A chemotaxonomic study of some Iranian *Linum* taxa

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Abstract. *Linum* is one of the most useful genera of *Linaceae* widely distributed in different parts of Iran. In the present study, petal flavonoids of 14 *Linum* taxa from three sections were examined by 2-dimentional paper and thin-layer chromatography. The extracted flavonoids belong to three main groups. The kind as well as number of flavonoids varied between the taxa. PCA-biplot confirmed that some of the studied taxa had unique flavonoids, which were useful in their identification. Some types of flavonoids such as kaempferol and quercetin were general and had no chemotaxonomic values. While other types of flavonoids had restricted distribution and were useful in identification of the studied taxa. The species classification in the UPGMA tree and also PCA and PCO plots did not confirm the traditional grouping of the species in sections. Apparently, that traditional infrageneric classification of the genus must be changed.

Key words: chemotaxonomy, chromatography, flavonoid, *Linum*

Introduction

Linum is the largest and most important genus of the flax family (*Linaceae* (DC.) Dumort.) and comprises nearly 200 taxa distributed across the temperate and warm-temperate areas of Europe, Asia and America (Winkler 1931). Different investigations (Hooker 1875; Heywood 1993) showed that many species of the genus are cultivated for their seed oils, as animal fodder and as ornamentals. Seeds and leaves of many species have been used in traditional and modern medicine as anti-cancer agents, laxatives, a good source of Ω-3 fatty acids, anti-inflammatory, sore throat, against burns and angina cures (Weiss & al. 1975; Muir & Westcott 2003).

Variation in the basic chromosome number, which ranged from n = 8 to 43 (Gill 1987) and is associated with the wide range of the species diversity typical for this genus poses a constant challenge to scientists for development of its taxonomic treatments (McDill & Simpson 2005).

Winkler (1931) made a thorough overview of *Linum* and divided the species of this genus into six taxonomic sections, namely: *Linum*, *Dasylinum*, *Linastrum*, *Cathartolinum*, *Syllinum*, and *Cliococca*. However, Gill (1966) believed that the mentioned classifications, which were based only on the morphological traits, are useful, but may not reflect the evolutionary relationships between the taxa. Nearly twenty-one species, subspecies and varieties of the genus belonging to five sections grow naturally in Iran (Sharifnia & Assadi 2001).

Flavonoids are polyphenolic secondary metabolites, which play various roles in plants. For example, the compounds are involved in flower pigment production and also in plant protection from different pathogens, such as microbes and insects. Some types of flavonoids, such as isoflavones, flavons and flavanones are antifungal agents, while other kinds (e.g. flavonoids and tannins) have key roles in the protection of plants from insects and herbivorous mammals (Harborne & Williams 2000). Several studies (e.g. Asen 1984; Harborne & Turner 1984; Van Sumere & al. 1985; Noori 2012) proved that flavonoids have chemical stability, as well as widespread occurrence; therefore, they are used as chemical markers in the classification of plants and are considered a useful tool for

identification and taxonomy of higher plants. Studies confirmed that various flavonoids existed in the different species of the genus *Linum*. For example, Stosic & al. (1989) reported iso-orientin from *L. capitatum*. Earlier studies (Volk & Sinn 1968; Wagner & al. 1972) showed that *L. maritimum* contains 3', 4'-dimethoxy-7-rhamnosyl-luteolin and linoside A 91 and linoside B 92. The main anthocyanin of *L. grandiflorum* cv. *scarlet* is anthocyanidin triglycoside (Toki & al. 1995). Talebi & al. (2016) studied flavonoid characters in three members of the genus *Linum* and used these data for introducing a new record of *Linum austriacum* var. *album* from Iran (Talebi & al. 2016).

Earlier studies (e.g. Harborne 1994; Moor & Giannasi 1994; Noori & al. 2009; Noori 2014) confirmed that patterns of plant phenolic compounds were very useful for studying the relationships within or between species. Harborne & al. (1975) stated that flavonoid compounds are very important in plant taxonomy and often exhibit correlations with available classifications at the family, genus and infra-specific ranks, while key traits are scarcely recorded since these components may be hidden in one or more members of the taxon and the same flavonoid may happen in an unrelated taxon.

In this study, we have investigated the floral flavonoids of fourteen *Linum* taxa using two-dimensional paper and thin-layer chromatography. The main purposes of the study were to identify whether the patterns of flavonoids agreed with the infra-generic classification of these taxa and to determine the species-specific patterns of flavonoids. As far as we know, the flavonoid composition of most studied taxa was identified for the first time in this paper.

