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Short communication

Determination of Total *Ortho*-Dihydroxycinnamic Acid Derivatives and Flavonoid Contents of *Ballota* Species Growing in Turkey

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Ballota species have been used in Turkish folk medicine for treatment of wounds and burns, suppression cough and upper respiratory inflammation. According to the previous phytochemical investigations terpenoids, flavonoids, phenylpropanoids, essential oils, tannins, and saponins were isolated from *Ballota* species. The aim of the present research is to evaluate total *ortho*-dihydroxycinnamic acid derivatives (TCC) and total flavonoid content (TFC) of *Ballota* species by spectrophotometrically according to European Pharmacopoeia. The results of this study show that; *Ballota* species have distinct phenolic contents. *Ballota nigra* subsp. *anatolica* extract contained the highest amount of *ortho*-dihydroxycinnamic acid derivatives, while highest proportion of flavonoids was found in *Ballota glandulosissima*. On the other hand, *Ballota nigra* subsp. *foetida* extract contained the lowest amount of *ortho*-dihydroxycinnamic acid derivatives, while lowest proportion of flavonoids was found in *Ballota rotundifolia*.

Key words: *Ballota, Ortho*-dihydroxycinnamic acid, Flavonoids, Spectrophotometry.

Türkiye'de Yetişen *Ballota* Türlerinin Toplam *O*-dihidroksisinnamik asit ve Flavonoit Miktarlarının Belirlenmesi

Ballota türleri Türkiye'de halk arasında yara ve yanıkların tedavisinde, öksürüğe karşı ve üst solunum yolu enfeksiyonlarında kullanılmaktadır. Daha önce yapılan fitokimyasal çalışmalarda *Ballota* türlerinden terpenoit, flavonoit, fenil propanoit, uçucu yağ, tanen ve saponozit yapısında bileşikler izole edilmiştir. Bu çalışmanın amacı, *Ballota* türlerinin içerdiği toplam *orto*-dihidroksisinnamik asit türevlerini (TSİ) ve toplam flavonoit (TFİ) içeriklerini Avrupa Farmakopesine göre spektrofotometrik olarak değerlendirmektir. Bu çalışmanın sonuçları *Ballota* türlerinin değişik miktarlarda fenolik içeriğe sahip olduğunu göstermiştir. *Ballota nigra* subsp. *anatolica orto*-dihidroksisinnamik asit türevi bileşikleri en yüksek oranda taşırken, *Ballota glandulosissima* flavonoitleri en yüksek oranda taşımaktadır. Diğer taraftan, *Ballota nigra* subsp. *foetida orto*-dihidroksisinnamik asit türevleri en düşük oranda taşırken, *Ballota rotundifolia* flavonoitleri en düşük oranda taşımaktadır.

Anahtar kelimeler: *Ballota, Orto*-dihidroksisinnamik asit, Flavonoit, Spektrofotometri.

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INTRODUCTION

The genus *Ballota* L. (Lamiaceae) is represented by 16 taxa in Turkey (1). *Ballota* species have been used in Turkish folk medicine as an antiulcer, antispasmodic, diuretic, choleretic, antihaemorrhoidal, and sedative agents (2,3), for treatment of wounds and burns, and suppress cough and upper respiratory inflammation (4,5). Previously, the antimicrobial, antinociceptive, anti-inflammatory, hepatoprotective, antilisterial,

and antioxidant activities of *Ballota* species growing in Turkey were investigated by us (6-16).

