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ANTIOXIDANT ACTIVITY OF TISSUE CULTURE-RAISED *BALLOTA NIGRA* L. PLANTS GROWN *EX VITRO*

JOANNA MAKOWCZYŃSKA*, IZABELA GRZEGORCZYK-KAROLAK and HALINA WYSOKIŃSKA

Department of Biology and Pharmaceutical Botany, Medical University of Łódź, 1 Muszyńskiego St., 90-151 Łódź, Poland

Abstract: Antioxidant properties and total phenolic and flavonoid contents were evaluated in methanolic extracts of shoots from *Ballota nigra* plants initiated *in vitro* (from nodal explants) and *in vivo* (from seeds). The plants were grown in greenhouse and in the field, and were analyzed at the vegetative and flowering stages. The shoot extract of wild-grown plants of *B. nigra* was also investigated. The results indicate that antioxidant potential of the *B. nigra* extracts seems to be due to their scavenging of free radicals (DPPH assay) and metal reducing (FRAP test), while they were less effective at the prevention of linoleic acid peroxidation (LPO test). The extracts from shoots of *in vitro* derived plants were found to exhibit the greatest antioxidant properties. The extracts were also characterized by the highest content of phenolic compounds and their level was affected by plant developmental stage. The extracts of shoots collected at the flowering period exhibited higher amounts of phenolics and flavonoids than in the extracts of immature plants. A close correlation between the total phenolic content and flavonoid content and antioxidant activity using the DPPH and FRAP assays was obtained. The results of the present study suggest the use *in vitro*-derived plants of *B. nigra* instead of using wild plants for pharmaceutical purposes.

Keywords: *Ballota nigra* L., total phenolic and total flavonoid contents, antioxidant activity, *in vitro* cultures

Ballota nigra L. (black horehound) is a species of perennial herb within the Lamiaceae that grows in Europe, the Middle East and North Africa. The plant is listed in the pharmacopoeias of many countries, including the Polish pharmacopoeia (1) and the European pharmacopoeia (2). The flowering aerial parts of *B. nigra* are commonly known to be a neurosedative drug (3). Good therapeutic results have been achieved using *Ballotae nigrae herba* in the treatment of depression, insomnia and neurasthenia (4, 5), as well as gastrointestinal disorders and it is used externally in the treatment of wounds and burns (6). Due to the anti-inflammatory and expectorant properties of black horehound, it is also used in the treatment of colds, coughs and influenza (7). This species is also known to have antioxidant, spasmolytic, antiemetic and antidiabetic properties (8, 9) and has demonstrated antibacterial activity against *Proteus mirabilis* and *Staphylococcus aureus* (10, 11).

Phytochemical studies have shown that the herb of *B. nigra* is rich in phenolic compounds such as flavonoids (luteolin-7-lactate, luteolin-7-glucosyl-lactate, apigenin-7-glucoside, vicenin-2, tangeretin, ladanein) (12), phenylpropanoids (alyssonoside, angoroside, arenarioside, ballotetroside, forsythoside B, lavandulifolioside, martynoside, verbascoside) (10, 13-15) and phenolic acids (chlorogenic, caffeic, caffeoyl malic acids) (4). Due to their chemical structures and redox properties, phenolic compounds have antioxidant activity and play an important role in protection from oxidative stress induced by reactive oxygen species (ROS). Recently, it has been shown that reactive oxygen species can induce degenerative diseases including cardiovascular diseases, diabetes, cancer, neurodegenerative disorders and ageing (16, 17). Also, as lipid peroxidation is initiated by ROS, and antioxidants retard the oxidative degradation of lipids, phenolics can be used to improve the nutritional value of food (18). In this respect, research into the identification of plants which may be used as non-toxic sources of natural antioxidants is very important in the promotion of public health.

The antioxidant activity of *B. nigra* herb has been characterized (4, 6, 8) but such studies only concern plants from the wild population. In contrast, the

* Corresponding author: e-mail: joanna.makowczynska@umed.lodz.pl

current study estimates phenolic antioxidant activity and its relation to total phenolic and flavonoid compound contents in methanolic extracts of *B. nigra* shoots from micropropagated plants at two different stages of maturity: the vegetative and flowering stages. For comparison, methanolic extracts of shoots from seed-derived plants were also analyzed. The plants were cultivated under identical conditions and harvested after the same period as *in vitro*-derived plants. The study also presents the antioxidant activity and total phenolic and flavonoid content of commercially available samples of *B. nigra* herb.

