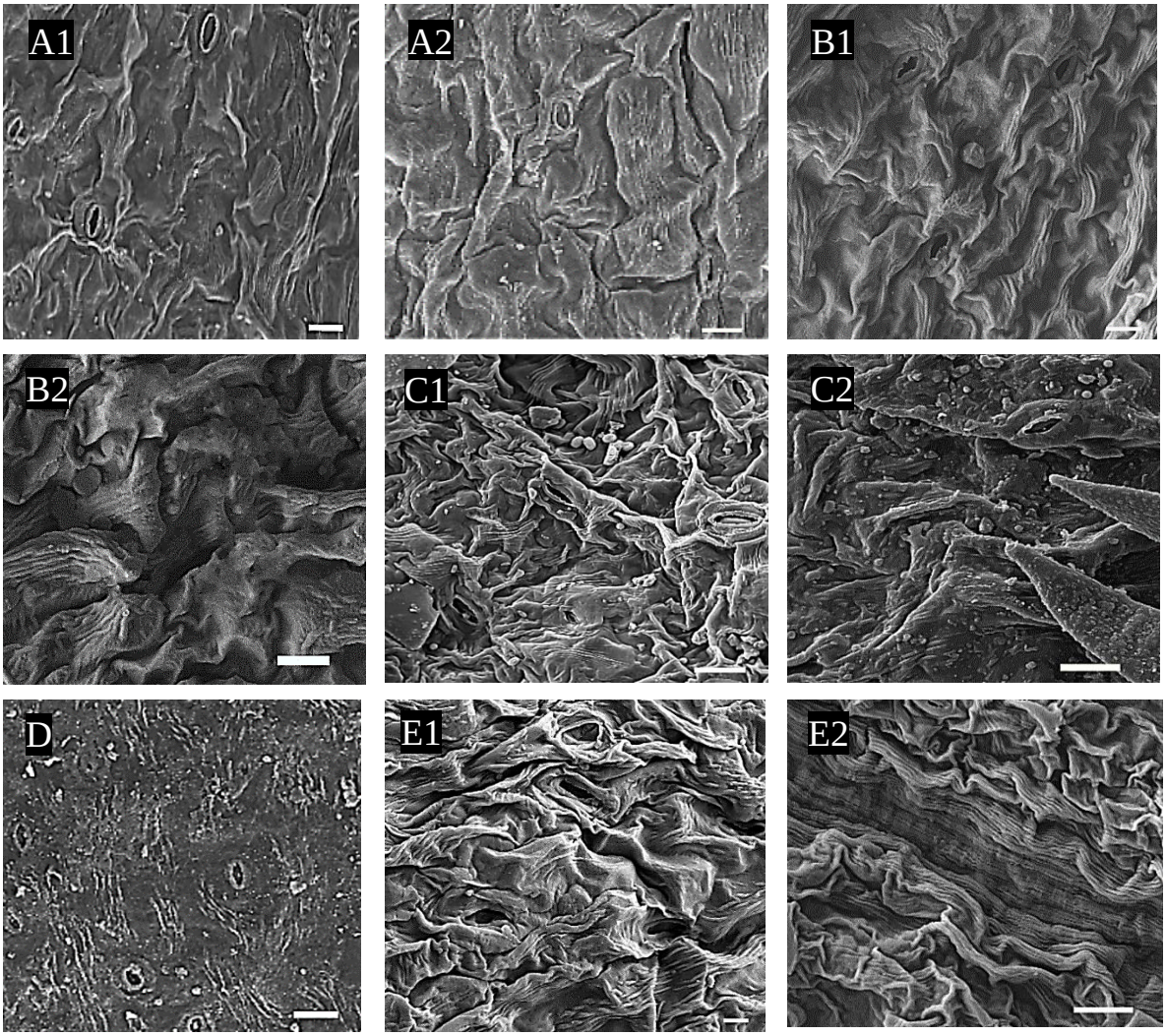

Fig. 2. SEM micrographs showing wax ornamentation types: A1-A2. *L. thesioides* (adaxial surface A1, abaxial surface A2), *L. thymifolia* (adaxial surface B1, abaxial surface B2), *L. salicaria* (adaxial C1, abaxial C2), *L. tribracteatum* (adaxial surface D), *L. silenoides* (adaxial surface E1), *L. hyssopifolia* (adaxial surface E2) (Bars = 50 µm).

Based on SEM observations, epicuticular wax type of the studied species composed of film (smooth layer and crust), and crystalloids (granule, irregular, scale, and rod like platelets). Wax is mainly syntopism (including crust+ granule; crust+granule+platelets; smooth layer+granule patterns) except in *L. virgatum* L. (having only irregular platelets). The current results also showed nine types and five sub-types of epicuticular wax. Ornamentations were identified as follows (Fig. 2, Table 3): Type I: Crust/striate epicuticular folding: This type includes two sub-types: Sub-type I: Short striate epicuticular folding with scattered granule e.g., *L. thesioides*, *L. thymifolia*, and *L. salicaria* (both surfaces) (Fig. 2 A1-C2). Sub-type II: Short striate epicuticular folding, with granule and scale like platelets e.g., in *L. tribracteatum* (both surfaces) (Fig. 2 D). Type II: Smooth layer with long and short striate epicuticular folding and granule e.g., *L. hyssopifolia* L. and *L. silenoides* (abaxial surface) (Fig. 2 E1-E2). Type III: Irregular platelets e.g., *L. virgatum* (both surfaces) (Fig. 3 A1-A2). Type IV: Tuberculate and granule of the leaf in *L. junceum* (both surfaces) (Fig. 3 B1-B2) and adaxial surface of the leaf in *L. hyssopifolia* (Fig. 3 C), and *L. silenoides* (Fig. 3 D). Type V: Crust, irregular, and rod shape platelets e.g., *R. indica* (abaxial surface) (Fig. 3 E). Type VI: Crust and granule e.g., *A. auriculata* Willd. (Abaxial surface) (Fig. 3 F) and *A. verticillata* (adaxial surface) (Fig. 3 G). Type VII: Alveolate, with striate epicuticular folding and granule e.g., *R. indica* (adaxial surface) (Fig. 4 A). Type VIII: Reticulate-Alveolate-Tuberculate. This type includes three sub-types: Sub-type

