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Antioxidative Properties of Cardoon (*Cynara cardunculus* L.) Infusion Against Superoxide Radical, Hydroxyl Radical, and Hypochlorous Acid

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Polyphenols are able to act as antioxidants by virtue of their hydrogen-donating and metal-chelating capacities. Cardoon (*Cynara cardunculus* L.) is a species containing considerable amounts of polyphenolic compounds, namely flavonoids and phenolic acids. This study examined the antioxidant activity of cardoon lyophilized infusion against superoxide radical, hydroxyl radical, and hypochlorous acid. Superoxide radical was generated either in an enzymatic system or nonenzymatically, and the scavenging ability was assessed by the inhibition of superoxide radical-induced reduction of nitroblue tetrazolium. Hydroxyl radical was generated by the Fe^{3+}-EDTA/ascorbate Fenton system, and scavenging capacity was estimated by evaluating the inhibition of hydroxyl radical-induced deoxyribose degradation into thiobarbituric acid-reactive substances. Inhibition of hypochlorous acid-induced 5-thio-2-nitrobenzoic acid oxidation to 5,5′-dithiobis(2-nitrobenzoic acid) was used in order to test the hypochlorous acid scavenging activity.

KEYWORDS: Cardoon; *Cynara cardunculus*; antioxidant activity; superoxide radical; hydroxyl radical; hypochlorous acid; polyphenols

INTRODUCTION

Antioxidants are of great interest because they may help to protect the body against damage by reactive oxygen species (ROS) (*1*). Active oxygen in the form of superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^{\bullet}$) is a byproduct of normal metabolism. It attacks biological molecules such as lipids, proteins, enzymes, DNA, and RNA, leading to cell or tissue injury associated with degenerative diseases (*2*−*4*). Excessive free radical production and lipid peroxidation are known to cause several pathological conditions including atherosclerosis, aging, nephrites, diabetes mellitus, rheumatic diseases, cardiac and cerebral ischemia, cancer, and adult respiratory distress syndrome (*5*−*7*). Although the organism possesses defense mechanisms to reduce the oxidative damage, such as enzymes and other antioxidant molecules to arrest the damaging properties of ROS (*1*, *8*, *9*), continuous exposure to chemicals and contaminants may lead to an increase in the amount of free radicals in the body beyond control, and cause irreversible oxidative damage. Therefore, antioxidants with free radical scavenging activities could have great relevance as prophylactic and therapeutic agents in diseases in which oxidants or free radicals are implicated (*9*, *10*). In fact, there is convincing epidemiological evidence that the consumption of fruits and vegetables is in general beneficial to health due to the protection provided by the antioxidant phytonutrients contained in them (*6*, *11*).

Furthermore, fats and oils are easily deteriorated by oxidation. The addition of antioxidants to food is an effective way to prevent the development of various off-flavors and undesirable compounds that result from lipid peroxidation (*2*, *10*). A number of synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) were extensively added to foodstuffs, although their use has begun to be questioned because of their toxicity (*8*, *12*, *13*).

Consequently, there is considerable interest in preventive medicine and in the food industry in the development of natural antioxidants from botanical sources, especially edible plants (*10*, *14*). In fact, some plants synthesize large amounts of the well-known antioxidants vitamin C, vitamin E, and carotenoids. Phenolic compounds are also widely distributed in plants and have been found to possess antioxidative potential as well (*1*, *6*, *11*, *14*−*16*).

