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Antioxidative properties and phytochemical composition of *Ballota nigra* infusion

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Abstract

The ability of *Ballota nigra* (Lamiaceae) infusion to act as a scavenger of DPPH radical, reactive oxygen species (superoxide radical, hydroxyl radical, hypochlorous acid) and nitric oxide was investigated. The tested infusion mainly exhibited a potent scavenging effect on DPPH, nitric oxide and superoxide radicals. In hydroxyl radical assay a potent pro-oxidant activity was noticed. No effect was found against hypochlorous acid. A phytochemical study was also undertaken, and seven phenolic compounds (chlorogenic, caffeic and caffeoylmalic acids, ballotetroside, forsythoside B, verbascoside and allysonoside) and eight organic acids (oxalic, aconitic, citric, ascorbic, malic, quinic, shikimic and fumaric acids) were identified and quantified by HPLC/DAD and HPLC/UV, respectively. Forsythoside B and quinic acid were revealed to be the main compounds in this infusion.

Keywords: *Ballota nigra*; DPPH; Reactive oxygen species; Nitric oxide; Phenolic compounds; Organic acids

1. Introduction

Ballota nigra (Lamiaceae), known as black horehound, is a perennial herb which is commonly distributed in most areas of the world that have mild climates. It is a small herb, from which flowered aerial parts are gathered, dried and used for medicinal purposes. Internally, *B. nigra* is traditionally used in the treatment of upset stomach, nausea and vomiting, in the symptomatic treatment of nervous disorders in adults and children, especially for mild sleep disorders and for the symptomatic treatment of coughs (Newall, Anderson, & Phillipson, 1996; PDR, 1998; Van Hellemont, 1986), and is also reported to treat inflammation (Saltan Citoğlu, Çoban, Sever, & Işcan, 2004). Externally, *B. nigra* is used in cases of gout (PDR, 1998). Most frequently, *B. nigra* is consumed as an infusion (10 g/l) (French Pharmacopoeia, 1989) and due to its strong taste it is often sweetened and mixed with other herbs or beverages. Regarding its chemical composition, previous studies on *B. nigra* aerial parts have revealed the presence of phenolic compounds, namely flavonoids (Bertrand, Tillequin, & Bailleul, 2000; Citoglu, Yilmaz, Tarikahya, & Tipirdamaz, 2005; Siciliano et al., 2005) and phenylpropanoid derivatives (Citoglu et al., 2005; Didry, Seidel, Dubreuil, Tillequin, & Bailleul, 1999; Seidel, Bailleul, Libot, & Tillequin, 1997; Seidel, Bailleul, & Tillequin, 1996; Seidel, Verholle, & Malard, 2000; Siciliano et al., 2005). As far as we know, nothing has been reported about its organic acids composition.

ROS are implicated in the development of many chronic disorders, such as cancer, arteriosclerosis, nephritis, diabetes mellitus, rheumatism and cardiovascular diseases, as well as in gastrointestinal tract disorders and inflammatory injury (Saltan Citoğlu et al., 2004). Since *B. nigra* has been traditionally used for the treatment of the two latter conditions, its antioxidant potential deserves to be explored. In

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this respect, the activity of an ethanol extract of *B. nigra* as a scavenger of superoxide radical, generated in a xanthine/xanthine oxidase system and as an inhibitor of lipid peroxidation, has already been investigated (Saltan Citoğlu et al., 2004). It has also been reported that some of its phenylpropanoids inhibited Cu^{2+}-induced LDL peroxidation (Seidel et al., 2000).

The aim of the present study was to evaluate the antioxidant potential of a *B. nigra* lyophilized infusion, since this is the most common form of using the species and it may constitute an interesting dietary source of health-protective compounds. Therefore, its capacity to act as scavenger of DPPH radical, reactive oxygen species (superoxide radical, hydroxyl radical, hypochlorous acid) and nitric oxide was investigated. In order to chemically characterize the infusion, the polar phenolic compounds already reported to occur in the species were determined by HPLC/DAD. Since, besides the phenolic compounds (Seidel et al., 2000; Siciliano et al., 2005), organic acids also have shown antioxidant capacity (Silva et al., 2004), the compounds from this latter class were also determined by HPLC/UV. A possible relationship between the chemical composition and the antioxidant potential was considered.

