

# High Performance Liquid Chromatographic Analysis of some Diterpenoids of the *Ballota* species

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## High Performance Liquid Chromatographic Analysis of some Diterpenoids of the *Ballota* species

**Summary :** In this study, the diterpenoids, namely, hispanolone, ballonigrine and dehydrohispanolone, in 16 *Ballota* taxa growing in Turkey have been determined qualitatively by High-performance Liquid Chromatography (HPLC). Analysis was performed on a normal phase column (Zorbax-CN, 5 µm, 250 x 4.6 mm i.d.). The solvent system consisted of n-Hexane-MeOH (98:2, speed gradient). Peaks were detected at 190-360 nm using Photo-diode array (PDA) dedector. The extracts of *Ballota acetabulosa*, *B. pseudodictamnus subsp. lycia*, *B. cristata*, *B. inaequidens*, *B. saxatilis subsp. saxatilis*, *B. saxatilis subsp. brachyodonta*, *B. larendana*, *B. latibracteolata*, *B. rotundifolia*, *B. macrodonta*, *B. nigra subsp. foetida*, *B. nigra subsp. uncinata* and *B. antalyense* were found to contain the diterpenoids.

**Keywords:** HPLC analysis, Diterpenoids, *Ballota*, Lamiaceae

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## *Ballota* Türlerinde Bulunan Bazı Diterpenlerin Yüksek Basınçlı Sıvı Kromatografisi ile Analizi

**Özet:** Bu çalışmada, diterpenoid yapısındaki hispanolon, ballonigrin ve dehidrohispanolon'un Türkiye'de yetişen 16 *Ballota* taksonundaki dağılımı yüksek basınçlı sıvı kromatografisi (YBSK) ile nitel olarak incelenmiştir. Analiz normal faz kolon (Zorbax-CN, 5 µm, 250 x 4.6 mm iç çap) kullanılarak gerçekleştirilmiştir. Kullanılan solvan sistemi n-Heksan-MeOH (98:2, hız gradiyenti) dir. Pikler foto -diod array (PDA) dedektör kullanılarak 190-360 nm'de değerlendirilmiştir. *Ballota acetabulosa*, *B. pseudodictamnus subsp. lycia*, *B. cristata*, *B. inaequidens*, *B. saxatilis subsp. saxatilis*, *B. saxatilis subsp. brachyodonta*, *B. larendana*, *B. latibracteolata*, *B. rotundifolia*, *B. macrodonta*, *B. nigra subsp. foetida*, *B. nigra subsp. uncinata* ve *B. antalyense* diterpenoid yapı maddeler içermektedir.

**Anahtar kelimeler:** YBSK analizi, Diterpen, *Ballota*, Lamiaceae

## INTRODUCTION

*Ballota* L. is a plant belonging to the *Lamiaceae* family and is represented by 16 taxa in Turkey<sup>1</sup>.

*Ballota* species have been used in Turkish folk medicine as an antiulcer, antispasmodic, diuretic, chole-retic, antihæmorrhoidal and sedative agent<sup>2</sup>. *Ballota nigra* is used externally in the treatment of wounds and burns. It is used internally to suppress coughs and upper respiratory inflammation<sup>3,4,5</sup>. Vural et al. reported that *B. nigra* subsp. *anatolica* and *B. larendana* have an antidepressant activity. *B. larendana* has also an anxiolytic activity<sup>6</sup>. Another study reported that *B. acetabulosa* is used for the

treatment of hæmorrhoids as an infusion in folk medicine<sup>7</sup>. The antimicrobial activities of all *Ballota* species growing in Turkey were investigated by us<sup>8</sup>. The genus *Ballota* is a rich source of diterpenoids. Previous investigations of the aerial parts of the plant resulted in the isolation of labdane diterpenoids -ballotinone, ballotenol, marrubiin, marrubeno<sup>9-11</sup>, ballonigrine, ballonigrinone, 7 $\alpha$ -acetoxymarrubiin<sup>12</sup>, 13-hydroxyballonigrinolide<sup>13</sup>, balloaucherolide<sup>14</sup>, preleosibirine<sup>15,16</sup>, 18-hydroxyballonigrine<sup>11,17</sup>, hispanolone, hispaninic acid and hispanoic acid<sup>18</sup>.

In our previous studies, three diterpenoids (hispanolone, ballonigrine, dehydrohispanolone) and ten

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flavonoids (kumatakenin, pakipodol, 5-hydroxy 7, 3',4' trimethoxyflavone, velutin, corymbosine, 5-hydroxy 3,7,4'trimethoxy flavone, retusin, 5-hydroxy 7, 4' dimethoxy flavone, flindulatine, lada-  
nein) were isolated, chemically characterized and analysed by HPLC in different species of *Ballota*<sup>19-22</sup>. The structures of isolated diterpenoids are shown in Fig 1.