Cardoon (*Cynara cardunculus* L.) is a Mediterranean species, with a long traditional use in Southwest Europe. The flowers are widely employed in the preparation of cheeses because of their proteases. Cardoon leaves are traditionally used for their diuretic, choleretic, and hepatoprotective effects (*17*). Several phenolic compounds have been characterized in its chemical composition, namely 3-, 4-, and 5-caffeoylquinic acids; cafeic

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acid; 1,3-dicaffeoylquinic acid (cynarin); 3,4-, 3,5-, 1,5-, and 4,5-dicaffeoylquinic acids; luteolin 7-glucoside; apigenin 7-glucoside; and luteolin (*18*−*20*). However, as far as we know, despite its wide use as a therapeutic agent, nothing has been reported about the antioxidant activity of this species. The purpose of the present study was to evaluate the antioxidant potential of cardoon leaves infusion since this is the most common use of the species. Therefore, the scavenging effect on $O_2^{\bullet-}$ generated either by enzymatic or chemical systems was studied as well as the effect on xanthine oxidase activity. The scavenging capacity of the lyophilized infusion on •OH generated by a Fenton system, in the presence and absence of EDTA or ascorbic acid, and on HOCl were also evaluated. As hydrophilic compounds, it is expected that some of cardoon's phenolic compounds will be present in the lyophilized infusion, among other constituents; and the antioxidant capacity exhibited by the infusion will be the sum of activities of its different components, for which the phenolic fraction will also contribute. To characterize the phenolic compounds we performed a HPLC/diode array detection (HPLC/DAD) analysis, and a correlation between the antioxidant observed effects and phenolic composition was made.

MATERIALS AND METHODS

Standards and Reagents. 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), ferric chloride anhydrous ($FeCl_3$), ethylenediaminetetraacetic acid disodium salt (EDTA), ascorbic acid, trichloroacetic acid, thiobarbituric acid, deoxyribose, sodium hypochlorite solution with 4% available chlorine (NaOCl), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), lipoic acid, and sodium borohydride were obtained from Sigma Chemical Co. (St. Louis, MO). Standards were from Extrasynthese (Genay, France). As 3- and 4-caffeoylquinic acids were not commercially available, they were prepared by transesterification of 5-caffeoylquinic acid using tetramethylammonium hydroxide (*21*, *22*). 1,3-dicaffeoylquinic acid (cynarin) was kindly supplied by Natiris (Lisbon, Portugal). All other reagents were of analytical grade. Ultrapure Milli-Q purified water was used throughout.

Plant Material. Cardoon leaves were collected in Arouca, Portugal, in May 1998, and dried at room temperature. The cultivation was under the responsibility of Direcção Geral de Agricultura de Entre Douro e Minho.

Sample Preparation. Cardoon infusion was prepared by pouring 200 mL of boiling water on 4 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 (Modulyo 4K freeze-dryer, Edwards). The yield of the lyophilized infusion was 1.17 g.

HPLC Analysis of Phenolic Compounds. Separation, identification, and quantification of phenolic compounds was achieved with an analytical HPLC unit (Gilson), using a reversed-phase Spherisorb ODS2 (5-μm particle size; 25.0 × 0.46 cm) column, as previously reported (*20*). For the mobile phase, water/formic acid (19:1) and methanol were used.

Evaluation of Superoxide Radical Scavenging Activity. Antiradicalar activity was determined spectrophotometrically in a 96-well plate reader (Ceres 900) by monitoring the effect of the lyophilized infusions on the $O_2^{\bullet-}$-induced reduction of NBT at 560 nm. The superoxide production was controlled by superoxide dismutase (SOD), which inhibited the reactions of NBT reduction in a concentration-dependent manner (data not shown).

Nonenzymatic Assay. Superoxide radicals were generated by the NADH/PMS system according to a described procedure (*23*). The lyophilized infusion was tested at the concentrations 52.1, 104.2, 208.3, 416.7, 833.3, and 1666.7 μg/mL. All components were dissolved in phosphate buffer (19 mM, pH 7.4). Four experiments were performed in triplicate.

Enzymatic Assay. Superoxide radicals were generated by the xanthine/xanthine oxidase (X/XO) system following a described procedure (*23*). The lyophilized infusion was tested at the concentrations 5.2, 10.4, 20.8, 41.7, 83.3, and 166.7 μg/mL. Xanthine was dissolved in NaOH (1 μM) and subsequently in phosphate buffer (50 mM) with EDTA (0.1 mM, pH 7.8), xanthine oxidase in EDTA (0.1 mM) and the other components in phosphate buffer (50 mM) with EDTA (0.1 mM, pH 7.8). Four experiments were performed in triplicate.