Material and methods

Plant collection and preparation

Randomly collected plants were studied of 14 *Linum* species, subspecies or varieties. Details of localities and voucher numbers are given in Table 1. Taxa were identified on the basis of the provided descriptions in *Flora Iranica* (Rechinger 1974) and also *Flora of Iran* (Sharifnia & Assadi 2001). The vouchers have been deposited in the Herbarium of Shahid Beheshti University of Tehran, Iran (HSBU). Flowers of the species were air-dried for the detection and identification of their flavonoids.

Table 1. Localities and voucher numbers of the studied *Linum* taxa from Iran.

Taxa	Locality	Voucher number
Section: *Linum*		
Linum austriacum L. var. *austriacum*	Saveh to Hamadan, After Nobaran, 1761m.	HSBU 2011103
Linum austriacum L. var. *album*	Hamadan, Famenin, 1700 m.	HSBU 2011336
L .nervosum Waldst & Kit. var. *nervosum*	Mazenderan, 90 km Karaj to Chalous, 2193 m.	HSBU 2011130
L. nervosum Waldst & Kit. var. *bungei* (Boiss.) Sharifnia	Mazenderan, 90 km Karaj to Chalous, 2193 m.	HSBU 2011129
L. usitatissimum L. var. *usitatissimum*	Markazi, 20 km Saveh to Salafchegan, Saleh Abad, 1320 m.	HSBU 2011165
L. glaucum	Kurdistan, 25 km Baneh to Saqqez, 1623 m.	HSBU 2011161
Section: *Syllinum* Griseb		
L. album Ky. ex Boiss	Kurdistan, Sanandaj to Kamyaran, 1329 m.	HSBU 2011114
L. mucronatum Bertol. ssp. *mucronatum*	Qazvin, Avaj, 2350 m.	HSBU 2011196
L. mucronatum ssp. *orientale* (Boiss.) P.H. Davis	Zanjan, 90 km Abhar to Zanjan, 1839 m.	HSBU 2011132
L. mucronatum ssp. *assyriacum* P.H. Davis	Khuzestan, Izeh, Atabaki Park 350 m.	HSBU 2011164
L. mucronatum ssp. *armenum* (Bordzil.) P.H. Davis	West Azerbaijan, Salmas, Ghoshchi, 1557 m.	HSBU 2011140
Sect. *Linastrum* (Planch) H. Winkler		
L. corymbulosum Reichenb.	Guilan, Rodbar, Darestan Gungle, 654 m.	HSBU 2011127
L. strictum L. var. *spicatum* Pers.	Hormozgan, Bandar Abbas, Geno, 1700 m.	HSBU 2011193
L. strictum L. var. *strictum*	Hormozgan, Bandar Abbas, Geno, 1600 m.	HSBU 2011198

Flowers extract preparation

For the comparative analysis of flavonoids, small extracts of all taxa were prepared by boiling 200 mg of powdered air-dried flowers for 2 min in 5 ml of 70 % EtOH. The obtained mixtures were cooled and left to extract for 24 h. Then, the extract was filtered and evaporated to dryness by means of rotary evaporation at 40 °C, and taken up in 2 ml of 80 % MeOH for 2-dimensional paper chromatography (2-DPC) analyses (Markham 1982).

Two-dimensional paper chromatography (2-DPC)

For the flavonoids identification, nearly 20 µl of each extract was placed on the corner of chromatography paper (Whatman No. 1) as a concentrated spot (10 applications of 2 µl). The sample chromatogram was developed in BAW (n-BuOH-AcOH-H2O=4:1:5; V/V; upper layer), first direction, and AcOH (=15 % aqueous acetic acid), second direction, with quercetin 3-O-

rutinoside as a standard. Then, the prepared chromatograms were viewed in long-wave UV light (366 nm). During this process, the dark absorbing and fluorescent spots were marked. R_f values in BAW and 15 % AcOH were estimated.

Flavonoids identification

The purified flavonoids were identified by UV spectroscopy with shift reagents to study the flavonoid substitution patterns (Mabry & al. 1970; Markham 1982). Acid hydrolysis was used to identify the aglycones and sugar moieties. Whenever possible, co-chromatography of standards was also done. Apigenin, hesperidin, luteolin (from Sigma Company), chrysin, genistein, isorhamnetin, kaempferol, morin, myricetin, naringenin (from Merck Company), quercetin, rhamnetin, rutin, tricine and vitexin (from Fluka Company) were used as available flavonoid standards for comparison during this study.