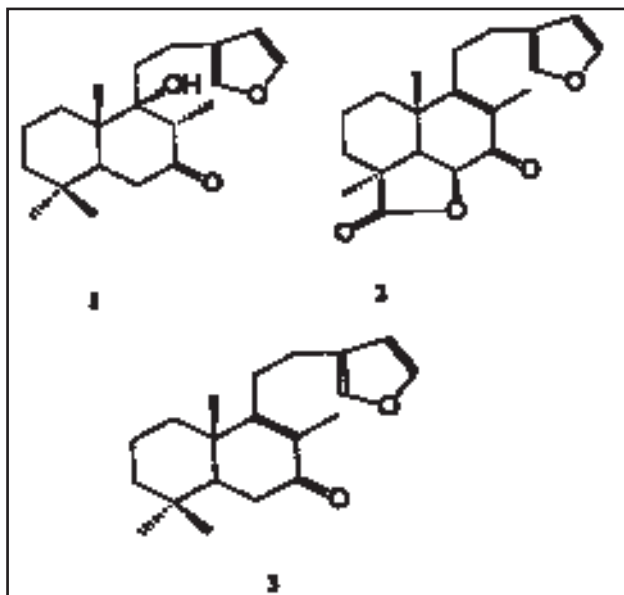


Fig. 1 The structures of hispanolone **1**, ballonigrine **2**, dehydrohispanolone **3**

This paper is a part of our on-going studies<sup>8,19-22</sup> on this genus in which we attempt to identify the diterpenoids present in the acetone extracts from sixteen species of the genus *Ballota*. Our aim is to contribute to their chemotaxonomic determination.

## EXPERIMENTAL

### Plant material.

Sixteen species of *Ballota* were collected from different parts of Turkey. Designation of the individual species and their origin are given in Table 1.

**Table 1.** The names and origins of *Ballota* species used in this study

|  |  |
|--|--|
| <i>B. acetabulosa</i>                          | B1 İzmir:Yeniçağa, 10m, 18.6.1998, AEF 21602           |
| <i>B. pseudodictamnus</i> subsp. <i>lycia</i>  | C2 Muğla:Fethiye, 20m, 12.6.1997 AEF 21603             |
| <i>B. cristata</i>                             | C3 Isparta:Eğirdir, 910 m, 17.7.1997, AEF19899         |
| <i>B. inaequidens</i>                          | C3 Antalya: Alanya, 200m, 20.7.1997, AEF 19901         |
| <i>B. saxatilis</i> subsp. <i>saxatilis</i>    | C4 İçel: Anamur, 1530m, 20.7.1997, AEF 19904           |
| <i>B. saxatilis</i> subsp. <i>brachyodonta</i> | C4 İçel: Silifke, 1400 m, 3.7.1998, AEF 21505          |
| <i>B. glandulosissima</i>                      | C3 Antalya:Kumluca, 500m, 19.7.1997, AEF 19900         |
| <i>B. larendana</i>                            | A4 Ankara:Kızılcahamam, 830m, 28.6.1998, AEF 21604     |
| <i>B. latibracteolata</i>                      | C3 Antalya: Gazipaşa, 425 m, 20.7.1997, AEF 19902      |
| <i>B. rotundifolia</i>                         | A8 Erzurum: Tortum Lake, 1200 m, 1.9.1998, AEF 21606   |
| <i>B. macrodonta</i>                           | B5 Kayseri:Yahyalı, 1150 m, 2.8.1997, AEF 19907        |
| <i>B. nigra</i> subsp. <i>nigra</i>            | A5 Sinop: Boyabat, 370 m, 9.10.1998, AEF 21607         |
| <i>B. nigra</i> subsp. <i>foetida</i>          | C2 Muğla: Doğuşbelen, 600m, 12.7.1999, AEF 21608       |
| <i>B. nigra</i> subsp. <i>uncinata</i>         | B1 İzmir: Gökçealan, 250 m, 19.6.1998, AEF 21607       |
| <i>B. nigra</i> subsp. <i>anatolica</i>        | B4 Ankara: Gölbaşı, 800 m, 28.6.1998, AEF 21601        |
| <i>B. antalyense</i>                           | C3 Antalya: Turunçova, 150 m, 19.7.1997, not published |