I: Without folding and granule e.g., *A. multiflora* (both surfaces) (Fig. 4 B1-B2). Sub-type II: With elevated muri, with short striate epicuticular folding and granule, e.g., *L. portula* (both surfaces) (Fig. 4 C1-C2). Sub-type III: Elevated muri, without short striate epicuticular folding, granule, and in some species with papilla e.g., *A. baccifera* (both surfaces) (Fig. 4 D1-D2). Type IX: Reticulate, with short striate epicuticular folding and granule, e.g., *A. verticillata* (abaxial surface) (Fig. 4 E) and *A. auriculata* (adaxial surface) (Fig. 4 F).

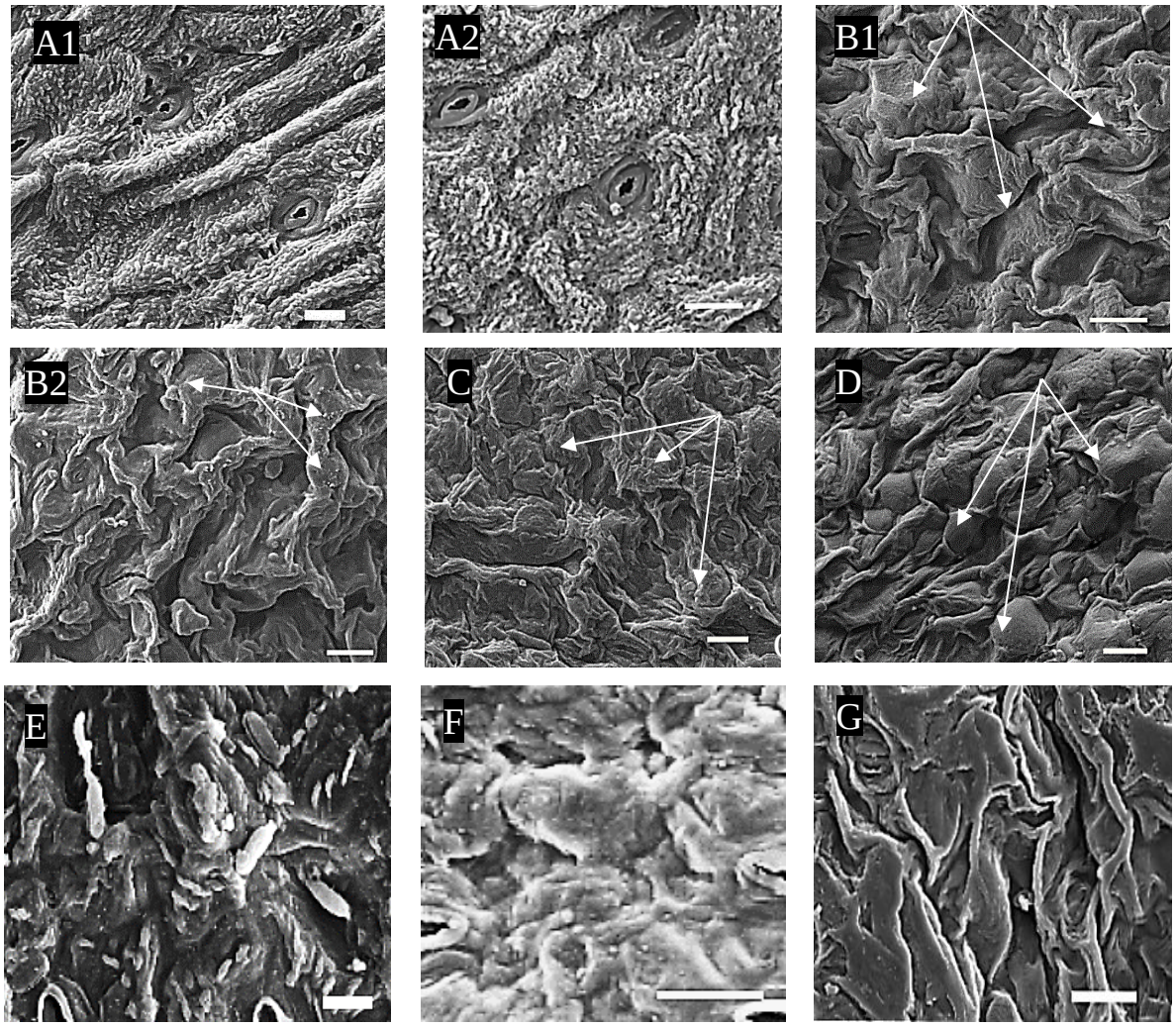

Fig. 3. SEM micrographs showing wax ornamentation types: A1-A2. *L. virgatum* (adaxial surface A1, abaxial surface A2), B1-B2. *L. junceum* (adaxial surface B1, abaxial surface B2), C. *L. hyssopifolia* (adaxial surface), D. *L. silenoides* (adaxial surface), E. *R. indica* (abaxial surface), F. *A. auriculata* (abaxial surface), G. *A. verticillata* (adaxial surface). Arrows indicate tubercles (Bars = 50 µm).