2. Materials and methods

2.1. Standards and reagents

Oxalic, citric, malic, quinic, shikimic and fumaric acids were purchased from Sigma (St. Louis, MO, USA). Aconitic, chlorogenic and caffeic acids were from Extrasynthése (Genay, France) and verbascoside and caffeoylmalic acid from PhytoLab (Hamburg, Germany). Ascorbic acid, methanol, acetonitrile, trifluoracetic and formic acids were obtained from Merck (Darmstadt, Germany) and sulphuric acid from Pronalab (Lisboa, Portugal). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH), xanthine, xanthine oxidase (XO) grade I from buttermilk (EC 1.1.3.22), β-nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), nitroblue tetrazolium chloride (NBT), anhydrous ferric chloride ($FeCl_3$), ethylenediaminetetraacetic acid disodium salt (EDTA), trichloroacetic acid, thiobarbituric acid, deoxyribose, sodium hypochlorite solution with 4% available chlorine (NaOCl), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and sulphanilamide were obtained from Sigma Chemical Co. (St. Louis, USA). Sodium nitroprussiate dehydrate (SNP) was obtained from Riedel-de-Haën and *N*-(1-naphthyl)-ethylene-diamine dihydrochloride from Merck (Darmstadt, Germany).

The ISOLUTE C18 non-end-capped (NEC) SPE columns (50 μm particle size, 60 Å porosity; 0.5 g sorbent mass/6 ml reservoir volume) were purchased from International Sorbent Technology Ltd. (Mid Glamorgan, UK).

2.2. Plant material and sampling

Aerial parts of three *B. nigra* plants were collected in September 2005, in the botanical garden of Faculty of Pharmacy, Charles University in Hradec Králové (Czech Republic), dried at room temperature for one week and reduced to powder (1600 μm). A voucher specimen was deposited at the Department of Pharmacognosy, Faculty of Pharmacy, Charles University, Hradec Králové, Czech Republic.

2.3. Sample preparation

B. nigra infusion was prepared by pouring 500 ml of boiling water on 5 g of plant material. The mixture was left to stand for 15 min and then filtered over a Büchner funnel. The resulting infusion was then lyophilized (Labconco 4.5, Kansas City, MO). The yield of the lyophilized extract was 1.22 g. The lyophilized extract was kept in a desiccator, in the dark.

2.4. Organic acids extract

1 mg of lyophilized infusion was redissolved in 2 ml acid water (pH 2 with HCl) and the aqueous solution was passed through an ISOLUTE C18 (NEC) column, previously conditioned with 2 ml of methanol and 5 ml of acid water (pH 2 with HCl). Organic acids were eluted with the aqueous solution. This aqueous extract was evaporated to dryness under reduced pressure (40 °C), redissolved in 0.01 N sulphuric acid (1 ml) and 20 μl were analysed by HPLC/UV. A mixture of standards was prepared by dissolving the compounds in 0.01 N sulphuric acid.

2.5. HPLC analysis of organic acids

The separation was carried out according to a described procedure (Ferreres et al., 2006) with an analytical HPLC unit (Gilson), using an ion exclusion Nucleogel® Ion 300 OA (Macherey-Nagel, Germany) column (300 × 7.7 mm) in conjunction with a column-heating device (Jones Chromatography) set at 30 °C. Elution was carried out isocratically with 0.01 N sulphuric acid at a flow rate of 0.2 ml/min. Detection was performed with a Gilson Holochrome UV detector set at 214 nm. Organic acids quantification was achieved by comparison of the peak area of the sample with that of the external standard.

2.6. HPLC analysis of phenolics

The lyophilized infusion (10 mg) was redissolved in 1 ml of water, passed by a 0.2 μm pore filter and 20 μl were subjected to HPLC analysis. This was performed using a HPLC unit (Gilson) and a Spherisorb ODS2 reversed-phase (Waters, Milford, USA) column (250 × 4.6 mm, 5 μm particle size) (Silva et al., 2000). The solvent system was a mixture of 5% formic acid (A) and methanol (B),