Sixteen samples of *Ballota acetabulosa* (L.) Benth., *B. pseudodictamnus* (L.) Benth. subsp. *lycia* Hub.-Mor., *B. cristata* P.H.Davis, *B. inaequidens* Hub.-Mor.&Patzak, *B. saxatilis* Sieber ex J.&C.Presl subsp. *saxatilis*, *B. saxatilis* Sieber ex J.&C.Presl subsp. *brachyodonta* (Boiss.) P.H. Davis&Doroszenko, *B. glandulosissima* Hub.-Mor.&Patzak, *B. larendana* Boiss.&Heldr., *B. latibracteolata* P.H. Davis&Doroszenko, *B. rotundifolia* C.Koch, *B. macrodonta* Boiss.&Bal., *B. nigra* L. subsp. *nigra*, *B. nigra* L. subsp. *foetida* Hayek, *B. nigra* L. subsp. *uncinata* (Fiori&Beg) Patzak, *B. nigra* L. subsp. *anatolica* P.H. Davis and *B. antalyense* F.Tezcan& H. Duman nom. nud. were obtained from the different parts of Turkey. Voucher specimens have been deposited at the Herbarium of Ankara University, Faculty of Pharmacy (AEF). All solvents were of HPLC grade. The reference substances hispanolone **1**, ballonigrine **2** and dehydrohispanolone **3** were isolated from *B. saxatilis* subsp. *saxatilis* and *B. inaequidens* as described in detail previously<sup>19,21</sup>.

### Extraction of plant materials:

Air dried and powdered specimens of the sixteen *Ballota* species (25 g of each) were extracted with acetone (500 ml of each) at room temperature for 3 days, respectively. After evaporation, the residue

was extracted with EtOAc and the extracts were washed with H<sub>2</sub>O and dried. The extracts were concentrated separately to dryness in *vacuo*. The concentrated extracts dissolved in the mobile phase (100 ml of each)<sup>18</sup>. Aliquots (20 µl of each) of these solutions were subjected to HPLC.

The plant extracts were also analysed for their hispanolone, ballonigrine and dehydrohispanolone contents by using thin layer chromatography (TLC) with CHCl<sub>3</sub>:MeOH (100: 0.5, v/v) solvent system<sup>21</sup>.

### HPLC analysis

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 detector (PDA). Data was analysed 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). The used gradient programme was as 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 out at 25 °C. The qualitative and quantitative determination of terpenes by HPLC is still problematic and limited. The complex matrix of *Ballota* extracts, containing polyphenolic compounds such as flavonoids as the major impurities, as well as the leak of standards, are the main problems<sup>23</sup>. Because of this feature, the eluates were monitored with a photo-diode array detector (λ=190-360 nm) to check purity. 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 α, 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.

### RESULTS AND DISCUSSION

On reviewing the literature regarding the analysis of diterpenoids, we found a lack of HPLC systems for studying these compounds, and this induced us to develop a new HPLC method to be applied to 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 diterpenoid contents by TLC in the presence of standards. The substances were also separated successfully by TLC. Accordingly, due to the insufficiency 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 PDA detector. 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 fact that there were widely variable amounts of compounds present in extracts and the existence of other groups of compounds such as flavonoids, the shifts in the retention times due to the concentrations were observed.

The diterpenoids in the ethanolic extracts of some *Ballota* species were identified by HPLC comparing their retention times (Rt) with those of authentic standards: a representative three dimensional image is shown in Figs 2, 3 and 4.

This modified method can be applied for the deter-

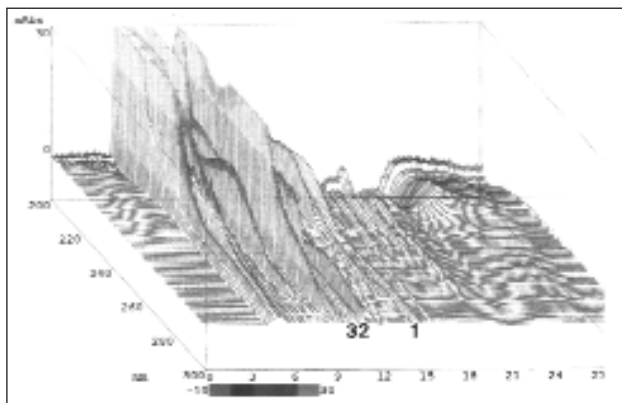


Fig. 2 Three dimensional image of *Ballota cristata*  
1 hispanolone, 2 ballonigrine, 3 dehydrohispanolone