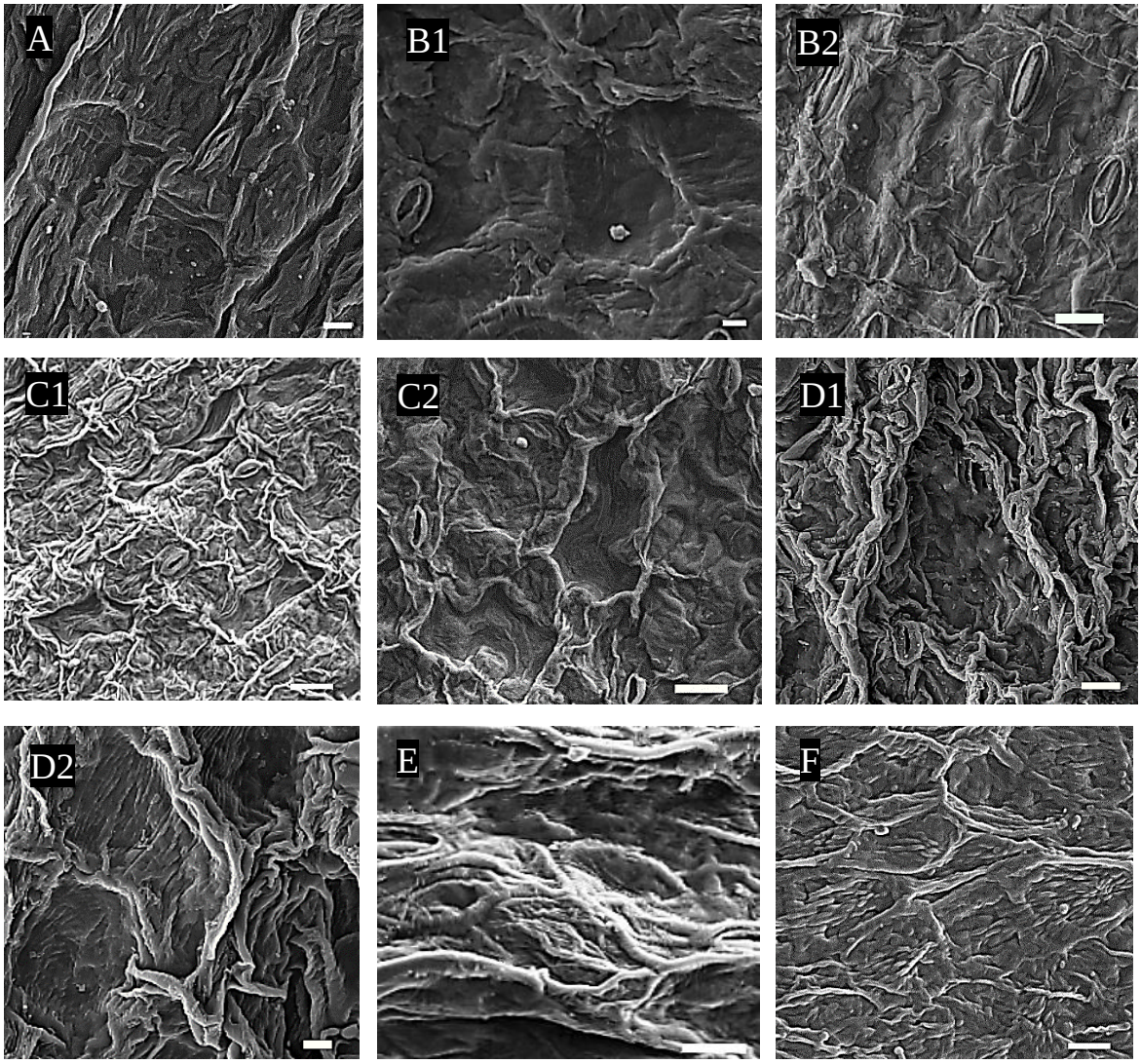

Fig. 4. SEM micrographs showing wax ornamentation types: A. *R. indica* (adaxial surface), B1-B2. *A. multiflora* (adaxial surface B1, abaxial surface B2), C1-C2. *L. portula* (adaxial surface C1, abaxial surface C2), D1-D2. *A. baccifera* (adaxial surface D1, abaxial surface D2), E. *A. verticillata* (abaxial surface), F. *A. auriculata* (adaxial surface) (Bars = 50 µm).

Anticlinal wall and outer periclinal wall layer vary from raised to oblate and depressed on leaf surfaces of the examined species. Anticlinal wall surface is raised in seven species viz. *L. salicaria*, *L. portula*, *A. verticillata*, *A. multiflora* and *A. baccifera* (both surfaces), *L. junceum* (adaxial surface), and *A. auriculata* (abaxial surface). It is depressed in eight species viz. *L. virgatum*, *L. thymifolia*, *L. thesioides*, *L. junceum*, and *R. indica* (both surfaces), *L. silenoides*, *L. hyssopifolia* and *A. auriculata* (adaxial surface), and oblate of *L. tribracteatum* (both surfaces). In addition, anticlinal wall of the studied species is smooth in *L. tribracteatum*, *L. virgatum* (both surfaces) and *A. auriculata* (abaxial surface), *A. multiflora* (adaxial surface), almost smooth in *R. indica* (both surfaces) and *A. verticillata* (adaxial surface), undulate in *L. junceum*, *L. thymifolia*, *A. baccifera* (both surfaces), *L. silenoides*, *L. hyssopifolia* and *A. auriculata* (adaxial surface), *A. verticillata* (abaxial surface), and almost undulate in *L. thesioides*, *L. salicaria*, *L. portula* (both surfaces), and *A. multiflora* (abaxial surface).

Table 3. Epicuticular wax ornamentation of the studied species of *Lythrum*, *Ammannia*, and *Rotala*