with a flow rate of 0.9 ml min^{-1}, and the gradient was as follows: 0 min – 5% B, 3 min – 15% B, 13 min – 25% B, 25 min – 30% B, 35 min – 35% B, 39 min – 45% B, 44 min – 50% B. Detection was achieved with a Gilson 170 diode array detector. Verbascoside and caffeoylmalic, chlorogenic and caffeic acids were identified on the basis of their chromatographic behaviour, by comparison of their retention times and UV–VIS spectra, in the range 200–400 nm, with those of authentic standards. The other phenylpropanoid derivatives were identified according to published data (Kirmizibekmez et al., 2005; Seidel et al., 2000): on using the same experimental conditions described by Kirmizibekmez et al. (2005), we obtained identical chromatograms in what concerns verbascoside, forsythoside B and alyssonoside, in which the compounds had the same order of elution and the same UV spectra. Ballotetroside was identified according to the characteristic shape and absorption maxima of its UV spectrum (Seidel et al., 2000). Peak purity was checked by means of the software contrast facilities. The data were processed with Unipoint system Software (Gilson Medical Electronics, Villiers le Bel, France). Phenolic compounds quantification was achieved by comparison of the peak area of the sample with that of the external standard at 320 nm. Chlorogenic, caffeic and caffeoylmalic acids and verbascoside were quantified as themselves and the other compounds as verbascoside.

2.7. DPPH·-scavenging activity

The antiradical activity of the extracts was determined spectrophotometrically in an ELX808 IU Ultra Microplate Reader (Bio-Tek Instruments, Inc), by monitoring the disappearance of DPPH· at 515 nm, according to a described procedure (Ferreres et al., 2006). For each extract, a dilution series (five different concentrations) was prepared in a 96 well plate. The reaction mixtures in the sample wells consisted of 25 µl of lyophilized infusion and 200 µl of 150 µM DPPH· dissolved in methanol. Three experiments were performed in triplicate.

2.8. Evaluation of superoxide ($O_2^{\cdot-}$) radica-scavenging activity

2.8.1. General

Antiradical activity was determined spectrophotometrically in an ELX808 IU Ultra Microplate Reader (Bio-Tek Instruments, Inc), by monitoring the effect of the lyophilized infusion on the $O_2^{\cdot-}$-induced reduction of NBT at 562 nm.

2.8.2. Non-enzymatic assay

Superoxide radicals were generated by the NADH/PMS system according to a described procedure (Valentão et al., 2001). All components were dissolved in phosphate buffer 19 mM, pH 7.4. Three experiments were performed in triplicate.

2.8.3. Enzymatic assay

Superoxide radicals were generated by the xanthine/xanthine oxidase (X/XO) system, following a described procedure (Valentão et al., 2001). Xanthine was dissolved in 1 µM NaOH and subsequently diluted in 50 mM phosphate buffer with 0.1 mM EDTA, pH 7.8, xanthine oxidase in 0.1 mM EDTA and the other components in 50 mM phosphate buffer with 0.1 mM EDTA, pH 7.8. Three experiments were performed in triplicate.

2.8.4. Effect on xanthine oxidase activity

The effect of the lyophilized infusion on XO activity was evaluated by measuring the formation of uric acid from xanthine in a double beam spectrophotometer (Helios e, Unicam), at room temperature. The reaction mixtures contained the same proportion of components as in the enzymatic assay for superoxide radical-scavenging activity, except NBT, in a final volume of 600 µl. The absorbance was measured at 295 nm for 2 min. Three experiments were performed in triplicate.

2.8.5. Hydroxyl radical (·OH) assay

The deoxyribose method for determining the effect of the infusion on hydroxyl radicals was performed according to a described procedure (Valentão et al., 2002). Reaction mixtures contained ascorbic acid, $FeCl_3$, EDTA, H_2O_2, deoxyribose and lyophilized extract. All components were dissolved in 10 mM KH_2PO_4–KOH buffer, pH 7.4. This assay was also performed, either without ascorbic acid, or EDTA, in order to evaluate the extracts' pro-oxidant or metal chelation potential, respectively. In each case, three experiments were performed in triplicate.

2.8.6. Hypochlorous acid (HOCl)-scavenging activity

The inhibition of HOCl-induced 5-thio-2-nitrobenzoic acid (TNB) oxidation to 5,5′-dithiobis(2-nitrobenzoic acid) was performed according to a described procedure (Valentão et al., 2002), in a double beam spectrophotometer (Helios e, Unicam). Hypochlorous acid and TNB were prepared immediately before use. Scavenging of hypochlorous acid was ascertained by using lipoic acid as a reference scavenger. The amount of TNB unchanged after incubation was calculated and expressed as a percentage of the initial value. Three experiments were performed in triplicate.

