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Phenolics as antioxidants in garlic (*Allium sativum* L., Alliaceae)

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ABSTRACT

The antioxidant activities of polar fractions of mature garlic bulbs and immature plants in four different model systems are presented. Antioxidant activity was evaluated as free radical-scavenging capacity (RSC), together with the effect on lipid peroxidation (LP). RSC was assessed by measuring the scavenging activity of garlic extracts on 2,2-diphenyl-1-picrylhydrazyl (DPPH·) and hydrogen peroxide. Effects on LP were evaluated by following the activities of examined garlic extracts in Fe^{2+}/ascorbate and Fe^{2+}/H_2O_2 systems of induction. Investigated extracts reduced the DPPH radical formation (IC_{50} ranging from 1.03 to 6.01 mg/ml) and neutralised H_2O_2 (IC_{50} ranging from 0.55 to 2.01 mg/ml) in a dose-dependent manner. Strong inhibition of LP in both systems of induction was observed for all tested garlic extracts. Various levels of phenolics (0.05–0.98 mg gallic acid equivalents/g of dry extract) and flavonoid aglycones (4.16–6.99 μg quercetin equivalents/g of dry extract) in the investigated extracts of garlic could explain the obtained differences in these results only partially.

1. Introduction

Garlic (*Allium sativum* L., Alliaceae) has been playing one of the most important dietary and medicinal roles in human beings for centuries. It has been cultivated since ancient times, used as a spice and flavouring and, due to its potential benefits in preventive and curative medicine, has been used in many cultures (Rivlin, 2001). Even today, the medical use of garlic is widespread and growing. Epidemiological, clinical, and preclinical studies have shown close relation between dietary habits, including garlic intake, and the occurrence of disease. Furthermore, garlic was investigated extensively for health benefits, which has resulted in more than 1000 publications over the past decade. It is considered to be one of the best disease-preventive foods, based on its potential and varied effects (Amagase, 2006). A wide array of therapeutic effects, such as hypolipidaemic, antiatherosclerotic, hypoglycaemic, anticoagulant, antihypertensive, antimicrobial, antidote (for heavy metal poisoning) and hepatoprotective, has been reported (Agarwal, 1996; Rivlin, 2001). Furthermore, it prevents cold and flu symptoms through immune enhancement and exhibits anticancer and chemopreventive activities (Agarwal, 1996; Amagase, 2006; Banerjee, Mukherjee, & Maulik, 2003; Thomson & Ali, 2003). However, the exact mechanisms of protection under these conditions are not well understood. Oxidative stress, arising as a result of imbalance between free radical generation and key endogenous antioxidant defence in tissues, plays a key role in the initiation and progression of almost all these conditions (Halliwell & Gutteridge, 1985). The antioxidant properties of garlic and different garlic preparations are well documented (Agarwal, 1996; Banerjee et al., 2003; Cho & Xu, 2000; Gedik et al., 2005; Gorinstein et al., 2006; Nuutila, Puupponen-Pimia, Aarni, & Oksman-Caldentey, 2003; Pedraza-Chaverri, Medina-Campos, & Segoviano-Murillo, 2007; Rabinkov et al., 1998; Saravanan & Prakash, 2004; Wang et al., 1996; Yin & Cheng, 1998). It is generally considered that health-related functions are mostly attributed to the fresh garlic content, rich in γ-glutamylcysteine and many other sulfur-containing compounds in it, giving a characteristic flavour formed during storage and processing (Agarwal, 1996; Banerjee et al., 2003; Cho & Xu, 2000; Rabinkov et al., 1998; Wang et al., 1996). However, additional constituents of garlic include a wide range of primary and secondary non-sulfur biomolecules, such are steroidal glycosides (Matsuura, Ushiroguchi, Itakura, Hayashi, & Fuwa, 1988), essential oil (Calvo-Gomez, Morales-Lopez, & Lopez, 2004), flavonoids (Harborne & Williams, 1996), anthocyanins (Fossen & Andersen, 1997), lectins (Kaku, Goldstein, Van Damme, & Peumans, 1992), prostaglandins, fructan, pectin, adenosine, vitamins B1, B2, B6, C and E, biotin, nicotinic acid, fatty acids, glycolipids, phospholipids and essential amino acids (Fenwick & Hanley, 1985). Many of these components work synergistically to provide different health benefits (Amagase, 2006; Banerjee et al., 2003), but their real importance in the explanation of health benefits of garlic remains to be resolved. According to our knowledge, there are very few data that characterise the potential antioxidant properties related to