Acid hydrolysis and identification of flavonoid aglycones

Nearly 0.5 mg of each purified flavonoid was dissolved in 0.5 ml of 80 % MeOH. Subsequently, 2 ml of 2M HCl was added to it and the obtained mixture was heated in a water bath at 100 °C for 0.5 h. Then the solution was cooled; 2 ml of EtOAc was added and thoroughly mixed by a mixer. The upper EtOAc layer was removed by a pipette and evaporated to dryness. The dried matter was dissolved in 0.5 ml of MeOH and applied as spots on thin-layer chromatograms (cellulose). The Harborne (1998) method was used to distinguish the aglycones moiety.

Statistical analysis

For clustering of the studied taxa, flavonoid data were standardized (mean = 0, variance = 1) and used for multivariate analyses, including UPGMA (Unweighted Pair-Group Method with Arithmetic mean) and principal coordinate analysis (PCO) (Podani 2000). One-way ANOVA test was used to assess the significant differences in quantitative characteristics between the taxa. MVSP ver. 3.1 (2004) and SPSS ver. 9 (1998) software was applied for the statistical analyses.

Results

In the present study, floral flavonoids of fourteen taxa of the genus *Linum* were examined. The identified flavonoids belong to three main groups; flavonoid sulphates, flavonoid *C*-&*C*-/*O*-glucosides and flavonoid aglycones (Table 2). The total flavonoids number varied between the studied taxa. The highest total numbers of flavonoid and also of flavonoid sulphates were recorded in *L. austriacum* var. *austriacum*, while *L. mucronatum* subsp. *assyriacum* has shown the smallest amounts of them. Furthermore, *L. nervosum* var. *nervosum* and *L. usitatissimum* var. *usitatissimum* had the highest and the

Table 2. Two-dimensional paper and thin-layer chromatographical data of flavonoids in the studied *Linum* taxa from Iran.

	Total flavonoids number	Flavonoid sulphates number	Flavon *C*-&*C*-/*O*-glucosides number	Flavonoid aglycones number	Apigenin	Chrysin	Isorhamnetin	Kaempferol	Myricetin	Quercetin	Rhamnetin	Rutin	Vitexin
L. austriacum var. *austriacum*	11	7	4	–	–	+	+	+	+	+	+	–	–
L. austriacum var. *album*	8	4	4	–	–	+	+	+	–	+	–	–	–
L. nervosum var. *nervosum*	10	4	6	–	–	+	+	+	+	+	+	–	–
L. nervosum var. *bungei*	7	3	4	–	–	–	–	+	+	+	–	–	–
L. usitatissimum	6	4	2	–	+	+	–	+	+	+	–	–	+
L. glaucum	6	2	4	–	–	+	+	+	+	+	–	–	–
L. album	8	3	5	–	+	+	–	+	+	+	–	–	+
L. mucronatum ssp. *mucronatum*	9	3	6	–	–	+	+	+	+	+	+	–	–
L. mucronatum ssp. *orientale*	9	4	5	–	–	–	–	+	–	+	–	–	–
L. mucronatum ssp. *assyriacum*	5	2	3	–	+	+	+	+	+	+	–	+	+
L. mucronatum ssp. *armenum*	6	2	4	–	+	+	+	+	+	+	–	+	+
L. corymbulosum	6	2	4	–	–	+	–	+	+	+	–	–	–
L. strictum var. *spicatum*	9	3	6	–	–	–	–	+	+	+	–	–	–
L. strictum var. *strictum*	6	2	3	+	+	+	–	+	+	+	–	–	+

lowest numbers of flavon *C*-&*C*-/*O*-glucosides, respectively. Furthermore, flavonoid aglycones were found only in *L. strictum* var. *strictum.*

The distribution patterns of the identified flavonoids differed between the taxa. Some of them, such as kaempferol and quercetin, were general and encountered in all studied taxa. In contrast, other types of flavonoids have been recorded only in some specific taxa. For example, all studied taxa had chrysin, with the exception of *L. strictum* var. *spicatum*, *L. nervosum* var. *bungei* and also, *L. mucronatum* ssp. *orientale.* Apigenin as well as vitexin were recorded in *L. album*, *L. strictum* var. *strictum, L. usitatissimum* var. *usitatissimum*, *L. mucronatum* ssp. *assyriacum,* and *L. mucronatum* ssp. *armenum.* Rhamnetin was found in three taxa (*L. austriacum* var. *austriacum*, *L. mucronatum* ssp. *mucronatum* and *L. nervosum* var. *nervosum*). Among the studied taxa, myricetin was absent only in *L. album* and *L. mucronatum* ssp. *orientale.*

