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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF SOME FLAVONOIDS OF *Ballota* SPECIES

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Ballota L. is a plant belonging to the Lamiaceae family and is represented by 16 taxa in Turkey [1].

Ballota species have been used in Turkish folk medicine as an antiulcer, antispasmodic, diuretic, choleretic, anti-haemorrhoidal, and sedative agents [2, 3]. The antimicrobial activities [4] and the antioxidant activities [5] of all *Ballota* species growing in Turkey was recently reported as well as the antifungal activities of some flavonoids isolated from *B. glandulossima* [6] and *B. inaequidens* [7]. The water extract of *B. glandulosissima* has been reported to have antinociceptive [8], anti-inflammatory, and hepatoprotective activities [9].

Previous investigations of the aerial parts of the *Ballota* species resulted in the isolation of some flavonoids apigenin and apigenin-7-glucoside [10, 11]; kumatakenin, ladanein [10]; genkwanin, nuchensin, isokaempferide, luteolin, luteolin-7-rutinoside, quercetin-3-glycoside, salvigenin, apigenin 6,8-di-C-glycoside [10]; luteolin-7-glycoside [10]; scutallerein-7,4′-dimethylether, acacetin-7-glycoside, chrysoeriol-7-glycoside [10], luteolin-7-lactate, and luteolin-7-glycosyl lactate [12].

In our previous studies, three diterpenoids (hispanolone, ballonigrine, dehydrohispanolone) and ten flavonoids (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′-tetramethoxyflavone, ladanein) were isolated, chemically characterized, and analyzed by HPLC in different species of *Ballota* [6, 7, 13, 14].

Thus, as a continuation of our research [3–9, 13–15] on this genus we now report on the flavonoids present in the acetone extracts from sixteen species of the genus *Ballota*. Our aim is to contribute to their chemotaxonomic determination and establish the flavonoid profiles of *Ballota* species. To our knowledge, no data are available with respect to the chemotaxonomic determination and HPLC analysis of this genus.

Sixteen *Ballota* species (*B. acetabulosa* (L.) Benth., *B. antalyense* F. Tezcan & H. Duman (nom.nud.), *B. cristata* P. H. Davis, *B. glandulosissima* Hub.-Mor & Patzak, *B. inaequidens* Hub.-Mor & Patzak, *B. larendana* Boiss. & Heldr., *B. latibracteolata* P. H. Davis & Doroszenko, *B. macrodonta* Boiss. & Bal., *B. nigra* L. subsp *anatolica* P. H. Davis, *B. nigra* L. subsp. *foetida* Hayek, *B. nigra* L. subsp. *nigra*, *B. nigra* L. subsp. *uncinata* (Fiori & Beg.) Patzak, *B. pseudodictamnus* (L.) Benth. subsp. *lycia* Hub.-Mor., *B. rotundifolia* C. Koch, *B. saxatilis* Sieber ex. J & C. Presl subsp. *brachyodonta* (Boiss.) P. H. Davis & Doroszenko, *B. saxatilis* Sieber ex. J & C. Presl subsp. *saxatilis*) were collected in different locations of Turkey.

Voucher specimens were deposited at the Herbarium of Ankara University, Faculty of Pharmacy (AEF). All solvents were of HPLC grade. The reference flavonoids were isolated from *B. saxatilis* subsp. s*axatilis* and *B. inaequidens* as described in detail previously [7, 13, 14].

Air dried and powdered samples of the sixteen *Ballota* species (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 dissolved in the mobile phase (100 mL each) [16]. Aliquots (20 mL each) of these solutions were subjected to HPLC.

The plant extracts were also analyzed for their 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′-tetramethoxyflavone, and ladanein contents by thin layer chromatography (TLC) with a $CHCl_3$–MeOH (100: 0.5, v/v) solvent system [14].

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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 photo-diode array dedector (PDA). Data were analyzed using LC10 software provided by Shimadzu. The column was a Zorbax–CN (5 μm, 250 × 4.6 mm i.d.). The solvent system consisted of *n*-hexane–MeOH (98:2, v/v, speed gradient).

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 qualitative and quantitative determination of terpenes and flavonoids by HPLC in many Lamiaceae plants is still problematic. The complex matrix of *Ballota* extracts, containing diterpenoids and other polyphenolics as the major impurities, as well as the peak of standards, are the main problem [15, 17]. Because of this feature, to check purity, the eluates were monitored with a photo-diode array dedector (λ 190–360 nm).

In addition, the retention times of each lack 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.

On reviewing the literature regarding the analysis of flavonoids, we found a lack of HPLC systems for studying these compounds, and this prompted us to develop a new HPLC method to be used in the study of the genus *Ballota*.

For the separation of the compounds indicated herein, we utilized reversed-phase chromatography, ion-pair chromatography, and normal-phase chromatography. On the basis of our preliminary studies the best separation of the constituents of the extracts was achieved by normal-phase HPLC. On the other hand, before HPLC analysis, the extracts were also screened for their flavonoid contents by TLC in the presence of standards. The substances were also separated successfully by TLC. Accordingly, due to the insuffiency of the reversed-phase chromatography and ion-pair chromatography in the separation process and the observation of the separation efficiency of the TLC, we modified and used normal-phase HPLC equipped with a PDA dedector. Successive assays were carried out to determine the optimum mobile phase. *n*-Hexane–MeOH (98:2, v/v) was found to be optimal, with speed gradient. Nevertheless, owing to the widely variable amounts of compounds present in the extracts and the existence of other groups of compounds such as diterpenoids, the shifts in the retention times due to the concentrations were observed.

In conclusion, the present study, in which the HPLC method has been developed, showed that extracts of *B. glandulossima* and *B. inaequidens* were the richest in flavonoid content. *Ballota antalyense, B. cristata, B. larendana, B. macrodonta, B. nigra* subsp. *nigra, B. nigra* subsp. *uncinata*, *B. pseudodictamnus* subsp. *lycia*, and *B. saxatilis* subsp. *brachyodonta* do not contain any of the flavonoids examined. On the other hand, *B. nigra* subsp. *anatolica, B. latibracteolata*, and *B. acetabulosa* contained one or all of the flavonoids. This modified method can be applied for the determination of the other flavonoids.

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