2.8.7. Nitric oxide (·NO)-scavenging activity

The ability of the lyophilized infusion to scavenge nitric oxide radical was determined spectrophotometrically in an ELX808 IU Ultra Microplate Reader (Bio-Tek Instruments, Inc.) according to a described procedure (Sumanont et al., 2004), with some modifications. A dilution series (five different concentrations) was prepared in a 96-well plate. The reaction mixtures in the sample wells consisted of extract and SNP dissolved in saline phosphate buffer, pH 7.4. The plates were incubated at 25 °C for 60 min under light. Afterwards, Griess reagent (1% sulphanilamide

and 0.1% naphthylethylenediamine dihydrochloride in 2% H_3PO_4) was added and the absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with naphthylethylenediamine was read at 540 nm. Three experiments were performed in triplicate.

3. Results and discussion

3.1. Phenolic compounds

The results obtained by the HPLC/DAD analysis of *B. nigra* lyphilized infusion revealed a fingerprint from which seven compounds were identified: chlorogenic, caffeic and caffeoylmalic acids, ballotetroside, forsythoside B, verbascoside and allysonoside (Fig. 1). All of these compounds have already been described in *B. nigra* (Newall et al., 1996; PDR, 1998; Seidel et al., 1996, 1997, 2000; Van Hellemont, 1986), with the exception of chlorogenic and caffeic acids, which are reported for the first time in this species. Other compounds were detected, namely compounds a, b and c, but were not identified (Fig. 1). These compounds showed UV spectra with maxima at 250 and 320 nm and a shape characteristic of hydroxycinnamic acid derivatives. The lyophilized infusion showed a high content of phenolic compounds (ca. 121 g/kg) (Table 1), with forsythoside B and compound c as the compounds present in higher amounts (ca. 20.3% and 22.1% of total compounds, respectively). Allysonoside and caffeic acids were the minor compounds, representing 1.0% and 0.7% of total phenolics, respectively (Table 1).

3.2. Organic acids

The HPLC/UV analysis allowed the identification and quantification of eight organic acids in *B. nigra* infusion: oxalic, aconitic, citric, ascorbic, malic, quinic, shikimic and fumaric acids (Fig. 2). All of these compounds are reported for the first time in *B. nigra*. A high content of organic acids was found (ca. 14 g/kg), with quinic acid as the main compound, corresponding to 58.9% of total acids (Table 2). Shikimic and fumaric acids were the compounds present in minor amounts, less than 0.1% of total organic acids (Table 2).

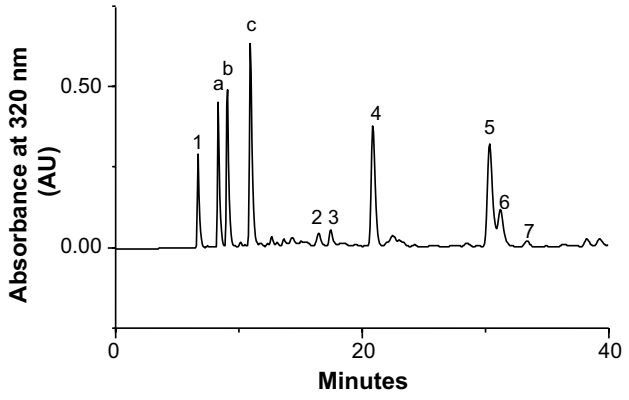

Fig. 1. HPLC phenolic profile of *Ballota nigra* aerial parts. Detection at 320 nm. Peaks: (1) ballotetroside; (2) chlorogenic acid; (3) caffeic acid; (4) caffeoylmalic acid; (5) forsythoside B; (6) verbascoside; (7) allysonoside; (a)–(c) unidentified compounds.

Table 1
Phenolic composition of *Ballota nigra* lyophilized infusion (mg/g)[a]

Compounds	Mean	SD
Ballotetroside (RT 6.7 min)	9.5	0.3
a (RT 8.3 min)	15.0	0.1
b (RT 9.1 min)	19.3	0.0
c (RT 10.9 min)	26.8	0.0
Chlorogenic acid (RT 16.5 min)	1.8	0.0
Caffeic acid (RT 17.4 min)	0.8	0.0
Caffeoylmalic acid (RT 20.9 min)	11.9	0.9
Forsythoside B (RT 30.3 min)	24.6	1.3
Verbascoside (RT 31.2 min)	10.3	1.4
Allysonoside (RT 33.4 min)	1.2	0.0
$\sum$	121.2	

[a] Results are expressed as means of three determinations. SD, standard deviation; $\sum$, sum of the determined phenolic compounds.

3.3. Antioxidant activity

The DPPH test is a simple non-enzymatic assay used to provide basic information on the ability of extracts to scavenge free radicals. *B. nigra* lyophilized infusion strongly scavenged $DPPH^{\cdot}$ in a concentration-dependent way ($IC_{25} = 4.81$ μg/ml) (Fig. 3).

