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Flavonoid Patterns and their Diversity in ten *Stachys* L. (Lamiaceae) Species from Iran

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ABSTRACT

Stachys genus with medicinal properties and high polymorphic features has been considered one of the largest genera of Lamiaceae. The aim of this study was to determine the flavonoid pattern variations and flavonoid groups in ten *Stachys* species belonging to two sections; *Fragilicaulis,* and *Aucheriana*. The studied species were collected from natural habitats in Iran and analysed for their flavonoid constituents using thin layer chromatography with silica gel. The purification of the flavonoid compounds of each species was carried out using column chromatography with sephadex LH20. The identification of flavonoid class was confirmed by spectral data. In order to study the flavonoid variations, cluster analysis was used with SPSS ver.20 software. The results of this study showed that the highest variations were found in *Stachys pilifera* Benth., *Stachys aucheri* Benth., *Stachys ballotiformis* Vatke and *Stachys benthamiana* Boiss. Based on the results, six flavonoid classes were identified. Most of the flavonoid classes were found to be flavones. The flavones and isoflavones were observed in section *Fragilicaulis* and flavanones, flavonols, isoflavones, dihydroflavonol, chalcones and flavones were in section *Aucheriana*. It can be concluded that the flavonoid compounds are appropriate markers in chemotaxonomic studies of the *Stachys* genus.

Keywords: flavonoid, thin layer chromatography, *Stachys*, Iran

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Introduction

Stachys L. is one of the largest genera of the Lamiaceae (Lamioideae sub-Family). It contains 275-300 species all over the world, displaying a remarkable range of variation, and represents an enormous and cosmopolitan distribution. Iran is an area extremely rich in taxa, including 34 species (1, 2). Some species are annual, biennial, perennial, subshrub or shrubby and are found in rocky regions and mountain steppes (3, 4). *Stachys* species are mainly distributed in the warm temperate regions of the Mediterranean and southwest of Asia. The main diversity centres are considered to be the south and east of Anatolia, Caucasia, northwest of Iran and north of Iraq, and the other centre is constrained to the Balkan Peninsula (2, 3). The Asiatic centre mainly contains Mediterranean and Irano-Turanian phyto-geographical elements (2).

Stachys species exhibit high morphological similarity with enormous morphological diversity and high polymorphism in infra-specific taxa, predominantly in different ecological environments (4). As a result, it is difficult to discriminate between the related species. This morphological diversity and the lack of identification of nomenclature types give rise to great uncertainty on their allocation (5). *Stachys* genus presents a wide range of variability, leading to several infra-generic classifications (3). On the whole, the taxonomy of this genus is extremely complicated. Differentiation and circumscription of specific and infra-specific taxa have been more problematic (6, 7). Various features including morphology, pollen, nutlet, trichome, isoenzyme and anatomical features have been proven to be useful for differentiating some *Stachys* taxa from Iran (4, 6, 7, 8, 9, 10). However, the arrangement of *Stachys* sections varies among different classifications (7, 8, 11).

Due to the high content of secondary compounds, *Stachys* species are used in traditional medicine for the treatment of cardiac disease and are incorporated into anti-inflammatory drugs, analgesics and anticonvulsants for treating genital tumours, sclerosis of the spleen, coughs and ulcers (11). The aerial parts are used in antispasmodic, diuretic, asthmatic, rheumatic and contained antibacterial and antioxidant compounds (12).

In recent years, studies on chemical compounds of plant species have been generally constrained to the essential oils, triterpenoids, fatty acids and phenolic compounds (13). Based on the literature, the compositions of essential oils have been identified in endemic *Stachys* in the Balkan Peninsula (14). Khanavi *et al.* (15), Roustaiyan *et al.* (16) and Safaei-Ghomi *et al.* (17) recognized the essential oils in some Iranian *Stachys* species. Radulovic *et al.* (14) reported on the 2-ethyl substituted fatty acids from *S. milanii* Petr. from the eastern region of the Balkan, which are significant as chemotaxonomic markers in this genus. Moreover, the flavonoid constituents have been generally identified in multiple reports on the *Stachys* species (5, 18, 19).

In terms of morphological characters and hybridization in infra-specific levels, *Stachys* species display high variability. Therefore, this genus is one of the great genetic resources for Iran. There have been few reports on the flavonoid compounds and no data about diversity in flavonoid investigations of this genus in Iran. Consequently, the aim of the present study is to identify the flavonoid class and its variations in ten *Stachys* species belonging to two sections for Iran, 1) section *Fragilicaulis*: *S. benthamiana* Boiss., *S. ballotiformis* Vatke, *S. megalodonta* Hausskn. &. Bornm. ex P. H. Davis, *S. kurdica* Boiss. &. Hohen., *S. asterocalyx* Rech. f., *S. kermanshahensis*

Rech. f., and 2) section *Aucheriana*: *S. acerosa* Boiss., *S. multicaulis* Benth., *S. pilifera* Benth., *S. aucheri* Benth. The flavonoid variations and some of the flavonoid classes were first reported for Iran.

Material and Methods

Ten *Stachys* species belonging to 50 accessions were collected from their natural habitats from Zagros-Iran (Table 1).