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phenolic and flavonoid fractions of garlic (Miller, Rigelhof, Marquart, Prakash, & Kanter, 2000; Nuutila et al., 2003). Furthermore, there are no data regarding antioxidant properties of immature garlic plants, which are widely used as salads and spices in most of the countries of the Balkan Peninsula.

With respect to this, the antioxidant activities of polar fractions of mature garlic bulbs and immature plants in four different model systems are presented in the study, together with the content of total phenolics and flavonoids in the investigated extracts.

2. Materials and methods

2.1. Plant material

The whole plants of cultivated immature garlic (*A. sativum* L., Alliaceae) – bulbs with steam and leaves, were collected in April, 2006, and the bulbs of mature garlic plants were collected in July, 2006, both in Padej, Vojvodina Province, Serbia. Voucher specimens of collected plants (immature garlic No. G-12/06 and mature garlic No. G-19/06) were confirmed and deposited at the Herbarium of the Laboratory of Pharmacognosy, Department of Pharmacy, Faculty of Medicine (HLPhM), University of Novi Sad.

2.2. Chemicals

Trichloracetic acid (TCA), 2-thiobarbituric acid (TBA), ethylene diamine-tetraacetic acid disodium salt dihydrate (EDTA), ascorbic acid and 2,2-diphenyl-1-picrylhydrazyl (DPPH·) were purchased from Sigma Co. (St. Louis, Mo, USA). Folin–Ciocalteu (FC) reagent was purchased from Merck (Germany). All other chemicals and reagents were of analytical grade.

2.3. Extracts preparation

The ground air-dried immature garlic plants (I), ground and air-dried garlic bulbs (II; prepared as Aged Garlic Extract) and fresh garlic bulbs (III) were extracted using a method of maceration with 80% methanol (MeOH) for 76 h at room temperature. After the maceration, the extracts were collected, filtered and evaporated to dryness under vacuum. The quantities of dry extracts were determined gravimetrically and, for extracts I, II and III, they were 3.38%, 9.26% and 6.01%, respectively. For the evaluation of the antioxidant activity, the residues were dissolved in water, making 5% (w/v) stock solutions.

2.4. Analysis of total phenolic content

The amount of total phenolic compounds in the extracts was determined colorimetrically with the Folin–Ciocalteu (FC) reagent, using a slightly modified method of Fukumoto and Mazza (2000). The reaction mixture contained 500 μl of 0.1% aqueous dilution of dry extract, 2.5 ml of freshly prepared 0.2 M FC reagent and 2 ml of sodium carbonate solution and was kept in the dark under ambient conditions for 30 min to complete the reaction. The absorbance of the resulting solution was measured at 760 nm in a UV–Vis spectrophotometer (model 8453 Hewlett Packard, Agilent Technologies, USA). The concentration of total phenolic compounds was expressed as mg of gallic acid equivalents (GAE) per g of dried extract (de), using a standard curve of gallic acid. All measurements were carried out in five replicates.

2.5. Estimation of total flavonoid content

Measurement of total flavonoid content in the investigated extracts was determined spectrophotometrically according to Jia, Tang, and Wu (1999), using a method based on the formation of complex flavonoid-aluminium with the absorbtivity maximum at 430 nm. The aqueous dilutions of samples, in the amount of 1 ml, were separately mixed with 1 ml of 2% $AlCl_3 \times 6H_2O$. After incubation at room temperature for 15 min, the absorbance of the reaction mixtures was measured at 430 nm. The flavonoids content was expressed as μg of quercetin equivalents (QE) per g of dried extract (de), by using a standard graph. All measurments were carried out in five repetitions.