Effect on Xanthine Oxidase Activity. The effect of the lyophilized infusion on xanthine oxidase activity was evaluated by measuring the formation of uric acid from xanthine in a double-beam spectrophotometer (Shimadzu 2600) at room temperature. The reaction mixtures contained the same proportion of components as those used 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. Four experiments were performed in triplicate.

Additionally, this procedure was repeated with several concentrations of xanthine (11, 22, 44, and 88 μM) and lyophilized infusion (89.4 μg/mL), to evaluate the inhibitory pattern of the extract. Three experiments were performed in triplicate.

Hydroxyl Radical Assay. The deoxyribose method for determining the scavenging effect of the lyophilized infusion on hydroxyl radicals was performed according to a described procedure (*24*). Reaction mixtures contained, in a final volume of 1 mL, ascorbic acid (50 μM), $FeCl_3$ (20 μM), EDTA (2 mM), H_2O_2 (1.42 mM), deoxyribose (2.8 mM), and lyophilized infusion (3.1, 12.5, 50.0, 200, or 800 μg/mL). All components were dissolved in KH_2PO_4/KOH buffer (10 mM, pH 7.4). After incubation at 37 °C for 1 h, 1 mL of 2.8% trichloroacetic acid (w/v) and 1 mL of 1% thiobarbituric acid (TBA) (w/v) were added, and the mixture was heated in a water bath at 100 °C for 15 min. The absorbance of the resulting solution was measured at 532 nm. This assay was also performed without ascorbic acid or EDTA, to evaluate the infusion's prooxidant and metal chelation potential, respectively. Four experiments were performed in duplicate.

Hypochlorous Acid Scavenging Activity. *Synthesis of Hypochlorous Acid.* For the assay, 75 μM HOCl was prepared immediately before use by adjusting a solution of NaOCl to pH 6.2 with diluted sulfuric acid. The concentration of HOCl was further determined spectrophotometrically at 235 nm using the molar absorption coefficient of 100 M^{-1} cm^{-1} (*24*).

Synthesis of 5-Thio-2-nitrobenzoic Acid (TNB). TNB was prepared according to a described procedure (*25*). Briefly, to a 1 mM solution of DTNB in 50 mM potassium phosphate buffer (pH 6.6), containing 5 mM EDTA, 20 mM sodium borohydride was added. The solution was incubated at 37 °C for 30 min. The concentration of TNB was determined by measuring the absorbance at 412 nm using the molar absorption coefficient of 13 600 M^{-1} cm^{-1}.

Hypochlorous Acid Scavenging Assay. The assay was performed at room temperature in a cuvette containing a 40 μM TNB solution, with or without the lyophilized infusion (0, 125, 250, 500, or 1000 μg/mL). The absorbance was measured at 412 nm before and 5 min after the addition of hypochlorous acid (40 μM). Scavenging of hypochlorous acid was ascertained by using lipoic acid as a reference scavenger, which inhibited TNB oxidation in a concentration-dependent manner. Four experiments were performed in duplicate.

RESULTS AND DISCUSSION

In the present work, the lyophilized infusion of cardoon leaves exhibited superoxide radical scavenging activity using the X/XO system (**Figure 1A**), with an IC_{50} at 35.0 μg/mL. Having in mind that an inhibitory effect on the enzyme itself would also lead to a decrease of NBT reduction (*1*), the effect of the extract on XO activity was checked. For this purpose a control experiment was performed evaluating the effect of the infusion on the metabolic conversion of xanthine to uric acid (**Figure 1A**). The results revealed that the infusion has a weak inhibitory effect on XO, (IC_{25} at 89.4 μg/mL), so it was not possible to show a clear-cut scavenging effect on superoxide radical. To determine the inhibitory mechanism of the lyophilized infusion

