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Phytochemical profile and ABTS cation radical scavenging, cupric reducing antioxidant capacity and anticholinesterase activities of endemic *Ballota nigra* L. subsp. *anatolica* P.H. Davis from Turkey

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PEER REVIEW

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Comments

This manuscript is a successful attempt to introduce a new source of antioxidant compounds. It partially clarifies the chemical composition of Ballota nigra and underlines its anti-Alzheimer's activity. It is well organized, well designed and could be a starting point for further investigations on this plant and the other members of this family.

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ABSTRACT

Objective: To evaluate the chemical compositions and biological activities of an endemic *Ballota nigra* L. subsp. *anatolica* P.H. Davis.

Methods: Essential oil and fatty acid composition were determined by GC/MS analysis. ABTS cation radical decolourisation and cupric reducing antioxidant capacity assays were carried out to indicate the antioxidant activity. The anticholinesterase potential of the extracts were determined by Ellman method.

Results: The major compounds in the fatty acid composition of the petroleum ether extract were identified as palmitic (36.0%) and linoleic acids (14.3%). The major components of essential oil were 1-hexacosanol (26.7%), germacrene-D (9.3%) and caryophyllene oxide (9.3%). The water extract indicated higher ABTS cation radical scavenging activity than α-tocopherol and BHT, at 100 μg/mL. The acetone extract showed 71.58 and 44.71% inhibitory activity against butyrylcholinesterase and acetylcholinesterase enzyme at 200 μg/mL, respectively.

Conclusions: The water and acetone extracts of *Ballota nigra* subsp. *anatolica* can be investigated in terms of both phytochemical and biological aspects to find natural active compounds.

KEYWORDS

Lamiaceae, *Ballota nigra* L. subsp. *anatolica*, Fatty acid, Essential oil, Antioxidant, Anticholinesterase

1. Introduction

Ballota nigra (*B. nigra*) L. subsp. *anatolica* P.H. Davis (Lamiaceae) is an endemic species that distrubuted northeast and inland of Turkey[1]. The local names of this species are Yalancı Isırgan, Leylim, Kara yer pırasası, Köpekotu[2], Arı otu, Bal otu, Ballık otu, Leylim yaprağı, Pembe oğul otu[3], Ballıbaba, Yavşan[4], and Grip otu[5] in Turkey. *B. nigra* subsp. *anatolica* has been used in folk medicine as diuretic, antispasmodic, digestive, worm reducer, regularize menstruation[2]. Also it is used for the treatment of anorexia, nausea, bronchitis[4], asthma, vasodilatation, jaundice,

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gastric disorders cold and flu[3-5]. In previous studies, diterpenoids and flavonoids were isolated and analysed by HPLC in different species of Ballota[6-9].

Since ancient times, people have benefited from plants not only as food supply, but also as smell, flavor, fuel, weapon and medicine. Especially extracts derived from medicinal plants have been used to treat many diseases and accordingly, healing has emerged as a profession. Nevertheless, in the 1800s, first active substances derived from plants, produced synthetically, as a result, pharmaceutical industry was born and the old traditional methods were left aside. However, especially in the last 30-35 years, an increased interest emerged towards traditional methods known as "alternative medicine" namely the therapeutic usage of plant extracts, since the treatment of synthetic drugs used in modern medicine failed to reach the desired success and despite having many negative side effects, synthetic drugs usually only have a positive impact[10-13].

A literature survey showed that there have been no previous ABTS cation radical scavenging, cupric reducing antioxidant capacity (CUPRAC) and anticholinesterase activities and fatty acid constituents reports on an endemic *B. nigra* subsp. *anatolica*. The aim of this study was to evaluate the antioxidant and anticholinesterase activities of the petroleum ether, acetone, methanol and water extracts of *B. nigra* subsp. *anatolica*. The petroleum ether extract was analysed to determine its fatty acid composition by GC/MS. The essential oils were analysed to determine its composition by GC/MS. ABTS cation radical decolourisation and CUPRAC assays were carried out to indicate the antioxidant activity. The anticholinesterase potential of the extracts was determined by Ellman method.

2. Materials and methods

2.1. General experimental procedures

A Thermo pH-meter, a BioTek Power Wave XS, an Elma S15 ultrasonic bath and a vortex (LMS Co. LTD) were used for the activity assays. Ethanol, hexane, chloroform, dichloromethane, methanol, ABTS, sodium acetate, butylated hydroxytoluene were purchased from Merck (Germany), acetic acid, sodium methoxide, copper (II) chloride dihydrate ($CuCl_2.2H_2O$), neocuproine, EDTA, acetylcholinesterase, butyrylcholinesterase from Sigma (Germany), α-tocopherol, acetylthiocholine iodide from Aldrich (Germany), galanthamine hydrobromide from Sigma-Aldrich (Germany), and petroleum ether, sodium dihydrogen phosphate, sodium hydrogen phosphate, ammonium acetate from Reidel de Haen (Germany).