Taxon	Ornamentation type and sub-type		Wax type
L. thesioides (both surfaces) *L. thymifolia* (both surfaces) *L. salicaria* (both surfaces) *L. tribracteatum* (both surfaces)	**Type I** Crust/striate epicuticular folding	**Sub-type I** Short striate epicuticular folding/scattered granule **Sub-type II** Short striate epicuticular folding/granule, scale like platelets	Film+crystalloid
L. hyssopifolia (abaxial surface) *L. silenoides* (abaxial surface)	**Type II** Smooth layer with long and short striate epicuticular folding and granule		Film+crystalloid
L. virgatum (both surfaces)	**Type III** Irregular platelets		Crystalloid
L. junceum (both surfaces) *L. hyssopifolia* (adaxial surface) *L. silenoides* (adaxial surface)	**Type IV** Tuberculate and granule		Film+crystalloid
R. indica (abaxial surface)	**Type V** Crust/platelets/rodlets		Film+crystalloid
A. auriculata (abaxial surface) *A. verticillata* (adaxial surface)	**Type VI** Crus/granule		Film+crystalloid
R. indica (adaxial surface)	**Type VII** Alveolate with striate epicuticular folding, granule		Film+crystalloid
A. multiflora (both surfaces) *L. portula* (both surfaces) *A. baccifera* (both surfaces)	**Type VIII** Reticulate-alveolate-tuberculate	**Sub-type I** Reticulate-alveolate-tuberculate **Sub-type II** Elevated muri, with short striate epicuticular folding and granule **Sub-type III** Elevated muri, without short striate epicuticular folding and granule	Film+crystalloid
A. verticillata (abaxial surface) *A. auriculata* (adaxial surface)	**Type IX** Reticulate	Reticulate with short striate epicuticular folding and granule	Film+crystalloid

Outer periclinal layer changes from raised to oblate and depressed in the studied species. It is raised in eight species viz. *L. virgatum*, *L. thymifolia*, *L. thesioides*, *L. junceum*, and *R. indica* (both surfaces), *L. silenoides* and *L. hyssopifolia* (adaxial surface). Depressed outer periclinal layer was identified in five species representatives viz. *A. verticillata* (both surfaces), *L. portula*, *A. multiflora*, *A. auriculata*, and *A. baccifera* (both surfaces). Two species displayed oblate outer periclinal layer viz. *L. tribracteatum* and *L. salicaria* (both surfaces). Stomata quantitative characters (stomata and stomata aperture length and width) were measured. Maximum stomata length (12.51–15.27 µm) and width (7.79–10.86 µm), stomata aperture length (10.31–11.96 µm), and width (5.27–6.14 µm) were identified in *L. tribracteatum* while, minimum stomata length (5.55–6.84 µm) and width (2.57–4.16 µm), stomata aperture length (3.96–4.56 µm), and width (1.28–2.08 µm) were recognized in *L. junceum* (Table 4).

Table 4. Stoma characters of the studied species of *Lythrum*, *Ammannia*, and *Rotala*

Taxon	Stomata length (µm)	Stomata width (µm)	Stomata aperture length (µm)	Stomata aperture width (µm)
L. silenoides	9.22–14.1	4.76–7.83	8.1–12.2	1.09–3.54
L. virgatum	5.75–11.8	3.17–6.24	2.08–5.75	1.48–3.57
L. salicaria	8.43–12.59	4.16–6.34	4.46–9.12	2.38–8.53
L. junceum	5.55–6.84	2.57–4.16	3.96–4.56	1.28–2.08
L. hyssopifolia	6.05–12.59	2.87–6.05	2.38–7.83	0.49–1.28
L. thymifolia	5.25–14.18	2.87–8.23	3.57–10.01	1.09–6.54
L. portula	8.92–14.68	3.67–6.84	5.25–11.3	1.28–3.37
L. thesioides	12.77–15.76	7.25–9.46	9.93–12.77	4.41–7.25
L. tribracteatum	12.51–15.27	7.79–10.86	10.31–11.96	5.27–6.14
A. verticillata	7.44–17.85	6.54–10.21	5.75–13.88	3.67–5.55
A. auriculata	6.54–16.67	3.96–8.63	5.55–13.09	2.38–4.76
A. multiflora	11.3–22.3	4.96–9.6	9.42–20.73	3.37–8.43
A. baccifera	7.04–15.4	3.96–9.42	5.25–11.5	1.88–5.74
R. indica	10.56–16.69	7.72–8.35	7.72–10.03	5.67–6.15

The current micromorphological analysis revealed four types of outer stomatal rim/peristomatal rim pattern on adaxial/abaxial surface of the leaves (Table 5): Type I: Overlapping/overlapping in *L. silenoides* (Fig. 5 A). Type II: Overlapping/overlapping-stout in *L. virgatum* (Fig. 5 B1-B2), *L. salicaria* (Fig. 5 C1-C2), *L. junceum* (Fig. 5 D1-D2), *L. hyssopifolia* (Fig. 5 E1-E2), *L. thymifolia* (Fig. 5 F), *A. baccifera* (Fig. 5 G1-G2), and *A. verticillata* (adaxial surface). Type III: Raised overlapping/ overlapping-stout in *L. thesioides* (Fig. 5 H1-H2), *L. portula* (Fig. 5 I1-I2), *L. tribracteatum* (Fig. 5 J1-J2), *A. auriculata* (Fig. 5 K1-K2), and *A. verticillata* (abaxial surface, Fig. 5 L). Type IV: Raised overlapping/stout-stout in *A. multiflora* (Fig. 5 M) and *R. indica* (Fig. 5 N1-N2). Stomatal aperture type changed from sinuolate-erose to sinuolate. The first type is the most dominant and recorded in nine species viz. *L. silenoides*, *L. virgatum*, *L. salicaria*, *L. thymifolia*, *L. thesioides*, *L. tribracteatum*, *A. verticillata*, *A. auriculata*, and *A. baccifera*. While the second type was identified in five species viz. *L. junceum*, *L. hyssopifolia*, *L. portula*, *A. multiflora*, and *R. indica*. Based on wax distribution on the epidermal cells of stomata rims and pore, two types were identified: Type I: Epidermal cells covered by wax, rim, and pore free in *L. silenoides*, *L. salicaria*, *L. thymifolia*, *L. portula*, *L. tribracteatum*, *A. multiflora*, *A. verticillata*, *A. baccifera*, *R. indica* and *A. auriculata* (adaxial surface), and *L. thesioides* (abaxial surface). Type II: Epidermal cells and rim covered by wax and pore free in *L. hyssopifolia*, *L. junceum*, *L. virgatum* and *L. thesioides* (adaxial surface), and *A. auriculata* (abaxial surface) (Table 5).