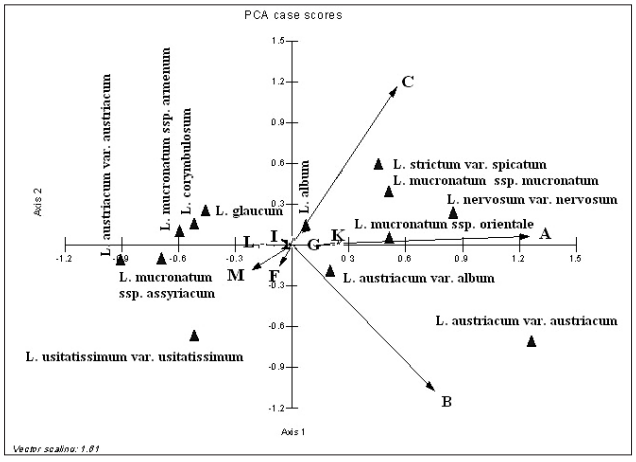

Fig. 2. UPGMA tree of the studied *Linum* taxa based on flavonoids data.

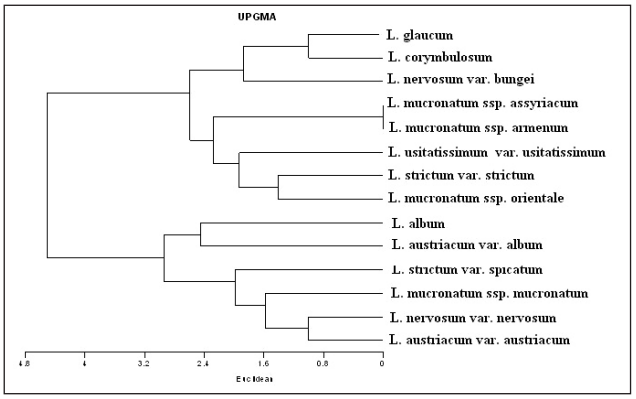

Fig. 1. PCA-biplot of phytochemical data and the studied *Linum* taxa. Abbreviations: **A**: total flavonoid number, **B**: flavonoid sulphates number, **C**: number of flavon *C*-&*C*-/*O*-glucosides, **F**: chrysin, **G**: isorhamnetin, **I**: myricetin, **K**: rhamnetin, **L**: rutin, **M**: vitexin.

The ANOVA test did not show significant variations in the total number of flavonoids between the studied taxa. A PCA-biplot showed that some of the studied taxa had unique chemical compounds, which were useful for their identification (Fig. 1). For example, flavonoid chrysin and vitexin separated *L. usitatissimum* var. *usitatissimum* from the other taxa. Flavonoid sulphates number and rhamnetin were used for distinguishing *L. austriacum* var. *album* and *L. austriacum* var. *austriacum*, respectively. The total flavonoid number was a good trait for *L. mucronatum* ssp. *orientale*, while the number of flavon *C*-&*C*-/*O*-glucosides had the same effect for *L. album.*

Our studied *Linum* taxa were clustered separately in a UPGMA tree (Fig. 2). Furthermore, PCA and PCO plots (Figs 3 & 4) produced similar results. The arrangement of taxa in this tree was discussed here; it had two branches. The smaller branch had six taxa and consisted of two sub-branches: in one *L. album* and *L. austriacum* var. *album* clustered together, and in the larger subgroup *L. strictum* var. *spicatum* and *L. mucronatum* ssp. *mucronatum*, *L. nervosum* var. *nervosum* and

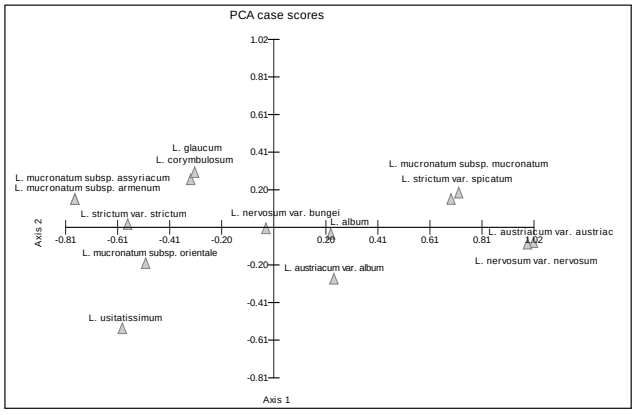

Fig. 3. PCA plot of the studied *Linum* taxa based on phytochemical studies.