2.2. Plant material

The whole plant of *B. nigra* subsp. *anatolica* P.H. Davis was collected from Western Turkey (İstanbul-Bayrampaşa) in July 2012 by one of us (Dr. A. Ertaş) and identified by one of us (Dr. Y. Yeşil). The specimen used to diagnose was stored in the Herbarium of Istanbul University (ISTE 98058).

2.3. Isolation of essential oil

Essential oil was obtained using a Clevenger apparatus from the whole parts of *B. nigra* subsp. *anatolica*, which were crumbled into small pieces and soaked in distilled water for 3 h. The obtained essential oils were dried over anhydrous Na_2SO_4 and stored at +4 °C for a sufficient period of time.

2.4. GC/MS and GC-FID conditions (essential oil)

The essential oils were diluted using CH_2Cl_2 (1:3 volume/volume) prior to GC/FID and GC/MS analysis. GC/FID performed using Thermo Electron Trace GC FID detector and GC/MS performed using same GC and Thermo Electron DSQ quadrupole for MS. A nonpolar Phenomenex DB5 fused silica column (30 m, 0.32 mm, 0.25 μm film thickness) was used with helium at 1 mL/min (20 psi) as a carrier gas. The GC oven temperature was kept at 60 °C for 10 min and programmed to 280 °C at a rate of 4 °C/min and then kept constant at 280 °C for 10 min. The split ratio was adjusted to 1:50, the injection volume was 0.1 μL, and EI/MS was recorded at 70 eV ionization energy. The mass range was *m/z* 35-500 amu. Alkanes (C8-C24) were used as reference points in the calculation of Kovats indices by the same conditions[14,15]. Identification of the compounds was based on comparing their retention times and mass spectra with those obtained from authentic samples and/or the NIST and Wiley spectra as well as data from the published literature. GC/FID and GC/MS were replicated three times (Mean RSD % <0.1).

2.5. Esterification of total fatty acids with GC/MS conditions

Esterification of the petroleum ether extract was prepared according to Sabudak *et al*[16]. Thermo Scientific Polaris Q GC-MS/MS was used. GC/MS procedure described by Sabudak *et al.* was applied[16].

2.6. Preparation of the extracts

Whole plants of *B. nigra* subsp. *anatolica* (100 g) were dried, powdered, and then sequentially macerated with petroleum ether, acetone, methanol, and water for 24 h at 25 °C. After filtration, the solvents were evaporated to obtain crude extracts. This yielded 0.25% petroleum ether extract, 0.72% acetone extract, 2.5% methanol extract, and 2.1% water extract (w/w).

2.7. Antioxidant activity of extracts

We used the ABTS cation radical decolourization and CUPRAC methods to determine antioxidant activity[17,18].

2.8. Anticholinesterase activity of extracts

A spectrophotometric method developed by Ellman, Courtney, Andres, and Featherstone was used to determine the acetyl- and butyryl-cholinesterase inhibitory activities[19].

2.9. Statistical analysis

The results of the antioxidant and anticholinesterase activity assays were mean±SD of three parallel measurements. The statistical significance was estimated using a Student's *t*-test, P values <0.05 were regarded as significant.

3. Results

3.1. Phytochemical identification by GC-MS analysis

3.1.1. Fatty acid composition

The fatty acid composition of the petroleum ether extract was determined by GC/MS analysis. As shown in Table 1, thirteen components were identified, constituting 99.8% of the petroleum ether extract. The main components of the fatty acid were found to be palmitic (36.0%), linoleic (14.3%) and oleic acids (10.6%). This study is the first report on *B. nigra* subsp. *anatolica* fatty acid composition.

Table 1

GC-MS analysis of *B. nigra* subsp. *anatolica* petroleum ether extract.

Rt (min)[a]	Constituents[b]	% Composition
14.39	10-undecenoic acid	1.5
18.60	Myristic acid	1.8
24.94	Palmitoleic acid	0.4
25.27	Palmitic acid	36.0
28.86	11,13-dimethyl-12-tetradecen-1-ol acetate	2.1
29.75	Phytol	4.6
30.64	Linoleic acid	14.3
30.77	Oleic acid	10.6
30.86	Linolenic acid	9.8
31.54	Stearic acid	9.2
37.38	Arachidic acid	4.1
38.19	6-hexadecenoic acid, 7-methyl	1.4
43.82	Behenic acid	4.0
	Total	99.4

[a]Retention time (as minutes); [b]A nonpolar Phenomenex DB-5 fused silica column.