Table 5. Wax distribution in outer stomatal rim/peristomatal rim/inner stomatal rim), epidermal cell/stomata rims/pore of the studied species of *Lythrum*, *Ammannia*, and *Rotala*

Taxon	Outer stomata rim adaxial/abaxial surface	Peristomatal rim adaxial/abaxial surface	Inner stomatal rim adaxial/abaxial surface	Epidermal cell, stomata rims, pore adaxial/abaxial surface
L. silenoides	Overlapping/-	Overlapping/-	Sinuolate-erose/-	Type I/type I
L. virgatum	Overlapping/ overlapping	Overlapping-stout/ overlapping-stout	Sinuolate-erose/ sinuolate-erose	Type II/type II
L. salicaria	Overlapping/ overlapping	Overlapping-stout/ overlapping-stout	Sinuolate-erose/ sinuolate-erose	Type I/type I
L. junceum	Overlapping/ overlapping	Overlapping-stout/ overlapping-stout	Sinuolate/ sinuolate	Type II/type II
L. hyssopifolia	Overlapping/-	Overlapping-stout/-	Sinuolate/-	Type II/type II
L. thymifolia	Overlapping/ overlapping	Overlapping-stout/ overlapping-stout	Sinuolate-erose/ sinuolate- erose	Type I/type I
L. portula	Raised overlapping/ raised overlapping	Overlapping-stout/ overlapping-stout	Sinuolate/ sinuolate	Type I/type I
L. thesioides	Raised overlapping/ raised overlapping	Overlapping-stout/ stout	Sinuolate-erose/ sinuolate-erose	Type II/type I
L. tribracteatum	Raised overlapping/ raised overlapping	Overlapping-stout/ overlapping-stout	Sinuolate-erose/ sinuolate-erose	Type I/type I
A. verticillata	Overlapping/raised overlapping	Overlapping-stout/ overlapping-stout	Sinuolate-erose/ sinuolate-erose	Type I/type I
A. auriculata	Raised overlapping/ raised overlapping	Overlapping-stout/ overlapping	Sinuolate-erose/ sinuolate-erose	Type I/type II
A. multiflora	Raised overlapping/ overlapping	Stout/ stout	Sinuolate/ sinuolate	Type I/type I
A. baccifera	Overlapping/ overlapping	Overlapping-stout/ overlapping-stout	Sinuolate-erose/ sinuolate-erose	Type I/type I
R. indica	Raised overlapping/ raised overlapping	Stout/stout	Sinuolate/sinuolate	Type I/type I

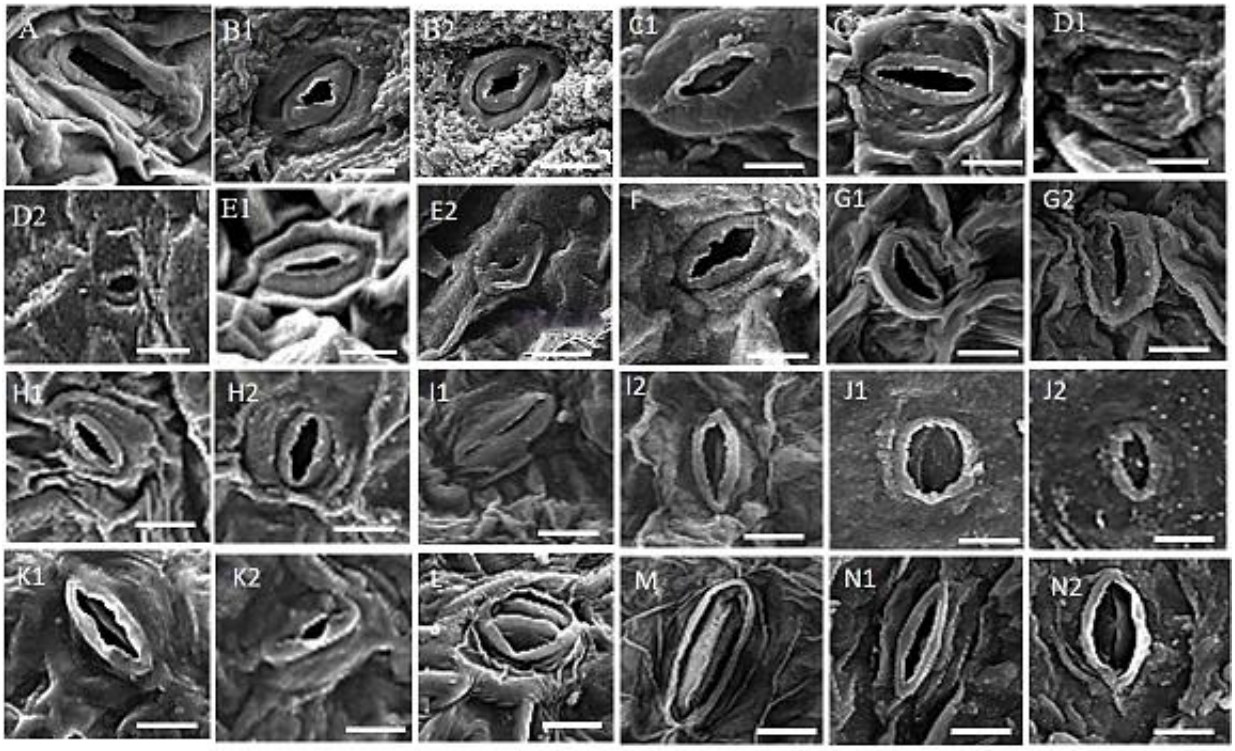

Fig. 5. SEM micrographs showing stomata: A. *L. silenoides*, B1-B2. *L. virgatum*, C1-C2. *L. salicaria*, D1-D2. *L. junceum*, E1-E2. *L. hyssopifolia*, F. *L. thymifolia*, G1-G2. *A. baccifera*, H1-H2. *L. thesioides*, I1-I2. *L. portula*, J1-J2. *L. tribracteatum*, K1-K2. *A. auriculata*, L. *A. verticillata*, M. *A. multiflora*, N1-N2. *R. indica* (Bars = 12.5 µm).