2.6. Antioxidant activity

2.6.1. DPPH· assay

The DPPH·-assay was performed as previously described (Mimica-Dukic, Bozin, Sokovic, & Simin, 2004). The samples (ranging from 5 to 50 μl/ml of stock solution) were mixed with 1 ml of 90 μM DPPH· solution and made up with 95% MeOH to a final volume of 4 ml. The absorbance of the resulting solutions and the blank (with same chemicals, except sample) was recorded after 1 h at room temperature. For each sample, four replicates were recorded. The disappearance of DPPH· was measured spectrophotometrically at 515 nm. RSC, expressed as a percentage, was calculated by the following equation:

$$\text{RSC}\ (\%) = 100 \times (A_{\text{blank}} - A_{\text{sample}}/A_{\text{blank}}).$$

2.6.2. Hydrogen peroxide-scavenging activity

The hydrogen peroxide-scavenging ability of examined extracts was determined according to the method of Ruch, Cheng, and Klaunig (1989). A solution of H_2O_2 (40 mM) was prepared in phosphate buffer (pH 7.4). Examined extracts in different concentrations (ranging from 10 to 50 μl/ml of stock solution) were added to 3.4 ml of phosphate buffer, together with 0.6 ml of H_2O_2 solution. The absorbance value of the reaction mixture was recorded at 230 nm. Blank solution contained only the phosphate buffer (4 ml). The precentage of H_2O_2 scavenging of examined extracts was calculated as

$$\%\ \text{of scavenged}\ H_2O_2 = [(A_0 - A_1)/A_0] \times 100$$

where A_0 is the absorbance of the control (phosphate buffer with H_2O_2) and A_1 is the absorbance of the examined extracts.

2.6.3. Determination of lipid peroxidation (LP)

The extent of LP was determined by measuring the colour of adduct produced in the reaction between thiobarbituric acid (TBA) and malondialdehyde (MDA) in the TBA assay, performed with small modifications (Mimica-Dukic et al., 2004). For investigation, two systems of induction, Fe^{2+}/ascorbate and Fe^{2+}/H_2O_2, were used. As a model system of biological membranes the commercial preparation of liposomes "PRO-LIPO S" (Lucas-Meyer, Hamburg, Germany), pH 5–7, was used. The liposomes, 225–250 nm in diameter, were obtained by dissolving the commercial preparation in demineralized water (1:10) in an ultrasonic bath. For the assay, the examined extracts were added in different concentrations (ranging from 10 to 50 μl/ml of stock solution).

In the Fe^{2+}/ascorbate-induced LP, 60 μl of suspension of liposomes were incubated, together with 20 μl of 0.01 M $FeSO_4$, 20 μl of 0.01 M ascorbic acid and appropriate amounts of extract samples in 2.89 ml of 0.05 M KH_2PO_4–K_2HPO_4 buffer, pH 7.4 (3 ml final solution).

The reaction mixture in Fe^{2+}/H_2O_2-induced LP contained 30 μl of suspension of liposomes, 0.125 ml of 9 mM $FeSO_4$, 0.125 ml of 0.88 M H_2O_2 and investigated extract samples in different concentrations in 2.71 ml of 0.05 M KH_2PO_4–K_2HPO_4 buffer, pH 7.4 (3 ml final solution).

Samples were incubated at 37 °C for 1 h. LP was terminated, both, by the reaction of 1.5 ml of TBA reagent and 0.2 ml of 0.1 M EDTA and by heating at 100 °C for 20 min. After cooling and centrifugation (4000 rpm for 10 min), in order to precipitate proteins, the content of the MDA (TBARS) was determined by measuring the absorbance of adduct at 532 nm.