Table 1. Habitats of *Stachys* species from Iran

Species	Locality	Height (m)
*S. benthamiana*35	Chaharmahalva Bakhtiari- Rostamabad	1899
*S. benthamiana*192	Isfahan- Gahrouye	2200
*S. benthamiana*122	Chaharmahal va Bakhtiari-Bajgiran	1950
*S. benthamiana*130	Isfahan- Vanak Semirom	2043
*S. benthamiana*187	Isfahan-Dalankuh	2080
*S. benthamiana*116	Chaharmahal va Bakhtiari- Tange Mahmud	1967
*S. benthamiana*191	Chaharmahal va Bakhtiari- Naghan, Chahartagh	2010
*S. benthamiana*25	Chaharmahal va Bakhtiari-Tange darkesh varkesh	1979
*S. ballotiformis*30	Isfahan- Dalankuh	1910
*S. ballotiformis*114	Chaharmahal va Bakhtiari- Helen forest	1817
*S. ballotiformis*157	Isfahan- Vanak semirom, Cheshmenaz	2080
*S. ballotiformis*302	Chaharmahal va Bakhtiari- Dehcheshme, Pireghar	2072
*S. magalodonta*26	Chaharmahal va Bakhtiari- roustaye Kaj	1743
*S. magalodonta*111	Chaharmahal va Bakhtiari- Helen forest	1817
*S. magalodonta*82	Chaharmahal va Bakhtiari- 5 km of Sudejan	2370
*S. asterocalyx*304	Chaharmahal va Bakhtiari- Dehcheshme, Pireghar	2100
*S. asterocalyx*115	Chaharmahal va Bakhtiari- Helen forest	1817
*S. asterocalyx*245	Chaharmahal va Bakhtiari- Seifabad, kuh-e Kalar	1868
*S. asterocalyx*221	Kohgiluye va Boyer Ahmad- chesme Mishi	1880
*S. asterocalyx*287	Kohgiluyeh va Boyer Ahmad-Yasouj, Sisakht	1500
*S. kermanshahensis*20	Chaharmahal va Bakhtiari- Sardabe Rostamabad	1771
*S. kermanshahensis*4	Chaharmahal va Bakhtiari- Lordegan	1900
*S. kermanshahensis*36	Chaharmahal va Bakhtiari- Tange darkesh varkesh	1967
*S. kurdica*152	Isfahan- Vanak Semirom, Cheshmenaz	2035
*S. kurdica*154	Isfahan- Dalankuh	2050
*S. kurdica*144	Isfahan- Vanak Semirom	1990
*S. pilifera*276	Isfahan- Dalankuh	1946
*S. pilifera*202	Chaharmmahal va Bakhtiari- Tange Chehrazgun, Sabzkuh	
*S. pilifera*119	Chaharmahal va Bakhtiari- Dopolan	2034
*S. pilifera*47	Chaharmahal va Bakhtiari- kuh-e Cheheldokhtar	1940
*S. pilifera*140	Isfahan- Ghale-Ghadam, roustaye Mokhtar	2555
*S. pilifera*91	Chaharmahal va Bakhtiari- Dehcheshme	2092
*S. aucherii*87	Isfahan- Dalankuh	2076
*S. aucherii*39	Chaharmahal va Bakhtiari- Shamsabad, Tang-e-Kharajy	2056
*S. aucherii*311	Chaharmahal va Bakhtiari- Dehcheshme	2155
*S. aucherii*136	Isfahan- Bordekan	2480
*S. aucherii*167	Chaharmahal va Bakhtiari- Gandoman	2335
*S. aucherii*101	Chaharmahal va Bakhtiari- Naghan	2081
*S. multicaulis*30	Kohgiluye va Boyer Ahmad- Sisakht	1971
*S. multicaulis*114	Chaharmahal va Bakhtiari- Helen forest	2020
*S. multicaulis*157	Isfahan-Vanak, cheshmehnaz	2000
*S. multicaulis*302	Chaharmahal va Bakhtiari- Dehcheshme	2135
*S. multicaulis*116	Chaharmahal va Bakhtiari- Tange Mahmud	1967
*S. multicailus*289	Kohgiluyeh va Boyer Ahmad- cheshme Mishi	2033
*S. multicaulis*290	Kohgiluyeh va Boyer Ahmad- kuh Gol	2012
*S. acerosa*268	Isfahan- Damaneh	1839
*S. acerosa*238	Chaharmahal va Bakhtiari- kouh-e Kelar	1889
*S. acerosa*207	Chaharmahal va Bakhtiari- Tange Chehrazgun, Sabzkuh	2000
*S. acerosa*176	Chaharmahal va Bakhtiari- Ughunsu, Hezardare	2455
*S. acerosa*172	Isfahan, Damaneh	2448

The voucher specimens were deposited in the Herbarium of Shahrekord University.

Extraction of flavonoids was based on the protocol suggested by Markham (20) and Rahman (21). The flavonoid solution was extracted from air-dried leaves (10.5 g) from ten *Stachys* species using crude 85% MeOH at 60°C. The extract was concentrated using a rotary evaporator at 70°C for total solvent removal. Removal of carotene and chlorophyll was performed by n-BuOH and subsequently analysed by silica gel 60F 254 (15 mg, 67.5 ml H_2O) thin layer chromatography (TLC; 3 µm, 20 ×20 cm). The chromatogram was transferred to BuOH-$C_2H_4O_2$-H_2O (3BuOH: $1C_2H_4O_2$:$1H_2O$) representing an organic system. Spot detection with natural product identifiers (5% H_2SO_4 in crude MeOH, and $C_{14}H_{16}BNO$ in 1MeOH:$1H_2O$) was achieved under UV-366 nm (20, 22). The purification of flavonoid compounds of each species was carried out using column chromatography (65 × 3 cm) on sephadex LH20 Sigma- Aldrich (Sephadex and 20% MeOH mixture) by 100 ml MeOH solution (MeOH content 20%, 40%, 60%, 80%, 100% and Acetone) and the fractions were extracted (the amount of packing material is 50 ml for each MeOH content). The fractions were transferred to a one-dimensional map (1DM) on silica gel plates (3 µm). Identification of purified compounds was carried out on the basis of their UV spectra (366 nm), MeOH solution, and shift reagents such as $AlCl_3$, $AlCl_3$/HCl, NaOAc, NaOAc/H_3Bo_3 and MeOH.

