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CHEMOTAXONOMY OF *Ballota* SPECIES

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Sixteen taxa of Ballota *were investigated by analyzing the contents of diterpenoid and flavonoid compositions, and the relationships were compared with their morphological properties. HPLC chromatograms of diterpenoids and flavonoids from acetone extracts of sixteen* Ballota *taxa revealed the presence of thirteen compounds. Isolated compounds from Ballota species were evaluated by the unweighted pair-group arithmetic average (UPGMA) clustering method.* B. glandulosissima *is distinct from all other taxa in the dendogram, and this species is morphologically different from other taxa by having a high number of glandular hairs. The second group is composed of* B. saxatilis *ssp.* saxatilis *and* B. inaequidens*; these two species are in close kinship as evidenced by their morphology (similar calyx shape). In the latter clusters at most, the affinities among taxa, as suggested by diterpenoid and flavonoid pattern, are only partially congruent with affinities based on other evidence. In general, morphologic, anatomic characters, distributions, and habitats are not concordant with the clusters. Also, no concordance was found between the sections, phylogenetic order [1], and those of the groups formed by cluster analyses.*

Key words: *Ballota*, diterperpenoids, flavonoids, *Lamiaceae*, chemotaxonomy.

Ballota species have been used in folk medicine as antiulcer, antispasmodic, diuretic, choleretic, antihemorrhoidal, and sedative agents [2, 3], for treatment of wounds and burns, and suppress coughs and upper respiratory inflammation [4, 5]. The antimicrobial, antiinflammatory, hepatoprotective, and antioxidant activities of all *Ballota* species growing in Turkey were investigated by us [6–11].

Patzak [12] put all *Ballota* species in the world (31 species) in to 10 sections. The 12 species belonging to *Acetabulosa*, *Pseudodictamnus, Microselidae,* and *Ballota* sections are present in Turkey (Table 1). *Ballota* (L.) is represented by 12 species and 16 taxa in Turkey [13]. Eleven of 16 taxa are endemic to Turkey [1, 13]. The phylogenetic order list of *Ballota* taxa prepared according to Tezcan [1] is presented in Table 1.

The genus *Ballota* is a rich source of diterpenoids and flavonoids. There are many reports about these compounds of *Ballota* [2, 6, 7, 14–26].

Flavonoids are primarily useful in assessing relationships among closely related species or in studies of infraspecific variation, and they are also occasionally useful in assessing phylogenetic relationships at higher levels [27–29]. Various diterpenoids (20-carbon), triterpenes (30-carbon), and steroids (triterpenes based on the cyclopentane perhydrophenanthrene ring system) are widely distributed and also have some systematic significance [30]. Terpenoids and flavonoids can be used for solving taxonomic problems [31], but no chemotaxonomic report is found in the literature about Turkish *Ballota* species.

The aim of this study is to contribute to their chemotaxonomic determination and establish the diterpenoid and flavonoid profiles of *Ballota* species. The taxonomic classification of plant species was assessed based on their morphological characteristics with diterpenoid and flavonoid contents.

Isolated diterpenoids and flavonoids in *Ballota* taxa are presented in Table 2. HPLC chromatograms of diterpenoids and flavonoids from acetone extracts of sixteen *Ballota* species revealed the presence of thirteen compounds.

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TABLE 1. Turkish Ballota Taxa (Tezcan, 2001) and the Section They Belong to (Patzak, 1959)