The extract exhibited superoxide radical-scavenging activity, using the X/XO system (Fig. 4a), and the observed effect was concentration dependent ($IC_{25} = 14.6$ μg/ml). The effect of the extract on XO activity was also checked, once the inhibitory effect on the enzyme itself could also lead to a decrease of NBT reduction (Valentão et al., 2002). For this purpose, a control experiment was performed to evaluate the effect of the infusion on the conversion of xantin to uric acid (Fig. 4b). The results showed that the infusion had a weak inhibitory effect on XO ($IC_{25} = 143$ μg/ml), so it was not possible to show a clear-cut scavenging effect on superoxide radical. To confirm the scavenging capacity of lyophilized infusion against superoxide radical, we performed an assay using a chemical system composed of PMS, NADH and oxygen for production of superoxide radical. The extract scavenged superoxide radical in a concentration dependent manner with an IC_{25} of 26.1 μg/ml. The different IC_{25} values obtained with the two systems might be due to the higher production of superoxide radical in the non-enzymatic assay.

For hydroxyl radical, practically no scavenging activity was observed (Table 3). If ascorbate is omitted in the reaction mixture, then, if some prooxidant compounds are present, they will be able to redox cycle the metal ion required for hydroxyl generation and thus increase the radical production (Valentão et al., 2002). In order to evaluate the pro-oxidant activity of the extract, we omitted ascorbic

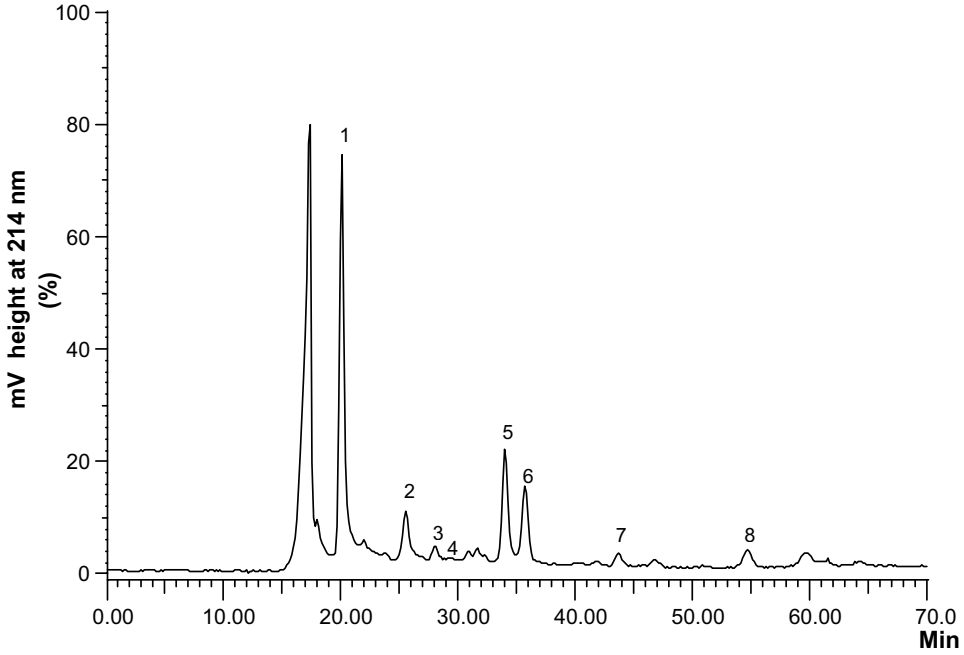

Fig. 2. HPLC organic acid profile of *Ballota nigra* aerial parts. Detection at 214 nm. Peaks: (1) oxalic acid; (2) aconitic acid; (3) citric acid; (4) ascorbic acid; (5) malic acid; (6) quinic acid; (7) shikimic acid; (8) fumaric acid.

Table 2
Organic acids composition of *Ballota nigra* lyophilized infusion (mg/g)[a]

Compounds	Mean	SD
Oxalic acid (RT 19.6 min)	0.83	0.02
Aconitic acid (RT 25.1 min)	0.07	0.00
Citric acid (RT 27.9 min)	0.48	0.05
Ascorbic acid (RT 29.6 min)	1.48	0.43
Malic acid (RT 33.9 min)	2.79	0.07
Quinic acid (RT 35.0 min)	8.01	0.85
Shikimic acid (RT 43.2 min)	0.01	0.00
Fumaric acid (RT 58.4 min)	0.01	0.00
$\sum$	13.68	

[a] Results are expressed as means of three determinations. SD, standard deviation; $\sum$, sum of the determined organic acids.