In order to assay the flavonoid variations among *Stachys* accessions, statistical methods such as cluster analysis with Euclidian Distance and Ward method using SPSS (SPSS V20, IBM) were applied. The presence and absence of spots was estimated for this process. Moreover, the retention factor (Rf) of each spot belonging to species was considered.

Results

The flavonoid patterns of crude extract from each *Stachys* species showed coloured spots on chromatography plates. The total numbers of spots obtained for each species were found to be:

1. *S. benthamiana* accessions 37 spots,
2. *S. ballotiformis* accessions 15 spots,
3. *S. kurdica* accessions 8 spots,
4. *S. asterocalyx* accessions 17 spots,
5. *S. megalodonta* accessions 9 spots,
6. *S. kermanshahensis* accessions 13 spots,
7. *S. acerosa* accessions 21 spots,
8. *S. pilifera* accessions 22 spots,
9. *S. aucheri* accessions 28 spots,
10. *S. multicaulis* accessions 13 spots.

The yellow, blue and violet spots were common in *Stachys* species (Table 2a). Orange, brown, dark yellow, light yellow, light blue, blue fluorescent, dark blue and blue-violet spots were found in some of these species (Table 2a). In some of the studied species, the colour variations and new colour spots after detection of natural products were observed, which were yellow, orange, blue and brown (Table 2a). Various new colours as yellow, orange, blue and brown were observed for the first time in Iran's *Stachys* species. Moreover, Rf values of each spot were identified from *Stachys* species (Table 2b). The highest Rf value was observed in *S. multicaulis* (Rf=1.9) and the lowest was in *S. ballotiformis* (Rf= 0.57) (Table 2b).

Table 2. Spot colors and Rf values in *Stachys* species

Part A) Presence and absence of spots before detection of natural product

Species	1	2	3	4	5	6	7	8	9	10	11
S. benthamiana	+	+	+	+	-	-	-	-	-	-	-
S. ballotiformis	+	-	+	-	-	+	+	-	-	+	-
S. asterocalyx	+	+	-	-	+	+	+	-	-	-	-
S. kurdica	-	-	+	+	-	-	-	-	-	-	-
S. kermanshahensis	-	+	-	+	-	-	-	-	-	-	-
S. megalodonta	+	-	-	-	+	-	+	-	-	-	-
S. pilifera	-	+	+	+	-	-	+	+	+	-	+
S. aucheri	+	+	-	+	-	-	+	-	+	-	+
S. multicaulis	+	-	+	-	+	+	-	-	-	-	+
S. acerosa	+	-	+	-	+	-	+	+	-	-	-

1: blue, 2: violet, 3: yellow, 4: orange, 5: dark yellow, 6: light yellow, 7: blue-violet, 8: light blue, 9: blue fluorescent, 10: dark blue, 11: brown.

Part B) Rf values of each spot in *Stachys* species

Species	Rf
S. benthamiana	1.33, 1.06, 1, 0.88, 1.3, 1.2, 0.95, 0.84, 1.3, 1.2, 0.95, 0.84, 1.29, 0.96, 0.86, 1.28, 1.16, 0.92, 0.84, 1.27, 0.95, 0.84, 1.27, 1.15, 0.94, 0.84, 1.24, 1.15, 1, 0.93, 0.84, 0.78
S. ballotiformis	1.11, 0.86, 0.71, 1.11, 0.75, 0.64, 1.11, 0.89, 0.78, 0.71, 0.64, 1.11, 0.78, 0.68, 0.57,
S. asterocalyx	1, 0.77, 0.7, 1, 0.82, 0.79, 0.73, 1, 0.77, 0.7, 1, 0.77, 0.7, 1, 0.77, 0.71,
S. kurdica	1.03, 0.71, 0.64, 1.03, 0.71, 0.64, 1.03, 0.71
S. kermanshahensis	1.03, 0.92, 0.82, 0.75, 0.66, 1.03, 0.85, 0.74, 0.68, 1.04, 0.74, 0.64
S. megalodonta	0.96, 0.82, 0.77, 0.72, 0.97, 0.79, 0.76, 0.98, 0.82
S. pilifera	1.07, 0.89, 0.74, 0.61, 1.04, 0.94, 0.75, 1.04, 0.92, 0.79, 0.67, 1.05, 0.92, 0.81, 1.07, 0.96, 0.81, 0.72, 1.09, 1, 0.85, 0.72
S. aucheri	1.12, 1.07, 1, 0.81, 0.7, 1.15, 1.1, 1, 0.9, 0.8, 1.15, 1.03, 0.9, 0.8, 1.2, 1.12, 1.04, 0.9, 0.83
S. multicaulis	1.11, 0.86, 0.71, 1.11, 0.75, 0.64, 1.11, 0.89, 0.78, 0.71, 0.64, 1.11, 0.78, 0.68, 0.57, 1.9, 1.7, 1.5, 1.3, 1.15, 1.9, 1.8, 1.5, 1.17, 1.9, 1.7, 1.5, 1.2
S. acerosa	1.02, 0.94, 0.82, 0.77, 0.62, 1.02, 0.96, 0.75, 0.61, 1.03, 0.94, 0.78, 0.62, 1, 0.89, 0.78, 0.62, 1.02, 0.89, 0.79, 0.61

Based on TLC flavonoid profiles, the presence and absence of the spots was estimated for ten *Stachys* species. These data were carried out for displaying the flavonoid variations among 50 accessions. The cluster analysis in each section is displayed in Figures 1 and 2. The section *Fragilicaulis* comprised two groups (Fig. 1). Two groups were also

found in section *Aucheriana* (Fig. 2).