All reactions were carried out in four replicates.

The percentage of LP inhibition was calculated by the following equation:

$$I\ (\%) = (A_0 - A_1)/A_0 \times 100$$

where A_0 was the absorbance of the control reaction (full reaction, without test compound) and A_1 was the absorbance of the examined samples.

2.7. Statistics

The data were reported as mean values ± standard deviation (SEM). Values representing the concentrations of investigated extracts that cause 50% of neutralization/inhibition (IC_{50}) were determined by the linear regression analysis of obtained RSC and values of the inhibition of LP (Microsoft Excel programme for Windows, v. 2000).

3. Results and discussion

3.1. Total phenolic and flavonoid contents

Although most antioxidant activities from plant sources are derived from phenolic-type compounds (Cai, Luo, Sun, & Corke, 2004), these effects do not always correlate with the presence of large quantities of phenolics. Therefore, both sets of data need to be examined together. With respect to this, the investigated garlic extracts were analysed for total phenolic and flavonoid contents. The amount of total phenolics varied widely in plant materials and ranged from 0.05 to 0.98 mg GAE/g (Table 1). Furthermore, the results obtained from evaluation of total flavonoid content also indicate great variations, especially regarding the plant phenophasis (Table 1). In the immature garlic plant, the content of quercetin equivalents was notably higher (6.99 μg QE/g) than it was in mature plant bulbs (5.78 and 4.16 μg QE/g). The decreases of total phenolic and flavonoid contents are most probably caused by the increase of sulphur compounds and terpenoid substances present in the essential oil of mature garlic bulbs.

3.2. Antioxidant activity

3.2.1. General

The antioxidant potential of different plant extracts and pure compounds can be measured using numerous *in vitro* assays. Each of these tests is based on one feature of the antioxidant activity, such as the ability to scavenge free radicals, or the inhibition of lipid peroxidation. However, a single method is not recommended for the evaluation of the antioxidant activities of different plant products, due to their complex composition (Chu, Chang, & Hsu, 2000; Nuutila et al., 2003). Therefore, the antioxidant effects of plant products must be evaluated by combining two or more different *in vitro* assays to get relevant data. With respect to this, the antioxidant properties of the examined garlic extracts were evaluated, both, as free radical-scavenging capacity (RSC) and as protective effect on the lipid peroxidation.

Table 1
Total phenol and flavonoid contents for the studied extracts of garlic

Garlic extract	Total phenolics (mg GAE/g)	Total flavonoids (μg QE/g)
I	0.98 ± 0.004	6.99 ± 0.01
II	0.18 ± 0.006	5.78 ± 0.09
III	0.05 ± 0.005	4.16 ± 0.03

3.2.2. Free radical-scavenging capacity (RSC)

The RSC was evaluated by measuring the scavenging activity of examined garlic extracts on 2,2-diphenyl-1-picrylhydrazyl (DPPH·) radicals and by neutralisation of hydrogen peroxide. The DPPH radical is one of the most commonly used substrates for fast evaluation of antioxidant activity because of its stability (in radical form) and simplicity of the assay. On the other hand, although hydrogen peroxide is a non-free radical species, it is the source of the very toxic hydroxyl radical, especially in the presence of metal ions such as copper or iron. Also, hydrogen peroxide can cross membranes and may slowly oxidise a number of cell compounds. Thus, the elimination of hydrogen peroxide, as well as hydroxyl radical, is important for both, human health and the protection of pharmaceutical and food systems.