3.1.2. Essential oil composition

The essential oil composition of *B. nigra* subsp. *anatolica* was determined by GC/MS analysis. As seen in Table 2, thirteen components were determined, constituting 99.4% of the essential oil. The major components were 1-hexacosanol (26.7%), germacrene-D (9.3%) and caryophyllene oxide (9.3%). Some previous studies have investigated the essential oil composition of *Ballota* species. Beta-pinene (39.0%), beta-caryophyllene (20.0%), α-cadinol (21.0%), linalool (14.6%), germacrene D (19.1%) and caryophyllene oxide (22.4%) were reported as the major components of the essential oil of *B. nigra*, *B. nigra* L. ssp *foetida*, *Ballota aucheri*, *Ballota saxsatilis*, *Ballota undulata* and *Ballota pseudodictamnus*, respectively[20-23]. According to report of Kazemizadeh *et al.*, twelve compounds were identified and the main constituents of the essential oil of *B. nigra* subsp. *anatolica* were germacrene D (18.1%), nerolidol epoxyacetate (15.4%), sclareol oxide (12.1%), linalyl acetate (11.5%), and β-caryophyllene (10.5%)[24]. The composition of the essential oil of *B. nigra* subsp. *anatolica* investigated by Kazemizadeh *et al.* was found to be quite different from our findings; it may be attributed to their different collected locations.

Table 2

Chemical composition of the essential oil from *B. nigra* subsp. *anatolica*.

RI[a]	Rt (min)[b]	Constituents[c]	% Composition
1 485	30.56	Germacrene-D	9.3
1 498	30.87	α-selinene	8.7
1 583	33.13	Caryophyllene oxide	9.3
1 800	36.45	Octadecane	3.0
1 890	36.74	2-methyl-1-hexadecanol	3.3
2 185	38.35	Z-8-octadecen-1-ol acetate	7.1
2 171	38.98	Butyl phthalate	3.0
2 109	40.00	Heneicosane	4.4
2 259	40.13	2,5-di-tert octyl-p-benzoquinone	7.3
2 366	40.59	Arachidic acid	6.0
2 407	40.84	Tetracosane	4.5
2 700	43.30	Heptacosane	4.3
2 852	43.64	1-hexacosanol	26.7
		Total	96.9

[a]RI Retention indices (DB-5 column); [b]Retention time (as minutes); [c]A nonpolar Phenomenex DB-5 fused silica column.

3.2. Antioxidant activity

The antioxidant activity of the petroleum ether (BNP), acetone (BNA), methanol (BNM) and water (BNW) extracts prepared from both the root and the aerial parts of *B. nigra* subsp. *anatolica* were investigated by using CUPRAC and ABTS cation radical decolourisation assays. As shown in Figure 1, the water extract exhibited over 80% inhibition in ABTS cation radical scavenging assay at 100 μg/mL. The water extract exhibited higher inhibition (88.00%) than the reference compounds, α-tocopherol and BHT, in ABTS cation radical scavenging assay at 100 μg/mL. The acetone and methanol extracts exhibited 70.10 and 72.60% inhibition in ABTS cation radical scavenging assay at 100 μg/mL, respectively. As shown in Figure 2, the acetone, water extracts and α-tocopherol treatment exhibited 0.92, 1.10 and 1.65 inhibition in CUPRAC at 100 μg/mL, respectively. Some previous studies have investigated the antioxidant activity of *B. nigra* subsp. *anatolica*. According to report of Citoglu *et al.*, the antioxidant activities of ethanolic extracts of *Ballota* species were examined for superoxide anion scavenging activity and inhibition of lipid peroxidation. *B. nigra* subsp. *anatolica* showed strong scavenging activity against superoxideanion formation and weak effects on lipid

peroxidation[25]. According to report of Erdogan-Orhan *et al*, the antioxidant activities of ethyl acetate, methanol, and water extracts of 16 *Ballota* species were examined for radical quenching activity, ferric-reducing antioxidant power and ferrous ion-chelating capacity. *B. nigra* subsp. *anatolica* extracts showed moderate activity on all methods[26].

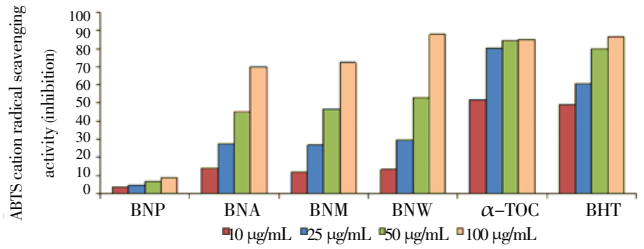

Figure 1. Inhibition (%) of ABTS cation radical scavenging activity of the extracts, α-tocopherol and BHT. Values are means±SD, n=3, P<0.05, significantly different with Student's t-test.