Based on these results, the flavonoid variation patterns in *Stachys* species exhibited more diversity, which is described as follows:

Each substitution was mainly found to be either hydroxylation, methoxylation, or other substitutions in different positions as shown in Table 3. The shift reagents such as $AlCl_3$, $AlCl_3$/HCl, NaOAc, NaOAc/H_3Bo_3 and MeOH in band I showed different spectral data which detected each flavonoid class respectively (Table 4). The highest flavonoid compounds in ten Stachys species were flavones (derivatives) and the lowest were dihydroflavonols, flavonols, flavanones, isoflavones and chalcones (Table 4). Consequently, in this research a total of six flavonoid classes were observed in leaves from ten *Stachys* species in Iran (Table 4).

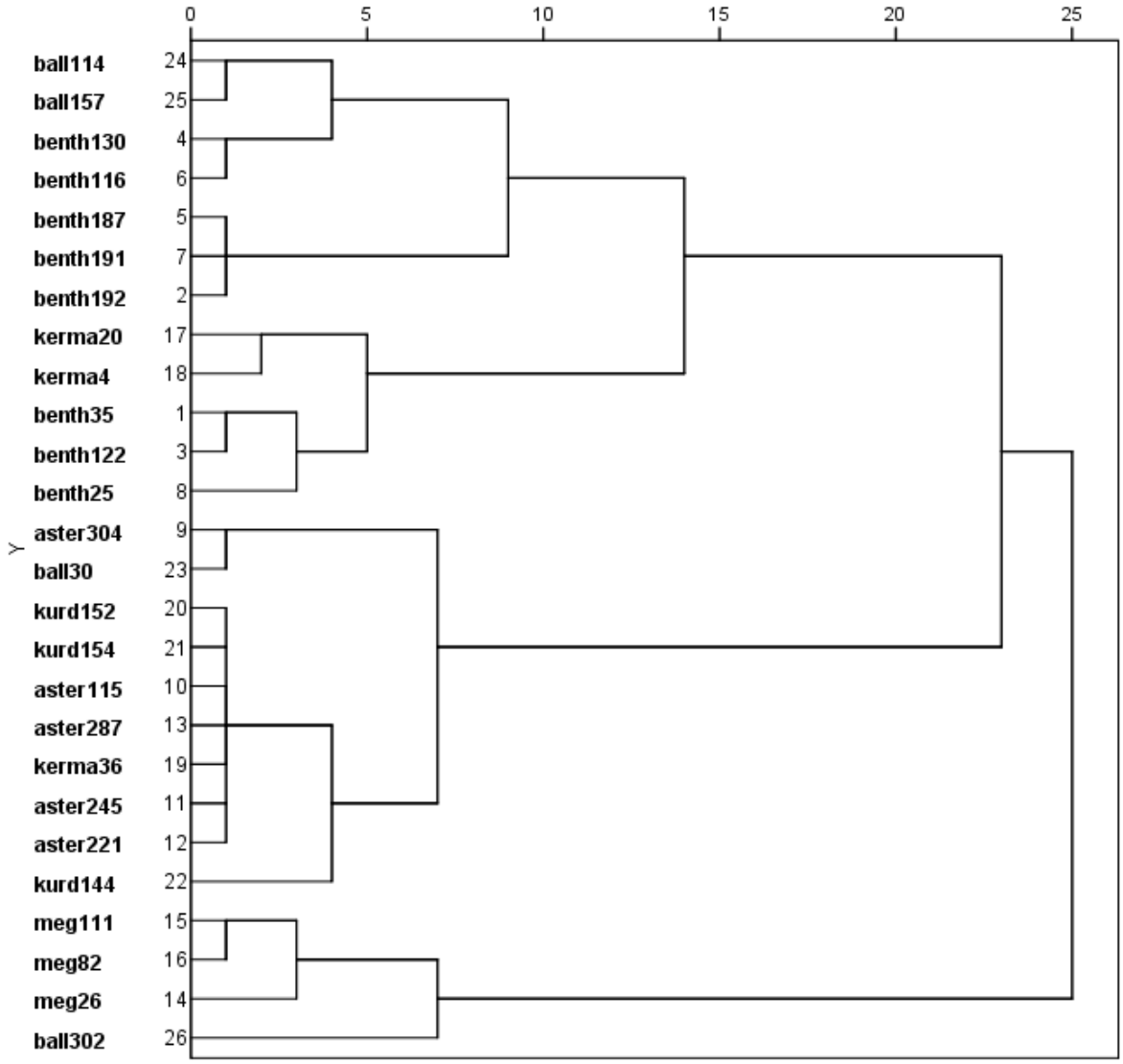

Figure 1. Cluster analysis in six *Stachys* species belonging to Section *Fragilicaulis*. ball: *ballotiformis*, benth: *benthamiana*, kerma: *kermanshahensis*, aster: *asterocalyx*, kurd: *kurdica*, meg: *megalodonta*. X-axis: Distance coefficient, Y-axis: number of each row including the samples.