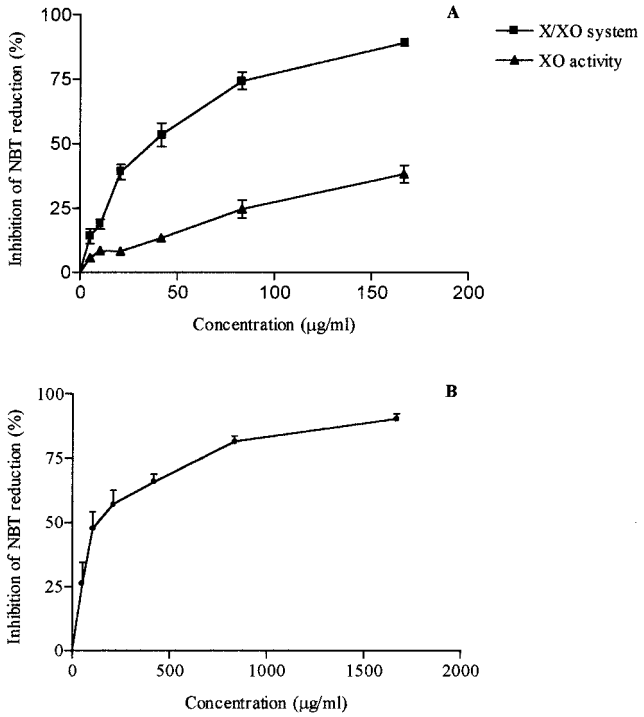

Figure 1. Effect of cardoon lyophilized infusion on (A) NBT reduction induced by superoxide radical generated in a X/XO system and XO activity; (B) NBT reduction induced by superoxide radical generated in a NADH/PMS system. Values show mean ± SE from 4 experiments performed in triplicate.

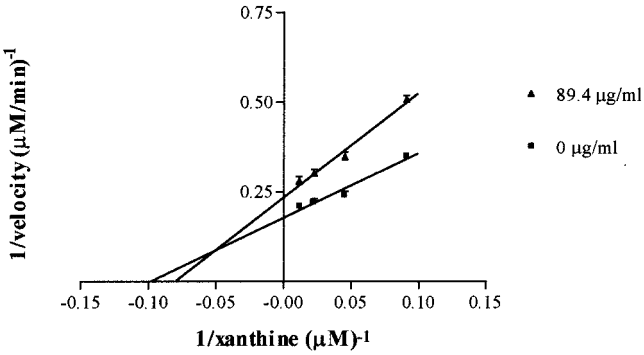

Figure 2. Lineweaver–Burk plots for the inhibition of XO by cardoon lyophilized infusion with xanthine as substrate. Values show mean ± SE from 3 experiments performed in triplicate.

against XO, its activity was tested at different substrate concentrations (**Figure 2**). In the presence of the extract V_{max} lowered from 5.6 to 4.3 μM/min and k_m raised from 10.2 to 12.5, suggesting a mixed noncompetitive inhibitory effect; that is, the constituents of the extract bind to both the enzyme and the X/XO complex, but with greater affinity for the first (*26*). In view of clarification, the effect of the infusion on superoxide radical generated by a nonenzymatic system was also evaluated (**Figure 1B**), which indicated an IC_{50} at 145.2 μg/mL. The difference found for the IC_{50} values obtained with the two systems might be due to the higher production of superoxide radical in the nonenzymatic system, as judged by the faster rate of NBT reduction and the need of much more superoxide dismutase for an effective scavenging activity in the NADH/PMS (data not shown).