MATERIALS AND METHODS

Plant materials

Ballota nigra nodal explants were derived from 6-week-old *in vitro* cultured shoots initiated from seeds obtained from The Medicinal Plant Garden of the Department of Pharmacognosy, Medical University of Łódź (Poland). Explants about 1 cm in length were cultured on Murashige and Skoog (MS) agar (0.7%) medium supplemented with 0.1 mg/L indole-3-acetic acid (IAA) and 0.5 mg/L 6-benzylaminopurine (BAP) for 12 cycles of 6 weeks each. Each subculture was performed to fresh medium of the same composition at the end of each cycle. The cultures were maintained in a growth chamber at 26 ± 2°C with a 16 h photoperiod (40 µmol/m^2 s^1 photosynthetic photon flux). The growth conditions were not altered all through the experiment.

For rooting, 6-week-old shoots, 3 to 4 cm in length, were transferred into MS agar medium supplemented with 0.5 mg/L indole-3-butyric acid (IBA). After 5 weeks, the rooted shoots were transferred into pots containing a sterile mixture of soil, sand and peat (4 : 3 : 3, v/v/v) and kept in a greenhouse for 3 months before subsequent transfer into the field at the Department of Pharmacognosy Medicinal Plant Garden, Medical University of Łódź.

Greenhouse- and field-grown *B. nigra* plants were also obtained from seeds derived from the same source as those used for shoot culture initiation. The classification of the plant specimen was confirmed by Prof. Jan Siciński (Department of Geobotany and Plant Ecology, University of Łódź). Voucher specimen was deposited at the Department of Biology and Pharmaceutical Botany, Medical University of Łódź, Poland.

Sample collection and extract preparation

For antioxidant assays and determination of total phenolic and flavonoid content, the following *B. nigra* materials were used:

- shoots of micropropagated plants (SV-1, SV-2) and seed-propagated plants (SS-1, SS-2). The shoots were harvested at two different developmental stages: at vegetative (after 3 months of growth in the greenhouse) (SV-1, SS-1) and at flowering (after 1 year of growth in open field conditions) (SV-2, SS-2).
- a commercially-available sample of *B. nigra* herb (WP) purchased from Nanga, Zlotów (Poland). The material was originally collected from plants growing wild in Poland, and is representative of material used for medicinal purposes.

The lyophilized and powdered plant materials (1.1 g each) were extracted three times with 96% methanol (3 × 50 mL) for 15 min at room temperature using an ultrasonic bath. The extracts were filtered, combined and evaporated to dryness under reduced pressure. Extraction yields (% w/w) were calculated by the following formula: weight of the dry extract (g) / weight of the original sample (g) × 100%. The percentage yields of methanolic extracts of *B. nigra* shoots were as follows: SV-1 – 20.4%; SV-2 – 15.9%; SS-1 – 15.4%; SS-2 – 17.27%; WP – 16.45%.

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay

The radical scavenging activity of plant extracts against DPPH free radical (Sigma Aldrich) was determined spectrophotometrically according to Grzegorczyk et al. (19). Briefly, 2 mL of extracts (at concentrations 2.0, 20.0, 100.0, 200.0, 500.0 and 1000.0 µg/mL) was added to 2 mL of 0.2 µM DPPH solution, and the absorbance was measured after 30 min at 517 nm (spectrophotometer Beijing Reyleigh, Corp. China). The results were expressed as EC_{50} (the concentration of sample at which 50% of maximum scavenging activity was recorded).

Ferric-reducing antioxidant power assay (FRAP)

The FRAP was determined according to the method modified by Pulido et al. (20). Three mL of fresh prepared FRAP reagent was mixed with 300 µL of redistilled water and 100 µL of the sample methanol extract. The FRAP reagent contained 2.5 mL 10 mM TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine) solution in 40 mM HCl, 2.5 mL of 20 mM aqueous $FeCl_3 \times 6\ H_2O$ solution and 45 mL of 0.3 M acetate buffer at pH 3.6. The reaction mixture was incubated at 37°C for 15 min. The absorbance was measured after 15 min at 595 nm relative to a blank: a sample containing methanol instead of extract. The antioxidant activity was determined against a standard of known FRAP value: ferrous sulfate calculated from a calibration curve of (0-2000 µM).