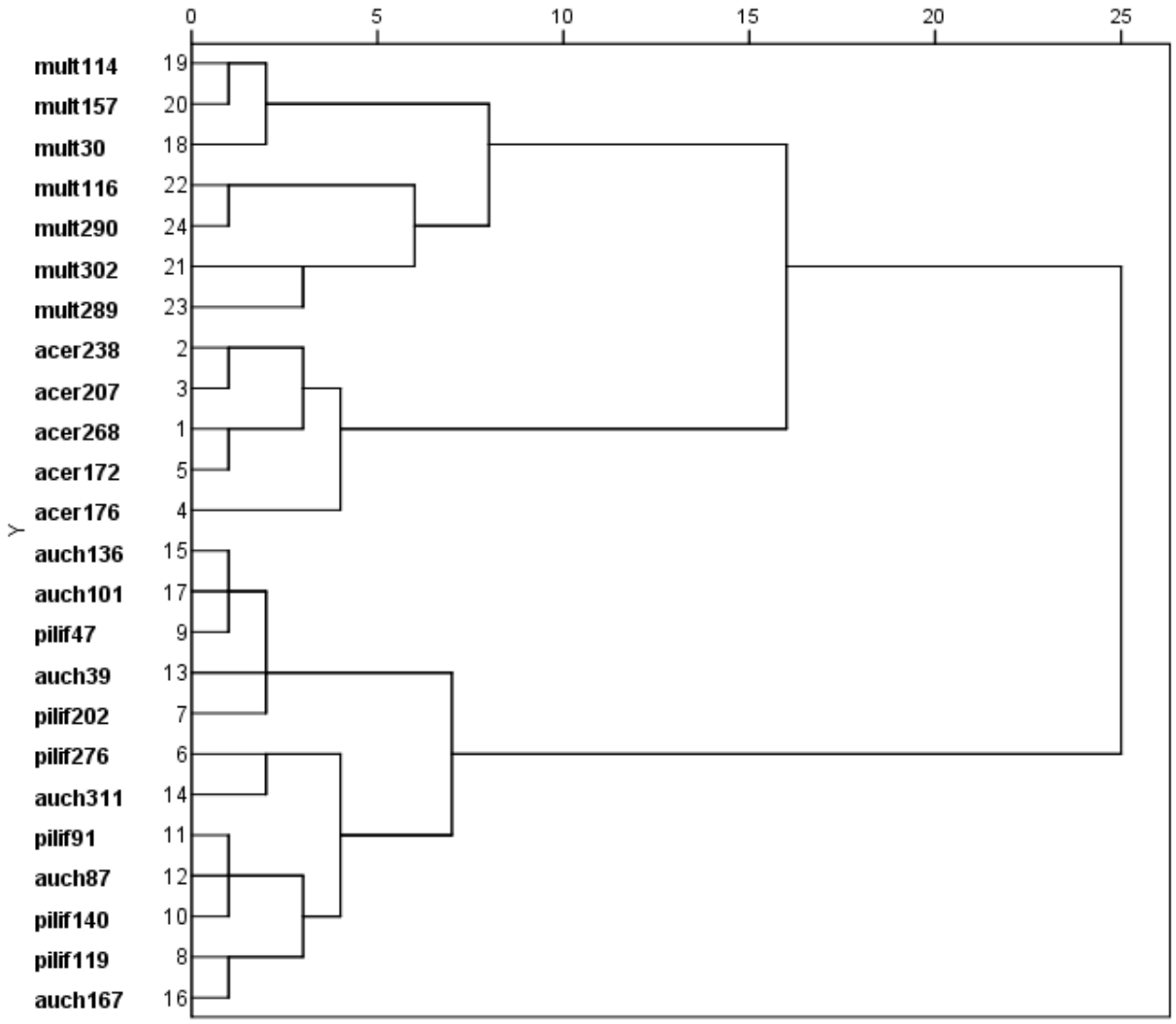

Figure 2. Cluster analysis in four *Stachys* species belonging to Section *Aucheriana*. mult: *multicaulis*, auch: *aucheri*, pilif: *pilifear*, acer: *acerosa*. X-axis: Distance coefficient, Y-axis: number of each row including the samples.

Discussion

The cluster analysis results showed that the flavonoid variations of section *Fragilicaulis* are more related to *S. benthamiana* (four groups) and *S. ballotiformis* (three groups). Similarly, the highest flavonoid variations of section *Aucheriana* were observed in *S. pilifera* and *S. aucheri*. The members of section *Fragilicaulis* are highly similar and these species are not easily discriminated using morphological characters (1, 6). Moreover, the morphological characters in this section are variable particularly in terms of leaf form, leaf, calyx and stem indumentum (1, 10, 23). The flavonoid patterns of *S. kermanshahensis* belonging to sub-section *Multibracteolate* are strongly related to the members of sub-section *Fragiles*, this species has different habits and includes rather polymorphic features (1, 9). The flavonoid patterns of *S. kermanshahensis* form the basis of the relationship between these two sub-sections, which are observed in the types of substitutions (Table 3) and the colour spots in TLC profiles (Table 2a).

Table 3. Flavonoid variation patterns (oxidation) in Stachys species

Variation patterns/ species	Meg.	Ballot	Benth.	Kurd.	Kerma.	Aster.	Pilif	Auch.	Mult.	Acer.
2-hydroxylation	-	-	-	-	-	-	+	-	-	+
3-hydroxylation	-	-	+	+	+	-	+	+	-	+
4-hydroxylation	-	-	-	-	-	-	-	-	-	-
5-hydroxylation	+	+	+	+	+	+	+	+	+	+
6-hydroxylation	-	-	+	-	+	+	-	-	-	+
7-hydroxylation	+	+	+	+	+	+	+	+	+	+
8-hydroxylation	+	+	+	+	+	+	+	+	+	+
2'-hydroxylation	-	-	-	-	-	-	+	-	-	+
3'-hydroxylation	+	-	+	+	+	+	+	+	+	-
4'-hydroxylation	+	+	+	+	+	+	+	+	+	+
5'-hydroxylation	-	-	+	+	-	-	+	+	-	-
3-methoxylation	-	-	+	+	-	-	+	+	-	-
4-methoxylation	-	-	-	-	-	-	+	-	-	-
5-methoxylation	-	-	-	-	-	-	+	+	-	-
6-methoxylation	+	+	+	+	+	-	+	+	-	+
7-methoxylation	+	+	+	+	+	-	+	+	-	+
8-methoxylation	+	-	-	-	+	-	+	+	-	+
3'-methoxylation	-	-	-	-	-	-	+	+	+	+
4'-methoxylation	+	+	+	+	-	+	+	+	+	+
2-carboxylation	-	-	-	-	-	-	+	+	-	-
7-*o*-rutinosyl	-	-	-	-	-	-	-	+	-	-
3-*o*-glucosyl	-	-	-	-	-	-	-	-	-	-
7-*o*-glucosyl	-	+	+	+	-	-	+	+	+	-
6-*c*-glucosyl	-	-	-	-	-	-	+	+	-	-
6,8-di-*c*-glucosyl	+	+	-	+	-	-	+	+	-	+
7-*o*-ß-*D*-glucosyl	+	-	+	-	-	-	-	-	-	-
7-*o*-diglucosyl	-	-	-	-	-	-	+	-	-	-
7-*o*-rhamnoglucosyl	-	-	-	-	-	-	-	-	-	+
7-o-ß-*D*-glucopyranosyl	+	-	-	+	-	+	+	-	-	+
7-*o*-ß-*D*-glucuronide	-	+	-	-	-	-	-	-	-	-
7-*o*-glucuronide	-	-	-	-	-	-	-	-	-	+