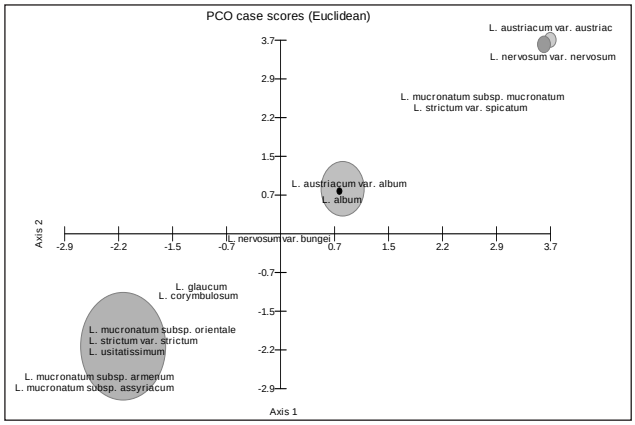

Fig. 4. PCO plot of the studied *Linum* taxa based on flavonoid data.

L. austriacum var. *austriacum* were observed. *L. strictum* var. *spicatum* and *L. mucronatum* ssp. *mucronatum* were placed separately and *L. nervosum* var. *nervosum* and *L. austriacum* var. *austriacum* were placed close together. The larger sub-branch consisted of eight taxa in two groups: *L. glaucum*, *L. corymbulosum* and *L. nervosum* var. *nervosum* constituted one group, while in the other two subgroups were formed. In one of them, *L. mucronatum* ssp. *assyriacum* and *L. mucronatum* ssp. *armenum* clustered close to each other, and the remaining taxa were grouped together.

Discussion

In this study, photochemical data were used for infraspecific and infra-generic classification of the studied *Linum* taxa. So far, different taxonomic tools such as palynology (Xavier & Rogers 1963; Talebi & al. 2012a), seed morphology (Talebi & al. 2012b), molecular biology (Sheidai & al. 2014), cytology (Gill 1987), and phylogenic data (Fu & al. 2010) have been used for taxonomic treatment of this genus, though most of them did not resolve its taxonomic problems. The main problems relate to species classification into sections and in some cases to infra-specific variations, which lead to alternation in taxonomic ranks. The phytochemical data used in the present study were flower flavonoids. There were many reasons for this. First, the petal color was a good characteristic in this genus and was stable in each taxon. Second, earlier studies (for example, Harborne 1967) showed that the flavonoid patterns in the petal tissue are qualitatively stable under the different conditions of selection. This uniformity might be due to the functioning of carotenoids as the main visible pigmentation, or to a conservative stability of floral pigmentation. Third, the study of Webb & Harborne (1991) showed that flavonoid data were meaningful in infra-generic classification, especially at sectional level. Furthermore, it is well documented that the flavonoid data on higher plants are often of value for resolving the evolutionary relationships among the plant taxa (Markham & al. 1970). For example, the studies of de Oliveira & al. (2017) have reported that flavonoids can be used as a taxonomic characteristic of the genus *Chromolaena*, and that chemotaxonomic significance of the compounds was useful for resolving the infra-generic complexity of the genus. Moreover, flavonoid data were used as chemotaxonomic traits in *Drosera* for the classification of species in sections and for the improvement of infra-generic taxonomy in this genus (Braunberger & al. 2015).

The obtained results showed that some types of flavonoids, such as kaempferol and quercetin, were general, and therefore had no chemotaxonomic values. Apparently, the mentioned compounds were also present in other taxa of the genus. For example, Ilić & al. (2004) reported rutin in *L. capitatum* Kit. and quercetin in *L. sulcatum* (Giannasi & Rogers 1970). These flavonoids have medicinal use. For example, flavonol quercetin is the most active flavonoid. Several medicinal herbs owe their functions to their high content of quercetin. Various investigations showed that the significant anti-inflammatory activity of quercetin is related to direct inhibition of the initial processes of inflammation. Experiments have proved that quercetin has antitumor effect, including in inhibiting the proliferation as well as migration of cancer cell (Lim & al. 2006). Among the different properties of kaempferol are: antioxidant, antitumor, anti-inflammatory, and antiulcer activity, as well as inhibitory effect on HIV protease (Vinson & al. 1995; Asif & Khodadadi 2013).