In the previous phytochemical investigations, terpenoids, flavonoids, phenylpropanoids, essential oils, tannins, and saponins were isolated from *Ballota* species. Likewise, in our study, we also isolated diterpene and flavonoid compounds from *Ballota saxatilis* subsp. *saxatilis, B. glandulosissima* and *B. inaequidens* (6-8,13). Moreover there are several studies which reports phenolic contents of other *Ballota* species which seem to be rich in flavonoids (e.g. methoxy flavone derivatives, retusin and pachypodol in *B. inaequidens*; kumatakenin, pachypodol, 5-hydroxy-7,3',4'-trimetoxyflavone, velutin, salvigenin retusin, corymbosin in *B. glandilosissima*; scutellarein, apigenin and chrysoeriol derivatives, eutigoside A in *B. acetabulosa*; eupatorin in *B. limbata*; kaempferol and quercetin derivatives, ladanein in *B. saxatilis*; apigenin and quercetin derivatives, vicenin 2, salvigenin, kumatakenin, genkwanin, ladanein, nuchensin, isokaempferide in *B. hirsuta*; apigenin-7-glucoside and vicenin 2 in *B. foetida*; apigenin and luteolin glucosides and rutin in *B. undulata*; luteolin and scutellarein derivatives in *B. andreuzziana*; apigenin glucosides in *B. larendana* and *B. pseudodictamnus*) and phenylpropanoids (e.g. forsythoside B, lydionotoside, verbascoside and betonyoside F in *B. undulata*; caffeoylmalic acid, acteoside, forsythoside B and two chrysoeriol heterosides in *B. pseudodictamnus*; verbascoside, forsythoside B and caffeoylmallic acid in *B. hirsuta* and *B. rupestris* (7,8,17-20).

Ballota nigra is the only *Ballota* species included in European Pharmacopoeia and according to the monograph of *Ballota nigra,* the determination of phenolic content is conducted through thre determination of *ortho*-dihydroxycinnamic acid derivatives.

Polyphenols are products of the secondary metabolism of plants. They arise biogenetically from two main synthetic patways: the shikimate patway and the acetate pathway. This is an extremely wide and complex group of plant substances. Polyphenols can be divided into at least 10 different classes depending on their basic chemical structure. Flavonoids, which constitute the most important single group, can be further subdivided into 13 classes, with more than 5000 compounds described. Phenylpropanoid derivatives (C6-C3) also are an important group of low-molecular-weight phenolics. The most important phenylpropanoids are hydroxycinnamic acids (*p*-coumaric, caffeic, ferulic, sinapic) and derivatives (21). Polyphenols may have important applications in the prevention and treatment of highly prevalent human disease, such as degenerative diseases particularly cardiovascular disease and cancer, as well as gastric and duodenal ulcer, allergy, vascular fragility, viral and bacterial infections (21,22).

In recent years, interest in food phenolics has increased owing to their roles as antioxidant, antimutagens, and scavengers of free radicals and their implication in the prevention of pathologies such as cancer and cardiovascular disease. Epidemiologic studies have shown a correlation between an increased consumption of phenolic antioxidants and a reduced risk of cardiovascular disease and certain types of cancer (21).

The aim of this study is to investigate whether the *Ballota* species which are growing in Turkey are good resources with respect to their phenolic contents.

EXPERIMENTAL

Plant material

Botanical name, collection sites, altitudes, dates, and herbarium numbers of the 16 *Ballota* taxons used in this study are given in Table 1. Voucher specimens of the plants were deposited in the Herbarium of the Faculty of Pharmacy, Ankara University (AEF), Ankara, Turkey (Table 1).

Preparation of extracts

Extracts, stock solutions and test solutions were prepared according to the method of European Pharmacopeia 7.0.