meg: megalodonta, ballot: ballotiformis, benth: benthamiana, kurd: kurdica, kerma: kermanshahensis, aster: asterocalyx, pilif: pilifera, auch: aucheri, mult: multicaulis, acer: acerosa.

Table 4. Flavonoid classes with UV spectral data λ max (nm) (based on band I, MeOH, $AlCl_3$, $AlCl_3$/HCl, NaOAc, NaOAc/H_3BO_3) in Stachys species.

Compounds/ Species	Meg	Ballot	Benth	Kurd	Kerma	Aster	pilif	Auch	Mult	Acer
Isoflavones1	-	-	-	-	-	-	-	-	-	1
Isoflavone2	2	-	2	2	2	2	2	2	2	2
Isoflavone3	-	-	-	-	-	-	-	-	-	3
Isoflavone4	-	-	-	-	-	-	4	-	-	-
Isoflavone5	-	-	-	-	-	-	5	5	-	-
Isoflavones6	-	6	-	-	-	-	-	-	-	-
Flavanones1	-	-	-	1	-	-	-	1	-	-
Flavanones2	-	-	-	-	-	-	-	2	-	-
Dihydroflavonols1	-	-	-	-	-	-	-	-	1	-
Flavonols1	-	-	-	-	-	-	1	1	-	-
Flavonols2	-	-	-	-	-	-	2	-	-	-
Flavonols3	-	-	-	-	-	-	3	-	-	-
Chalcones1	-	-	-	-	-	-	1	-	-	1
Chalcones2	-	-	-	-	-	-	2	-	-	-
Flavones1	-	-	-	-	-	-	1	-	-	-
Flavones2	2	2	2	2	2	2	2	2	2	2
Flavones3	-	-	3	3	-	-	3	3	-	-
Flavones4	-	-	4	4	-	-	-	4	-	-
Flavones5		5	5	5					5	
Flavones6	6	6	6	-	-	-	-	-	-	-
Flavones7	-	-	-	-	-	-	7	-	-	-
Flavones8	8	8	-	8	-	-	8	8	-	8
Flavones9			9		9	9				
Flavone10					10					
Flavones11	-	-	-	-	11	-	-	-	-	11
Flavones12	12	-	-	-	12	-	-	12	-	12
Flavones13	-	-	-	-	-	-	-	-	-	13
Flavones14	-	-	-	-	-	-	-	-	-	14
Flavones15	-	-	-	-	-	-	-	-	-	15
Flavone16	-	-	-	-	-	-	-	-	-	16
Flavones17	-	-	-	-	-	-	17	-	-	-
flavonoid aglycone18	-	-	-	18	-	18	18	-	-	18
flavonoid aglycones19	19	-	-	-	-	19	-	-	-	19
Flavones20	-	-	-	20	-	-	20	-	-	-
Flavones21	-	-	-	-	-	-	21	-	-	21
Flavones22	-	-	-	-	-	-	22	-	-	-
Flavones23	-	-	-	-	-	-	23	-	-	-
Flavones24	-	-	-	-	-	-	24	-	-	-
Flavones25	-	25	-	-	-	-	-	-	-	25

meg: *megalodonta*, ballot: *ballotiformis*, benth: *benthamiana*, kurd: *kurdica*, kerma: *kermanshahensis*, aster: *asterocalyx*, pilif: *pilifera*, auch: *aucheri*, mult: *multicaulis*, acer: *acerosa*.

Isoflavones: 1) 311,301,301,334,303, 2) 353,284,285,352,352, 3) 303,300,302,330,303, 4) 306,305,305,306,306, 5) 323,324,317,331,309, 6) 315,367,367,327,317, Flavanones 1: 326,375,371,323,332, 2: 326,383,379,328,326, Dihydroflavonols:1) 302, Flavonols:1) 340,396,402,396,343, 2) 350,430,401,372,367, 3) 340,393,391,364,332, Chalcones: 1) 344,348,347,345,345, 2) 323,384,391,344,325, Flavones: 1) 331,385,382,359,330, 2) 364,366,395,274,287, 3) 335,398,403,336,339, 4) 336,384,381,376,338, 5) 333,386,382,387,340, 6) 334, 359,341,390,350, 7) 345,392,382,394,351, 8) 333,398,381,389,355, 9) 323,375,346,405,333, 10) 344,393,400,405,407, 11) 333,394,393,335,334, 12) 332,407,408,390,336, 13) 342,396,387,366,346, 14) 325,327,327,386,328, 15) 307,307,372,358,309, 16) 338,338,340,334,335, 17) 311,347,408,334,312, 18) 332, 19) 325,347,345,329,327, 20) 325,350,347,386,327, 21) 334,359,357,371,337, 22) 350, 23) 349,357, 24) 347,390,386,396,349, 25) 328,360,350,376,329