Myricetin was found in most studied taxa, therefore, the absence of this flavonoid is a taxonomic trait for identification of *L. mucronatum* ssp. *orientale* and *L. austriacum* var. *album.* Godse & al. (2010) demonstrated that this flavonoid has antihypertensive effect. Myricetin hindered the progression of high blood pressure and also turned the metabolic variations in rats on fructose-induced diet. Furthermore, it was proven to suppress body weight increment and accumulation of fat by maximizing the fatty acids oxidation, which was due to enhance the regulation of hepatic peroxisome proliferator activated receptor and reduce the regulation of hepatic sterol regulatory element-binding protein expressions in in rats on high-fat diet (Chang & al. 2012).

In contrast, some other kinds of identifying flavonoids had restricted the distribution and were useful in identification of the studied taxa. For example, the presence of rutin is a good characteristic for identification of *L. mucronatum* ssp. *assyriacum*, as well as of *L. mucronatum* ssp. *armenum* from the other taxa. Of the studied members of section *Syllinum*, only these subspecies had this compound. Apparently, rutin is a specie-specific flavonoid found in some taxa of section *Syllinum,* because it was previously recorded in *L. capitatum* (Ilić & al. 2004). Earlier studies have shown

that rutin is a more effective antioxidant than vitamins and has antioxidant and free radical scavenging effect in foods (Vinson & al. 1995; Hollman & al. 1997).

Although, *L. usitatissimum* var. *usitatissimum* with four other taxa had vitexin, this flavonoid was considered a good trait for identification of *L. usitatissimum* var. *usitatissimum* from the other members of section *Linum*. Of the six studied taxa of this section, *L. usitatissimum* var. *usitatissimum* was the only one to have this flavonoid. Our results were in agreement with Dubois & Mabry (1971), who also reported vitexin in this species. Giang & al. (2004) demonstrated that vitexin has good antioxidant and also antimicrobial properties. Furthermore, it protects the function of the liver, reduces oxidative stress, and improves histopathological structures in rats (Manikya Kumari & al. 2012).

L. austriacum and *L. glaucum* are very similar morphologically. Thus, in some sources such as *Flora of Turkey* (Davis 1967), *L. glaucum* was defined as a subspecies of *L. austriacum* and the main difference between them related to the stem leaf shape. In our study, these species were clustered far from each other. This confirmed that *L. austriacum* and *L. glaucum* were sibling species. These types of species have different genetical structures, with the exception of regulatory genes, which lead to likeness in their morphology. Flavonoid data were successfully used in the taxonomic treatments of different genera. For example, Lucimar & al. (2009) isolated and identified 12 kinds of flavonoids in *Camarea* species. The results of their work confirmed that flavonoids may be useful traits for taxonomical investigations of this genus, both at generic as well as at infra-generic level.

Our results did not confirm the traditional classifications of this genus in *Flora Iranica* (Rechinger 1974) and *Flora of Iran* (Sharifnia & Assadi 2001). In the traditional classification of *Linum*, the species were grouped into five sections, while the different studies showed that these arrangements pose many difficulties. Finally, we know that plant flavonoid pattern depends on genetic factors and ecological conditions. These parameters are effective in flavonoid production and it is believed that flavonoid patterns cannot always reveal the differences of the taxa. Presumably, for more subtle results, study of other biosystematic characters would be required. Furthermore, application of molecular markers along with the current research strategies could be useful and is recommended.

Conclusion

There are many discussions about infra-generic classification of the genus *Linum* and different taxonomical patterns were proposed for it, but most of them cannot clarify its complexity. In the present study, flavonoid data were used for taxonomic treatment of the genus and a flavonoid profile of the studied taxa was set, for some of them for the first time. Some types of the identified flavonoids were encountered in all studied taxa and were not useful in taxonomic studies, but some other types had a limited distribution and were of taxonomic value. Like in the recent studies, the species arrangement in sections did not confirm the infra-generic/ specific classification of the genus. Besides, most of the identified flavonoids had medicinal uses and our findings showed that many species of *Linum* can be used as remedial plants.

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