Linoleic acid peroxidation inhibition

Linoleic acid peroxidation (LPO) inhibition was determined by the TBARS test according to Choi et al. (21), with some modifications. Briefly, 150 µL plant extracts at concentrations of 250 µg/mL were mixed with 100 µL ascorbic acid (2 mM), 500 µL linoleic acid (20 mM) and 500 µL TRIS-HCl buffer (100 µM, pH 7.5). Peroxidation was initiated by the addition of 100 µL $FeSO_4$ (4 mM). The mixture was incubated for 90 min at 37°C. The reaction was terminated by the addition of 1.5 mL of ice cold 10% trichloroacetic acid (TCA) (POCh) in 0.5% HCl. Following this, 3 mL of 2-thiobarbituric acid (TBA) (Sigma Aldrich) in 50 mM NaOH was added. The mixture was heated at 95°C for 60 min. After cooling, each sample (4 mL) was vortexed with an equal volume of butanol. The mixture was centrifuged for 4000 rpm for 15 min and the upper colored layer was decanted into spectophotometric cuvettes. The absorbance was read at 532 nm. The percentage of linoleic acid peroxidation inhibition was calculated using the following equation:

% inhibition = (Abs control - Abs sample - Abs extract) × 100/Abs control

where Abs control is the absorbance of methanol instead of sample.

Total phenolic determination

Total phenolic content was measured using Folin-Ciocalteu method described by Singleton and Rossi (22). The extracts (400 µL) were mixed with 2 mL of Folin-Ciocalteu reagent (POCh, Poland) (diluted 10-fold) and 1.6 mL of 7.5% sodium carbonate. The absorbance was determined after 30 min of incubation at room temperature at 765 nm. The results were expressed as gallic acid mg equivalents (GAE) per gram of dry extract. The calibration curve was obtained by preparing gallic acid solution in range of concentration 1-400 mg/L.

Total flavonoid determination

The flavonoid content was estimated using colorimetry according to Lamaison and Carnat (23). Briefly, 2 mL of extracts were mixed with 2 mL of 2% $AlCl_3$. The absorbance of the reaction mixture was measured after 15 min at room temperature at 415 nm. Quantification was performed with respect to a standard calibration curve of quercetin at concentrations ranging from 1 to 100 mg/L. The results were expressed as quercetin mg equivalents per gram of dry extract.

Statistical analysis

The estimated values are calculated as the means (n = 6) ± standard error. The Kruskal-Wallis test was used to assess the significance of the effects of treatment, at $p \leq 0.05$. The calculation was performed using Statistica 10.0 software (Statsoft, Poland). EC_{50} values and correlation coefficients between antioxidant assays and the total phenolic

Table 1. The antioxdant properties of *in vitro*- and *in vivo*–derived plants and wild-grown plants of *Ballota nigra* L. methanolic extracts.

Plant material	Assay		
	DDPH (EC_{50})* (µg/mL)	FRAP**(µmol Fe (II)/g DW) %	Inhibition of linoleic acid (LA) peroxidation***
Shoots of *in vitro* propagated plants			
SV-1	56.00 ± 0.44^{a}	642.42 ± 1.72^{a}	30.11± 5.18^{ab}
SV-2	61.16 ± 0.35^{c}	641.38 ± 0.93^{a}	29.47± 3.49^{ab}
Shoots of *in vivo* propagated plants			
SS-1	202.62 ± 3.29^{b}	331.46 ± 2.08^{b}	23.30± 3.53^{a}
SS-2	80.12 ± 1.75^{d}	470.99 ± 1.61^{c}	36.05± 2.35^{b}
Shoots of wild-grown plants			
WP	117.99 ± 3.91^{e}	424.53 ± 3.07^{d}	20.97± 4.0^{a}

Plants were propagated from nodal explants (*in vitro* derived) and from seeds (*in vivo* derived). SV-1, SS-1 shoots harvested at vegetative stage (after 3 months of growth in greenhouse), SV-2, SS-2 shoots harvested at flowering stage (1 year of growth in the field), WP shoots of wild-grown plants from Świętokrzyskie Province. *EC_{50} - the concentration of sample showing 50% of maximum DPPH radical scavenging activity; ** ferric reducing antioxidant power, 15 min of incubation; ***extract concentrations: 250 µg/mL. Values are the mean of 6 replicates ± standard error. Mean values followed by different letter in column are significantly different according to the Kruskal-Wallis test ($p \leq 0.05$).

and flavonoid contents were calculated using MS-Excel software.