Moreover, the trichome morphological studies have shown the stalked glandular trichomes in *S. kermanshahensis* (sub-section *Multibracteolate*) and *S. benthamiana* (sub-section *Fragiles*) (8). Noticeably, the micro-sculpture of nutlets in *S. kermanshahensis* was similar to the members of sub-section *Fragiles*. However, the nutlet and pollen morphology did not demonstrate the separation of these two sub-sections but showed a close relationship between the species (4, 6), which is coincident with our results. On the contrary, *S. kermanshahensis* is an exclusive species based on anatomical features (9). Evidently, the hybridization and gene flow may be accountable for the effectiveness of its chemical constituents as flavonoids within sections (5). In previous reports of morphological characters such as leaf, stem and calyx indumentum and calyx form, *S. ballotiformis* is similar to *S. benthamiana,* which displays high variations in *S. ballotiformis* and might be due to the hybridization between these species (23). This is further confirmed by our flavonoid results showing colour spots and substitutions in different positions (Table 2a, Table3). Investigations of flavonoid results have revealed that *S. megalodonta* was separately clustered as a distinct group since having low variations in TLC profiles and the type of flavonoid class, while Salmaki *et al.* (7) using isoenzyme markers, defined a close relation between *S. benthamiana* and the latter species. This might be related to the geographical conditions, which are observed in flavonoid results. Salmaki *et al.* (7) also provided that the isoenzyme in two accessions of *S. kurdica* showed little variation, and in our results fewer flavonoid variations were detected. In addition, *S. kurdica* is separated from *S. benthamiana* (7). Although Jamzad (23) reported high similarity between them, in flavonoid results *S. kurdica* accessions were not closely grouped with *S. benthamiana* and *S. ballotiformis*. These data have been proven by spot colours in TLC profiles (Table 2a). Certainly, some flavonoid classes and variations in flavonoid patterns confirm this evidence. In one case using isoenzyme, Salmaki *et al.* (7) proved that *S. kurdica* can be closely grouped with *S. ballotiformis*. Excluding the case of nutlet morphology, these two species were exactly distinguished (6), which supports our results. In the taxonomical treatments of previous research, *S. asterocalyx* was introduced as a subspecies of *S. kurdica* (10). However, *S. asterocalyx* was closely grouped with *S. ballotiformis*. *S. kurdica* and *S. asterocalyx* comprised a complex group that belongs to the flavonoid profiles. Furthermore, *S. kurdica* 144 and *S. asterocalyx* 304 demonstrate variations in the aforementioned complex. In the literature, contradictory observations have been reported concerning this section; it seems that intra-specific variability and geographical distribution could have an effect on flavonoid profiles in *Stachys* species (5). Obviously, the trichome and nutlet features in these species seem to be age-dependent in Lamiaceae (6, 8).

All species of section *Aucheriana* are endemic to Iran (1). The flavonoid profiles of *S. pilifera* are closely related to *S. aucheri*, which are observed in our clustering results. Furthermore, the flavonoids of both species were observed with high variations, especially in the type of flavonoid class and other substitutions. Morphologically, these two species present great variations in leaf form, leaf and stem indumentum (1, 10, 23). Based on the distributions of these species, it appears that there was high hybridization between them (23). Noticeably, the flavonoid classes of *S. multicaulis* and *S. acerosa* were

different and strongly separated, although these two species belong to one original cluster. From the viewpoint of nutlet morphology, it is not feasible to find any considerable differences between the species mentioned in this section representing more of a relation between them and the high variation in related species. However, with trichome morphology and anatomical studies, the species in this section were distinguishable (8, 9). Nevertheless, our flavonoid cluster showed the differences between *S. acerosa* and *S. multicaulis*. In contrast, pollen morphology indicates the homogeneity of section *Aucheriana* and does not provide strong evidence for restriction of the two species mentioned above (4). Moreover, it seems that the studied parts such as leaves will produce the different flavonoid compounds. These results are of interest since they support division of taxa in each section in agreement with previous results. Moreover, flavonoid markers are useful in taxonomic problems within this genus.

Based on the flavonoid compounds in *Stachys* species, El-Ansari *et al.* (24) identified 24 flavonoid compounds in *S. aegyptiaca* Pers. Some of which revealed types of flavones and flavone glucosides such as luteolin, apigenin, apigenin-7-*o*-glucoside, isoscutellarin, xanthomicrol, vicenin-2 (apigenin-6,8-di-*c*-glucoside), Lucenin-2, 5,4′-dihydroxy-6,7,8,3′-tetramethoxyflavone, 5,3′,4′-trihydroxy-3,6,7,8-tetramethoxyflavone, 5,4'-dihydroxy-6,7,8,3'-tetramethoxyflavone, 5-hydroxy-6,7,8,3',4'-pentamethoxyflavone, 5-hydroxy-3,6,7,8,4'-pentamethoxyflavone. Other methoxylate flavones and flavanones as naringenin were also identified. Apigenin-7-*o*-glucoside has been observed in *S. bizantina* C. Koch. (25). Conspicuously, the flavones apigenin and luteolin are specific constituents of the Lamiaceae; particularly in *Stachys* genus (24). The root flavonoid from *S. tibetica* Vatke and *S. schtschegleevii* Sosn. ex Grossh. also showed apigenin derivatives, luleolin or flavonoid glycosides (26, 27). These findings strongly agree with our results, since the UV spectral data (λ max, nm) (Table 4) in five types of shift reagents detected a range of flavonoid classes as flavones, flavone glucosides, methylated flavones and flavanones, which were obtained from different spectral data. In these cases we observed spectral data in MeOH band I for different flavones compounds, flavone glucosides, different methylated flavones and for flavanones (Table 4). Other UV spectral data from shift reagents also have been identified in Table 4. Noticeably, the range of UV spectral data in five shift reagents strongly supported the previous research mentioned above. It appears that apigenin and its derivatives occurred in our flavonoid compounds. The presence of flavonoid aglycones in our investigation of *Stachys* species (Table 4) is consistent with previous flavonoid reports; Meremeti *et al.* (28) and Komissarenko *et al.* (29) identified aglycones such as scutellarin, 4'-*o*-methylisoscutellarein, isoscutellarin and isoscutellarein 7-*o*-ß-*D*-glucopyranoside for *S. ionica* Halácsy and *S. inflata* Benth. Moreover, the flavonoid compounds namely hesperidin (flavanones), chryseriol derivatives (flavones), luteoline 7-*o*-ß-*D*-glucoside (flavones), salvigenin (flavones), baicalein derivatives (flavones), eupatorin (flavones) and penduletin (flavonols) were identified in *S. swainsonii* Benth. (5), *S. sylvatica* L., *S. ionica* and *S. palustris* L. (28, 30) and in some of Croatian *Stachys* taxa (31). Ghaffari *et al.* (32) also detected quercetin in *S. lavandulifolia* Vahl. and Bilusic Vundac *et al.* (31) distinguished isoquercetin from some Croatian *Stachys*