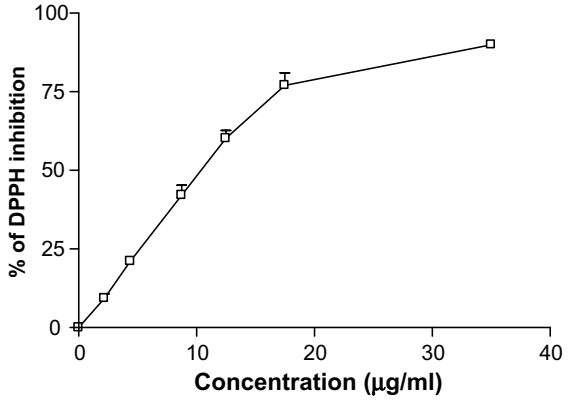

Fig. 3. Antiradical activity of *Ballota nigra* infusion against DPPH· radical. Values show means ± SE from three experiments performed in triplicate.

acid. As can be seen in Table 3, *B. nigra* lyophilized infusion was found to be a very effective substitute for ascorbic acid, especially at concentrations above 2.25 µg/ml. This pro-oxidant effect certainly also conditioned the results observed previously in the deoxyribose assay. Damage to deoxyribose also occurs if the Fe^{3+}-ascorbate-H_2O_2-induced generation of hydroxyl radicals is performed in the absence of EDTA, since omission of the chelator allows iron ions to bind directly to the sugar. Under such conditions, compounds inhibit deoxyribose degradation, not by reacting with hydroxyl radicals, but because they present ion-binding capacity and can withdraw the iron ions and render them inactive or poorly active in Fenton reactions (Payá, Halliwell, & Hoult, 1992). The assay performed in the absence of EDTA showed that *B. nigra* lyophilized infusion was not able to chelate Fe^{3+} ions (Table 3). The results obtained in the hydroxyl radical assay can be of great relevance, since the hydroxyl radical is the most reactive radical known in chemistry: it can attack and damage almost every molecule found in living cells (Halliwell, 1991). Hydroxyl radicals are produced *in vivo* by Fenton-type reactions, in which transition metals are involved. In addition, reducing agents, such as ascorbic acid, accelerate hydroxyl radical formation (Puppo, 1992). It seems that *B. nigra* infusion stimulates the generation of hydroxyl radical without ascorbic acid and these findings can be important in the case of pathological situations such as Wilson's disease or hemochromatosis, in which high levels of non-chelated transition metal contents are involved (Powell et al., 1991; Scheinberg et al., 1991).

Reactive oxygen species produced *in vivo* by activated phagocytic cells include HOCl. Hypochlorous acid is