The cardoon lyophilized infusion also exhibited a potent scavenging activity for hydroxyl radical in a concentration-dependent manner (**Table 1**). Deoxyribose is degraded into

Table 1. Absorbances and Scavenging Effect Obtained in the Deoxyribose Assay in the Presence and Absence of Ascorbic Acid (–AA) or EDTA (–EDTA)

conc. of cardoon extract (μg/mL)	ABS	scavenging ratio (%)	ABS (–AA)	ABS (–EDTA)
0.0	0.401		0.151	0.212
3.1	0.325	19.0	0.124	0.205
12.5	0.235	41.5	0.093	0.247
50	0.182	54.8	0.092	0.261
200	0.134	66.7	0.104	0.201
800	0.115	71.5	0.106	0.092

malonaldehyde on exposure to hydroxyl radicals generated by Fenton systems. If the resulting mixture is heated under acid conditions, malonaldehyde may be detected by its ability to react with thiobarbituric acid to form a pink chromogen (*27*). However, some compounds may act as pro-oxidants because of their ability to redox cycle the metal ion required for hydroxyl generation and thus increase the radical production (*28*). To evaluate the pro-oxidant potential of the infusion we omitted ascorbic acid. As can be seen in **Table 1**, no pro-oxidant effect was observed at the concentrations tested. 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, because omission of the chelator allows iron ions to bind directly to the sugar. Compounds which can inhibit deoxyribose degradation in the absence of EDTA are those with iron ion-binding capacity and which can withdraw the iron ions and render them inactive or poorly active in Fenton reactions (*24*). The assay performed in the absence of EDTA showed that cardoon infusion was able to chelate iron ions only for concentrations above 200 μg/mL.

HOCl is a powerful oxidant which reacts readily with many biologically important molecules. It is produced in the organism by oxidation of Cl^- ions at sites of inflammation by the neutrophil enzyme myeloperoxidase (*29*). The oxidizing properties of HOCl induce the conversion of TNB (λ_{max} = 412 nm) to DTNB (λ_{max} = 325 nm) (*30*). A HOCl scavenger inhibits the conversion of TNB to DTNB by this species. Cardoon lyophilized infusion exhibited a weak antioxidant protective activity against damage by HOCl, as shown in **Figure 2A**. In 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 95% at 500 μM (**Figure 2B**).

To estimate a putative correlation of the observed antioxidant activity with the phenolic composition of cardoon infusion, an aliquot of the lyophilized infusion was submitted to HPLC/DAD analysis. The results obtained revealed a phenolic fingerprint composed of seven identified compounds, namely 3-caffeoylquinic acid, 4-caffeoylquinic acid, 5-caffeoylquinic acid, 1,3-dicaffeoylquinic acid (cynarin), 1,5-dicaffeoylquinic acid, luteolin 7-glucoside, and apigenin 7-glucoside (**Figure 4**). Coumpound **a** revealed a UV spectra characteristic of a luteolin derivative but was not identified. The quantification of the identified polyphenols present in the lyophilized infusion showed that luteolin 7-glucoside was the compound present in higher amounts (1290 mg/kg), followed by 5-caffeoylquinic acid (380 mg/kg) and 1,5-dicaffeoylquinic acid (120 mg/kg). Cynarin and apigenin 7-glucoside exhibited lower amounts (70 and 20 mg/kg, respectively). The others were minor compounds, which were not quantified. These results suggest that luteolin 7-glu-