taxa. On the whole, the previous reports of flavonoid *Stachys* are in close agreement with our results (Table 4). Obviously, the flavones no. 9, 22, 24 and 25, flavanones no. 1 and 2, and flavonols no. 1, 2 and 3 in MeOH band I were closely confirmed by the above results. It especially seems that the luteolin derivative, chryseriol, salvigenin and baicalein consist of our flavonoid compounds.

In our results, other flavonoid classes with MeOH band I as flavones, penthamethoxyflavone, tetramethoxyflavone, trihydroxyflavone, and isoflavones were confirmed by previous researches on *Stachys* species and some related genera of Lamiaceae as baicalein, norwogonin, pomiferin, cirsimaritin, other hydroxyflavones and methoxyflavones (13, 21, 28,). The new flavonoid classes such as two chalcone derivatives (chalcone no. 1 and 2), four types of isoflavones (isoflavone no. 1, 3, 4 and 6), flavonol no. 3, and flavone no. 17 with five shift reagents appear to be reported here for the first time for the *Stachys* species (Table 4). It can be concluded that the chemical differentiations are correlated to the geographical distribution of the *Stachys* species (28).

From the findings on the final fraction and the UV absorption spectra, it appears tendencies to hydroxylation, methoxylation, glucosylation, diglucosylation, glucopyranosylation, rutinosylation, glucuronidation and rhamnoglucosylation were based on the previous results in *Stachys* species and other related genera of Lamiaceae (5, 13, 19, 21, 24, 29, 33). The highest substitution patterns were found in 5, 7, 8, 3', 4'-hydroxylation and 6 and 7-methoxylation (Table 3). Noticeably, flavone mono-glucoside is mainly distributed in this genus.

On the whole, six flavonoid classes were observed in both *Stachys* sections belonging to ten species, namely 25 flavones. Other classes were six isoflavones, three flavonols, two flavanones, two chalcones and one dihydroflavonol (Table 4). Flavones are the most frequent constituents to have greater amounts of these flavonoid products, and this is in fact the main external compound in ten *Stachys* species. Flavone no. 2 and isoflavone no. 2 with UV spectra in band I and five shift reagents were commonly observed in *Fragilicaulis* and *Aucheriana* taxa. In section *Fragilicaulis* we only observed flavones and isoflavones, and in section *Aucheriana* we recognized flavones, isoflavones, dihydroflavonols, flavanones, chalcones and flavonols. With the exception of flavones, these compounds are less widely distributed within this section. Both sections mainly showed different substitutions in hydroxylation and methoxylation (Table 3). In section *Aucheriana* the substitutions such as glucosyl seem to be effective and differentiate the taxa as *S. acerosa* and *S. multicaulis,* which are also determined in the type of flavonoid constituents namely as isoflavones, chalcones and flavones. Whereas, rutinosyl and glucosyl substitutions caused the similarity of *S. aucheri* and *S. pilifera*. The two mentioned species were different in some types of flavone derivatives, flavone aglycone derivatives and chalcone derivatives. Within section *Fragilicaulis* taxa, in spite of the high similarity of flavonoid profiles, particularly in hydroxylation, other compounds as flavones, isoflavones, flavanones and flavonols and oxidation variations such as methoxylation, glucosyl, glucopyrosyl and glucuronyl substitutions mainly described them. Moreover, the classes of flavone derivatives and flavone aglycones were commonly detected in *S. kermanshahensis, S. asterocalyx, S. kurdica* and *S. benthamiana*. Obviously, the taxa belong to section *Fragilicaulis* mainly

contained flavones in the form of apigenin derivatives. Based on the distribution of flavonoids under taxonomical levels, different flavonoid compounds were identified in different taxonomical statuses (5). Indeed, the *Stachys* genus exhibits considerable variability in its chemical compositions depending on the location and stage of development (34). Moreover, flavonoid glycosides identified the other taxonomic levels such as genus (35).

In conclusion, the secondary metabolites are of great value in identifying the relationships between plants and classification. Moreover, flavonoid compounds have been used effectively for interpreting the taxonomic status among angiosperms. The usefulness of flavonoid in systematic aims has been recognized by many studies (36). Flavonoid constituents in the Stachys species show excessive diversity in the Zagros region of Iran, and these compounds strongly differentiated them. A correlation between the flavonoid patterns and morphology has been frequently found (37). These flavonoids play an important role in the adaptation of plants to different habits. Our research showed that hydroxylation, methoxylation and glycosylation patterns may be considered to be specific to the Stachys species. Their presence could be significant in the taxonomy of this genus and TLC methods give additional information that is useful in identification.

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