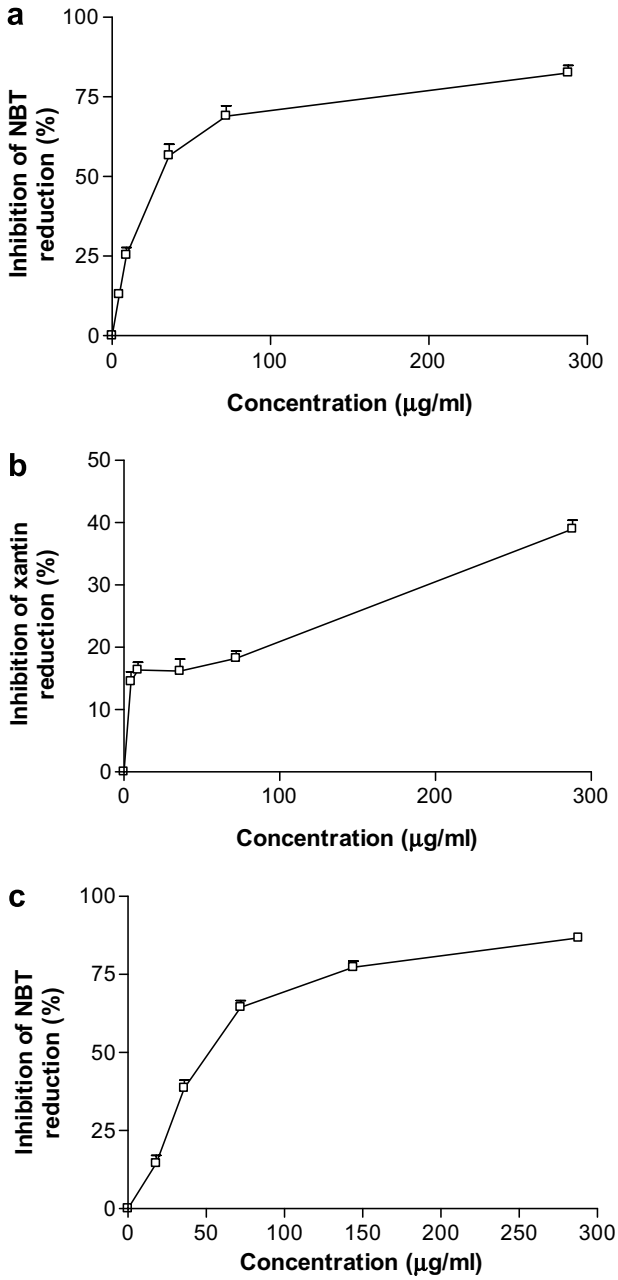

Fig. 4. Effect of *Ballota nigra* infusion on: (a) NBT reduction induced by superoxide radical generated in a X/XO system; (b) on XO activity; (c) NBT reduction induced by superoxide radical generated in a NADH/PMS system. Values show means ± SE from three experiments performed in triplicate.

Table 3
Absorbance and scavenging effect obtained in the deoxyribose assay in the presence and absence of ascorbic acid (-AA) or EDTA (-EDTA)

Ballota nigra lyophilized infusion (μg/ml)	ABS	Scavenging ratio (%)	ABS (-AA)	ABS (-EDTA)
0.00	0.396	0.0	0.402	0.302
1.13	0.370	7.1	0.411	0.325
2.25	0.386	3.0	0.427	0.347
4.50	0.391	1.1	0.480	0.348
9.00	0.386	2.9	0.499	0.331
18.0	0.422	–	0.573	0.327

produced in the organism at sites of inflammation by the oxidation of Cl^- ions, catalysed by neutrophil-derived myeloperoxidase, in the presence of H_2O_2 (Aruoma, Halliwell, Hoey, & Butler, 1989). HOCl is a powerful oxidant, which reacts readily with many biologically important molecules. Thiol groups are easily oxidized by HOCl (Ching, De Jong, & Bast, 1994). Thus, HOCl damages and induces target cell lysis, caused by sulfhydryl oxidation in plasma membrane proteins (Cochrane, 1991), inactivates α_1-antiprotease, activates collagenase and gelatinase, depletes antioxidant vitamins, such as ascorbic acid, and inactivates antioxidant enzymes such as catalase (Payá et al., 1992; Visioli, Bellomo, & Galli, 1998). In the present assay, a HOCl scavenger inhibits the oxidation of TNB into DNTB (Künzel, Zee, & Ijzerman, 1996). *B. nigra* lyophilized infusion exhibited no activity against HOCl (Fig. 5a). Under the assayed conditions, lipoic acid was used as a reference compound, which scavenged HOCl effectively in a concentration-dependent manner, presenting a protective effect of 82% at 500 μg/ml (Fig. 5b). So, the traditional uses of *B. nigra* in inflammatory processes cannot be attributed to the scavenging of HOCl.

Nitric oxide, a short-lived free radical endogenously generated, has many physiological functions, including vasodilatation, neurotransmission, synaptic plasticity and