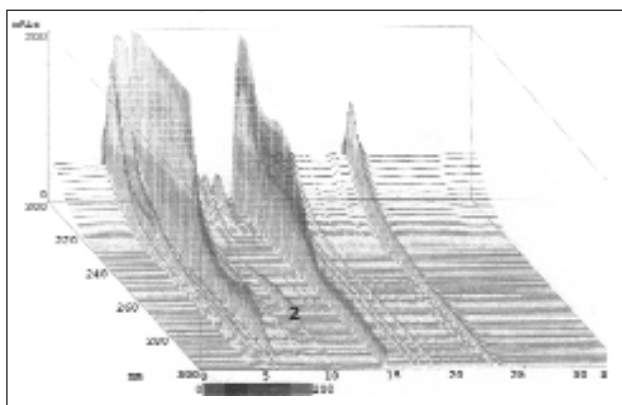


Fig 3. Three dimensional image of *Ballota nigra* subsp.  
*foetida* 2 ballonigrine

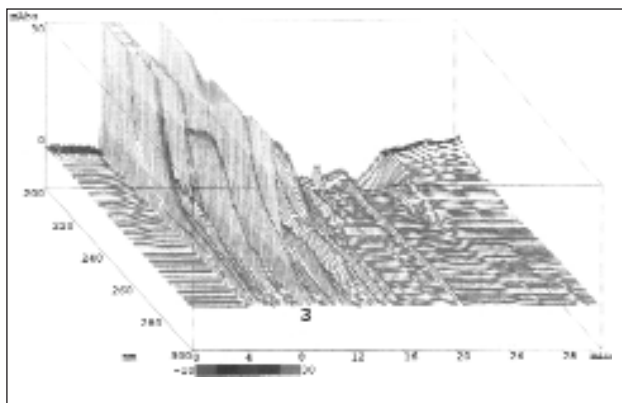


Fig 4. Three dimensional image of *Ballota macrodonta*  
3 dehydrohispanolone

mination of the other labdane-type diterpenoids.

The diterpenoid contents of the plant extracts are shown in Table 2. *Ballota glandulosissima*, *B. nigra* subsp. *nigra* and *B. nigra* subsp. *anatolica* do not contain any of the diterpenoids examined. *B. acetabulosa*, *B. cristata* and *B. saxatilis* subsp. *saxatilis* contain all of the diterpenoids investigated. *B. pseudodictamnus* subsp. *lycia*, *B. inaequidens*, *B. saxatilis* subsp. *brachyodonta*, *B. larendana*, *B. rotundifolia* and *B. antalyense* contain two of the diterpenoids and *B. latibracteolata*, *B. macrodonta*, *B. nigra* subsp. *foetida* and *B. nigra* subsp. *uncinata* contain only one of the diterpenoids.

**Table 2.** Diterpenoid contents of *Ballota* species

| Samples  | 1 | 2 | 3 |
|--|---|---|---|
| <i>B. acetabulosa</i>                          | + | + | + |
| <i>B. pseudodictamnus</i> subsp. <i>lycia</i>  | + | - | + |
| <i>B. cristata</i>                             | + | + | + |
| <i>B. inaequidens</i>                          | + | + | - |
| <i>B. saxatilis</i> subsp. <i>saxatilis</i>    | + | + | + |
| <i>B. saxatilis</i> subsp. <i>brachyodonta</i> | - | + | + |
| <i>B. glandulosissima</i>                      | - | - | - |
| <i>B. larendana</i>                            | - | + | + |
| <i>B. latibracteolata</i>                      | - | - | + |
| <i>B. rotundifolia</i>                         | + | - | + |
| <i>B. macrodonta</i>                           | - | - | + |
| <i>B. nigra</i> subsp. <i>nigra</i>            | - | - | - |
| <i>B. nigra</i> subsp. <i>foetida</i>          | - | + | - |
| <i>B. nigra</i> subsp. <i>uncinata</i>         | - | - | + |
| <i>B. nigra</i> subsp. <i>anatolica</i>        | - | - | - |
| <i>B. antalyense</i>                           | - | + | + |

In conclusion, the present study, for which the HPLC method has been developed, showed that the extracts of *B. acetabulosa*, *B. pseudodictamnus* subsp. *lycia*, *B. cristata*, *B. inaequidens*, *B. saxatilis* subsp. *saxatilis*, *B. saxatilis* subsp. *brachyodonta*, *B. larendana*, *B. latibracteolata*, *B. rotundifolia*, *B. macrodonta*, *B. nigra* subsp. *foetida*, *B. nigra* subsp. *uncinata* and *B. antalyense* contained one to all of the diterpenoids.