Table 1. *Ballota* taxons studied in this research

Plant	Collection site, altitude and date	Herbarium number
Ballota acetabulosa Benth.	B1: İzmir: Yenifoça, 10 m 18.6.1998	AEF No: 21602
Ballota pseudodictamnus Bentham subsp. *lycia* Hub.-Mor.	C2: Muğla: Fethiye, 20 m, 12.6.1997	AEF No: 21603
Ballota inaequidens Hub.-Mor.&Patzak	C3: Antalya: Alanya, 200 m, 20.7.1997	AEF No: 19901
Ballota cristata P.H. Davis	C3: Isparta: Eğirdir, 910 m, 17.7.1997	AEF No: 19899
Ballota saxatilis Sieber ex J.&C. Presl subsp. *saxatilis* Banks&Sol.	C4: İçel: Anamur, 1530 m, 20.7.1997	AEF No: 19904
Ballota saxatilis Sieber ex J.&C. Presl subsp. *brachyodonta* Davis&Doroszenko	C4: İçel: Silifke, 1400 m, 3.7.1998	AEF No: 21505
Ballota glandulosissima Hub.-Mor.&Patzak	C3: Antalya: Kumluca, 500 m, 19.7.1997	AEF No: 19900
Ballota larendana Boiss.&Heldr.	A4: Ankara: Kızılcahamam, 830 m, 28.6.1998	AEF No: 21604
Ballota latibracteolata Davis&Doroszenko	C4: Antalya: Gazipaşa, 425 m, 20.7.1997	AEF No: 19902
Ballota rotundifolia C. Koch.	A8: Erzurum: Tortum Lake, 1200 m, 11.9.1998	AEF No: 21606
Ballota macrodonta Boiss.&Bal.	B5: Kayseri: Yahyalı, 1150 m, 2.8.1997	AEF No: 19907
Ballota nigra L. subsp. *nigra* Sw.	A5: Sinop: Boyabat, 370 m, 9.10.1998	AEF No: 21609
Ballota nigra L. subsp. *foetida* Hayek	Muğla: Döğüşbelen, 600 m, 12.7.1999	AEF No: 21608
Ballota nigra L. subsp. *uncinata* Patzak	C6: Kahramanmaraş, şehir içi 650-700 m, 16.7.1998	AEF No: 21607
Ballota nigra L. subsp. *anatolica* P.H. Davis	B4: Ankara: Gölbaşı, 800 m, 28.6.1998	AEF No: 21601
Ballota antalyense F.Tezcan & H.Duman	C3: Antalya: Finike; Turunçova-Elmalı yolu 5. km makilik 170 m	GAZI F. Tezcan 1701

Determination of total ortho-dihydroxycinnamic acid derivatives content

Preparation of stock solution: 1 gram powdered drug was extracted in 90 mL ethanol (50% *v/v*) under reflux condenser, on water bath. After 30 minutes of extraction, the mixture was cooled, filtered and diluted to 100 mL with 50% *v/v* ethanol.

Preparation of test solution: 2 mL 0.5 M hydrochloric acid, 2 mL of a solution containing sodium nitrite (100 g/L) and sodium molybdate (100 g/L), and 2 mL of dilute sodium hydroxide solution were added

into 1 mL of stock solution successively. The mixture was shaken after each addition and diluted to 10 mL with water.

Preparation of compensation liquid: 2 mL 0.5 M hydrochloric acid and 2 mL diluted sodium hydroxide solution were added into 1 mL of stock solution and diluted to 10 mL with water.

The absorbance of the test solution was measured immediately at 525 nm by comparison with the compensation liquid.

The following equation was used to calculate the percentage content of total *ortho*-dihydroxycinnamic acid derivatives, expressed as acteoside:

$$\frac{A \times 1000}{185 \times m}$$

i.e. taking the spesific absorbance of acteoside to be 185.
A = absorbance at 525 nm;
m = mass of the substance to be examined, in grams (23).

Determination of flavonoid content

Preparation of stock solution: 2 g powdered drug was boiled in 1 mL hexamethylenetetramine solution (5 g/L), 20 ml acetone and 2 mL hydrochloric acid (0.37 g/L), under a reflux condenser for 30 minutes. The mixture was filtered and the liquid was extracted with 20 mL acetone for 10 minutes two more times. After cooling, the mixture was filtered and diluted to 100 ml with acetone. 20 mL water was added into 20 mL of the filtrate in a separating funnel. The mixture was extracted with 15 mL ethyl acetate and then 10 mL ethyl acetate three times. The ethyl acetate phases were combined and rinsed with 2 quantities of 50 mL water in a separating funnel. The extract was filtered over 10 g of anhydrous sodium sulfate and diluted to 50 mL with ethyl acetate.