- Cluster analysis

UPGMA dendrogram (Fig. 6) obtained from similarity matrix demonstrated a cophenetic correlation of 66% for the character data set. The results represented perfect agreement between the two triangular distance matrices and the dendrogram (Sneath & Sokal 1973, Rohlf 2000). The results showed that, the 14 taxa were isolated into two main clusters (A and B) (Fig. 7) and five groups. Cluster A includes six species arranged in two sub-clusters A1 and A2. Sub-cluster A1 comprises *L. virgatum*, *L. junceum*, and *L. hyssopifolia* (group 1) and sub-cluster A2 contains *L. salicaria*, *L. silenoides* and *L. thymifolia* (group 2). Cluster B comprises eight species (including *A. multiflora*, *L. thesioides*, *L. tribracteatum*, *L. portula*, *A. verticillata*, *A. auriculata*, *A. baccifera*, *A. auriculata*, and *R. indica*) divided into two sub-clusters (B1 and B2) and three groups.

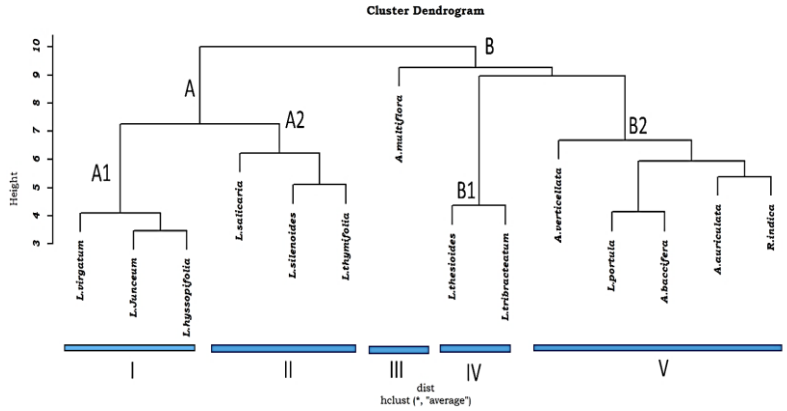

Fig. 6. Unweighted pair-group method with arithmetic averages (UPGMA) dendrogram of the 14 studied species of *Lythrum*, *Ammannia*, and *Rotala*.

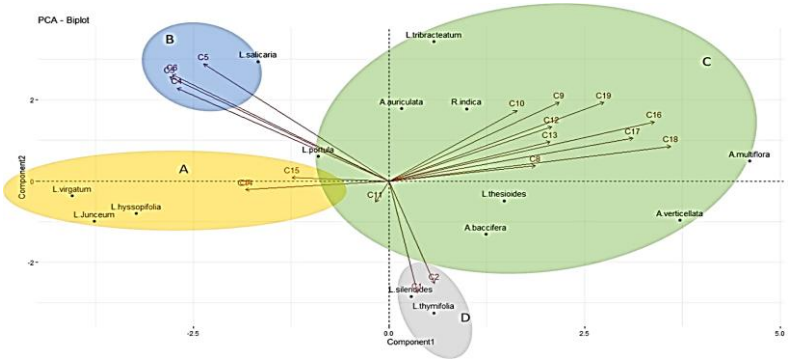

Fig. 7. PCA of the 14 studied species of *Lythrum*, *Ammannia*, and *Rotala* characters: C1. Presence or absence trichome, C2. Hair type, C3. Presence or absence papilla, C4. Papilla position on the leaf.

- Principal component analysis

The Principal Components Analysis (PCA) described 77.63% of the total variation of the character data set (Fig. 7). Based on the results, character data set accounted for 54.44% of the variation on the PC1 axis and 23.18% of the variation on PC2 and led to the formation of the following four groups (A-D): Group A includes three species viz. *L. virgatum*, *L. junceum*, and *L. hyssopifolia*, having identical peristomatal rim wax distribution pattern (C14) and epidermal cell wax distribution pattern (C15). Group B composed of one species, *L. salicaria*, separated by its unique presence or absence papilla (C3) and papilla ornamentation (C6). Group C comprises eight representatives viz. *L. tribracteatum*, *R. indica*, *A. auriculata*, *A. multiflora*, *A. verticillata*, *L. thesioides*, *A. baccifera*, and *L. portula*, sharing similar epicuticular wax ornamentation pattern (C8) raised or depressed anticlinal wall (C9), anticlinal wall type (C10), outer stomata rim wax distribution pattern (C12), inner stomata rim wax distribution pattern

(C13), stomata length (C16), stomata aperture length (C18), stomata aperture width (C19), and group D consists of two species viz. *L. silenoides* and *L. thymifolia* having similar presence or absence trichome (C1), and hair type (C2). The results of multivariate analysis showed that, the species combination obtained from UPGMA analysis was largely consistent with PCA analysis.

Identification key based on studied micromorphological traits for 14 studied species of *Lythrum*, *Ammania*, and *Rotala*