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## REFERENCES

1. Davis PH. Flora of Turkey and the East Aegean Islands. 7, Edinburgh, Edinburgh University Press, pp 156-160, 1982.
2. Baytop T. Türkiye'de Bitkilerle Tedavi (Therapy with Medicinal Plants in Turkey), İstanbul, Sanal Matbaacılık, pp 371, 1984.
3. Yeşilada E, Honda G, Sezik E, Tabata M, Goto K, Ikehira Y. Traditional medicine in the Mediterranean subdivision, *J. Ethnopharmacol.*, 39, 31-38, 1993.
4. Yeşilada E, Honda G, Sezik E, Tabata M, Fujita T, Tanaka T, Takeda Y, Takaishi Y. Traditional medicine in Turkey V. Folk medicine in the inner Taurus Mountains, *J. Ethnopharmacol.*, 46, 133-152, 1995.
5. Tuzlacı E, Tolon E. Turkish folk Medicinal plants, part III: Şile (İstanbul), *Fitoterapia*, 71, 673-685, 2000.
6. Vural K, Ezer N, Erol K, Şahin FP. Anxiolytic and antidepressant activities of some *Ballota* species, *J. Fac. Pharm. Gazi*, 13, 29-32, 1996.
7. Meriçli AH, Meriçli F, Tuzlacı E. Flavonoids of *Ballota acetabulosa*, *Acta Pharm. Turcica*, 30, 143-144, 1988.
8. Çitoglu GS, Yılmaz BS, Altanlar N. Antimicrobial activity of *Ballota* species growing in Turkey, *J. Fac. Pharm. Ankara*, 32, 93-97, 2003.
9. Savona G, Piozzi F, Hanson JR, Siverns M. Structures of ballotinone, a diterpenoid from *Ballota nigra*, *J. Chem. Soc. Perkin I*, 3, 1607-1609, 1976.
10. Savona G, Piozzi F, Hanson JR, Siverns M. The structures of ballotenol, a new diterpenoid from *Ballota nigra*, *J. Chem. Soc. Perkin I*, 5, 497-499, 1977.
11. Savona G, Bruno M, Piozzi F, Barbagallo C. Diterpenes from *Ballota* species, *Phytochemistry*, 21, 2132-2133, 1982.
12. Savona G, Piozzi F, Hanson JR, Siverns M. Structures of three new diterpenoids from *Ballota* species, *J. Chem. Soc. Perkin I*, 1, 322-324, 1977.
13. Savona G, Piozzi F, Hanson JR. 13-hydroxyballonigrinolide, a new diterpenoid from *Ballota lanata*, *Phytochemistry* 17, 322-324, 1978.
14. Rustaiyan A, Mosslemin-Kupaii MH, Zdero C. Furo-labdanes and related compounds from *Ballota aucheri*, *Phytochemistry*, 31, 344-346, 1992.
15. Bruno M, Savona G, Pasqual C, Rodriguez B. Preleosi-birin, prefuranic labdane diterpene from *Ballota nigra* subsp. *foetida*, *Phytochemistry*, 25, 538-539, 1986.
16. Seidel F, Bailleul F, Tillequin F. Isolation from *Ballota nigra* L. of 13-hydroxyballonigrinolide, a diterpene useful for the standardization of the drug. *J. Pharm. Belg.*, 51, 72-73, 1996.
17. Bruno M, Bondi ML, Piozzi F, Arnold NA, Simmonds MSJ. Occurrence of 18-hydroxyballonigrine in *Ballota saxatilis* subsp. *saxatilis* from Lebanon, *Biochem. Syst. Ecol.*, 29, 429-431, 2001.
18. Rodriguez B, Savona G, Piozzi F. Two new unusual diterpenoids from *Ballota hispanica*, *J. Org. Chem.*, 44, 2219-2221, 1979.
19. Çitoğlu G, Tanker M, Sever B, Englert J, Anton R, Altanlar N. Antibacterial activities of diterpenoids isolated from *B. saxatilis* subsp. *saxatilis*, *Planta Med.*, 64, 484, 1998.
20. Çitoğlu G, Tanker M, Sever B. Flavonoid aglycones from *Ballota saxatilis* subsp. *saxatilis*, *Pharm. Biol.*, 37, 158-160, 1999.
21. Sever B. The investigation of diterpenoid and flavonoid contents of *Ballota* species growing in Turkey, PhD Thesis, Ankara, 2000.
22. Çitoğlu GS, Sever B, Antus S, Baitz-Gacs E, Altanlar N. Antifungal flavonoids from *Ballota glandulosissima*, *Pharm. Biol.*, 41, 2003 (in Press)
23. Gomez-Serranillos P, Palomino OM, Villarrubia AI, Cases MA, Carreteo E, Villar A. Analysis of diterpenoids from *Sideritis* species by reversed-phase high performance liquid chromatography, *J Chromatog A*, 778, 421-425, 1997.