Sections	Addreviation	Name	Locality
Acetabulosa	**1**	*Ballota acetabulosa* L. Benth.	B1 Izmir: Yenifoca, 10 m, 18.6 1998, AEF 21602
Pseudodictamnus	**2**	*B. pseudodictamnus* L. Benth. subsp. *lycia* Hub.-Mor	C2 Mugla:Fethiye, 20 m, 12.6 1997 AEF 21603
Microselidae	**3**	*B. cristata* P. H. Davis	C3 Isparta:Egirdir, 910 m, 17.7, 1997, AEF 19899
	4	*B. antalyense* F. Tezcan&H. Duman (not pressed)	C3 Antalya: Turuncova, 150 m, 19.7. 1997, not published
	5	*B. saxatilis* Sieber ex. J.& C. Presl subsp. *saxatilis*	C4 Icel: Anamur, 1530 m, 20.7. 1997, AEF 19904
	6	*B. saxatilis* Sieber ex. J.&C. Presl subsp. *brachyodonta* (Boiss.) P. H. Davis&Doroszenko	C4 Icel: Silifke, 1400 m, 3.7. 1998, AEF 21505
	7	*B. inaequidens* Hub.-Mor&Patzak	C3 Antalya: Alanya, 200 m, 20.7. 1997, AEF 19901
	8	*B. glandulosissima* Hub.-Mor&Patzak	C3 Antalya: Kumluca, 500 m, 19.7. 1997, AEF 19900
	9	*B. larendana* Boiss. & Heldr.	A4 Ankara: Kizilcahamam, 830 m, 28.6. 1998, AEF 21604
	10	*B. latibracteolata* P. H. Davis&Doroszenko	C3 Antalya: Gazipasa, 425 m, 20.7. 1997, AEF 19902
	11	*B. rotundifolia* C. Koch	A8 Erzurum: Tortum Lake, 1200 m, 1.9. 1998, AEF 21606
	12	*B. macrodonta* Boiss. & Bal.	B5 Kayseri: Yahyali, 1150 m, 2.8. 1997, AEF 19907
Ballota	**13**	*B. nigra* L. subsp. *nigra*	A5 Sinop: Boyabat, 370 m, 9.10. 1998, AEF 21607
	14	*B. nigra* L. subsp. *foetida* Hayek	C2 Mugla: Dogusbelen, 600 m, 12.7. 1999, AEF 21608
	15	*B. nigra* L. subsp. *uncinata* (Fiori&Beg.) Patzak	B1 Izmir: Gokcealan, 250 m, 19.6. 1998, AEF 21607
	16	*B. nigra* L. subsp. *anatolica* P. H. Davis	B4 Ankara: Golbasi, 800 m, 28.6. 1998, AEF 21601
	17	*B. nigra* L. subsp. *kurdica* P. H. Davis	We could't find this species at the mentioned localities

The simplified dendrogram created by a simplified unweighted pair-group method with arithmetic averages (UPGMA) and flavonoid and diterpenoid data. According to this dendogram the flavonoid and diterpenoid pattern of *B. glandulosissima* is distinct from all other taxa, which yields kumatakenin, pachypodol, 5-hydroxy-7,3′,4′-trimethoxyflavone, velutin, corymbosin, and retusine, (Table 2) and, lacking diterpenoids, is morphologically different from other taxa by having a high number of glandular hairs. Two main clusters are depicted among the remaining taxa. One group is composed of *B. saxatilis* ssp. *saxatilis and B. inaequidens.* These two species are in close kinship as evidenced by their morphology (like similar calyx shape). The other cluster contains all the remaining other 13 taxa. In the latter clusters at most, the affinities among taxa, as suggested by the diterpenoid and flavonoid pattern, are only partially congruent with affinities based on other evidence. In general, morphologic, anatomic characters, distributions, and habitats are not concordant with the clusters. Also no concordance was found between the sections and those of the groups formed by cluster analyses. But, of course, there are meaningful links; for example, *B. nigra* ssp. *anatolica* and *B. nigra* ssp. *nigra* have clear affinities because they are subspecies of the same species. Also, the clusters are not congruent with the phylogenetic order of Turkish *Ballota* species (Table 1). Therefore more investigations are needed to determine whether their chemical properties are useful taxonomic characters or not for the genus *Ballota*.