In the DPPH· assay, the ability of the investigated garlic extracts to act as donors of hydrogen atoms or electrons in transformation of DPPH radical into its reduced form DPPH·-H was investigated. All the assessed extracts were able to reduce the stable, purple-coloured radical, DPPH·, into the yellow-coloured DPPH·-H reaching 50% of reduction. The IC_{50} values were as follows: 1.03 mg/ml for extract of immature garlic plants (I), 4.41 mg/ml for extract of air-dried garlic bulbs (II) and 6.01 mg/ml for extract of fresh garlic bulbs (III). The most powerful extracts were those obtained from immature garlic plants (I) (Table 2). The extracts from mature bulbs (II and III) expressed similar but significantly lower scavenging capacity than did those obtained from the immature plant (I), and this activity was dose-dependent. The earlier published data (Miller et al., 2000; Nuutila et al., 2003) also indicated great differences in results of scavenging activities of garlic bulb extracts, relating them partially to the polarity of extraction medium (50–100% MeOH) and consequently to the content of different phenolic compounds.

The ability of examined garlic extracts to scavenge hydrogen peroxide is shown in Table 3. All extracts were able to neutralise H_2O_2 in a dose-dependent manner (IC_{50} was 0.55 mg/ml for extract I, 0.65 mg/ml for extract II and 2.01 mg/ml for extract III). Strong scavenging effects were observed, especially in the extract obtained from the immature garlic plants (I) and the one prepared similarly to commercial aged garlic extract preparations (II). Relatively slight neutralisation of hydrogen peroxide, exhibited by extract III, could be explained partially by the chemical composition and relatively low content of total phenolics (0.05 mg GAE/g) and flavonoids (Table 1).

3.2.3. Lipid peroxidation (LP)

The protective effects of the examined garlic extracts on peroxidation of lipids have been evaluated by the TBA assay using two systems of induction, Fe^{2+}/ascorbate and Fe^{2+}/H_2O_2. The inhibition of LP was determined by measuring the formation of MDA, using liposomes as an oxidizable substrate. However, the thiobarbituric acid test is non-specific for MDA and therefore non-lipid substances present in plant extracts, together with peroxidation products other than malondialdehyde, can react positively with TBA (Janero, 1990). These interfering compounds distort the results; therefore, all final results of investigated garlic extracts have been corrected using the absorbances of those after the TBA test (without liposomes) (Nuutila et al., 2003).

In Fig. 1, results of antioxidant activities of the examined garlic extracts in the Fe^{2+}/ascorbate system of induction are presented. All extracts exhibited notable antioxidant activity, ranging from

Table 2
Percentage of neutralisation of DPPH radical by garlic extracts in the DPPH· assay

Garlic Extract	Concentrations (mg/ml)						IC_{50} (mg/ml)
	0.25	0.625	1.25	1.875	2.50	5.00	
I	36.0 ± 0.16	45.9 ± 0.07	56.9 ± 0.15	66.9 ± 0.27	75.6 ± 0.06	84.7 ± 0.08	1.03
II	8.21 ± 0.08	13.8 ± 0.06	21.2 ± 0.32	29.9 ± 0.09	40.3 ± 0.25	57.6 ± 0.54	4.41
III	30.7 ± 0.46	35.2 ± 0.33	37.8 ± 0.27	40.0 ± 0.16	42.7 ± 0.12	44.8 ± 0.15	6.01

Table 3
Percentage of scavenging activity of hydrogen peroxide by garlic extracts

Garlic Extract	Concentrations (mg/ml)					IC_{50} (mg/ml)
	0.1	0.125	0.187	0.25	0.5	
I	10.2 ± 0.11	24.1 ± 0.82	30.5 ± 0.69	37.1 ± 0.27	42.2 ± 0.45	0.55
II	19.7 ± 0.49	25.6 ± 0.37	32.0 ± 0.67	34.9 ± 0.86	40.7 ± 0.77	0.65
III	24.8 ± 0.56	25.8 ± 0.10	26.7 ± 0.99	27.7 ± 0.53	30.3 ± 0.93	2.01

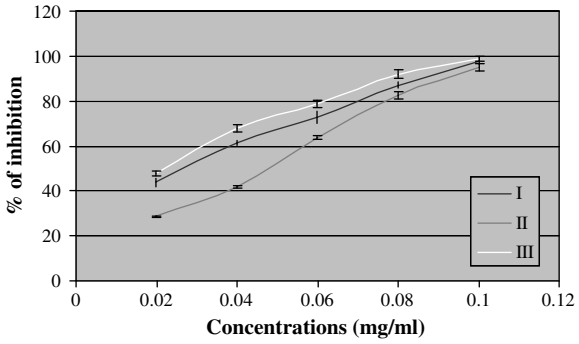

Fig. 1. Inhibition of lipid peroxidation (LP) in Fe^{2+}/ascorbate system of induction by the investigated garlic extracts.