RESULTS AND DISCUSSION

In vitro plant regeneration

The *in vitro* propagation of *B. nigra* plants was established by using nodal explants. Axillary shoots were multiplied from nodal explants on MS medium supplemented with 0.5 mg/L BAP and 0.1 mg/L IAA. An average number of 11.5 buds and shoots, which were about 3 cm in length, were formed from one explant within 6 weeks. The explant type as well as the type and concentration of growth regulators were selected on the base of our preliminary studies (data not shown). The shoots developed roots after 5 weeks on MS medium containing 0.5 mg/L IBA. The plantlets were transferred into soil and grown in pots in the greenhouse for 3 months. Following this, they were transplanted into the field, where flowered the following year.

Antioxidant activity

The antioxidant potentials were assessed of methanolic extracts from shoots of *in vitro*-raised *B. nigra* plants grown under *ex vitro* conditions and harvested at two maturity stages: after 3 months of growth in the greenhouse (immature plants at vegetative stage) and after 1 year of growth in the field (mature plants at flowering stage). The activity was compared to that of extracts from shoots of seed-derived plants of *B. nigra*, which were at the same developmental stages and grown under identical conditions as *in vitro* derived plants. Additionally, the methanolic extract of aerial parts of wild-grown plants (commercially available sample of *B. nigra* dried herb) was also investigated. As there are several mechanisms by which antioxidants can act (24-26), three different types of *in vitro* assays were used to determine the antioxidant capacities of the tested types of plant materials. The free radical scavenging activity was evaluated by the discoloration of DPPH, based on the reaction where a purple-colored stable free radical DPPH (2,2-diphenyl-β-picrylhydrazyl) is converted into a colorless form, DPPH-H, (2,2-diphenyl-β-picrylhydrazine) in the presence of hydrogen-donating antioxidants (16, 27). The degree of discoloration indicates the scavenging potential of the antioxidant extract (28). The FRAP assay was employed to measure the ability of the antioxidant reductants present in the extracts to reduce Fe^{3+} to Fe^{2+} (20). Finally, the LPO method was used to determine the capacity of the *B. nigra* extracts to inhibit LA peroxidation by chain-breaking peroxyl radical scavenging. The results are summarized in Table 1.

As observed with DPPH, the methanolic extracts *in vitro*-derived plant shoots (SV-1, SV-2) were found to have a significantly ($p \leq 0.05$) higher free radical scavenging capacity than other tested plant materials. The highest free radical scavenging activity, with an EC_{50} value of 56 µg/mL, was exhibited by the SV-1 extract (greenhouse-grown *in vitro*-derived plants) followed by SV-2 extract (field-grown *in vitro*-derived plants) with an EC_{50} value of 61 µg/mL. These values were 30-40% higher than those achieved for the SS-2 extract (field-cultivated seed-derived plants) with EC_{50} = 80 µg/mL, and 4-times higher than the SS-1 extract (greenhouse-grown seed-derived plants) with EC_{50} = 203 µg/mL and the wild-grown *B. nigra* (WP) extract (EC_{50} = 118 µg/mL). Increasing radical scavenging activity has been observed in the regenerated shoots of *Piper nigrum* (29), *Silybum marianum* (30) and *Aloe arborescents* (31). Perveen et al. (32) suggest that micropropagated plants develop an antioxidant protective system to avoid oxidative stress during establishment under *ex vitro* conditions. Similar to the DPPH assay, the highest ferric reducing potential was observed in shoot extracts of micropropagated plants of *B. nigra*, with a mean reducing ability of 640 µmol Fe (II)/g DW, which was significantly ($p \leq 0.05$) higher than those remaining shoot extracts tested (Table 1). However, when compared to BHT and tocopherol, all studied extracts were less effective than these synthetic antioxidant agents (data not shown).

The present study demonstrates that the inhibition of LA peroxidation in *B. nigra* extracts was not very strong, ranging between 21-36% at a concentration of 250 µg/mL. No significant difference was observed in the type of antioxidant activity between *in vitro*- and *in vivo*-derived shoot extracts (Table 1).

Total phenolic and flavonoid contents

The differences between the responses of methanolic extracts of *B. nigra* prepared from *in vitro* and *in vivo* propagated plants could be attributed to the differences in the chemical constituents. Hence, colorimetric determinations of total phenolic content and total flavonoid content in *B. nigra* methanolic extracts were performed. The results in Table 2 showed that extracts of tissue culture-derived plants exhibited greater amounts of phenolic compounds, expressed in gallic acid equivalents, than extracts of plants propagated from seeds, in spite of the fact that both plants were grown under

Table 2. Total content of phenolics and flavonoids in methanolic extracts of *B. nigra*.