TABLE 2. Diterpenoid and Flavonoid Contents of *Ballota* Species

Compound	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Diterpenoids																
Hispanolone	+	+	+	-	+	-	+	-	-	-	+	-	-	-	-	-
Ballonigrine	+	-	+	+	+	+	+	-	+	-	-	-	-	+	-	-
Dehydrohispanolone	+	+	+	+	+	+	-	-	+	+	+	+	-	-	+	-
Flavonoids																
Kumatakenin	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	+
Pachipodol	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-
5-Hydroxy-7,3′,4′-trimethoxyflavone	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-
Velutin	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
Corymbosin	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
5-Hydroxy-3,7,4′-trimethoxyflavone	-	-	-	-	+	-	+	-	-	-	+	-	-	+	-	-
Retusine	-	-	-	-	+	-	+	+	-	-	-	-	-	+	-	-
5-Hydroxy-7,4′-dimethoxyflavone	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-
5-Hydroxy-3,6,7,4′-tetramethoxy flavone	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-
Ladanein	+	-	-	-	+	-	+	-	-	+	+	-	-	-	-	-

EXPERIMENTAL

Sixteen taxa of *Ballota* genus were collected from different localities in Turkey. Designation of the taxa, their localities, sections [12], and abbreviations are given in phylogenetic order [1] Table 1.

Diterpenoids and flavonoids of *Ballota* taxa have been isolated and identified according to Citoglu et al. [2, 24], Sever, [25], and Citoglu et al. [7]. For this aim, air-dried and powdered leaves of the sixteen *Ballota* taxa (25 g each) were extracted with acetone (500 ml each) at room temperature for 3 days. After evaporation, the residue was extracted with EtOAc and the extracts were washed with H_2O and dried. The extracts were concentrated separately to dryness *in vacuo*. The concentrated extracts were dissolved in the mobile phase (100 mL each) [19]. Aliquots (20 mL each) of these solutions were subjected to HPLC.

The extracts were also analyzed for their hispanolone, ballonigrine, dehydrohispanolone, kumatakenin, pachypodol, 5-hydroxy-7,3′,4′-trimethoxyflavone, velutin, corymbosine, 5-hydroxy-3,7,4′-trimethoxyflavone, retusin, 5-hydroxy-7,4′-dimethoxyflavone, 5-hydroxy-3,6,7,4′-tetramethoxyfalavone, and ladanein contents by using thin layer chromatography (TLC) with the $CHCl_3$–MeOH (100:0.5, v/v) solvent system [25].

Chromatography was performed on a Shimadzu LC 10 (Japan) consisting of a Shimadzu LC 10 AD pump, an automated gradient controller, a UK 6 injector, and a Shimadzu SPD-M10 AVP photodiode array detector (PDA). Data were analyzed using LC10 software provided by Shimadzu. The column was a Zorbax–CN (5 μm, 250 × 4.6 mm i.d.) column. The solvent system consisted of *n*-hexane–MeOH (98:2, v/v, speed gradient). The gradient program used is shown below:

Time, min	Total flow rate, mL/min
0.00-9.59	1.3
9.60-13.0	1.7
13.1-22.0	2.5
22.1-35.0	3.2
35.0	stop

n-Hexane and methanol (Merck, Darmstadt, Germany) were of HPLC grade and were filtered through a 0.5 μm filter before use. Elution was carried at 25°C. The eluates were monitored with a photodiode array detector (λ 190–360 nm). The four spectra corresponding to the up-slope, apex, and down-slope of each peak were computer-normalized and superimposed. Peaks were considered pure when there was exact coincidence between the four spectra (match factor ~99.5). On the other hand, the evaluation of each extract content was performed based on the wavelength screening between 190–360 nm, the peak purity, the three dimensional image, and a, *k*, and *N* values.

In addition, the retention times of each peak in the extracts were determined in the presence of standards. After examination of the purity of each peak the presence of every peak in the extract was verified.

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Clustering analysis was performed by Euclidian distance combined with unweighted pair group average linking (UPGMA) by using the NTSYS program written for the IBM PC by Rohlf [32].

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