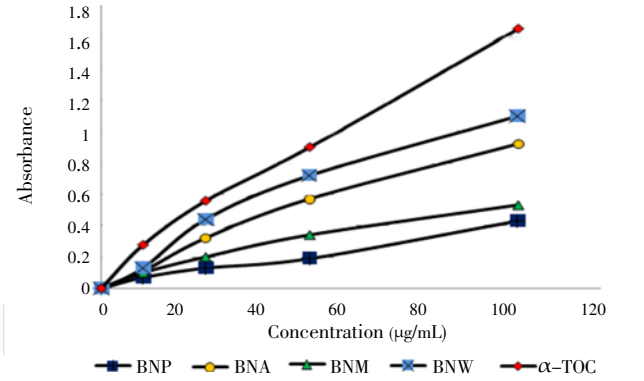

Figure 2. Cupric reducing antioxidant capacity of extracts and α-tocopherol. Values are means±SD, n=3, P<0.05, significantly different with Student's t-test.

3.3. Anticholinesterase activity

As shown in Table 3, the acetone extract showed 71.58% inhibitory activity against butyrylcholinesterase and 44.71% inhibitory activity against acetylcholinesterase enzyme at 200 μg/mL. Furthermore, the acetone extract indicated higher inhibitory effect against butyrylcholinesterase enzyme than the reference compound, galanthamine.

Table 3

Anticholinesterase activity of *B. nigra* subsp. *anatolica* extracts at 200 μg/mL[a].

Extracts	Inhibition % against AChE	Inhibition % against BChE
Petroleum ether extract	12.32±0.76	20.53±1.90
Acetone extract	44.71±1.22	71.58±1.09
Methanol extract	23.90±0.30	19.05±0.67
Water extract	NA	NA
Galanthamine[b]	85.09±0.40	70.22±1.46

[a]Values expressed are means±SD of three parallel measurements (P<0.05); [b]Standard drug; NA: Not active.

4. Discussion

The present study is the first ABTS cation radical scavenging, CUPRAC and anticholinesterase activities and fatty acid constituents reports on an endemic *B. nigra* subsp. *anatolica*. It is noteworthy that the water extract of *B. nigra* subsp. *anatolica* exhibited stronger ABTS cation radical scavenging activity than the standard compounds, α-TOC and BHT. Also, the acetone extract of *B. nigra* subsp. *anatolica* exhibited strong butyryl-cholinesterase inhibition (71.58%). Thus, the water and acetone extracts of *B. nigra* subsp. *anatolica* as the most active extract should be investigated in terms of both phytochemical and biological aspects to find natural active compounds responsible for ABTS cation radical scavenging and butyryl-cholinesterase activities.

Conflict of interest statement

The authors have declared that there is no conflict of interest.

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Comments

Background

Antioxidant compounds are of interest of scientific researches during last decades because of their important roles in preventing numerous important diseases such as cancer and Alzheimer's disease. Therefore, searching for the new sources of antioxidant compounds specially exploring the natural sources of these subjects is very important.

Research frontiers

The main aim of this paper is to define the biological activity of an endemic plant distributed northeast and inland of Turkey, *B. nigra*. This attempt is precious because of two reasons. First of all, the chemical components and biological activity of this plant have not been reported previously and the second, this paper not only reports the antioxidant activity of this plant but also clarifies the mechanism of action by performing several tests.

Related reports

Previously it has been well established that natural products are the best and the safest sources of antioxidant compounds. In addition, it has been shown that ABTS and CUPRAC tests are the most indicative tests for antioxidant

activity of natural compounds and anticholinesterase activity is the main mechanism of anti-Alzheimer effect.

Innovations and breakthroughs

This paper is reporting an innovative approach to antioxidant activity of a natural source since *B. nigra* is endemic to Turkey and no other researcher except a Turkish group would be able to report its chemical composition and biological activity.

Applications

B. nigra is a well-known herbal medicine used for treatment of several diseases. Thus, exploring its antioxidant activity would help to introduce a safe and nontoxic new source.

Peer review

This manuscript is a successful attempt to introduce a new source of antioxidant compounds. It partially clarifies the chemical composition of *Ballota nigra* and underlines its anti-Alzheimer's activity. It is well organized, well designed and could be a starting point for further investigations on this plant and the other members of this family.

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