Preparation of test solution: 10 mL stock solution and 1 mL aluminium chloride reagent were mixed and diluted to 25 mL with glacial acetic acid solution in methanol (5% *v/v*).

*Preparation of compensation liquid:*10 mL stock solution was diluted to 25 mL with glacial acetic acid solution in methanol (5% *v/v*).

The absorbance of the test solution was measured after 30 minutes by comparison with the compensation liquid at 425 nm.

The following equation was used to calculate the percentage content of flavonoids, expressed as hyperoside:

$$\frac{A \times 1.25}{m}$$

i.e. taking the spesific absorbance of hyperoside to be 500.
A = absorbance at 425 nm;
m = mass of the substance to be examined, in grams (23).

RESULTS AND DISCUSSION

Total ortho-dihydroxycinnamic acid derivatives content

The results of total *ortho*-dihydroxycinnamic acid derivatives content determination in 16 *Ballota* taxa evaluated according European Pharmacopeia method, were presented in Table 2. The content of total *ortho*-dihydroxycinnamic acid derivatives in the extracts, expressed as acteoside varied between 2.678% and 1.057%. The highest quantity of *ortho*-dihydroxycinnamic acid derivatives was observed in *Ballota nigra* subsp. *anatolica*. The lowest quantity of *ortho*-dihydroxycinnamic acid derivatives was found in *Ballota nigra* subsp. *foetida*.

According to European Pharmacopeia (EP), the dried flowering tops of *Ballota nigra* should have minimum 1.5% of total *ortho*-dihidroxycinnamic acid derivatives expressed as acteoside. This method is applied to the other *Ballota* species growing in Turkey, *Ballota nigra* subsp. *anatolica, Ballota macrodonta, Ballota acetabulosa, B. antalyense, Ballota larendana, Ballota inaequidens, Ballota saxatilis* subsp. *saxatilis, Ballota pseudodictamnus* subsp. *lycia, Ballota saxatilis* subsp. *brachyodonta, Ballota nigra* subsp. *nigra* meet the requirement of EP (*Ballota nigra* monographs) whereas *Ballota*

glandulosissima, Ballota nigra subsp. *uncinata, Ballota rotundifolia, Ballota latibracteolata, Ballota cristata, Ballota nigra* subsp. *foetida* do not.

Flavonoid content

The results of flavonoid contents (expressed as hyperoside equivalents) were given in Table 2. The highest quantity of flavonoids was found in *Ballota glandulosissima* (0.862% followed by *B. nigra* subsp. *nigra* (0.680%) and *B. uncinata* (0.580%). The lowest quantity of flavonoids was found in *B. rotundifolia* (0.141%).

In EP, generally calculated percentage content of total flavonoids is expressed as of hyperoside. Congruent with the EP, total flavonoids of *Ballota* species growing in Turkey were evaluated using spectroscopical method. The results of this study reveal that especially *Ballota glandulosissima, B. nigra* subsp. *nigra and, B. nigra* subsp. *uncinata* are rich in flavonoid content.

Table 2. TPC, TFC and ratio of total flavonoid content (TFC) to total phenol content (TPC) in hyperoside equivalents (HE) and acteoside equivalents (AE).

Plant	TPC (%)	TFC (%)	TFC (%)/TPC(%)
Ballota acetabulosa	2.172	0.250	0.115
Ballota pseudodictamnus subsp. *lycia*	1.954	0.298	0.153
Ballota inaequidens	2.030	0.273	0.134
Ballota cristata	1.063	0.280	0.263
Ballota saxatilis subsp. *saxatilis*	1.976	0.174	0.088
Ballota saxatilis subsp. *brachyodonta*	1.943	0.231	0.119
Ballota glandulosissima	1.466	0.862	**0.588**
Ballota larendana	2.042	0.225	0.110
Ballota latibracteolata	1.182	0.247	0.209
Ballota rotundifolia	1.296	0.141	0.109
Ballota macrodonta	2.219	0.180	0.081
Ballota nigra subsp. *nigra*	1.701	0.680	0.400
Ballota nigra subsp. *foetida*	1.057	0.312	0.295
Ballota nigra subsp. *uncinata*	1.399	0.580	0.415
Ballota nigra subsp. *anatolica*	2.678	0.355	0.133
Ballota antalyense	2.079	0.218	0.105