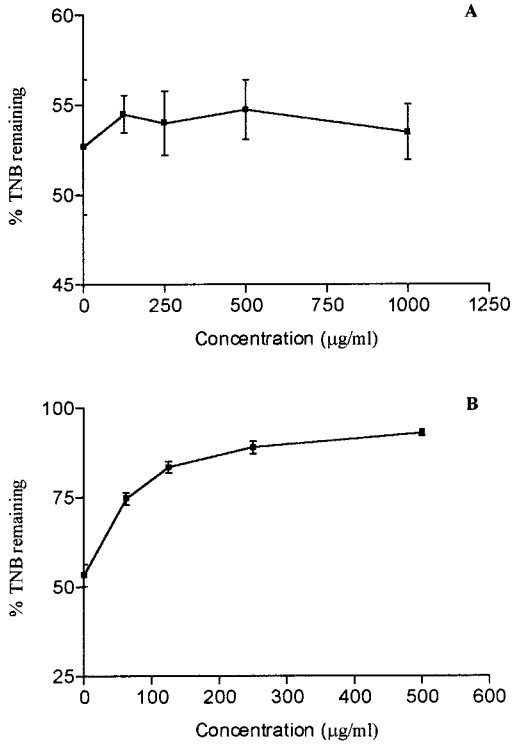

Figure 3. Effect of cardoon lyophilized infusion (A) and lipoic acid (B) on the oxidation of TNB by HOCl. The amount of TNB unchanged after incubation is calculated and expressed as percentage of the initial value. Values show mean ± SE from 4 experiments performed in duplicate.

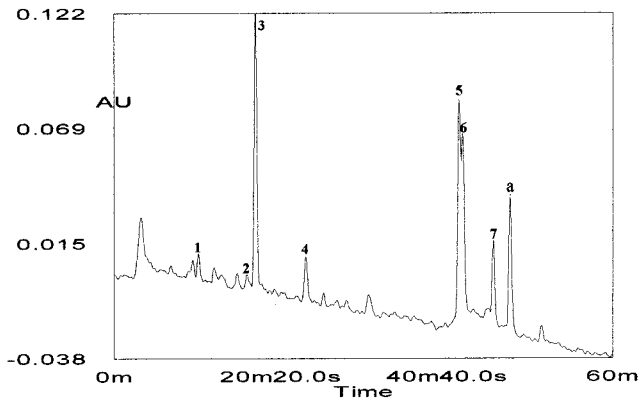

Figure 4. HPLC profile of cardoon lyophilized infusion. Detection at 320 nm. (1) 3-caffeoylquinic acid; (2) 4-caffeoylquinic acid; (3) 5-caffeoylquinic acid; (4) 1,3-dicaffeoylquinic acid; (5) 1,5-dicaffeoylquinic acid; (6) luteolin 7-glucoside; (7) apigenin 7-glucoside; and (a) unidentified compound.

coside is, most probably, the main compound contributing to the antioxidant properties exhibited by the infusion.

Hydroxycinnamic acids are present in plants, either esterified or in glycosilated form. The physiological role of the molecule bound to the phenolic hydroxycinnamic acid is usually secondary (*31*). Furthermore, flavonoids can be absorbed both at the gastrointestinal tube as the free aglycon and glycoside, and have been detected in blood and urine in the nonesterified form (*32*). The antioxidant activity of 5-*O*-caffeoylquinic acid and of its isomers has already been observed in several experimental models and for different ROS, including the in vitro lipoprotein oxidation model (*16*), the free radical method using 2,2-diphenyl-1-picrylhydrazyl (DPPH•) (*33*), scavenging activity on superoxide radicals enzymatically generated, and inhibitory effect against oxidation of methyl linoleate (*34*). The antioxidant capacity of 1,5-dicaffeoylquinic acid was also determined in several assays, such as inhibition of chemiluminescence (*35*) and inhibition of methyl linoleate hydroperoxide formation (*36*). The antioxidant properties of luteolin 7-glucoside and of the respective aglycon, luteolin, have already been observed against low-density lipoprotein oxidation (*11*), scavenging effect on hydroxyl radicals generated by UV photolysis (*37*), DPPH free radical scavenging activity and radical cation $ABTS^{\bullet+}$ scavenging effect (*13*), and by measuring the coupled oxidation of β-carotene and linoleic acid (*38*). The antioxidant effectiveness of apigenin was also determined in models such as the in vitro lipoprotein oxidation model (*16*) and against hydroxyl radicals generated by UV photolysis (*37*).

In conclusion, considering the results obtained and regarding the presence of several phenolic compounds in the lyophilized infusion of cardoon, the scavenging activities observed against superoxide radical, hydroxyl radical, and hypochlorous acid probably is due to the presence of these compounds, which contribute to the protective effects observed in this study.

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