Sample	Phenolics*	Flavonoids**
Shoots of *in vitro* propagated plants		
SV-1	61.54 ± 0.75[a]	16.68 ± 0.14[a]
SV-2	93.41 ± 0.13[c]	28.37 ± 0.22[c]
Shoots of *in vivo* propagated plants		
SS-1	28.09 ± 0.77[b]	12.41 ± 0.38[b]
SS-2	68.3 ± 1.47[a]	29.72 ± 0.63[c]
Shoots of wild-grown plants		
WP	56.04 ± 0.18[d]	17.42 ± 1.05[a]

*expressed as gallic acid equivalents in mg per g of dry extract; ** expressed as quercetin equivalents in mg per g of dry extract. Plants were propagated from nodal explants (*in vitro* derived) and from seeds (*in vivo* derived. SV-1, SS-1 shoots harvested at vegetative stage (after 3 months of growth in greenhouse), SV-2, SS-2 shoots harvested at flowering stage (1 year of growth in the field), WP shoots of wild-grown plants from Świętokrzyskie Province. Values are the mean of 6 replicates ± standard error. Mean values followed by different letter in column are significantly different according to the Kruskal-Wallis test ($p \leq 0.05$).

Table 3. Correlation between phenolic and flavonoid contents and antioxidant activities of methanolic extracts of *B. nigra* plants.

Parameter	DPPH (EC_{50} µg/mL)	FRAP (µmol Fe (II)/g DW)	Inhibition of linoleic acid peroxidation
Total phenolic content (GAE mg/g DW)	- 0.862	0.804	0.529
Total flavonoid content (quercetin mg/g DW)	- 0.670	0.665	0.455

DW = dry weight

the same environmental conditions, i.e., in the greenhouse or the field. Elevated levels of total phenolics have been also reported in other *in vitro* obtained plants, such as *Aloe arborescens* (31), *Ceropegia santapaui* (28) and *Merwilla plumbea* (33). Amoo et al. (31) reported that the growth regulators (auxins, cytokinins) applied during the *in vitro* propagation process demonstrated a significant carry-over effect on the level of the secondary metabolites upon transfer of plants to an *ex vitro* environment. Our observations also demonstrate that the capacity for *B. nigra* shoots to synthesize phenolic compounds varied based on stage of maturity. Shoots harvested during vegetative growth, i.e., 3-month-old greenhouse-grown plants, were found to have a lower level of phenolic compounds than in field-grown plants during their flowering period. Similarly, flavonoid levels, increased significantly by 70% from 16.7 to 28.4 quercetin equivalents in mg/g of plant material, from immature to mature *B. nigra* shoots. Similar observations were reported by Sreelatha and Padma (34) in *Moringa oleifera*, who note the presence of highest phenolic and flavonoid contents in mature leaf extract. Pandjaitan et al. (35) also reported changes in the accumulation of total phenolics and total flavonoids in spinach leaves at different stages of maturation.

A linear regression analysis was performed to study the relationship between estimated values of antioxidant activity of *B. nigra* extracts and total phenolic and flavonoid contents (Table 3). A strong correlation observed between DPPH and FRAP assays and phenolic content (-0.862 and 0.804, respectively) indicates that the phenolic compounds in the methanolic extracts of *B. nigra* were responsible for their radical scavenging and reducing activities. Seidel et al. (15) and Deals-Rakotoarison et al. (3) have reported that phenylpropanoids (verbasco-

side, ballotetroside, forsythoside B, arenarioside) isolated from a hydroalcoholic *B. nigra* extract exert an influence on ROS content.

Our results are also consistent with numerous previous findings which identify a correlation between total phenolic content and antioxidant activity for other plant species (19, 36). The moderate correlation observed between total phenolic and flavonoid content in *B. nigra* extracts and the results of the inhibition of LA peroxidation test (0.529 and 0.455, respectively) suggests that also the non-phenolic compounds, such as organic acids (4) and diterpenoids (7, 13, 37) detected in *B. nigra* may demonstrate capacity to inhibit LA peroxidation.

A few studies have previously been reported on the antioxidant potential of extracts from aerial parts of wild grown plants of *B. nigra* (4, 6, 8, 15). However, it is difficult to make any direct comparison of our antioxidant results with the literature data, given the varying assay protocols and different extraction solutions. From the pharmaceutical point of view, the most important finding of the current study is that extracts of *in vitro* raised plants display higher antioxidant capacities and greater total of phenolic compounds content compared to extracts taken from a commercial sample of *B. nigra* herb. This is advantageous in practical terms because micropropagation allows better quality material to be obtained for medicinal purposes.

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