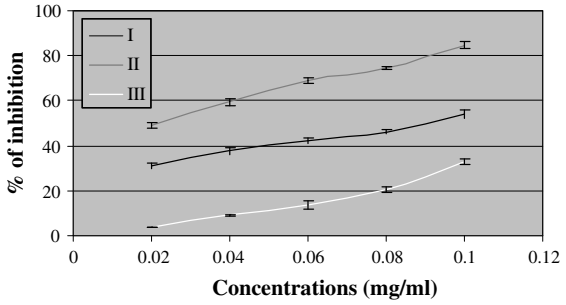

Fig. 2. Inhibition of lipid peroxidation (LP) in Fe^{2+}/H_2O_2 system of induction by the examined garlic extracts.

28.5% to 98.7% of inhibition of lipid peroxidation, and this activity was dose-dependent. Values of 50% of inhibition (IC_{50}) for extracts I, II and III were 0.03 mg/ml, 0.05 mg/ml and 0.02 mg/ml, respectively. In this model system the strongest antioxidant activity was exhibited by the extract from fresh garlic bulbs (III), which is contrary to the results previously shown.

In the LP induced by Fenton reaction (Fe^{2+}/H_2O_2), examined extracts exhibited different antioxidant activities (ranging from 3.97% to 84.6%) (Fig. 2). The extract prepared as an aged garlic extract (II) expressed the strongest protective effect on lipid peroxidation (IC_{50} = 0.02 mg/ml). For the other two examined extracts (I and III), IC_{50} values were notably higher (0.09 and 0.16 mg/ml, respectively). However, these findings are in accordance with earlier published data about antioxidant activities of garlic extracts (Nuutila et al., 2003).

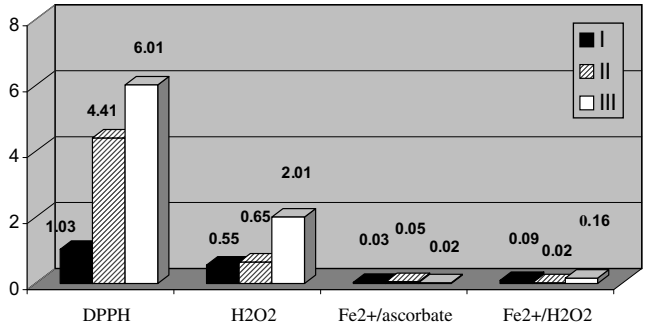

Fig. 3. Comparison of antioxidant activity (IC_{50} values) exhibited by examined garlic extracts (I, II and III) in different model systems. Concentrations of extracts are shown in mg/ml.

In conclusion, the measurement of antioxidant activity was used as a method for the evaluation of garlic extracts in this study. The comparison of antioxidant activity (IC_{50} values) of investigated garlic extracts (Fig. 3) showed variable effects, depending on the model system used for evaluation. Generally, the strongest activity, with very small variations between different garlic extracts, was observed in evaluation of LP. By contrast, great distinctions were noticed by testing RSC. However, these findings are in accordance with data published earlier (Miller et al., 2000; Nuutila et al., 2003). Various levels of phenolics and flavonoids in the investigated extracts (Table 1), together with additional constituents of garlic, could partially explain the differences in obtained results. Moreover, the responsibility of phenolics for antioxidant activity, estimated by various methods, depends on their chemical structures, too. Also, the contradictory results are most probably due to differences in the experimental conditions (different reaction mechanisms) used in different assays.

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