1. Wax sculpturing type V and VII, leaf glabrous on both surfaces…….. *Rotala* (*R. indica*)
- Wax sculpturing other types (I-IV, VI, and VIII-IX), leaf with hair and papilla on one or both surfaces ……..…. 2
2. Wax sculpturing type I-IV, and VIII sub-type II ……..……..……....……....… *Lythrum* 3
- Wax sculpturing type VIII (sub-types I and III), and type IX ……...…….…...…….……..… *Ammannia* 11
3. Leaf with hair ……..…….……..……..……..……..……..……. 4
- Leaf glabrous ……..……..……..……..……..……..……..…… 5
4. Hair short and long, flat and verrucate, appressed to semi erect, wax sculpturing type I sub-type I, stomata length and width 8.43–12.59 µm and 4.16–6.34 µm, stomata aperture length 4.46–9.12 µm ……..……..…. *L. salicaria*
- Hair short, flat, acute, smooth, appressed, wax sculpturing type I sub-type II, stomata length and width 12.51–15.27 µm and 7.79–10.86 µm, stomata aperture length 10.31–11.96 µm ……...…….... *L. tribracteatum*
5. Wax sculpturing type I sub-type I ……...… 6
- Wax sculpturing type II, III IV, and VIII sub-type II ……..……..……..……..…. 7
6. Papilla type II (leaf margin and adaxial surface), papilla ornamentation verrucate, anticlinal wall almost undulate ……..……... *L. thesioides*
- Papilla type I (adaxial surface), papilla ornamentation striate, anticlinal wall undulate ……..……...… *L. thymifolia*
7. Papilla type I ……..……..……..……..……..……. 8
- Papilla absent or type III ……..……...…...……..……. 10
8. Wax sculpturing type II (abaxial surface) and type IV (adaxial surface) ……..……..……..... 9
- Wax sculpturing type III (adaxial/abaxial surface) ……..……..……..……..…….. *L. junceum*
9. Outer stomatal rim/peristomatal rim type pattern overlapping/overlapping, stomata aperture length 8.1–12.2 µm and width 1.09–3.54 µm ……..…..…… *L. silenoides*
- Outer stomatal rim/peristomatal rim type pattern overlapping\overlapping-stout, stomata aperture length 2.38–7.83 and width 0.49–1.28 µm ...…..……..……..……..……..……..……... *L. hyssopifolia*
10. Wax sculpturing type III, outer stomatal rim/peristomatal rim overlapping/overlapping-stout (adaxial/abaxial surface) ……..……..……..……..……..……..…… *L. virgatum*
- Wax sculpturing type VIII sub-type II, outer stomatal rim/peristomatal rim raised overlapping/overlapping-stout (adaxial/abaxial surface) ……..……...…..……..……..……..…… *L. portula*
11. Wax sculpturing types VI and IX ……...……..……..……..…. 12
- Wax sculpturing type VIII (adaxial/abaxial surface) ……..……..……..……..……..…… 13
12. Anticlinal wall depressed/raised (adaxial/abaxial surface) and outer periclinal layer raised/depressed (adaxial/abaxial surfaces), wax sculpturing type VI (abaxial surface) and IX (adaxial surface) .....… *A. auriculata*
- Anticlinal wall raised/depressed (adaxial/abaxial surface) and outer periclinal layer depressed (both surfaces), wax sculpturing type VI (adaxial surface) and IX (abaxial surface) ……...……..... *A. verticillata*

13. Wax sculpturing type VIII sub-type I, outer stomatal rim/peristomatal rim raised overlapping/stout-stout (adaxial/ abaxial surface) ……..……...…….....……..……... *A. multiflora*

- Wax sculpturing type VIII sub-type III, outer stomatal rim/peristomatal rim overlapping/overlapping-stout (adaxial/ abaxial surface) ……..……..…….. *A. baccifera*

Discussion

The present results revealed the existence of diversity of leaf micromorphological evidence in *Lythrum*, *Ammannia*, and *Rotala* in Iran. It also underlines the taxonomic importance of leaf micromorphology in the family *Lythraceae*, in contrast to leaf morphological traits, which were rarely used for classification of the family (except leaf arrangement) (Polatschek & Rechinger 1968), used to separate *Lythrum* (with opposite leaves) and *Ammannia* (with alternate leaves). Presence or absence of trichome and papilla, their type, anticlinal wall and outer periclinal layer type, epicuticular wax ornamentation, wax type, wax distribution in outer stomatal, peristomatal, inner stomatal rim/epidermal cell, stomata rims, and pore are important diagnostic characters that provide strong support for identification of species of *Lythraceae.* Based on SEM observation, the trichome type helps to identify the two hairy species of *L. salicaria* and *L. tribracteatum*.

The results support the taxonomic treatments of Polatschek & Rechinger (1968) and Yousef Naanaie (2010). In addition, papilla, or hair-like structures (Metcalfe & Chalk 1957) and their characteristics (e.g., presence or absence of papilla, their position, shape, and surface ornamentation) have taxonomic value (Neinhuis & Barthlott 1997, Koch & Barthlott 2009, Faghir *et al*. 2018). According to the results, leaves of nine species studied here have papillae, and three papilla types were recorded (Table 2). Differences in papilla type can be used for interspecific and intergeneric identification. These are types I and II in the studied *Lythrum* species (type I in *L. silenoides*, *L. junceum* and type II in *L. thesioides*), and type III in *Ammannia.* Several previous authors indicated the remarkable effect of wax sculpture in classifying different taxonomic levels (Fehrenbach & Barthlott 1988, Barthlott *et al*. 1998, Wissemann 2000, Neinhuis & Barthlott 1997). Similarly, the present findings showed the diagnostic value of wax ornamentation in identification (Table 3), for example, wax ornamentation types I-IV and VIII (sub-type II) were restricted within the studied species of *Lythrum*, while VI, VIII (sub-types I and III), and type IX were observed in *Ammannia* and types V and VII in *R. indica*, respectively. The anticlinal walls and outer periclinal layer types (raised, oblate and depressed) on both surfaces of the leaf showed variation in the studied species, so it can be used as a good identification tool. Previous studies highlighted the importance of the taxonomic application of stomata features (Shaheen *et al.* 2009, Ergen Akin *et al*. 2013, Kumar & Murgan 2015). The present findings also showed that, stomata features (especially wax distribution at the outer stomatal border/peristomatal border/inner stomatal border, epidermal cell/stomatal border/pore) are meaningful criteria and can be used for species isolation.