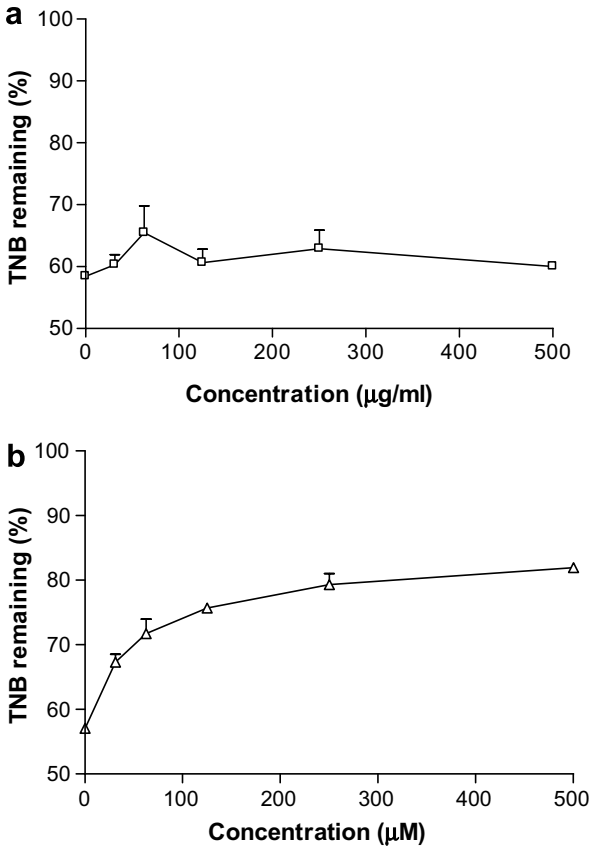

Fig. 5. Effect of *Ballota nigra* infusion (a) and lipoic acid (b) on the oxidation of TNB by HOCl. Values show means ± SE from three experiments performed in triplicate.

memory, in the central nervous system. In contrast, the NO radical has been implicated in pathogenesis of several diseases (Sumanont et al., 2004). One of the mechanisms by which excess of nitric oxide radical can cause tissue injury is by contributing to the generation of highly reactive oxygen radicals. In addition, the reaction of nitric oxide with superoxide radicals leads to the production of the two highly reactive species, hydroxyl radical and peroxynitrite (Rehman, Whiteman, & Halliwell, 1997). In the present work *B. nigra* extract exhibited NO radical scavenging activity, in a concentration dependent manner, with an IC_{25} at 122 μg/ml (Fig. 6).

The antioxidant activity observed in this study is due, most probably, to the presence of phenolic glycosides. The antioxidant capacity of verbascoside has been thoroughly studied, as this compound has been reported to have strong protective effects in several experimental models, such as the suppression of NADPH/CCl_4^- induced lipid peroxidation in rat liver microsomes (Xiong et al., 1998), inhibition of Cu^{2+}-induced LDL oxidation (Seidel et al., 2000), the scavenging of $O_2^{\cdot-}$ and ·OH using spin trap methodology (Wang et al., 1996), scavenging of $O_2^{\cdot-}$ generated in the NADH/PMS (Zhou & Zheng, 1991) or in the X/XO systems (Xiong, Kadota, Tani, & Namba, 1996), scavenging of $ABTS^{\cdot+}$ (Siciliano et al., 2005) or protection of DNA from Fenton reaction (Zhao et al., 2005). The antioxidant activity of forsythoside B has been previously determined by measuring the scavenging of $ABTS^{\cdot+}$ (Siciliano et al., 2005) or DPPH· (Delazar et al., 2005) and the protective effect against free radical-induced impairment of endothelium-dependent relaxation was also investigated (Ismailoglu, Saracoglu, Harput, & Sahin-Erdemli, 2002). Caffeic and chlorogenic acids have also been studied and these compounds were found to have effective antioxidant capacity using FRAP and ABTS (Nilsson et al., 2005) or β-carotene bleaching and DPPH methods (Fukumoto & Mazza, 2000). Organic acids may also contribute to the effects observed, since they are reported to have antioxidant capacity (Silva et al., 2004). The pro-oxidant activity observed in the hydroxyl radical assay can be partly attributed to the caffeic and chlorogenic acids, which have previously been reported as pro-oxidants (Laranjinha, Almeida, & Madeira, 1995; Yamanaka, Oda, & Nagao, 1997).

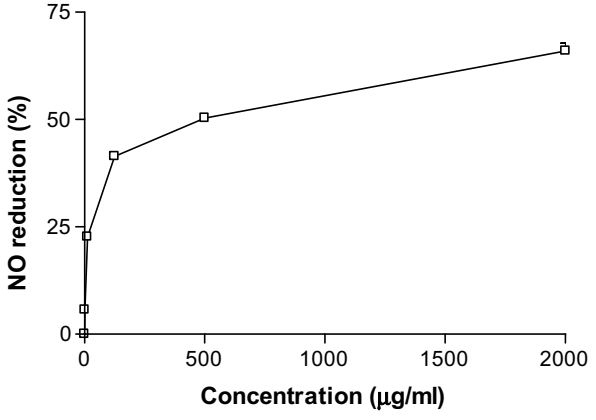

Fig. 6. Antiradical activity of *Ballota nigra* infusion against NO radical. Values means ± SE from three experiments performed in triplicate.

In conclusion, a potent antioxidant activity of *B. nigra* infusion was shown, considering the scavenging abilities displayed against superoxide radical, DPPH· and nitric oxide. This scavenging activity can be mainly attributed to the phenolic derivatives present in the infusion.

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