Acteoside and hyperoside are very effective natural compounds which are used as identity of total *ortho*-dihydroxycinnamic acid derivatives and flavonoid content according to EP.

Acteoside is a well-studied phenylethanoid glycoside and is widely distributed in the plant kingdom. Many studies have shown that acteoside has various kinds of biological activities.

Among those so far reported, its antioxidant activity, a modulating activity of nitric oxide (NO) production, cytotoxicity against various tumor cells, antimetastatic effect on lung metastasis using a mouse model injected with B16 melanoma cells to inhibit tumor metastasis, protective effect on carbon tetrachloride-induced hepatotoxicity and inhibition amyloid β-protein which is important protein for Alzheimer's disease patogenesis (24-26).

On the other hand, hyperoside, a flavonoid compound shows remarkable anti-inflammatory properties, beneficial cardiovascular effects, such as anti-ischemic and antidepressant activities, vasoprotective effect and cytoprotective properties against oxidative stress by scavenging intracellular reactive oxygen species as well as enhancing antioxidant enzyme activity. Hyperoside inhibits the free radical-induced oxidation of vitamin E in human low-density lipoproteins and lowers total cholesterol, thereby increasing superoxide dismutase activity and high-density lipoproteins. Hyperoside also prevents gastric mucosal injury in mice

induced by ethanol, and it has been shown to inhibit Ca^{2+} influx induced by the activation of G-protein-coupled receptors, block voltage dependent calcium to attenuate KCl-induced increase in [Ca^{2+}], and block N-methyl-D-aspartate receptor-linked Ca^{2+} channels to reduce [Ca^{2+}] in neonatal rat brain cells. The antioxidant effect of hyperoside in ECV-304 cells has been reported (27).

In our previous study, *Ballota glandulosissima* was found as the richest species with respect to flavonoids (kumatakenin, pachypodol, 5-hydroxy-7,3′,4′-trimethoxyflavone, salvigenin, velutin, corymbosin and retusine) which were previously isolated by us (8). Our research team previously reported that (10) *Ballota nigra* subsp. *anatolica*, *B. macrodonta*, *B. antalyense*, *B. larendana*, *B. inaequidens* and *B. saxatilis* subsp. *saxatilis* showed strong antioxidant activity. The content of *ortho*-dihydroxycinnamic acid derivatives which is determined in our study is in accordance with these antioxidant activity results.

Our results are in line with those of Erdoğan-Orhan et al. (16) in regard to the total phenol and total flavonoid contents of *Ballota* species.

CONCLUSION

Ballota species have been reported to contain several classes of pyhtochemicals such as diterpenes, flavonoids, phenylpropanoids, essential oils, tannins, and saponins (28). Regarding the chemical composition of *Ballota nigra*, which is the only *Ballota* species included in European Pharmacopoeia, previous investigations have so far identified phenolic compounds, mainly flavonoids and phenylpropanoids (chlorogenic, caffeic, and caffeoylmalic acids, ballotetroside, forsythoside B, verbascoside, and allysonoside) (29).

Among 16 *Ballota* species, which were analysed in this study, 10 species were found to be meet the requirements of EP with regard to their *ortho*-dihydroxycinnamic acid contents. Besides, *B. glandulosissima* is remarkable in point of TFC (%) / TPC(%) ratio.

Our current screening results demonstrated that most of *Ballota* species have rich phenolic contents. *Ballota* species seem to have potential for the treatment of oxidative stress cause diseases due to owing these active compounds.

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