The results of the multivariate analysis showed that, the majority of the *Lythrum* studied species (eight representatives) were grouped as follows: Cluster A includes six *Lythrum* species arranged in two sub-clusters A1 and A2. In the first sub-cluster, *L. hyssopifolia* and *L. junceum* formed a small group based on type II wax sculpture. The two species show morphological similarities such as stem branching type, leaf shape, and single flower without pedicel. Both species are distributed from Europe to Turkey, Palestine, and Iran, especially in humid areas (Polatschek & Rechinger 1968, Yousef Naanaie 2010). In this group, *L. virgatum* was found on an isolated branch, mainly because of its unique wax ornamentation of irregular platelets. This light green, glabrous, perennial species is well known for its linear-lanceolate leaves and loose, racemose inflorescences, it is widely distributed in Europe and north and northwest Iran, especially in flooded meadows, riverbanks, lakeshores, and bog edges (Afanas'ev 1949, Yousef Naanaie 2010).

Sub-cluster A2 consists of three representatives. Among them, *L. salicaria* formed an isolated branch because of its distinct pubescence type and no papilla. *L. salicaria* is called "Khon-fam" in Iran (Khanavi *et al.* 2011). This invasive species is very common in moist areas near riverbanks (Polatschek & Rechinger 1968, Dech & Nosko 2004, Hoffmann & Broadhurst 2016, Hanna 2017). In this sub-cluster, *L. silenoides* was found untied with *L. thymifolia*. These two species have several similar micromorphological characters (e.g., glabrous leaves with triangular papillae, raised outer periclinal layer and undulate depressed anticlinal wall, overlapping outer stomata rims, sinuolate-erose inner stomata; type I epidermal cell, stomata rims, pore wax distribution pattern). They also resemble for their linear sessile leaves, single flowers, 3 mm long hypanthium that is cylindrical at fruiting time, 2–6 stamens, and a small corolla (Polatschek and Rechinger 1968). *Lythrum thesioides* and *L. tribracteatum* were correlated in cluster B and subgroup B1. This result supports current classification. Both species have 2–8 stamens and usually grow in wetlands and riverbanks in Europe, Iran, Caucasus, Central Asia, and Afghanistan (Yousef Naanaie 2010).

The results of multivariate analysis also showed that, the micromorphological characteristics of leaves overlap in *Ammannia*, *Rotala* (*R. indica*) and three species of *Lythrum*. Three studied species of *Ammannia*, *R. indica*, and *L. portula* (in sub-cluster B2 correlated based on their wax sculpturing types of VII to IX, and absence of trichome. In this group, *A. baccifera* and *L. portula* were found correlated. However, there is no other report showing the correlation of the two species. *Lythrum portula* (Webb 1967, De vos 1971) is an annual prostrate herb, recognized by its glabrous, slightly fleshy, reddish stem, may root in branches, spatulate to obovate leaves, single flowers, often white or pink petals, and a spherical capsule fruit (Webb 1967), whereas, *A. baccifera* can be identified by its glabrous hypanthium, subs sessile stigma, and style up to 0.5 mm long (Polatschek & Rechinger 1968).

Ammannia auriculata and *R. indica* also formed a second small group in sub-cluster B2, because of their wax distribution on epidermal cells, rim, and pore (abaxial surface), and inner stomata rim, anticlinal wall, and outer periclinal layer types (abaxial surface) as well. *Ammannia auriculata* is classified in subg. *Euammannia* Koehne, sect. *Eustylia* Koehne (with long exerted style) (Afanas'ev 1949, Koehne 1880), distinguished by its style 1.5–3 mm long, peduncle 2–5 mm long, petals 1.5–2 mm long, (Polatschek & Rechinger 1968), flowers (usually 7), in axillary cymes and capsule 1.5–3.5 mm in diameter (Qin *et al*. 2007). It is reported from Tehran, Qazvin, Lorestan, and Bakhtiari provinces (Yousef Naanaie 2010, Polatschek & Rechinger 1968).

Rotala indica is an annual herb with a maximum height of 40 cm, which is very common in various parts of the world (Afanas'ev 1949, Polatschek & Rechinger 1968). This species closely associated with rice cultivation (Afanas'ev 1949, Qin *et al.* 2007) and has been reported from paddy fields of northern Iran, especially from Gilan province (Yousef Naanaie 2010, Joharchi *et al.* 2007). *Rotala indica* can be recognized by the shape of the leaves, obovate or oblong-spatulate, with a cuneate base, pinnately veined; calyx without appendages, capsules bivalved (Afanas'ev 1949, Qin *et al*. 2007). In addition, dendrogram shows two *Ammannia* species, *A. multiflora* (cluster B, group III) and *A. verticillata* (cluster B, subgroup B2, group V) in two isolated branches. *A. multiflora* was separated, based on its unique morphological features, such the type of wax sculpture (type VIII sub-type I), wax distribution patterns, large stomata length (11.3–22.3 µm) and aperture length (9.42–20.73 µm). It is classified with *A. auriculata*, in subg. *Euammannia* Koehne, sect. *Eustylia* Koehne (Graham 1985, Afanas'ev 1949, Koehne 1880) because of the similar stem length (style 1.5–3 mm) (Polatschek & Rechinger 1968). Many flowered *Ammannia*, has an almost cosmopolitan distribution, very common in rice fields and swampy regions, in different parts of the world (Afanas'ev 1949, Qin *et al*. 2007, Upadhyay *et al*. 2014). In Iran, it was reported from Lorestan and Esfahan

provinces (Polatschek & Rechinger 1968, Yousef Naanaie 2010). While *A. verticillata* was separated, due to its outer periclinal layer type, it is classified with *A. baccifera* in subg. *Euammannia*, sect. *Ammannia* (Afanas'ev 1949, Koehne 1880), on the base of its short style (0.5 mm long). *A. verticillata* grows in southwest Asia and southeast of the former USSR. It has been reported from western Iran (Lorestan province), in the rice fields and on riverbanks (Polatschek & Rechinger 1968, Ghahreman & Veiskarami 2000).

In conclusion, the present study reveals the diversity of micromorphological characteristics of the leaves of *Lythrum*, *Ammannia*, and *Rotala* species. These characters have diagnostic value and help in the identification of different species. Based on the distinguishing features, an identification key was constructed. Numerical analysis of these traits showed the correlation of the eight *Lythrum* species, however, it was unable to determine the delimitation of the three studied genera.

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